The 7th International Symposium on the Biosafety of Genetically Modified Organisms
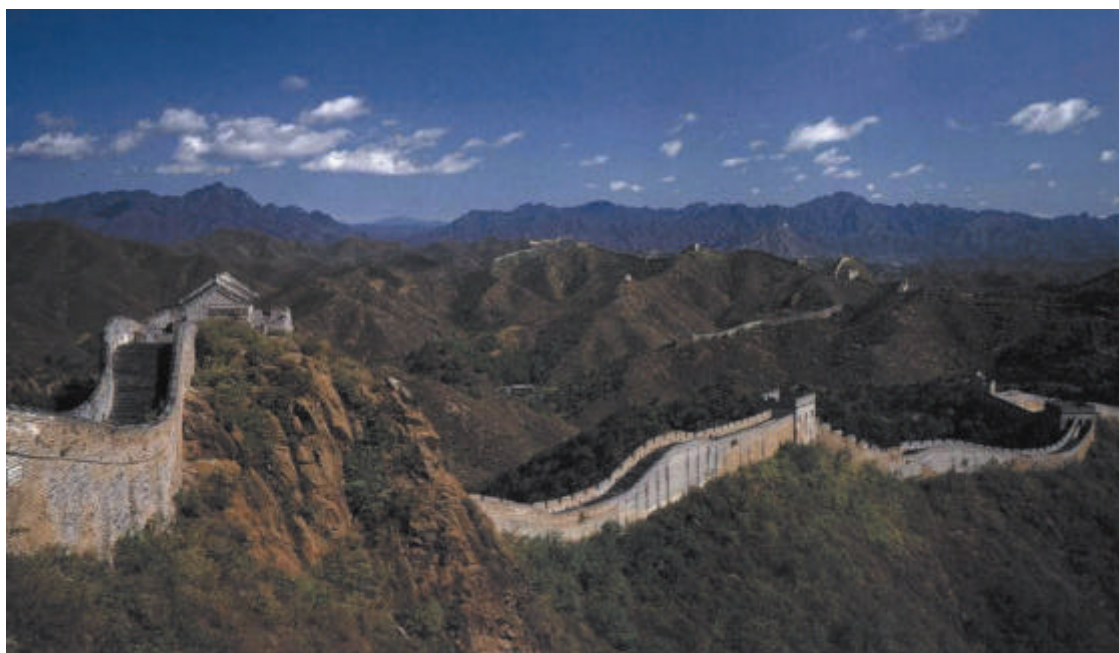
Beijing, China October 10-16

The 7th International Symposium on the Biosafety of Genetically Modified Organisms

Beijing, China

Schedule for Main Activities:

OCTOBER 10, 2002	
8: 00 21: 00	*Registration at the Meeting Hall, Friendship Hotel*
OCTOBER 11, 2002	
Morning:	
8: 00 13: 00	*Registration at the Meeting Hall, Friendship Hotel*
10: 00	*ISBR Executive meeting at Room 103, Meeting Hall*
Afternoon:	
14: 0018: 00	*Opening ceremony and the first plenary session, Meeting Hall*
Evening:	
19: 00	*Welcome reception* (Banquet at the Multifunction Hall of Friendship Hotel)
OCTOBER 12, 2002 *All following sessions are held at the Meeting Hall*	
Morning:	
8: 30 12: 10	*New science for enhanced biosafety* **Chair:** Dr. Joachim Schiemann, Germany
Afternoon:	
13: 00 18: 00	*Poster session*
OCTOBER 13, 2002	
Morning:	
8: 30 12: 10	*Consequences of gene flow.* **Chair:** Dr. Allison Snow, USA
Afternoon:	
13: 30 17: 30	*Possible implication of the release of transgenic crops in centers of origin or diversity* **Chair:** Dr. Ariel Alvarez-Morales, Mexico
OCTOBER 14, 2002	
Morning:	
8: 30 12: 00	*Why regulate and how* **Chair:** Dr. Julian Kinderlerer, UK
Afternoon:	
13: 30 17: 30	*Research and regulation on Biosafety of GMO's in China* **Chair:** Dr. Hongguang Wang, China
OCTOBER 15, 2002	
Morning:	
8: 30 12: 10	*Transgenic insects for pest management programs: status and prospects.* **Chair:** Dr. Marjorie Hoy, USA
Afternoon:	
13: 30 17: 30	*Effects of GMOs on microbial communities* **Chair:** Dr. Kornelia Smalla, Germany
18: 30	Banquet at Ju He Restaurant, Friendship Hotel
OCTOBER 16, 2002	
Morning:	
	Field trip Fieldtrip to Langfang, Hebei Province, to visit transgenic cotton field
Afternoon:	
15: 00	*Closing session* (Meeting Hall, Friendship Hotel)

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Chair:

Prof. Zhangliang CHEN (Peking University)

Member:

Contents

The 7th International Symposium on the Biosafety of Genetically Modified Organisms

Beijing, China
October 10   16

PROGRAM

OCTOBER 10, 2002

    8: 00   21: 00   *Registration at the Meeting Hall, Friendship Hotel*

OCTOBER 11, 2002

Morning:

    8: 00   13: 00   *Registration at the Meeting Hall, Friendship Hotel*
    10: 00   *ISBR Executive meeting at Room 103, Meeting Hall*

All following sessions will be held in the Meeting Hall, Friendship Hotel

Afternoon: *Opening ceremony*

14: 00 14: 10	Welcome address by Mr. Xueyong Li, Vice Minister of China Ministry of Science and Technology
14: 10 14: 20	Welcome address by Mr. Baowen Zhang, Vice Minister of China Ministry of Agriculture
14: 20 14: 30	Remarks from the representative from UNESCO
14: 30 14: 50	Dr. Charles Kessler, the representative from EC
14: 50 15: 00	**Coffee Break**
15: 00 15: 45	*The status of agriculture biotechnology in China* Dr. Zhang-Liang Chen, China
15: 45 16: 30	*Contrasts in the international risk debate scheme* Dr. Alan McHughen, USA
16: 30 17: 15	*The new journal, environmental biosafety Research* Drs. Mark Tepfer and Klaus Ammann, France and Switzerland
17: 15 18: 00	*Biotechnology in agriculture* Dr. Robert Fraley, USA

Evening: *Welcome reception*

    19: 00   **Banquet and a Chinese traditional performance the Multifunction Hall of Friendship Hotel**

OCTOBER 12, 2002

Morning: *New science for enhanced biosafety*
Chair: Dr. Joachim Schiemann, Germany

8: 30 9: 10	*Site-specific genetic modifications for the post-genomic era* Dr. David Ow, USA
9: 10 9: 50	*Elimination of marker genes by site-specific recombination* Dr Jianru Zuo, China
9: 50 10: 30	*Means of preventing gene flow due to outcrossing* Dr. Ralph Bock, Germany
10: 30 10: 50	**Coffee Break**
10: 50 11: 30	*Biosafety aspects for molecular farming* Dr. Rainer Fischer, Germany
11: 30 12: 10	*Science-based approach to assessing the ecological risk of crops derived through modern biotechnology* Dr. Thomas E. Nickson, USA

Afternoon: *Poster session*

    13: 00 18: 00   Poster session

OCTOBER 13, 2002

Morning: *Consequences of gene flow.*

    Chair: Dr. Allison Snow, USA

    8: 30 9: 10   *Crop-to crop gene flow: dispersal of transgenes during field tests and commercialization*
                      Dr. Baltazar Baltazar, Mexico

    9: 10 9: 50   *Gene flow from crops to wild relatives in Asia: case studies and general expectations*
                      Dr. Baorong Lu, China

    9: 50 10: 30   *Ecological effects of pest resistance genes that disperse into weed populations*
                      Dr. Allison Snow, USA

    10: 30 10: 50   Coffee Break

    10: 50 11: 30   *Agronomic effects of gene flow: multiple herbicide resistance in volunteer crop plants.*
                      Dr. Linda Hall, Canada

    11: 30 12: 10   *Mitigating gene flow: herbicide resistant rice, introgression to weedy red rice, and mitigation strategies.*
                      Dr. Jonathan Gressel, Israel

Afternoon: *Possible implication of the release of transgenic crops in centers of origin or diversity.*

    Chair: Dr. Ariel Alvarez-Morales, Mexico

    13: 30 14: 15   *Transgenes in maize landraces in Oaxaca: official report on the extent and implications*
                      Dr. Ariel Alvarez-Morales, Mexico

    14: 15 15: 00   *Farmers' management of maize landrace diversity*
                      Dr. Julien Berthaud, Mexico

    15: 00 15: 45   *Concerns about the effect of transgene introgression in maize landraces and teosinte*
                      Dr. José de Jesús Sánchez-González, Mexico

    15: 45 16: 00   Coffee Break

    16: 00 16: 45   *Possible effects of transgenes on genetic diversity*
                      Dr. Paul Gepts, USA

    16: 45 17: 30   Discussions

OCTOBER 14, 2002

Morning: *Why regulate and how*

    Chair: Dr. Julian Kinderlerer, UK

    8: 30 9: 15   *The precautionary principle*
                      Dr. Eric Schoonejans, France

    9: 15 10: 00   *The trigger is novelty*
                      Dr. Desmond Mahon, Canada

    10: 00 10: 30   Coffee Break

    10: 30 11: 15   *Biosafety regulation in China*
                      Mr. Jingen Cheng, China

    11: 15 12: 00   *Criteria for evaluating biosafety frameworks: objectives and standards.*
                      Dr. Terry Medley, USA

Afternoon: *Research and regulation on Biosafety of GMO's in China*

    Chair: Dr. Hongguang Wang, China

    13: 30 14: 15   *Strategic approaches to biosafety studies in China*

Dr. Yufa Peng, China

14: 15	15: 00	*Studies on gene flow in China* Prof. Shirong Jia, China
15: 00	15: 45	*Research and development of recombinant microbes and safety considerations in China* Prof. Dafang Huan, China
15: 45	**16: 00**	**Coffee Break**
16: 00	16: 45	*Economic impacts of plant biotechnology in China* Dr. Jikun Huang, China
16: 45	17: 30	*Environmental impact of Bt cotton: a case study from China* Dr. Kongming Wu, China

OCTOBER 15, 2002

Morning: *Transgenic insects for pest management programs: status and prospects.*
Chair: Dr. Marjorie Hoy, USA

8: 30	9: 10	*Analysis of risks of transgenic insects for pest management: past and future guidelines* Dr. Marjorie Hoy, USA
9: 10	9: 50	*Transformation of mosquito vectors of disease: goals and risk analyses* Dr. Chris Curtis, UK
9: 50	10: 30	*Transgenic pink bollworms: evaluation of risks of releases in genetic control projects.* Dr. John Peloquin, USA
10: 30	**10: 50**	**Coffee Break**
10: 50	11: 30	*Chagas disease vectors that do not transmit the disease agent* Dr. Ravi Durvasula, USA
11: 30	12: 10	*Transgenic Mediterranean fruit flies for sterile insect release programs* Dr. Alan Robinson, Austria

Afternoon: *Effects of GMOs on microbial communities*
Chair: Dr. Kornelia Smalla, Germany

13: 30	14: 15	*Exploitation of genetically modified Pseudomonas for industrial ecology applications* Dr. Fergal O'Gara, Ireland
14: 15	15: 00	*Why monitoring the fate of microbial inoculants and their impact on soil microbial communities is needed* Dr. Kornelia Smalla, Germany
15: 00	15: 45	*Monitoring microbial inocula, activity and impact on ecosystem function and soil microbial diversity* Dr. Mark. Bailey, UK
15: 45	**16: 00**	**Coffee Break**
16: 00	16: 45	*Fate of GM rhizobial inoculants: lessons from Europe and elsewhere* Dr. Penny Hirsch, UK
16: 45	17: 30	*Monitoring the fate and ecosystem effects of genetically modified Pseudomonas putida producing phloroglucinol and phenazine in wheat rhizosphere* Dr. Eric Smit, The Netherlands
18: 30		**Banquet at Ju He Restaurant, Frendeship Hotel**

OCTOBER 16, 2002

Morning: *Field trip*

Fieldtrip to Langfang, Hebei Province, to visit transgenic cotton field
(Bus starts at Friendship Hotel)

| 12: 00 | | Lunch |

Afternoon: *Closing session*

| 15: 00 | | Closing session at Meeting Hall, Friendship Hotel |

The Status of Agriculture Biotechnology in China

Zhang-Liang Chen and Li-Jia Qu

Peking University, Beijing 100871, P. R. China

As the most-populated country (close to 13 billion people) and one of the largest agriculture countries in the world, China is being challenged by great demand for food, with only about 7% of the world's arable land feeding over 20% of the world's population. For this reason, food security for the people is highly concerned in China. Chinese scientists, for many years, have been taking great efforts to improve the crop yields by traditional breeding techniques, e.g. breeding the hybrid rice, which made a great contribution to the agriculture production. Since transgene technology, when being applied to breeding, is much easier and powerful enough to breed crops with good traits (such as salinization-tolerance, drought-tolerance, disease-resistance and insect-resistance) compared with conventional breeding method, agricultural biotechnology has been considered as an important tool to achieve food security. It is even more important for a large agriculture country like China to develop its agriculture by transgene technology.

From 1986 on, R & D of agribiotechnology in China has been strongly supported and more than 100 laboratories around the country have been working for the past sixteen years to integrate biotechnology into conventional agriculture in order to improve yield and quality of crop plants. By the year 2001, many transgenic organisms (more than 130 species) with more than 100 different trait genes including insect-resistance, bacterial-, fungus- and virus-resistance, salt-tolerance, drought-resistance, nutrition enrichment, quality improvement, production of edible oral vaccines and recombinant pharmaceuticals, were obtained.

Field tests, environmental releases and commercialization of transgenic plants are strictly regulated in China. In November, 1993, the State Science and Technology Commission of China issued the Safety Administration Regulation on Genetic Engineering, which was the first law on biosafety in China. According to this Regulation, three years later, the Safety Administration Implementation Regulation on Agricultural Biological Genetic Engineering was issued in July and entered into effect in December, 1996 by the Ministry of Agriculture (MOA), China. In the same year (1996), MOA established the Office of Genetic Engineering Safety Administration (OGESA) to regulate field tests, environment releases and commercialization of transgenic organisms in China. From 1997 on, OGESA started to process biosafety evaluation applications twice a year. On May 23th of 2001, the Guideline for Biosafety Management of Agricultural GMO was issued by Chinese Government. On January 5th, 2002, MOA issued three managing documents according to the Guideline. They are Biosafety Evaluation Regulation for Agricultural GMOs, Import Regulation for Agricultural GMOs and Labelling Regulation for Agricultural GMOs. These three Regulations were implemented from March 20th of this year.

Since 1997, the Ministry of Agriculture of China has received a total of 703 applications for biosafety evaluation on agricultural genetically engineered organisms and their products, among which 517 applications were approved. 52 domestic applicants (including universities and academic institutions) and 4 foreign companies were equally treated. By the year 2001, 10 spieces of transgenic plants including rice, corn, cotton, soybean, oilrape, potato and poplus were approved to conduct environmental releases.

In 1997, China started to commercialize transgenic crops and four licenses for commercialization of transgenic plants were granted. Another two licenses were granted in the next year. Therefore, six licenses for commercialization altogether have been issued so far, i.e. Bt cotton (Monsanto, USA), Bt cotton (Chinese Academy of Agricultural Sciences, China), delayed ripening tomato (Central-China Agriculture University, China), color-altered petunia (Peking University, China), CMV-resistant sweet pepper (Peking University, China) and tomato (Peking University, China). Since China's license for commercialization is location-dependent, the six licenses will allow commercialization of the GM plants at 35 different locations by the July of 2000. Moreover, there are several lisences being released to recombinant vaccines for animals.

China is one of the countries planting largest area of transgenic plants in the world. Among the transgenic crops being planted in China, the acreage of transgenic insect-resistance cotton is the largest. In 1998, one hundred and twenty thousand hectares of transgenic insect-resistant Bt cotton (mainly Bollguard, Monsanto) were planted. During the period of 1999-2000, the Bt cotton planting acreage (including both CAAS Bt cotton and Monsanto Bt cotton) reaches three hundred and fifty thousand hectares. It is estimated that, due to the planting of Bt cotton, the number of insecticide spray is dramatically reduced from 15-20 times to 1-2 times per season, and that the benefit from this GM crop reaches one hundred and twenty five million US dollars during the year 1999-2000. This year, the planting acreage is over six hundred thousand hectares.

Although the other four licenses for commercialization were granted three or four years ago, the planting acreage of these GM plants is much smaller than that of transgenic Bt cotton. The color-altered petunia is planted in Guangdong province while the market for shelf-time-altered tomato is being developed in Hubei province. Neither transgenic virus-resistant tomato or sweet pepper was planted into large scale, nor were they sold in the market.

Due to the debates on environmental and food safety issues of GM plants, which brought about import and export problems, commercialization of transgenic plants in China has been slow down. Although the biological and biosafety research on GM major crop plants were both intensively and extensively carried out, and the importance of increasing the yield of major crops were widely recognized, not even a single major GM food crop was approved for commercialization up to date in China. This policy is thought to bring about enormous effects to the agricultural production of China in the future, which is crucial to the food security for China in the new century.

Contrasts in the international risk debate schemes

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Welcome to the 7th International Symposium on the Biosafety of GMOs. Much has changed since our first Symposium at Kiawah Island, USA, twelve years ago. Long before any genetically modified crops were grown commercially, and long before any consumers were presented with genetically modified foods, our predecessors recognized the necessity of scientifically sound assessment of risks brought by this new technology. At that time, discussions were based largely on hypothetical scenarios. True, GM microbes were already pumping out commercially available pharmaceuticals, and GM plants were in small scale field trials. But the concern then, and remains so with some people today, relates to the unexpected, the unknown, the lack of familiarity. Perhaps genetic engineering will result in some entirely new and hazardous phenomenon that no one had been able to predict, and that new hazards took a terrible toll on human life or the environment.

Much has changed. In a few short years, the introduction of GM crops has had a dramatic impact on farmers wherever they had been allowed. In spite of dire warnings that GM crops 'might' harm the environment, or they 'could' inadvertently poison consumers, or that markets will be lost, and that consumers will refuse to buy the products, farmers have embraced GM technology. In the US, three quarters of the soybeans are GM cultivars. A third of the maize, over 70% of the cotton. In Canada, 80% of canola farmers have at least tried GM cultivars, and only 10-15% revert back to conventional cultivars afterwards. This represents an amazing rate of uptake of a new technology, and one that demands appropriate biosafety scientific analyses and debate. Yes, farmers may be making more money while producing more food, and yes, there may be less pesticide load on the environment, and yes, there's less environmental damage in growing some of these crops. But if there are unique risks associated with products of genetic technologies, the sheer amount and distribution of GMOs in commercial production represents a major vulnerability. It is our job to identify potential and actual risks and analyze them to inform mitigation or management policy.

Even since we bid farewell at our last symposium in Saskatoon in 2000, a substantial amount of data has been collected and evaluated from researchers around the world. In the interim, The EC issued their report on their sponsored studies on the safety of GMOs[1]. The report summarized the results from 81 studies, conducted by 400 teams of predominantly public sector scientists over a 15 year period at a cost of about 70 Million Ecus. In the US, the National Academy of Sciences sponsored a study of the environmental effects of transgenic plants[2]. In both cases, the reports support what the results of tens of thousands of field trials with GMOs have indicated all along- that GMOs present no new types of risk relative to conventional technologies, and that risks, when they are present, are associated with the traits of the organism, regardless of the method of how the traits were introduced into the organism. This is the basis of the 'product vs, process' dichotomy, and a fundamental concept in scientifically valid and pragmatic risk assessment and management.

Wherever I travel, when I talk with people, whether scientists, politicians, regulators, or ordinary citizens, I notice we all want the same thing; a better world for our children.

Of course, exactly what constitutes a 'better world' differs from place to place, and from person to person, whether it entails food security, improved education, better health care or whatever.

Regulatory agencies in different countries also want the same thing. They want to be able to

provide assurances to their political masters and to the public that products in their charge won't devastate the ecology or kill consumers. At the same time, they don't want to block release of useful products. Unfortunately, and contrary to the assertions of extremists at both ends of the spectrum, this is not a question of 'all good' or 'all bad'. Few products are entirely bad, with no redeeming qualities; they wouldn't be brought before regulators in the first place. And even the best products carry some risks. So regulators know they cannot demand zero risk, or demand proof that the product will never cause harm. If a policy were to make such demands, as advocated by some groups, every product-- not just biotech products-- would have to be removed from or denied access to the market- a fact that seems beyond the comprehension of those demanding groups. Instead, in the real world regulators have to assess the benefits, and the risks, then evaluate the balance between the two and make a judgment.

Different nations have different regulatory philosophies in respect to GMOs. Three major bureaucracies with considerable experience dealing with GMOs include the US, Europe and Canada. All have mature bureaucratic structures. They have all modified their regulatory procedures over the years. And they're all dedicated to protecting the public good.

USDA APHIS, regulates transgenic plants in the US. The trigger for initiating a regulatory assessment is the process of rDNA, but the evaluation is based primarily on the features of the organism. As the US system continues to mature, it is recognizing the scientific basis of risk assessment is a combination of the novelty of the trait combined with the nature of the species in which the trait is expressed, combined with the environment in which the plant will be growing. The deficiency with the US system is that new cultivars carrying potentially hazardous features are exempt if they were developed using non-rDNA methods. This may change in the not too distant future.

The European system is similar in that it is also a process triggered one, meaning that new cultivars developed using rDNA are scrutinized, and also suffers the same deficiency, that potentially hazardous cultivars produced using, for example, ionizing radiation, traditional breeding, etc, are exempt from the regulatory process designated for GMOs. Whether Europe sticks with the process-based trigger remains to be seen.

Canada, another nation with a relatively mature regulatory system, triggers their assessment based on the novelty of the trait in the organism. This is a product based assessment, which means they require prior regulatory review on a new cultivar developed using traditional plant breeding methods if the resultant cultivar expresses traits novel to the crop.

As a scientific community, what is the basis for concern with GMOs and the regulatory philosophy governing their release?

What are the current deficiencies in regulatory theory?

The most important one is that there's no rational definition of GM that stands up to scientific and legal scrutiny. A consequence of the fuzzy definition of GM, is that two identical products, presenting identical risks, could be treated differently, with one receiving minimal regulatory oversight before being unleashed upon the world, and the other coming under such stringent scrutiny that it may not be grown at all- certainly the case in Europe currently, with the moratorium on new releases of GMOs.

For example, we can all appreciate a scorpion gene in a strawberry producing the scorpion toxin. Such a product would demand severe regulatory restrictions, if it is to be allowed at all. Unlikely to be any argument under any current regulatory regime.

However, consider a soybean into which a gene from another soybean was inserted, using a particle gun and without any other non-soybean DNA. The final cultivar is virtually identical to what

could arise using conventional cross pollination. In some countries, this GM soybean would face the substantial regulatory scrutiny, in others, it wouldn't. But the risk presented by this soybean would be the same, because it's the same organism. Obviously, either one nation is over regulating, or the other is under-regulating.

If we define a GM as having 'foreign DNA, DNA of another species', as some suggest, we exempt the soybean with transferred soybean DNA, even though it was produced using rDNA technologies. At the same time, we include mundane things like ordinary wheat, because many cultivars carry chromosomal translocations from rye, a different genus, without having undergone any rDNA manipulations. Does the mere presence of 'foreign' DNA constitute a risk sufficient to warrant intense regulatory scrutiny? If so, we need to start regulating wheat, tomatoes, and, in fact, all foods carry DNA from other species if only from the ubiquitous microbes in and on the plant.

Consider now a GM soybean into which T-DNA is inserted, conferring, say herbicide tolerance. For whatever reason, the breeder decides not to pursue regulatory approval for the GM soybean. Instead, the breeder notices some other attribute and uses the GM soybean as a parent and selects away from the herbicide tolerance, away from the T-DNA but in favor of the other attribute. The resulting cultivar has a new attribute, but no T-DNA. Scientifically, should the cultivar be regulated as a GM variety?

Here's an example we'll have to deal with soon. Many tree fruits, and other products, involve grafting of the scion, the fruit producing portion of the tree, onto rootstock. Often the rootstock is a different species. Even without rDNA technologies, some would view the fruits as 'transgenic', as it required DNA from another species to grow, even if the final food product is devoid of the foreign DNA.

Now let's add real foreign DNA to the rootstock, and transform only the rootstock. Does that change the consideration?

I give these examples to provoke thought. I hope it is as clear to you as it is to me that there is no simple, blanket definition of GMO and no blanket solution to risk assessment and management. Of course there are risks with GMOs, as there are with every type of organism. I know we are going to have plenty to think about in the next few days, and I encourage you to share your thoughts and philosophies on how we may better protect our citizens and our environments while allowing the same to enjoy the benefits of appropriately regulated products of biotechnology.

References

1. Kessler, C and I. Economidis,, eds. 2001. EC sponsored research on safety of genetically modified organisms.

2. NAS, 2002. Environmental effects of transgenic plants. The scope and adequacy of regulation. National Research Council. National Academy Press, Washington, DC.

Recombinase-directed transgene placement

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Abstract

The exact placement of foreign DNA into the plant genome produces transgenes with greater structural fidelity and faithful expression. Recombinase-mediated site-specific integration has been reported for several plant species, including rice and maize. The next challenge will be to develop an integrated strategy to stack and translocate DNA. Being able to append new DNA sequentially to a target site permits the continual use of a previously characterized chromosome location, which justifies the initial investment costs in identifying favorable chromosome targets. Stacking transgenic traits at a limited number of target sites is also preferable to scattering transgenes all over the genome, as the clustering of transgenes expedites the introgression of bundled traits to elite cultivars, through a process mediated by the translocation of transgenes from one chromosome to another.

Key Words

Site-specific recombination, DNA integration, transgene expression, gene stacking, line conversion

Introduction

In plants, the targeting of DNA by homologous recombination has had limited successes (for review, see Puchta, 2002). A most recent article, however, may be a breakthrough, with about 1% of the transformed rice harboring the integrated DNA at the chosen site (Terada *et al.*, 2002). Despite the encouraging news, this still means generating hundreds of transgenic lines to recover a few site-directed insertions. More importantly, the precise placement of a transgene in itself does not guarantee suitable transgene expression, as current knowledge cannot predict how a chromosome location affects newly introduced DNA. Hence, even if homologous recombination were practical, the screening of a collection of random integration events may still be a preferred option. Given that a "favorable" integration site is found empirically and through considerable labor, this begs the question of whether subsequent DNA deliveries can also be directed to the same location.

Recombinase-mediated gene targeting has been achieved in a number of plant species (for review, see Ow, 2002). The general scheme requires a first recombination site to be introduced into the genome to serve as the target site for the subsequent insertion of a second DNA molecule. Reports to date show that recombinase-directed site-specific integration can place a single-copy non-rearranged DNA fragment into the target site in 1 out of 3 selected events, a rate that is significantly higher than those reported for homology-dependent insertions (Albert *et al*., 1995; Srivastava & Ow, 2001). Moreover, half of the precise insertions express the transgene at a predictable and reproducible level (Day *et al*., 2000). This means that once a suitable target site is found, the plant line can be used for the subsequent delivery of trait genes. What is lacking thus far is a convenient way to append additional trangenes to the target locus after the initial insertion event. In this paper, the first section proposes a strategy that permits the sequential and repeated delivery of new DNA to the genomic target, as might be expected if a transgenic plant line were to be improved over time through the sequential addition of new transgenic traits. Appending DNA onto existing target sites justifies the initial investment in screening for suitable chromosome locations. The second section follows with the description of a recombinase-directed introgression strategy to move the clustered transgenes from laboratory to field cultivars.

Transgene stacking

The idea of gene stacking rests on a concept that the integrating DNA brings along a different recombination site, such that after insertion of the new recombination site into the genome, the new recombination site then becomes the new target for the next round of integration. While some recombination systems catalyze freely reversible reactions, others do not. Instead, the substrate sites, typically known as *attB* and *attP*, are not identical. This necessitates that the product sites generated from an *attB* x *attP* reaction, *attL* and *attR*, are dissimilar in sequence to *attB* and *attP*. The recombination enzyme that promotes the *attB* x *attP* reaction, often referred to as the integrase, by itself does not recombine *attL* x *attR*. The lack of a readily reversible reaction gives a distinct advantage for employing such a system in DNA integration since integrated molecules are stable. Most importantly, an irreversible system permits a novel gene stacking strategy that is not achievable using only freely reversible systems. In fact, this is the underlying reason for this laboratory's interest in the C31 recombination system (Thomason *et al*., 2001).

Figure 1 shows a strategy to stack genes sequentially using a non-reversible system along with a reversible system, as exemplified, respectively, by the C31 and the Cre-*lox* systems. Shown are BB', PP', BP' and PB' as *attB*, *attP*, *attL* and *attR*, respectively, filled arrowhead as *lox* site, *G1*, *G2*, *G3*, *G4*, *G5*, as trait genes, and *M1*, *M2* as marker genes (gene promoters and terminators not shown). The process begins with a single copy trait gene linked to a marker: *lox-M1-lox-G1*-BB'-(inverted *lox*). The single copy locus may be obtained by molecular screening. Alternatively, a complex multicopy integration pattern may be resolved by Cre-*lox* site-specific recombination into a single copy state (Srivastava *et al*., 1999). If a resolution-based strategy were used, the marker *M1* would have been deleted, leaving a configuration consisting of *lox-G1*-BB' (inverted *lox*). To append *G2* to the *G1* locus, the integrating plasmid with the PP'-*G2*-PP'-*lox-M2* configuration recombines with the genomic BB' target (Figure 1a). The integrase can be provided, for example, by transient expression from a cotransformed plasmid. Since either PP' can recombine with the single BB', two different integration structures would arise that are distinguishable by molecular analysis. Figure 1b shows only the structure useful for further stacking, consisting of *lox-M1-lox-G1*-BP'-*G2*-PP'-*lox-M2*-plasmid backbone-PB'-(inverted *lox*). The Cre recombinase is introduced into the system to remove the unneeded DNA (indicated by dotted lines). The resulting structure becomes *lox-G1*-BP'-*G2*-PP'-(inverted *lox*). To stack *G3*, the construct BB'-*G3*-BB'-*lox-M2* is introduced (Figure 1c). Analogous to the previous steps, the genome has only a single PP' site to recombine with either of the BB' sites on the plasmid. Recombination with the *G3* upstream site produces the structure shown in Figure 1d. After removing the unneeded DNA, the locus containing *G1*, *G2*, and *G3* is ready for the stacking of *G4* (Figure 1e). In another variation, sets of inverted *attB* and *attP* sites, rather than sets of directly oriented sites, can also be used. The sequence of events is analogous to those described for Figure 1.

There are several features worth noting. First, the vector for delivery of *G4* is the same as the vector for delivery of *G2*. Likewise, the vector for delivery of *G5* (Figure 1g) is the same as the vector for delivery of *G3*. In principle, the stacking process can be repeated indefinitely, alternating between the uses of two simple vectors. Second, the stacking of *G2* onward requires only a single marker gene, and if *M1* is first removed, a single marker can be used throughout. This bypasses the need to continually develop new selectable markers. Third, the trait genes, such as *G1*, *G2* and so on, should not be narrowly interpreted as a single promoter-coding region-terminator fragment. Not only could each DNA fragment be composed of multiple transgenes, but could also include border DNA that insulate its (their) expression from surrounding regulatory elements. This may be useful when clustering transgenes that bring with them dominant *cis*-regulatory sequences.

Transgene translocation

Crop improvement through genetic engineering requires that the transgene be introduced into cultivated varieties, also referred to as elite lines. In principle, this can be accomplished through direct gene transfer into the cultivated lines. However, this may not be an option as transformation protocols, especially those involving the tissue-cultured regeneration of plants, are often specific for a plant variety where DNA uptake and cell regeneration procedures have been worked out.

Therefore, in many instances, the transgene is first introduced into a transformable laboratory line and subsequently converted into cultivated lines through backcrosses to cultivated varieties. This may seem less efficient, but it does offer one advantage in that the steps involved in the gene transfer and selection for transgene expression are conducted once, rather than repeatedly with each and every locale-specific plant variety.

The major drawback to a line conversion approach is the length of the backcrossing program. Segregating away the DNA closely linked to the transgene is time consuming. Only a small fraction of the progeny would have a recombination event between the transgene and a tightly linked marker, a phenomenom known as "linkage drag". Take for example a transgene, *G1*, situated between two undesirable genetic traits *x'* and *y'*. If it were 0.1 genetic map units from *x'* and *y'*, a progeny pool size of 1 million would be needed to find a recombinant with both *x'* and *z'* segregated away, or in other words, the desired *x-G1-y* genotype. Consequently, linkage drag can make line conversion a rate-limiting step for crop improvement, with up to 10 backcross generations to produce commercially acceptable varieties.

The gene stacking strategy presented above incorporates features that permit the use of site-specific recombination to unlink a transgenic locus from its closely flanked DNA. Removing linkage drag of adjacent DNA would reduce the number of backcrosses since both the transgenic and the adjacent non-transgenic DNA would segregate as unlinked entities. In the example described above, if *x'*, *G1* and *y'* were to assort independently, an *x-G1-y* genotype would arise with a probability of 0.125 (0.5^3). In theory, an entire collection of desirable elite traits could be recovered in an individual from a single backcross.

Figure 2 depicts the series of events for a recombinase-mediated line conversion strategy. As in Figure 1a, Figure 2a shows the genomic BB' target line that also carries *G1* and *M1*. This target line is introgressed by conventional backcrosses to an elite line to establish a target line in an elite genetic background (Figure 2b). Some of the DNA adjacent to the transgenic locus may still be derived from the laboratory line, but as long as the linked undesirable traits (*x'* and *y'*) are segregated out, the remaining laboratory line DNA is inconsequential. Note also that the *M1* transformation marker can be removed by site-specific recombination to generate a selectable marker-free *G1* elite line, which could be a more consumer-friendly product.

As before (Figure 1a, b), the stacking of new transgenes is conducted using the laboratory line where genetic transformation is practical (Figure 2c, d). To convert the product shown in Figure 2d to the elite genetic background, it is crossed to the elite target line shown in Figure 2b. The progeny from this cross contains both the *G1*/elite line chromosome and its homologous *G1,G2*/lab line chromosome (Figure 2e). Should the recombinase be present, the two homologous chromosomes can recombine by site-specific recombination. For instance, the introduction of a Cre recombinase by constitutive or transient means can promote the recombination between *lox* sites. Most likely, the sequence of events will begin with the intramolecular deletion of unneeded DNA (Figure 2e, f) since closely linked recombination sites are most efficiently recombined. Intermolecular site-specific recombination should follow, resulting in a reversible but reciprocal translocation of the transgenic DNA (Figure 2f, g). This latter event breaks the linkage drag of nearby undesirable genetic entities. Without linkage drag, a much smaller progeny pool would be needed to find the recombinant with the desired set of relevant elite traits, in this example, the *x-G1-G2-y* combination. Therefore, even though the initial construction of the elite target line requires some 6 to 10 backcrosses, subsequent introduction of newly stacked transgenes should require substantially fewer generations. For instance, if each of 10 elite traits segregates without linkage drag, the cosegregation of all 10 traits would be 1 in 1024 individuals (0.5^{10}), a population size readily obtained in a single progeny generation.

Concluding remarks

The stacking strategy described above requires the use of one non-reversible site-specific recombination system, such as the C31 system, and one freely reversible system, such as the Cre-*lox*, the FLP-*FRT* or the R-*RS* system. Other systems with similar properties may also be developed for this use. The gene stacking protocol takes into consideration the issue of selectable markers in commercial products (Ow, 2000; Hohn *et al.*, 2001; Hare & Chua, 2002). Avoiding the use of

antibiotics resistance genes is possible, but alternative markers may not necessarily be free of public scrutiny either. For genes that are not relevant to the intended traits to be introduced, a prudent approach in dealing with the controversy is to just get rid of them. Hence, site-specific DNA deletion is used to eliminate as much as possible the DNA not needed for an engineered trait. Only short recombination sequences are necessarily co-introduced along with the trait genes, but most become non-recombinogenic BP' or PB' sites. Note that this DNA removal step need not be conducted independently, as it is part of the line conversion strategy (Figures 2e, f). Hence, the removal of selectable markers should not be viewed as a step that takes extra time or effort.

The conversion from a laboratory line to independent elite lines can also be conducted in parallel, provided that each independent elite line is first introduced with an appropriate target construct through the introgression process shown in Figures 2a and 2b. In this fashion, once a useful trait is engineered into a laboratory variety, a multitude of elite cultivars can rapidly be developed to host the new transgene. This should speed up the introduction of new traits in crop plants in different parts of the world, and in a much more precise and predictable fashion.
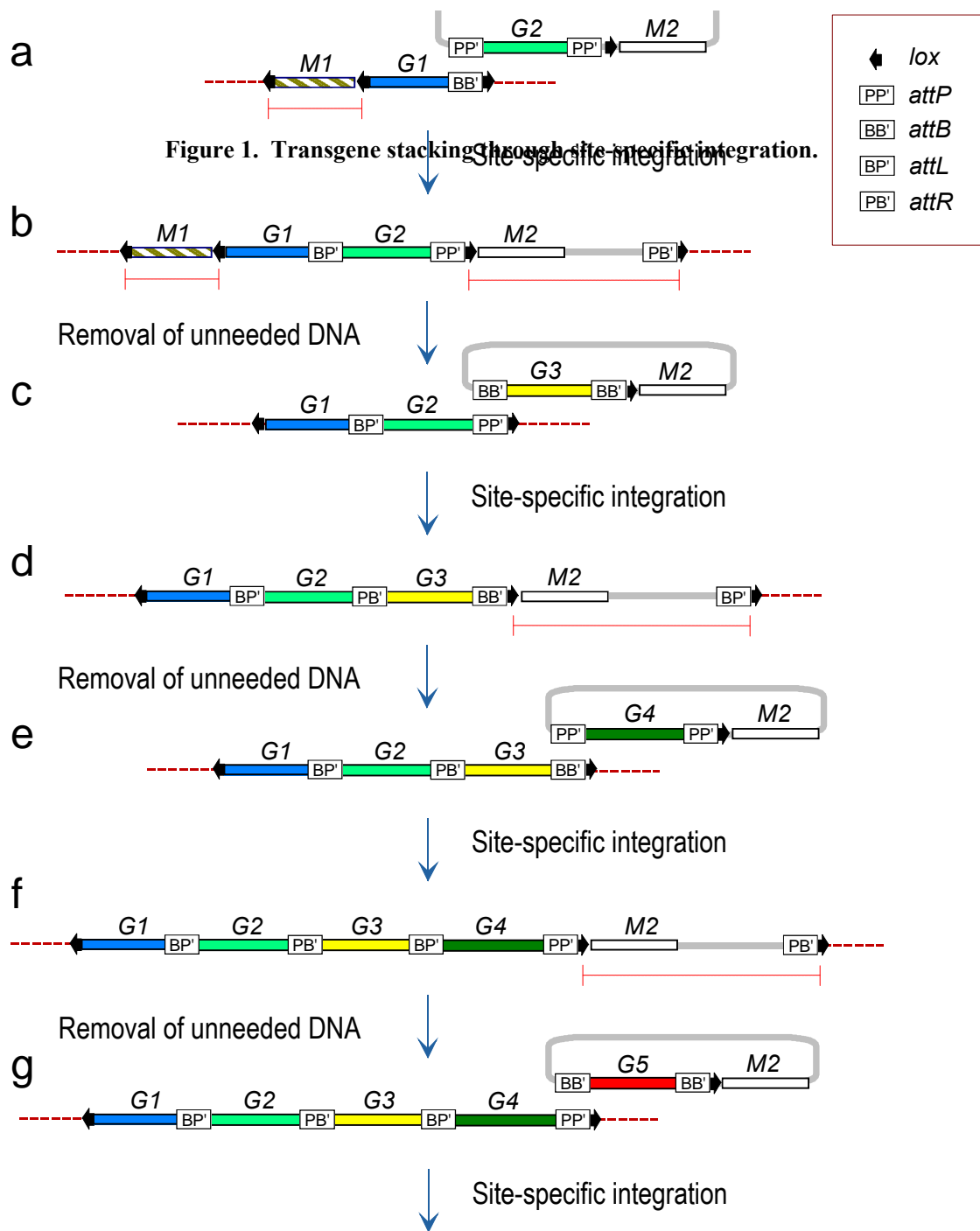
It is interesting to note that the development of new technologies for the precision engineering of plants has often been viewed as little more than attempts to address current public concerns in GMOs. No doubt this is an important consideration, but it is not the only consideration. New tools that permit greater precision in genetic manipulations will invariably improve the efficiency of introducing new traits for crop improvement. With an ever-growing wealth of genomic data, it will not be long before crop plants will be engineered with multitudes of useful traits. How these genes are integrated and introgressed into cultivated varieties can expedite or impede the growth of the transgenic era.

The immediate task ahead is to test the efficacy of the transgene stacking and translocation strategies. Providing that they be successful, suitable target lines in crop plants would need to be generated. This could be a major undertaking given the large number of different crop species where this technology may be applicable, and the large number of different cultivated varieties within a given crop. A concerted effort by interested parties would be much more preferable to independent efforts. How target sites are constructed dictates future stacking options. If engineered with common elements, they can be shared among research and commercial communities.

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Figure 1. Transgene stacking through site-specific integration.

Elimination of Marker Genes by Site-Specific DNA Recombination in Higher Plants

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We have developed a chemical-regulated, site-specific DNA excision system. In this system, a kanamycin-resistance marker gene was placed between two copies of *loxP* sequence, which can be specifically recognized and cleaved by a DNA recombinase Cre. Expression of Cre was tightly controlled by a chemical inducible prompter *LexA-46*. The latter can be specifically activated by a chimeric transcription factor XVE, whose activity, in turn, is controlled by the mammalian hormone estrogen, a chemical with no detectable non-physiological effects on plant growth and development. When this test DNA construct was introduced into the model plant *Arabidopsis thaliana* by a standard transformation protocol, application of -estradiol to the resulting transgenic plants led to the activation of XVE. The XVE transactivator then promoted a high level expression of Cre, which subsequently excised the *loxP*-sandwiched kanamycin-resistance marker gene and other "used" components of the system. Upon site-specific DNA excision and recombination, a promoter-less *GFP* (green fluorescent protein) reporter gene was brought directly downstream of a strong promoter, leading to *GFP* expression in marker-free transgenic plants. Genetic and molecular analyses indicated that the system is tightly controlled, showing high-efficiency inducible DNA excision in all tested transgenic events. An additional advantage of this system is that it is feasible to use any conventional marker genes, thus providing a convenient method to remove selectable markers from transgenic plants generated with different approaches (e.g., organogenesis or somatic embryogenesis).

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Production of Antibodies and Vaccines in Plants and Their Use for Global Health

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Antibodies and vaccines are critical tools in human and animal health care, enabling us to treat many life-threatening diseases. Until now, the engineering and large scale production of recombinant antibodies and vaccines has been time consuming and expensive, prohibiting the wide spread use of these proteins throughout medicine. Recent developments in protein engnineering and production technologies have contributed to overcome many of these problems. Using molecular farming, i.e. the production of recombinant proteins in transgenic organisms, we can use plants to synthesize antibodies and vaccines on an agricultural scale. This technology will help to bring recombinant antibody and protein therapeutics down in cost, without sacrificing their quality or safety, enabling us to broaden our concept of what they can be used for.

Different antibody variants and vaccines are all produced in an active form and they join a growing list of recombinant proteins that can be functionally expressed in plants. The highest production yields can be seen with recombinant proteins that are retained within the cell's secretory pathway, and the lowest yields are seen in the cytosol. Importantly, recombinant protein expression can be used to modify the inherent properties of plants, for example by using expressed anti-pathogen antibodies to increase disease resistance. Plant transformation is technically straightforward for model plant species and some cereals and the functional expression of recombinant proteins can be rapidly analysed using transient expression systems in intact or virally infected plants. Protein production can then be increased using plant suspension cell production in fermenters, or by the propagation of stably transformed plant lines in the field. Transgenic plants can be exploited to produce organs rich in a recombinant protein for its long-term storage.

This presentation will focus on discussing the challenges involved in engineering of antibodies and vaccines and their expression in plants, how these challenges can be overcome and efforts to produce a series of recombinant proteins in different plant species. Issue relating to safety of GMO and their impact on consumer and environemnt will be adressed- Our long term perspective is that recombinant protein production in crop plants may create an opportunity to distribute these diagnotic and therpeutic proteins beyond the developed and into the developing world.

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Science-based Approach to Assessing the Ecological Risk of Crops Derived through Modern Biotechnology

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New agricultural technologies need to be developed and prudently implemented since current practices have significant ecological and environmental impacts (Raven *et al.* 1998). Loss of the earth's basic resources to produce food through inefficient production practices has to be addressed now and into the future. The development of new tools that enable farmers to produce food, more efficiently and with less environmental impact should be priority for technology providers and public policy administrators. Recent advances in molecular biology and genetics have led to the introduction of a new generation of pest control tools for farmers that may represent a solution to these problems. The use of genetic engineering techniques to transfer traits useful in insect, disease and weed control have provided farmers with pest control solutions that are highly effective and yet very specific. In addition, some authors have noted that farmers are realizing greater flexibility in crop management practices (Schuler *et al.* 1998, James 2000). Obtaining regulatory approval is an essential part of the introduction of transgenic crops. A thorough, science-based assessment of a modified crop includes a rigorous ecological risk assessment. The process of evaluating modified crops for regulatory review also includes a detailed characterization of the product prior to conducting the risk assessment. This presentation describes an approach that has been developed over the years to assess the ecological risks associated with crops derived through biotechnology. The approach is science-based, utilizing the basic framework of ecological risk assessment as developed by the United States Environmental Protection Agency (US EPA 1998). In particular, the potential risks associated with the modified plant, such as altered weediness, and the introduced trait, for example nontarget impacts and potential effects associated with gene flow, are evaluated in a systematic manner. Key questions concerning potential hazards and exposures are addressed and, where necessary, a tiered experimental approach is used to characterize risk. This presentation will give an overview of the ecological risk assessment model along with some specific examples of data from products within Monsanto's plant biotechnology portfolio.

Crop-to-Crop Gene Flow: Dispersal of Transgenes in Maize, During Field Tests and Commercialization

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Abstract

Gene flow in maize (*Zea mays* L. ssp. *mays*), among genotypes with varying levels of hybridization and stages of evolution, including the wild relative teosinte (*Zea mays* ssp. *mexicana* (Schader) Iltis), is not new. Considerable research exists that evaluates the impact of improved, conventional maize cultivars on traditional landraces and teosinte in Mexico. Considerable research also exists concerning the ability of plant breeders and seedsmen to prevent the undesired transfer of genes from unimproved varieties or wild relatives to elite germplasm via stringent pollen control techniques. Recently, the results of these investigations have received renewed interest due to the possibility that transgenes may somehow affect the landraces and wild relatives. The purpose of this presentation is to provide a review of the literature on gene flow and pollen control in maize. Included will be a discussion of our research on maize pollen biology, flowering dynamics, and evaluation of several practical techniques for controlling pollen and therefore gene flow on a research scale. Results to date are consistent with observations that maize pollen is desiccation intolerant and loses water and viability due to desiccation rapidly after dehiscence as is found in *Gramineae* generally. Teosinte pollen generally desiccated more rapidly than maize pollen although the duration of shedding was typically longer due to the existence of multiple staminate inflorescences per plant. Stigma or 'silk' elongation in landraces and improved maize varieties was rapid and growth continued for approximately 10 days after initial emergence. Crossing occurred among improved cultivars and among improved cultivars and landraces equally in either direction. However, crossing of maize with teosinte typically involved teosinte plants fertilizing maize plants. The evaluation of two research scale methods of pollen control indicated successful pollen control could be obtained. Prior literature regarding maize pollen and silk biology and the use of spatial isolation to control maize hybridization was consistent with our results. Our research demonstrates and documents that effective tools for managing research scale pollen flow exist and that these results are consistent with the floral biology of the crop. The only way to determine impact of transgenic gene flow is to be able to conduct further research. The extent to which precautions need to be applied to the pollen flow depends ultimately on the implications of the flow of novel genes. If the consequences of novel gene flow are biologically significant, more precaution will need to be exercised than if experiments demonstrate no significant biological impact of the novel genes beyond that of traditional breeding activities.

Key words: maize, *Zea mays*, transgenes, pollen, teosinte, landraces

Likelihood of Gene Flow in Maize

Gene flow in maize (*Zea mays* L.) is closely associated with the biology of the staminate and pistillate inflorescences. Maize is a cross - wind pollinated specie, with pollen produced in copious quantities. A hybrid tassel of normal size can produce up to 25 million pollen grains (Kiesselbach, 1999). Dispersal of maize pollen is determined by a diversity of environmental and physical factors. Wind direction, turbulence and velocity are directly linked to pollen movement (Jones and Brooks, 1950; Di-Giovanni and Kevan, 1991; Di-Giovanni et al., 1995). Likewise, other factors such as pollen density, air density and viscosity, pollen sedimentation velocity, and pollen radius seem to influence pollen transport and deposition (Di-Giovanni et al., 1995). Once in the atmosphere, pollen grains have to maintain pollen viability long enough to be able to complete the pollination process (Luna et al., 2001).

Maize pollen viability is directly influenced by water content. At the time of dehiscence, maize pollen contains a high percentage of water, approximately 60% (Kerhoas et al., 1987). However, pollen grains lose water as soon as they are released from the anthers into the atmosphere and this water loss affects the longevity of viability. In a two-year field study, we demonstrated that hybrid maize pollen lost 100% of viability after two hours of atmospheric exposure (Luna et al., 2001). On average, 80% of the pollen was not viable after one hour, with a range of 96% under conditions of high temperature and low relative humidity to 58% under the more favorable conditions of high relative humidity and lower temperatures. In our experience, the combination of relative humidity and temperature is crucial to the duration of pollen viability. As temperature increases and relative humidity decreases, pollen grains have to face more arid atmospheric conditions decreasing the likelihood of cross-pollination (Baltazar and Schoper, 2002). Teosinte pollen generally desiccated more rapidly than maize pollen although the duration of shedding was typically longer due to the existence of multiple staminate inflorescences per plant.

The silks typically emerge from the husk sheath one to three days after initial pollen shed. An ear of hybrid maize can produce up to an average of 1000 silks (Kiesselbach, 1999). In the absence of fertilization and under normal, well-watered conditions maize silk elongation continues for approximately 7 days before they begin to senesce (Bassetti and Westgate, 1993a; Bassetti and Westgate, 1993b). Similar silk elongation patterns were observed in our experiments conducted in Mexico with local landraces, temperate hybrids and teosintes. Our observations indicated, silks from landraces tended to stop elongation after 10 days when compared to hybrids that kept growing although at slower rates. Silk elongation varied among landraces, hybrids and teosintes with Bolita having the most rapidly elongating silks. Typically, silks provide the pollen grain with moisture and other nutrients, which causes them to germinate. Growth of the pollen tube is usually visible within 30 minutes of the pollen grain landing on a receptive silk and fertilization normally occurs within approximately 24 hours (Kiesselbach, 1999).

During the process of product development plant breeders have improved the synchronization between pollen shed and silking in breeding material and hybrids at the individual plant and on a hybrid population basis (Burris, 2002). However, this is not so true for open heterogeneous landraces and open pollinated varieties, particularly if they are grown under stress conditions. When well watered, silks continue elongation and presumably are receptive for up to 10 days. If the initiation or duration of pollen shedding is insufficient to pollinate all emerging silks, the unpollinated silks have a higher probability of being pollinated by pollen from other sources. Under drought conditions silking can be significantly delayed compared to pollen shedding thus preventing synchronization and pollination (Hall et al., 1981; Hall et al., 1982).

Variability in the amount of pollen that is shed exists. Over the years, the amount of pollen that hybrids shed has decreased (Duvick and Cassman, 1999). As synchronicity of the flowering and drought tolerance have improved, less pollen is needed for optimal yields. As a result, most open pollinated varieties and landraces have larger and denser tassels when compared to modern hybrids as indicated by the total number of spiklets per tassel. A teosinte plant with at least 15 tassels distributed along the stems is also a large pollen producer compare to a hybrid plant, with a large number of spiklets, 3921 per plant as compared to 769 for a modern maize hybrid. This is an important characteristic when hybrids, landraces and teosinte plants are grown at the same time and in the same vicinity. Producing more pollen grains over a longer time period will increase the probability of outcrossing. Considering that landraces, open pollinated varieties, and teosinte produce more pollen over a longer timeframe suggests that gene flow would move in the direction of the modern, commercial hybrid. However, this tendency would be at least partially off-set by the

generally larger scale and higher density of commercial scale plantings. Additionally, the increased inclination of landraces and open pollinated varieties to have asynchronous flowering would also tend to increase the probability of gene flow from commercial hybrids to these varieties.

Gene flow involving teosinte

Gene flow between hybrid maize and its closest relative, teosinte (*Zea mays* ssp. *mexicana*) is only possible in Mexico and Central America. In the Central Plateau and Valley of Mexico, maize (*Zea mays* ssp. *mays*) can grow sympatrically with teosinte (*Zea mays* ssp. *mexicana*) providing the opportunity for hybridization (Wilkes, 1967; Sanchez et al., 1998; Blancas, 2001). The genetic exchange between maize and teosinte is dependant on (1) the spatial isolation of the two species, (2) the seasonal isolation of the two species and (3) the fitness of the hybrids combined with the types of selection operating in the teosinte populations.

Wilkes, 1967 and Sanchez et al., 1998, made a thorough review of teosinte distribution and characterization in Mexico. They describe dates of planting across the Mexican Republic, probability of outcrossing with maize due to synchronization and other parameters important for gene flow of maize with teosinte. Typically, the growing season for teosinte in Mexico is June through November. Seeds germinate with the beginning of the summer rains and growth parallels, but is later than the local cultivated maize. Flowering then occurs in September-October and the seeds mature in November. As a result, teosinte and maize can be thought of as seasonally isolated at most of the sites where they occur together, however, the isolation is not complete (Wilkes, 1967). As a result, the earliest flowering teosinte plants tend to overlap the end of the flowering period for maize. The presence of hybrids under natural conditions in Central Plateau and Valley of Mexico is an unequivocal indication of the cross compatibility between at least *Zea mays* ssp. *mexicana* and *Zea mays* ssp. *mays* (Doebley, 1990; Kato, 1997; Blancas, 2001). Based on our experience (Baltazar and Schoper, 2001) and physiological incompatibility reports (Evans and Kermicle, 2001) we hypothesize that most probably hybrids between teosinte and maize are formed by early shedding teosinte plants fertilizing late silking maize plants.

It is essential to highlight that Wilkes's (1997), best estimates of current distribution of teosinte is about half of what it was in 1900, as evidenced by herbarium specimens and written accounts. He attributes the disappearance of remaining populations mainly to road construction and intensified land use and to a lesser extent to biological factors such as the cultivation of other cash crops such as sorghum that make the presence of teosinte as a weed more obvious (Wilkes, 1997). Further, he concluded that the single most decisive fact for the survival of teosinte in a region is the widespread planting of maize over most of the land. Other forms of land use are far more devastating to the continued presence of teosinte.

Gene flow involving landraces

Gene flow between improved hybrids and open pollinated varieties to local landraces occurs largely in Mexico and Central America and less intensively in other parts of the world, e.g. South America. A widely held misconception about maize landraces is that what we find in the remote areas of Mexico today is essentially the same as the maize found in the same location 100 years ago. It is not (CIMMYT, 2002). Research has indicated that the present diversity in maize is the result of relatively controlled introductions of genetic material and not of geographic isolation (Bellon and Brush, 1994; Louette et al., 1997; Louette and Smale, 1998). As a result, landraces themselves are not static but are constantly evolving, while maintaining the traits desired by the farmers.

There are a number of ways gene flow may occur under farmer-field conditions. Current evidence suggests that many small-scale subsistence-oriented farmers have planted improved varieties alongside their local variety (Bellon and Brush, 1994). By design and by accident, these farmers have promoted hybridization between the varieties. The breeding process, through which materials that result from commercial and governmental breeding programs are crossed with local landraces, is termed "creolization" or rustication. In general, creolized varieties (variedades acriolladas or criollos) are appreciated because they combine desirable traits of improved varieties with those of landraces. The criollos are perceived as requiring less intensive management and are therefore more useful to the input constrained, resource poor farmers.

A second scenario whereby gene flow may occur is when neighbors of individual farmers plant their maize varieties within outcrossing distance of an individual's field (Bellon and Brush, 1994;

Louette et al., 1997). It is common in Central and Southern Mexico to find mostly subsistence farmers. They own no more than one hectare and in one hectare they plant at least four different landraces or improved landraces of maize that derive from local landraces or from neighbor's seed lots. Similarly, surrounding farmers plant not one, but a combination of local landraces that meet the farmer's needs. Farmers will use both distance and temporal isolation as it is available, but this is not always possible. Due to the inability to completely control pollination in all fields, farmers will have promoted gene flow and the creation of new open pollinated varieties.

A third situation is the farmer's interest in promoting gene flow between improved open pollinated varieties and hybrids into their local landraces. Mixing the genetics between different maize populations is the objective of a practice in which farmers mix seeds from two different varieties with the express purpose of improving one of them (Aguirre-Gomez et al., 2000). The creolized varieties are thought to maintain the advantages of the improved varieties, but retain fewer disadvantages. This introduction of new germplasm can be viewed more as a source of morphological and agronomic diversity than a cause of genetic erosion (Brush, 1995; Louette et al., 1997; Louette and Smale, 1998). Creolized varieties are commonly grown throughout Mexico. Hybridization between local and improved maize is also highly valued throughout Central America (Almekinders et al., 1994).

Gene flow between improved open pollinated varieties and hybrids and landraces therefore regularly occurs in Mexico and Central America (Bellon and Risopoulos, 2001). In general, maize breeding relies on the existence of genetic variability. Farmers have intentionally managed hybridization and gene flow in their empirical on-farm breeding for a long time trying to generate and maintain genetic variability. This practice is utilized when farmers detect a valuable trait in an improved open pollinated variety or hybrid in an attempt to have it in their local landraces. There are examples where farmers, through selection, have eliminated undesirable genes from a population. Most genes in maize are independent and considered not to be linked. This means that they will segregate independently of each other and farmers can thereby select for only the trait of interest. This approach to controlled introgression can be thought of as increasing diversity rather than causing any decrease in genetic diversity. In contrast to volunteer or weedy plants, in the long term, farmers manage the genetics of the landraces.

Gene flow during breeding, testing and commercialization

Gene flow can occur at different levels during the product development and characterization process. These levels include breeding material, parent seed production, commercial seed production or commercial fields. The scale of seed production ranges from very small scale, e.g. a small fraction of a hectare for research, to very large scale, e.g. millions of hectares for commercial release. All biological principles of pollen and silk apply to gene flow at any stage of testing. The expected result is minimizing flow of unwanted genes regardless of source, whether genes are from genetically enhanced maize, or derived from conventional plant breeding.

The field experiment level within research is the first opportunity for gene flow to occur. Field studies in Mexico have shown that properly managed detasseling of an inbred carrying a specific gene can provide control of cross pollination and therefore gene flow in small scale plantings (Garcia et al., 1998). The two mating designs that were tested were isolated crossing blocks and triplets. These mating designs represent two of the most common breeding methodologies used in small-scale seed production. The results from these experiments indicated we could remove all risk of outcrossing by detasseling. The plots were small enough that adequate care could be provided to eliminate all of the tassels. Additionally, even when plants were allowed to open pollinate we observed only 0.01% outcrossing to adjacent rows or 1 in 10,000 kernels. This level was quite low, likely the result of the size of plantings and calm, arid environmental conditions. Both of these factors would tend to limit pollen movement and viability.

A second set of experiments designed to investigate distance isolation as a means of controlling gene flow were also conducted at the same site as the other experiments in Mexico (Luna et al., 2001). Our results showed that cross pollinations occurred at a maximum distance of 200 m from the source planting and that very limited cross-pollinations occurred at 100 m. No cross-pollinations were observed at 300 m. The relatively high settling rate of maize pollen combined with low wind speeds experienced in our location apparently led to relatively short dispersal distance. The atmosphere was also very arid so, to the extent pollen moved laterally in the atmosphere, it would have been desiccated quickly. Our results indicated that distance isolation

could be a useful tool for controlling gene flow via pollination in research scale plantings in environments similar to these tested. Our results were consistent with previous reports where pollen flow was less than 200 m in a location with low wind speed and arid climates (Cervantes, 1998).

Controlling gene flow at the parent and commercial seed production levels is critical to provide farmers with the levels of purity required by international trade agencies and is a useful reference for researchers (Bateman, 1947; Raynor et al., 1972; Jemison and Vayda, 2001). In the USA, state seed certifying agencies are responsible for official standards for certified seed. The standards, which vary somewhat by state, generally set minimum distances for isolation. These distances are modified by 1) additional border rows, 2) size of the field and production block, 3) adequate natural barriers (in some states), and 4) differential flowering dates (in some states).

When either zero or one-border rows are used, minimum distances from 125-200 mts are typically required between the female parent of the hybrid being produced and any other corn of the same seed color, maturity, or endosperm type. If the corn has different kernel color or endosperm type, an isolation distance of 200 mts. is required. Embedded in these recommendations is the recognition that some adventitious presence is inevitable in large-scale plantings. As a result, the OECD established purity levels for parent seed at 99.0-99.5% and for hybrid maize seed at 98-99%. These widely accepted international standards for certification of varietal purity could also be useful when trying to establish thresholds for purity of non-transgenic maize.

It is important to realize, however, that these same isolation standards may result in much higher purity in commercial grain production fields where hybrid seed is planted. A field of a typical modern maize hybrid produces a pollen load that is many times, perhaps 10X to 100X, greater than that of a single-cross hybrid seed production field. In addition, the timing of pollen shed in a hybrid field is usually synchronized with silk emergence. At times this is not the situation in a seed production field. The large pollen load and synchronous timing of pollen and silks in a hybrid field serve to greatly limit the impact of pollen from outside sources.

The use of transgenes typically involves a gene that is used in a heterozygous or hemizygous dominant manner. As a result, only half of the pollen produced in a commercial planting is transgenic because only one parent contains the transgene. This reduces the chance of outcrossing from the transgenic field to another field by one half of what would otherwise be expected if all of the pollen carried the transgene.

The use of border rows is another technique widely used to minimize the probability of transgenic pollen drift into a non-transgenic field. The minimum number of border rows that can be substituted for distance in the isolation of a non-transgenic field from a transgenic field varies with the size of the source planting and the desired distance of isolation.

Segregation of grain at harvest also can improve purity. If non-transgenic and transgenic varieties are grown in plots next to each other, harvesting a number of rows (12-16) from the side of the non-transgenic plot that is nearest the transgenic field, and then separating this grain from the rest of the non-transgenic grain can help the grower achieve a much higher purity level in the grain harvested from the remainder of the non-transgenic field.

In many locations, one can also use temporal isolation. If the plantings are not sympatric, no gene flow can occur. This approach is not so convenient in locations that have a winter season but it is an important isolation tool in geographies where plantings can have significant temporal spread.

Finally, all of these methods can be used in various combinations to help ensure gene flow is managed. Collectively they offer considerable flexibility for controlling gene flow in small-scale research and achieving minimum purity standards for larger scale plantings were adventious presence is expected.

Conclusions

The principal objective of this presentation was to review relevant literature on gene flow and our research on the subject in Mexico. Considerable research exists that evaluates the impact of improved, conventional maize cultivars on traditional landraces and teosinte. Considerable research also exists concerning the ability of plant breeders and seedsmen to prevent the undesired transfer of genes from unimproved varieties or wild relatives to elite germplasm via stringent pollen control techniques. Neither area of research directly addresses the impact of novel genes on landraces or teosinte or documents the ability of researchers to control gene flow from their elite material to landraces or teosinte. By reviewing the underlying biology of the staminate and pistillate inflorescences, prior research on related areas of research and our own research we provide insight

into how to best approach allowing research to be resumed on the possible impacts of transgenes on the Center of Origin of maize.

The underlying floral biology and pollen control evaluations were consistent with each other and the literature. Our research location is very arid at the time flowering occurred. As a result maize pollen, which is very desiccation sensitive, desiccated rapidly with consequent loss of viability. The location is also relatively calm and maize pollen has a high sedimentation velocity. Together, these factors resulted in little outcrossing and no outcrossing beyond 200m. Detasseling of research scale plantings where one could be assured of complete manual detasseling also proved to be effective at preventing outcrossing. Rows adjacent to shedding males in typical mating designs experienced a relatively low outcross percentage thus reinforcing our observations of relatively little pollen movement. Collectively these results indicated pollen and therefore gene flow could be controlled in research scale plantings at this location.

Studies involving teosinte, landraces and improved hybrids documented likely pathways of gene flow. The frequency of gene flow from teosinte to maize was considerably higher than the frequency of gene flow from maize to teosinte. This result suggests that some level of backcrossing would be required for introgression to occur in teosinte. Similarly, this may help to explain the observation made in the literature about the ability of teosinte to maintain its genetic integrity in spite of substantially larger populations of maize being grown. This disparity was likely due to large differences in pollen/stigma sizes and/or of genetic barriers to crossing. Landraces and improved hybrids were found to cross more freely although the literature documents some level of genetic barriers to crossing in some landraces.

We feel it is important to be able to resume research involving transgenes in Mexico (Serratos et al., 1997; Alvarez-Morales, 2000), now that transgenes have been detected in some geographies in Mexico. An improved understanding of the how to best manage the current situation and how new technologies are managed in the future is needed. The extent to which precautions need to be applied to pollen control in Mexico ultimately depends on the implications of the flow of novel genes (Crawley et al., 2001). If the consequences of novel gene flow are biologically significant, more precaution will need to be exercised than if experiments demonstrate no significant biological impact of the novel genes beyond that of traditional breeding activities.

Acknowledgments

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Gene flow from crops to wild relatives in Asia: case studies and general expectations

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Abstract Gene flow is the major pathway for transgene escape from crops to their wild relatives (including weedy biotypes). Alien transgenes that escape to and persist in the environment will probably lead to ecological risks. Those alien genes resistant to biotic and abiotic stresses in particular could significantly enhance ecological fitness of the wild relative species, causing unpredictable environmental disasters. There are several major crop species such as rice, soybean, oilseed rape, bread wheat, and millets grown in Asian countries. These crops have their wild relative species and weedy types available in the agriculture ecosystem. If transgenic varieties of these crops are released into environment, alien transgene escape to wild relatives through outcrossing will likely occur. In the origin and diversity centers of crop species and wild relatives, the possibility of transgene escape to the wild species will be high, and as a consequence, the ecological risks caused by the transgene escape will also be high. It is general understanding that the possible crop-to-wild transgene escape must meet three conditions, these are (i) spatially, transgenic crops and their wild relatives should have an overlapped distribution and be in close contact; (ii) temporally, the flowering time of transgenic crops and their wild relatives should encounter with each other; and (iii) transgenic crop and the target wild relative species should have sufficiently close biological relationships and non-significant reproductive barriers. This paper presents studies of crop-to-wild gene flow in Asia using rice as an example, and discusses the general expectations of transgenic escape. Our research results on geographic distribution, flowering habit, interspecific hybridization, and gene flow of cultivated rice (*Oryza sativa*) and its closely related wild relatives, are used to estimate the opportunity of transgene escape to the wild relatives.

Key words Environment safety, gene flow, *Oryza* species, distribution, interspecific hybridization, molecular marker

Introduction

Since the birth of the first successful transgenic plant in the beginning of 1980's, tremendous accomplishments associated with transgenic biotechnology have been achieved and rapid application of the biotechnology in agriculture has substantially benefited crop genetic improvements. As a consequence, a great number of genetically modified crops (GMC) have been released into the environment or have entered commercial markets (Barber 1999; Fernadez-Cornejo and McBride 2000; Liu and Zhu 2001; Huang et al. 2002). Undoubtedly, the so-called "gene revolution" and transgenic biotechnology offer many more new possibilities for the global food security. However, the transgenic biotechnology and its products have also brought serious problems of biosafety (Liu and Zhu 2001). The biosafety issues raised in relation to transgenic biotechnology and its products have been most extensively debated worldwide in recent years (Bergelson et al. 1998; Crawley et al. 2001; Ellstrand 2001; Prakash 2001; Schiermeier 2001). Biosafety *per se* has become the "bottle-neck" to further development of transgenic biotechnology and wider application of transgenic products.

Will transgenic products pose a safety problem on environment? Can alien transgene escape occur through outcrossing and will the alien genes persist in the environment? Will transgene escape cause significant ecological risks? All these questions relating to the biosafety of transgenic crops need to be addressed scientifically. Effective strategies to minimize transgene escape and its ecological risks can only be made when a better understanding of the pathways and consequences of transgene escape is achieved, which will in turn lead to a more safe and efficient use of transgenic crops. The present paper will discuss whether or not the release of genetically modified crops into the environment in Asia will result in transgene escape and consequently lead to ecological risks, and what is the general expectation of crop-to-wild gene flow.

1 Gene flow from crops to wild relatives and its ecological impacts

Asia is the largest continent in terms of population and more than 55% of the world's population lives there. The continent is also an important origin and diversification center for some major crops, such as rice, soybean, wheat, vegetables, and fruit trees. During the co-evolutionary process, the cultivated species and their wild relatives are in close contact, and gene flow between them is relatively common. Some of the wild relatives have a large distribution area and more significant contact with their crop species at many locations, whereas the others have a relatively small or fragmental distribution and much less contact with their cultivated species at only a few locations. In addition, the breeding systems of different crop species and their wild relatives vary significantly. A great variation in gene flow between different crops and their wild relatives is observed; therefore, the risks of transgene escape from different crop species to their wild relatives should also be different. Table 1 lists some of the major crops and their wild relatives in Asia with their predicted opportunities gene flow.

Crop species often have their weedy biotypes in addition to the wild relative species, particularly for those crop species, of which their wild relatives share the common genomes and have the same ploidy level. For example, the cultivated rice (*O. sativa*) has its weedy type *O. spontanea*, and cultivated soybean (*Glycine max*) has the weedy type *G. gracilis*. Normally, the weedy biotypes of cultivated species occur simultaneously in the same field with crops and have very close genetic relationships with the crops, so gene flow from crop to weedy type is relatively frequent. It is considered that gene flow among crops and their weedy and wild relative species keeps the evolutionary process of these species continuing. It is very difficult to generalize gene flow from different crops to their wild relatives, therefore, we shall take cultivated rice and its wild relative species as an example to investigate the crop-to-wild gene flow.

When alien transgenes escape to and express normally in wild relatives and weedy species of rice, the transgenes will persist and disseminate within the wild or weedy populations through sexual reproduction and/or vegetative propagation. If the transgenes are encoding for traits, such as high protein content, special vitamins, and better grain quality, which are not associated with the ecological fitness of the wild or weedy species and not related by natural selection to the survival of wild plant species, the ecological risks caused by escape of these genes will be minimum. However, if the transgenes are responsible for resistance to biotic and abiotic stresses (such as drought and salt tolerance, and herbicide resistance), and the genes significantly enhance ecological fitness of wild and weedy species, escape of these genes will probably cause ecological problems. If several of such fitness enhancing genes are stacked in the same individual wild or weedy species, the ecological consequences might become more significant through formation of aggressive weeds that can escape human control and cause unpredictable damage to local ecosystems. On the other hand, when transgenes escape to populations of wild relatives through outcrossing, the persistence and rapid spread of the resulting hybrids and their transgene carrying progeny will lead to contamination of the original populations of the wild relatives, and even to the extinction of endangered populations of the wild relatives in local ecosystems (Kiang et al. 1979; Ellstrand and Elam 1993). The escape and persistence of transgenes in environment will make effective *in situ* conservation of wild genetic resources more difficult. In addition, perennial hybrids of cultivated species and their wild relatives carrying transgenes may serve as a bridge to spread their transgenes through outcrossing to other wild related species causing even more significant ecological risks.

2 Transgenic rice and the possible transgene escape

Rice is one of the most important world's cereal crops, providing staple food for nearly one half of the global population (Lu 1998). More than 90% of rice is grown and consumed in Asia (Lu 1996), reflecting the importance of rice in Asian people's daily life. Rice is one of the earliest of the world's crop species to which transgenic biotechnology has been effectively used for genetic improvement (Ajisaka et al. 1993; Yahiro et al. 1993). Although no transgenic rice varieties have yet been officially approved for extensive commercial cultivation anywhere in the world, genes conferring traits such as high protein content, disease and insect resistance, virus resistance, herbicide resistance, and salt tolerance, have been successfully transferred into different rice varieties through transgenic techniques (Ajisaka et al. 1993; Yahiro et al. 1993; Matsuda 1998; Messeguer et al. 2001). Transgenic biotechnology has rapidly developed and been extensively applied in rice breeding in Asian countries, particularly in China. To date, transgenic rice varieties resistant to three major diseases and insects, namely, rice stem borers (using *Bt* and *CpTI* genes), rice

hopper, and rice bacterial blight (using the *Xa21* gene isolated from an African wild rice, *Oryza longistaminata*), have been developed and released into the environment for testing. In addition, some herbicide resistant and salt tolerant transgenic rice varieties have also been produced (Tu et al. 2000; Huang et al. 2002). We are confident that, as an important world cereal crop, transgenic rice varieties will be released into environment for commercial production in the near future.

Crop-to-wild transgene escape refers to a gene or a group of genes introduced to a rice variety by genetic engineering moving to wild relative species (including weedy rice) through gene flow. Cross-pollination between transgenic and wild relatives is the major pathway for transgene escape, which may ultimately cause possible ecological risks. Normally, for transgene escape to happen the following requirements need to be met: (i) spatially, transgenic rice and its wild rice relatives should be sympatrically distributed, i.e. grow in the same vicinity; (ii) temporally, the flowering time (including flowering duration within a year and flowering time within a day) of transgenic rice and its wild relatives should overlap; and (iii) biologically, transgenic rice and its wild relative species should have a sufficiently close relationship, also the resulting interspecific hybrids should be able to reproduce normally. It is therefore necessary to know geographic distribution patterns and flowering habits of cultivated and wild rices, and to understand genetic relationships and actual gene flow frequencies between the cultivated and wild rice species. This will facilitate the effective prediction of transgene escape and its potential ecological risks, and the development of strategies to minimize the escape of alien transgenes.

3 The close wild relatives of rice in Asia

Cultivated rice is classified in the genus *Oryza* L. of the tribe *Oryzeae* in the grass family (Poaceae). The genus *Oryza* includes two cultivated species and over 20 wild species widely distributed in the pan-tropics and subtropics (Lu 1996). The Asian cultivated rice *O. sativa* had its origin in South and Southeast Asia, and is grown worldwide in the tropics, subtropics and some temperate regions, whereas the African cultivated rice *O. glaberrima* was domesticated in western Africa and is now cultivated only in local agricultural ecosystems in West Africa (Lu 1996). The cultivated rice that we discuss here in this paper is only referred to as *O. sativa*.

Species in the genus *Oryza* included ten different genome types, i.e. the AA, BB, CC, BBCC, CCDD, EE, FF, GG, JJHH, and JJKK genomes (Vaughan 1994; Ge et al. 1999; Lu 1999). Species containing different genomes have significant reproductive barriers. Therefore, genetically they are distantly related and spontaneous hybridization between species with different genomes is extremely rare. Asian cultivated rice contains the AA genome and is relatively easy to cross with its close relative species (including weedy rice) that also contain the AA genome. Theoretically speaking, transgene escape from transgenic rice varieties will only occur to species with the AA genome. Therefore, this paper only concerns the AA genome *Oryza* species.

There are eight diploid (2*n*=2*x*=24) *Oryza* species containing the AA genome. Apart from the two cultivated rice species, the perennial common wild rice *O. rufipogon* and annual common wild rice *O. nivara* from Asia, the perennial *O. longistaminata* and annual *O. barthii* from Africa, the perennial *O. glumaepatula* from Latin America, and the annual *O. meridionalis* from northern Australia and New Guinea are all comprised of the AA genome (Lu 1996, 1998; Lu and Silitonga 1999). Weedy rice occurring in Asia usually has its origin from hybridization between cultivated and wild rice species or degenerated individuals of cultivated rice. It is mostly found in the rice field alongside cultivated rice, but also occurs in the vicinity of rice fields, in ditches, or in sympatric regions of cultivated and wild rices (Vaughan 1994). It is evident in Asia that these wild relatives of rice, i.e. *O. rufipogon*, *O. nivara*, and *O. spontanea* (weedy rice) will be the target species for crop-to-wild transgene escape through gene flow. Our study clearly demonstrates that *O. rufipogon*, *O. nivara*, and *O. spontanea* are distributed across a significantly wide geographic region, and cultivated rice is grown sympatrically with these wild rice species in many areas of South and Southeast Asia (Lu et al. 2002). It is concluded from the geographic distribution data that spatially transgenes from cultivated rice have a great potential to escape to its wild relative species through gene flow.

4 Flowering habits of cultivated rice and its wild relatives

Flowering habits of cultivated rice grown in different parts of the world vary considerably depending on local cultivation time and seasons, and differences between varietal types (such as photoperiod and thermal sensitivity). The flowering and pollinating time of different wild rice

species or different populations of the same species also varies significantly across different geographic regions. In general, the flowering habit of wild rice species is characterized by a protracted flowering period. In other words, different individuals within the same population, and different tillers and spikelets of the same individuals, will flower at a considerably different time. For example, *O. rufipogon* usually starts its flowering at the beginning of September and terminates its flowering in December or towards the end of January in the next year; O. nivara has a relatively earlier and shorter flowering period from the middle of August to the end of October; and the weedy rice mimics the flowering time of the cultivated rice in the same field, but usually one to two week earlier. Obviously, the comparison of flowering habits becomes meaningful only when specific wild rice species and cultivated rice varieties from the same location are selected for flowering studies under the same conditions. Data from the selected *O. rufipogon* population, and two cultivated rice varieties (a late-maturing local variety and an improved variety, Minghui-63) at a field site in Chaling of Hunan Province, China, showed that both the flowering period in a year and flowering time in a day had considerable overlap for *O. rufipogon* and the two rice varieties (Table 2). Our additional experimental data further demonstrated that pollen grains of *O. rufipogon* and a cultivated rice variety (Minghui-63) could be actively alive in the air for more than 60 minutes (Song et al. 2001). In summary, it is essentially possible that cross-pollination between *O. rufipogon* and cultivated rice will occur, if the two species have sympatric distribution and are grown near to each other.

5 Biosystematic relationships of rice and its close relatives

Biosystematic relationships of the AA genome *Oryza* species can be estimated from the following aspects: (i) crossability between cultivated rice and its wild relatives; (ii) meiotic chromosome pairing in the F_1 interspecific hybrids; and (iii) fertility of the F_1 hybrids. If the cultivated rice has relatively high crossability with its wild relatives, normal meiosis formation in the F_1 hybrids, where chromosomes from different parental species will pair and genetic recombination will take place, observed in F_1 hybrids, and if the F_1 hybrids have comparatively high fertility, the transgenes will easily escape to wild relative species through cross pollination and persist in environment. The transgenes will also spread out through reproductive procedures or through vegetative propagation if the hybrids and their progeny are perennial.

Research on crossability between cultivated and wild rice species has extensively been reported (Nezu et al. 1960; Pental and Barnes 1985; Langevin et al. 1990). We also conducted interspecific experiments between eight AA genome *Oryza* species under greenhouse conditions (Naredo et al. 1997, 1998). The results from interspecific hybridization show that most of the AA genome wild rice species have relatively high compatibility with the cultivated rice, although with comparatively large variations among species. Only the Australian *O. meridionalis* had low crossability with cultivated rice (<5%). *Oryza rufipogon* had 6-10% and *O. nivara* had higher than 10% of crossability with cultivated rice.

The chromosome pairing ability at metaphase-I in meiosis of the F_1 hybrids reflects the genetic relationships of the parental species. The high frequency of meiotic pairing indicates a close genetic relationship between their two parents. In this case, exchange of genetic materials between the parents will occur during meiosis through genetic recombination. Extensive studies showed that very high frequency of meiotic pairing was found in F_1 hybrids between cultivated rice and its AA genome wild relatives (Nezu et al. 1960; Pental and Barnes 1985; Majumder 1997). Our cytogenetic studies also indicated that nearly all chromosomes from parental species formed 12 ring bivalents in meiosis of the hybrids with the AA genome (Lu et al. 1997, 1998).

Spikelet fertility of the F_1 hybrids indicates whether the hybrids will continue to survive through sexual reproduction. A great variation in spikelet fertility of F_1 hybrids between cultivated rice and its AA genome wild relatives was reported (Nezu et al. 1960; Langevin et al. 1990). Results from our spikelet fertility investigation in F_1 hybrids between cultivated and wild AA genome rice species show a similar rate of panicle fertility (Naredo et al. 1997, 1998; Lu et al. 2000). Our data indicated that spikelet fertility of the F_1 hybrids from all available combinations was relatively high under bagged self-pollination conditions. Hybrids of the cultivated rice with *O. rufipogon* and *O. nivara* produced highly fertile spikelet with fertility over 11%.

6. Gene flow between cultivated rice and *O. rufipogon*

It is evident that cultivated rice and *O. rufipogon* have sympatric distribution and overlap in

flowering in many Asian countries and regions, and genetically the two species have close relationships and low reproductive isolation. As a consequence, introgression between the two species occurs frequently in nature (Oka and Morishima 1969; Chu and Oka 1970; Langevin et al. 1990). Figure 1 shows the outcome of natural interspecific hybridization between *O. rufipogon* and *O. sativa* in Nepal. Although crossability between the two species obtained under artificial hybridization is considerably high, our knowledge on gene flow between the two species is limited. Information on gene flow frequency between the two species becomes essential for the assessment of transgene escape from transgenic rice varieties to wild relatives. In order to obtain actual data for the maximum gene flow frequency between cultivated rice and *O. rufipogon* under natural conditions, we designed an experiment where gene flow between a cultivated rice variety, Minghui-63 and *O. rufipogon* were examined under controlled conditions. Four different experimental designs were made with 12 treatments for gene flow detection, in which the cultivated Minghui-63 was planted to encompass the wild *O. rufipogon*, or be surrounded by the wild rice species, Minghui-63 and *O. rufipogon* were planted alternatively in rows, and *O. rufipogon* was planted at the downwind direction of Minghui-63. The simple sequence repeat (SSR) was used as molecular markers to determine cross-pollination rates between Minghui-63 and *O. rufipogon* under different designs. The results demonstrated that the maximum frequency of gene flow from Minghui-63 to *O. rufipogon* could reach as high as 3% in natural habitats of Chaling in Hunan Province, indicating clearly that gene flow between cultivated rice and the widely distributed *O. rufipogon* occurs with a considerable rate under natural conditions.

7 General expectations of gene flow and ecological risks

Transgene escape and its environmental impacts have become increasingly challenging biosafety issues worldwide, and should receive serious and long-term attention by the public, scientists and government agencies, because it is difficult monitor ecological problems caused by the transgene escape within a limited period. Most alien genes carried by genetically modified agricultural products are not from crops, instead, they are from other organisms or microorganisms, even from an artificially synthesized origin. These genes may completely alter the natural habit of crop species and significantly change wild relatives of the crop species when transgene escape happens. As a consequence, the environmental safety, particularly the agricultural ecosystems might be under their negative influence.

The crop-to-wild gene flow is a common phenomenon and is a part of plant evolution. Results from our own studies confirmed that cultivated rice and its wild relatives *O. rufipogon* have sympatric distribution and overlapping flowering times, which meets the spatial and temporal conditions for transgene escape from cultivated rice to its wild relatives. Similar situation is found in many other crop species and their wild relatives (Ellstrand et al. 1999). Our experimental data further show that most of the AA genome wild *Oryza* species have relatively close biosystematic relationships and high crossability with cultivated rice, particularly *O. rufipogon*, *O. nivara*, and weedy rice (*O. spontanea*). They do not have a significant reproductive isolation with the cultivated rice, and spontaneous introgression with the cultivated rice occurs with considerable frequency in the field. Also, it was reported by Japanese scientists that outcrossing rates between *O. rufipogon* (including weedy rice) and cultivated rice could be as high as 50% in nature, although with a considerable variation (Oka and Morishima 1967). Data obtained from our experiment further showed that the frequency of gene flow from cultivated rice (Minghhui-63) to *O. rufipogon* in Chaling of Hunan Province could reach as high as 3%. All experimental data from previous reports and our studies support the indisputable fact that crop-to-wild transgene escape through gene flow will occur if transgenic rice varieties are grown in the vicinity of the wild relative species, and if no effective isolation measures are taken.

It is general knowledge that pollen flow is the principal pathway for transgene escape because pollen can act as a vehicle to disseminate transferred alien genes in nature. Data from our studies on pollen flow of cultivated rice—a typical wind pollinating specie—showed that the dispersal range of rice pollen grains increased with the increase of wind speed and that the maximum distance of rice pollen flow could be as far as 110m at downwind direction when the wind speed reached 10m per second. Therefore, to effectively avoid or minimize transgene escape through pollen flow to the wild relatives of rice, it is recommended to have a buffering isolation zone wider than 110m or use tall crops such as sugarcane as an effective buffer objective between transgenic rice and its wild relatives, given the fact that the spatial, temporal, and biological conditions for rice transgene escape

are satisfied in many rice producing countries or regions.

It is generally recognized that a better understanding of species biosystematic relationships, pollen flow, and gene flow will facilitate efficient prediction of transgene escape and its potential ecological risks, as well as appropriate management of ecological risks. Although an effective buffering isolation zone between transgenic crops and their closely related wild species is an important biosafety strategy to avoid or significantly minimize transgene escape temporarily, implementation of transgene containment at large scales is nearly impossible. Therefore, more scientific questions regarding biosafety issues relating to transgenic agricultural products particularly the ecological impact of transgene escape need to be properly addressed and thoroughly studied. These questions include whether transgenic hybrids and their progeny have better ecological fitness than parental species if introgression between transgenic rice and its wild relative species does occur? Whether transgenes can be normally expressed in the interspecific hybrids and their progeny? Whether transgenes will change genetic structures and population dynamics of the natural wild rice populations? Will the wild rice hybrids carrying transgenes become a "genetic bridge" that further passes transgenes to other wild plant species? What kind of ecological consequences and risks will the individuals and populations carrying alien transgenes cause? What is the effective method for assessing and managing the biosafety risks related to environmental change? The appropriate answers to all these scientific questions will assist us to effectively assess and manage the potential ecological risks resulting from rice transgene escape through gene flow, which will also promote the possibility of safe utilization of transgenic rice varieties in the future.

Acknowledgement

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Table 1. Major crops and their wild relatives with reference of gene flow in Asia

Crop	Cultivation area	Main wild relatives	Distribution	Frequency of gene flow
Rice	Tropical and subtropical Asia	*Oryza rufipogon*, *O. nivara*, *O. spontanea*	South & Southeast Asia	Medium to high
Soybean	Most Asian countries	*Glycine soja*, *G. gracilis*	China, Far East of Asia	Low
Oil rape	Temperate Asia	*Brassica juncea*, *B. campestris*, *B. rapa*	Temperate Asia	Medium to high
Wheat	Temperate Asia	*Aegilops tauschii*, *Ae. cylindrica*	Temperate Asia	Low
Millets	India, China	Weedy biotypes	India, China	Low

Table 2. Flowering time of *Oryza rufipogon*, a late maturing local variety, and Minghui-63 in Chaling, Hunan Province

Species/variety	Sowing time	Flowering period in a year	Flowering time in a day
O. rufipogon	--	Beginning of September ~ Mid. November	9:30 am ~ 4:30 pm
The late maturing local variety	Mid. June	Mid. September ~ Beginning of October	8:30 am ~ 2:00 pm, 4:00 pm ~ 5:30 pm
Minghui-63	Mid. June	Mid. September ~ Mid. October	9:00 am ~ 3:00 pm

Figure legends

Figure 1. The natural hybrids (middle) between *Oryza sativa* (right) and *O. rufipogon* (left) commonly found in the rice field in Nepal.

Ecological effects of pest resistance genes that disperse into weed populations.

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Abstract

Gene flow from transgenic crops to cultivated or free-living plants has been the subject of much recent research, but little is known about the ecological and evolutionary consequences of this process. Here we focus on the question of whether transgenes that confer resistance to herbivores or diseases are likely to affect the fitness and population dynamics of free-living plants. An important first step in this research is to determine whether natural populations that can hybridize with crops are exposed to the types of herbivores and diseases that would be thwarted by resistance genes from the crop. In many cases, the fitness consequences of particular resistance genes may be negligible in wild populations. Wild plants may be unaffected by the pest, or they may not be exposed to it. However, our studies of wild sunflowers (*Helianthus annuus*) show that a *Bt* gene for lepidopteran resistance can be associated with reduced herbivory and enhanced fecundity under natural levels of insect pressure. Moreover, we did not detect any fitness costs of this transgene. Once it is known that resistance genes can enhance the fitness of wild or weedy plants, further studies are needed to assess whether these populations could become more widespread or invasive. In general, little empirical information is available about the extent to which various herbivores and diseases limit populations of wild or weedy relatives of crop plants. Due to the difficult, long-term nature of research on plant population dynamics, we recommend fitness studies as a key element in assessing the ecological effects of pest resistance genes. From a regulatory standpoint, it is also useful to examine whether new transgenic constructs could have greater ecological effects than ongoing gene flow involving nontransgenic resistance traits.

Key words: crop-wild hybridization, gene flow, transgenic resistance, herbivory, disease, fitness effects, population-level effects, ecological consequences of gene flow, *Bacillus thuringiensis* (*Bt*)

Introduction

Worldwide, many cultivated plants hybridize spontaneously with wild or weedy relatives (Small 1984, Ellstrand et al. 1999). In the USA, for example, this occurs in more than twenty species, including sunflower, sorghum, squash, canola, rice, sugar beet, poplar, turf grasses, and forage grasses (NRC 2000). In addition, many crops can become naturalized and persist as feral weed populations (Ellstrand et al. 1999). Thus, transgenes conferring novel traits that enhance survival and reproduction may inadvertently disperse from cultivated plants to wild or weedy populations that lack these traits. In the short term, the spread of transgenic herbicide resistance is likely to pose challenges for controlling weeds and unwanted "volunteer" crop plants (Snow et al. 1999, Hall et al. 2000). Over the longer term, we need to know whether the spread of transgenes coding for other fitness-related traits could exacerbate weed problems in agricultural settings and affect the population dynamics of wild relatives in unmanaged areas (Snow and Morán Palma 1997, NRC 2000, Snow 2002). This raises fundamental questions about the extent to which herbivores, diseases, and stressful abiotic conditions regulate populations of wild and weedy plants.

A transgenic trait that increases a crop plant's survival or yield has the potential to enhance the fitness of free-living crop relatives. Crop species with transgenic resistance to certain insects and diseases have already been commercialized, so we focus on these types of resistance genes in this paper. With regard to herbivores, studies of both native and exotic species suggest that herbivores can have a dramatic impact on plant population dynamics (e.g., Crawley 1997, Rees and Paynter 1997, Marvier and Kareiva 1999). Also, several recent studies have reported negative impacts of viral or fungal diseases on the growth, survivorship, and reproduction of plants in natural populations (e.g., Friess and Maillet 1996, Packer and Clay 2000, Funayama et al. 2001, Power

2001). However, little is known about the extent to which herbivores and diseases affect the population dynamics of wild or weedy relatives that hybridize with cultivated plants.

Previously, little attention has been paid to the effects of nontransgenic resistance genes on populations of wild relatives. One reason for this may be related to the fact that useful agronomic traits have often been obtained from wild relatives in the first place, creating the impression that these taxa are already very well adapted to local conditions. In some cases, however, agricultural breeding has resulted in crops with single-gene resistance traits that are easily transferred to wild populations lacking these traits. For example, resistance to a fungal disease (*Puccinia* spp.) has been bred into cultivated sunflowers (*Helianthus annuus*) using wild germplasm (Seiler 1992). This trait is not ubiquitous in wild/weedy sunflower populations (also *H. annuus*) and can spread to wild populations via crop-to-wild gene flow (Snow et al. 1998). A field experiment with wild-crop hybrids showed that naturally infected plants produced ~20% fewer seeds per plant than plants that lacked *Puccinia* symptoms (Snow et al. 1998). In general, it is not known which fitness-related crop genes have spread to wild populations and persisted, so it is difficult to generalize about this process and its ecological consequences. Single-gene resistance traits, especially dominant ones, are expected to move from crops to wild populations more easily than polygenic, quantitatively inherited resistance traits.

Another reason that the fitness consequences of crop-to-wild gene flow have not been examined is that crop genes are often considered to be harmful to wild plants. Traits such as short flowering periods, lack of seed dormancy, lack of seed dispersal mechanisms, and a lack of secondary compounds that deter insects would likely be detrimental to wild plants, leading to the conclusion that many crop genes would have deleterious effects on the fitness of wild or weedy relatives (e.g., National Research Council 1989). Moreover, the very low fertility of some wild-crop hybrids could impede the flow of crop genes into wild populations. However, recombination and introgression can allow deleterious crop genes to be purged from wild populations, while other crop genes that confer neutral or beneficial effects persist. This can occur even when the fertility of F_1 wild-crop hybrids is much lower than that of wild plants. Based on principles of population genetics, we expect that beneficial genes that are tightly linked to strongly deleterious crop genes will be lost from wild populations (e.g., Gressel 1999), while other beneficial crop genes will increase in frequency. It is these types of crop genes, including transgenes, that have the potential to have unintended and unwanted ecological consequences. Thus, it is useful to determine whether particular transgenes, and specific transgenic events, are associated with fitness costs or benefits in wild populations.

General Approaches to Fitness Studies

Fitness is typically defined as the product of survival and lifetime seed production of a given group of genotypes, such as transgenic wild plants, relative to the survival and reproduction of another group of genotypes, such as control plants lacking a particular transgene (e.g., Silvertown and Charlesworth 2001, Gurevitch et al. 2002).   A rigorous way to examine fitness effects of a given transgene is to conduct field experiments involving two groups of wild plants that differ in the presence or absence of the transgene, but otherwise have the same genetic composition. This approach is described in the case study below, but resistance transgenes generally are not available to ecological researchers to prior to commercialization of a crop, when risk assessments are carried out. Therefore, it is often necessary to begin with ecological studies of the prevalence of target pests in wild populations. In any event, it is useful to have a broad understanding of ecological factors that could affect the survival and reproduction of crop relatives when evaluating risks associated with novel crop genes.

A first step in this research is to determine whether pests that are the target of particular resistance (trans)genes occur in natural populations, and whether natural populations are susceptible to these pests. For example, wild cabbage (*Brassica oleracea*) in the United Kingdom is commonly infected by several viruses that reduce survival, growth, and reproduction, so transgenic resistance to these viruses could be beneficial (Maskell et al. 1999, Raybould et al. 1999). In contrast, wild carrot (*Daucus carota*), appears to be resistant to a common fungal disease, *Alternaria dauci*, in the Netherlands (Schouten et al. 2002), in which case wild plants are not expected to benefit from obtaining a transgene that confers resistance to this disease. Similar surveys can be carried out for insect groups (e.g., lepidoptera or coleoptera) that are the target pests of various Bt genes.

In many cases, quantifying the prevalence and effects of naturally occurring herbivores and

diseases on plant populations is challenging. Few researchers study plant-pathogen interactions in wild and weedy plants, so little previous information is available. Moreover, site-to-site and year-to-year variation in pest populations can be considerable, making it difficult to generalize from short-term and small-scale field studies (NRC 2000, 2001). Assessing the importance of infrequent outbreaks and/or patchily distributed pest populations may be impractical within the time frame that is available for regulatory decision-making. A lack of empirical data on the prevalence of target insects or diseases in populations of crop relatives calls for a cautious approach to the deregulation of novel transgenic resistance traits (NRC 2000, 2001).

When pest populations are known to be fairly common, a further challenge is to determine the extent to which these herbivores and diseases affect the fitness and population dynamics of wild plants. Sometimes it is possible to exclude plant pests in experimental field plots, for example by using insecticides (e.g., Louda and Potvin 1995), to quantify possible increases in seed production and seedling recruitment. But it is often difficult to mimic the effects of specific transgenes, such as constitutively produced Bt toxins or viral coat proteins, on the growth and reproduction of wild plants. To gain an understanding of how a given transgene or group of transgenes will affect the fitness of wild relatives, it is useful to carry out field and greenhouse experiments using transgenic wild plants. These experiments should be carried out under careful confinement procedures in much the same way as field tests of experimental transgenic crops are performed. Ideally, these two types of research should proceed simultaneously so that results pertaining to risk assessment can be made available to regulatory agencies and the public in a timely manner.

Bt Wild Sunflower: A Case Study
Note: A more detailed description of this case study can be found in a workshop contribution by Pilson et al. at http://www.biosci.ohio-state.edu/~lspencer/gene _flow.htm

Wild sunflower (*Helianthus annuus*) represents an excellent model system with which to address these questions. Wild sunflower is a native, self-incompatible, annual plant that is widespread throughout much of the USA, reaching its greatest abundance in midwestern states where most cultivated sunflower is grown. The process of crop-to-wild introgression has been well documented in sunflowers. Field experiments have shown that pollinators can transfer crop pollen to wild plants as far as 1,000 m away, with the frequency of hybrid seeds being greatest (up to 42%) at the crop margin (Arias and Rieseberg 1994, Whitton et al. 1997). Additional studies have shown that first generation wild-crop hybrids usually produce fewer seeds per plant than their wild counterparts, but the magnitude of this difference varies a great deal among plants, regions, and growing conditions (Snow et al. 1998). Under some field conditions, seed production of F_1 crop-wild hybrids is comparable to that of purely wild plants, and in several cases hybrids produce at least 50% as many seeds per plant as wild genotypes. Furthermore, selectively neutral crop markers have persisted for many generations in wild plants sampled in California, Kansas, North Dakota, and Canada (Whitton et al. 1997, Linder et al. 1998). These studies demonstrate that introgression of neutral or beneficial crop genes into wild gene pools can be an ongoing process wherever these taxa occur sympatrically. Clearly, both genetic and geographic barriers to gene flow from crop to wild sunflower are minimal.

Wild sunflower is a host for many insect herbivores (Pilson 2000), several of which are also pests of the crop. Wild sunflowers often are damaged by lepidopteran and coleopteran insects that feed inside the plant on seed heads, stems, and roots (Pilson 2000; Snow et al. 2002). The most damaging insect pests of cultivated sunflower are those that infest developing seed heads (weevil, moth, and midge larvae) and those that transmit disease (e.g., stem weevils that transmit phoma black stem; Schneiter 1997). Polygenic resistance to insects has been documented in other species of *Helianthus*, but efforts to introgress strong resistance into the crop have been unsuccessful (Seiler 1992). For these reasons, cultivated lines with transgenic resistance conferred by Bt toxins are being developed by a number of seed companies, and several field trials have been approved by regulatory agencies (http://www.isb.vt.edu). Different Bt-toxins are specific to different groups of insects, including lepidoptera, coleoptera, and diptera. Bt-induced resistance to coleoptera was first field-tested in the US in 1996 and resistance to lepidoptera was approved for field-testing in 1999, although none have been commercialized to date. Additional field trials have taken place in the Netherlands and Argentina (http://www.isb.vt.edu , http://siiap.sagyp.mecon.ar/http-hsi/english /conabia/liuk4.htm). Broad-spectrum resistance involving multiple Bt genes and other genes for insect resistance may also be developed in the future.

We studied a crop-developed *Bacillus thuringiensis* (Bt) transgene, *cry*1Ac, in backcrossed wild sunflower populations (Snow et al., 2002). To simulate the effects of introgression of a Bt transgene from the crop, male-sterile wild plants from a population near the Cedar Point Biological Station in Nebraska were bred with transgenic cultivars to create BC_1 progeny that segregated for both the Bt transgene (Bt+ or Bt-) and for male-sterility (male-sterile or male-fertile). However, to prevent the accidental escape of the transgene we did not use Bt+/male-fertile plants in the field. BC_1 progeny were planted in the field in 1999 at the Cedar Point Biological Station in western Nebraska and in an agricultural field in eastern Colorado, near Burlington. The effect of the transgene was examined by comparing insect damage and fecundity between Bt+/male-sterile and Bt-/male-sterile plants. These experiments were carried out under USDA-APHIS Permits 99-096-01N and 99-095-07N. All wild and BC_1 seed heads were collected, and any sunflower seedlings that appeared at the sites after tilling in 2000 and 2001 were destroyed.

Transgenic resistance to lepidopterans appears to be a dominant trait because BC_1 plants that were hemizygous for this gene had very low levels of lepidopteran damage (50% of the BC_1 plants inherited the transgene, as expected). Lepidopteran damage on transgenic plants was strongly reduced relative to control plants at our two study sites, while damage by several weevil and fly species was unaffected. As a result of reduced herbivory, transgenic plants produced an average of 55% more seeds per plant relative to nontransgenic controls at the field site in Nebraska. A similar but non-significant trend was seen at the site in Colorado (14% more seeds per plant). At both sites, plants that were male-sterile had less lepidopteran damage and more seeds per plant than control plants that were male-fertile (based on comparisons between non-transgenic plants, with or without pollen). This could be due to the fact that lepidopterans also feed on sunflower pollen (Delisle et al. 1989, Korman and Oseto 1989) and may prefer pollen-producing plants. If this is the case, we may have underestimated the advantage of a Bt transgene because this estimate was based on comparisons between two groups of male-sterile plants, with or without the transgene.

In any study of a single transformation event, it is not clear whether phenotypic effects (e.g., greater fecundity) are caused by the transgenic construct or by other mechanisms, such as position effects, pleiotropy, or close physical linkage with other crop genes. Thus, it is useful to determine whether effects associated with the Bt transgene can occur in the absence of lepidopteran herbivores. We performed a greenhouse experiment using BC_1 plants to examine this possibility, while recognizing there are many biotic and abiotic differences between field and greenhouse conditions. The Bt transgene had no effect on the number of inflorescences or seeds per plant in the greenhouse, regardless of whether the plants were grown under water-stressed, drought-stressed, or control conditions, and regardless of whether they were male-fertile or male-sterile. This suggests that the transgene was not associated with an inherent fitness cost or benefit. It would be preferable to employ a wider range of growing conditions and several transgenic events in this type of study, but our results suggest that the fecundity advantage of transgenic plants in the field was due to protection from lepidopteran herbivores.

To summarize, this study shows that selection favoring an increase in the frequency of a Bt transgene has the potential to be quite strong. Therefore, we expect that subsequent generations of Bt wild plants would produce more seeds per plant than non-transgenic individuals in many locations and growing seasons, depending on the abundance of lepidopteran herbivores. If so, the transgene is expected to increase in frequency. When this occurs, we expect that this very effective Bt transgene would be expressed in many wild plants and would kill susceptible, native lepidopterans that feed on these plants. Thus, proteins from pest resistance genes could potentially affect non-target organisms and ecological communities when these genes become common in wild sunflower populations. It is possible that specialist herbivores would eventually evolve resistance to transgenic Bt toxins, but this has not been reported yet in target pests of transgenic Bt cotton or corn (e.g., Carriere et al. 2001).

Prior to this experiment, we predicted that Bt wild sunflowers would gain a fecundity benefit of about 10-15% at the Nebraska field site, based on preliminary ecological surveys of lepidopteran damage within seed heads. Without access to a constitutively expressed Bt transgene, we would not have known that a much larger fecundity benefit could occur under field conditions. It would be

very instructive to repeat this experiment in other years and locations, using advanced generations of Bt wild sunflowers, and following accepted methods for confinement of the transgene construct. However, the biotechnology companies that developed this transgene have not allowed us to continue our research. Thus, legal constraints associated with patented genes can hinder ecological studies involving non-commercialized transgenic crops.

Moving Beyond Small-Scale Fitness Studies

Once it is known that a given transgene has the potential to enhance the fitness of wild relatives, we need to know whether transgenic plants that produce more seeds per plant are likely to give rise to larger populations, more populations, and/or more extensive seed banks. Experimental manipulations of local population dynamics and models of metapopulation dynamics are needed to understand these processes. We are currently pursuing these approaches with wild sunflower. In addition, to gain a better understanding of how lepidopteran herbivores affect wild sunflower populations, experiments similar to those described above should be repeated over several study sites and seasons. Fitness studies are an essential first step in understanding the ecological and evolutionary effects of gene flow because it is important to know the magnitude of presumed fecundity effects of a given transgene. This knowledge, together with an evaluation of the ecological effects of transgenes, is critical for biosafety risk assessments. Due to the difficult, long-term nature of research on the effects of naturally occurring herbivores and diseases on plant population dynamics, we recommend fitness studies as a key element in assessing the ecological effects of pest resistance genes. From a regulatory standpoint, it is also useful to examine whether new transgenic constructs could have greater ecological effects than ongoing gene flow involving nontransgenic resistance traits.

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Agronomic effects of gene flow: multiple herbicide resistance in volunteer crop plants

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Abstract

Gene stacking in volunteer crop plants was first reported in canola, *Brassica napus.* Herbicide resistant *Brassica napus* resistant to glyphosate, glufosinate, bromoxynil or imidazolinone herbicides has been widely accepted by Canadian producers since 1996, occupying approximately 85% of *B. napus* acres. Volunteer canola is also a significant weed, ranking between 16th and 20th in relative abundance amongst weeds in western Canada in recent post-herbicide weed surveys. It survives in the seed bank for 4 or 5 years following the canola crop. Plant-to-plant outcrossing rates in *B. napus* are approximately 20%. Outcrossing diminishes with distance to less than 1% at boundaries between adjacent fields and further to less than 0.2% at greater distances. Outcrossing has been identified up to 3 km distance from the pollen source. Gene flow via pollen, in combination with volunteer survival results in multiple herbicide resistant volunteers, with two or three different resistance genes stacking in a single plant. The longevity of volunteer canola in the seed bank, outcrossing rates and multiple herbicide resistance prohibit the easy extinction of traits once they have been released. Resistant and multiple resistant volunteers have necessitated the modification of agronomic practices for their control. Most producers rely on herbicide mixtures containing auxinic herbicides like 2, 4-D which control all canola volunteers. The risks of gene flow and modified agronomic practices must be balanced against the gains to the industry and the environment. The use of herbicide resistant canola has reduced the amount of herbicide used in western Canada by 6,000 tonnes annually, and decreased tillage and consequent use of diesel fuel by 31.2 million liters. While much can be learned from the Canadian example, decisions on the introduction of future traits for crops must continue to be trait, environment and crop specific.

Key words: *Brassica napus*, *Brassica rapa*, glyphosate, glufosinate, imidazolinone herbicides, transgenes, plants with novel traits**,** ALS.

Canadian canola growers have readily adopted herbicide resistant canola. Approximately 85% of the canola grown on 4.0 million ha in 2001 in Canada was herbicide resistant. Their experiences over the last five years can provide considerable insight into the agronomic benefits and constraints of herbicide resistant canola and the occurrence and consequence of gene flow.

Canola, the Canadian Oilseed

Two species, *Brassica napus* and *B. rapa,* are grown in Canada to produce canola quality oil. In Eastern Canada, which grows 11 % of the canola crop, *B. napus* is grown exclusively, while in Western Canada, *B. napus* is grown on approximately 95% of the acres and *B. rapa* grown on the balance.

Four types of herbicide-resistant *B. napus* are grown in Canada, glyphosate, glufosinate and bromoxynil resistant canola; all created using transgenic techniques, and imidazolinone-resistant canola, produced by mutagenesis (Table 1). In Canada, all four types were evaluated prior to release and were regulated identically as 'plants with novel traits' (PNT's). The Canadian Food Inspection Agency defines PNTs as 'plant varieties/genotypes that are not considered substantially equivalent, in terms of their specific use and safety both for environment and for human health, to plants of the same species in Canada, having regard to weediness potential, gene flow, plant pest potential, impact on non-target organisms and impact on biodiversity. PNTs may be produced by conventional breeding, mutagenesis, or more commonly, by recombinant DNA techniques' (Canadian Food Inspection Agency 2002).

Table 1. Herbicide resistant canola grown in Canada, the type of novel trait and proportion of the acreage upon which it is grown.

Herbicide Resistance	Year of Introduction	Resistance Trait	% of Total Seeded Area
Glyphosate	1996	*Epsps + gox* transgene[1]	50
Glufosinate	1996	*Pat* or *bar* transgene[2]	17
Imidazolinones	1996	ALS modified by mutagenesis[3]	17
Bromoxynil	1999	*Nitrilase* transgene[4]	<1

[1]CFIA 1996, [2]CFIA 1995b, [3]CFIA 1995a, [4]CFIA 1998

Approximately 15% of producers continue to seed only conventional canola; generally because they grow *B. rapa* or specialty oil varieties, they are satisfied with conventional weed control, or because of a philosophical disagreement with the technology.

Volunteer Canola as Weeds

Volunteer canola is an important weed in Eastern and Western Canada. Because canola is a small seeded crop, prone to shattering, harvest losses are significant, averaging 5 to 10% of the seed harvested. In the year following canola, volunteer density can be several hundred per m^{-2} prior to herbicide application. Seeds may remain viable in the seed bank for 4 years in Western Canada and 5 years in Eastern Canada (Légère et al. 2001). Volunteer canola density declines with years following the canola crop. After herbicide application in Western Canada, *B. napus* and *B. rapa* (confounded) volunteers averaged 7 and 0.5 plants m^{-2} after 1 and 4 years, respectively (Thomas and Leeson 1999), and in Eastern Canada *B. napus* volunteers averaged 2.4 and 0.21 plants m^{-2} after 1 and 5 years, respectively (Simard et al. 2002). Averaged across all crops, volunteer canola was the 16th most abundant weed reported in a recent post herbicide weed survey in Alberta (Leeson et al. 2002). Herbicide resistance profiles were not assessed in canola volunteers, but should reflect the proportion of the herbicide resistance types grown (Table 1) over the last 4 to 5 years.

Canola and Canadian Cropping Systems

Prior to the release of herbicide resistant canola, yield loss by weeds was considered the agronomic factor most limiting to canola production. Conventional canola is tolerant to few herbicides. The product of choice was a soil applied dinitroanaline herbicide that had a narrow weed spectrum, was subject to environmental variability and required tillage for incorporation. The tillage requirement prevented many producers from taking advantage of direct seeding techniques, known to reduce soil erosional losses and carbon and nutrient losses. Many broadleaf weeds were not controlled, resulting in yield losses, increased weed seed banks and yield losses in subsequent crops. In Eastern and Western Canada, herbicide resistant weeds and multiple resistant weeds, in particular wild oat (*Avena fatua*), have been selected through repeated use of herbicides (Beckie et al. 2001a,b). However there are no reports of weeds resistant to glyphosate or glufosinate in Canada and these herbicides are considered relatively low risk for the selection of herbicide resistance. Glyphosate and glufosinate offered options for in-crop weed control, for enhanced herbicide rotations to delay selection for resistant weeds and for control of existing resistant weeds.

Therefore, when herbicide resistant canola varieties become available, they were rapidly adopted. Herbicide resistant canola increased profitability of canola production by an estimated $14.32 per ha (Canola Council of Canada 2001). Reduced tillage resulted in 31.2 million liters less fuel used by farm machinery in 2000. Replacing high-use-rate dinitroanaline herbicides with lower-use-rate products decreased the total herbicide used in Western Canada by 6,000 tonnes in each of 1999 and 2000 (Devine and Buth 2001).

Gene Movement by Pollen in B. napus

B. napus is a partially outcrossing species, with plant to plant outcrossing rates of approximately 20% in Western Canada (Rakow and Woods 1987). When herbicide resistant varieties were released, it was predicted that cross pollination would occur and these volunteers may be resistant to more than one type of herbicide (CFIA Decision Documents, 1995 a and b, 1996, 1998). However, the extent and distance of potential pollen flow was not well defined.

Multiple Herbicide Resistant Volunteers

Several studies from Canada and Australia have examined pollen movement between fields. In a study of 11 fields pairs of adjoining glyphosate and glufosinate resistant canola fields, Beckie et al. (2001c) reported an average of 1.1% cross pollination at field boundaries, diminishing to less than 0.2 % at 50 meters. From 50 to 800 meters, outcrossing frequency was relatively stable at less than 0.2%. In Australia, 63 fields were investigated in 2000, the first year of introduction of imidazolinone resistant canola (Rieger et al. 2002). Cross pollination at low frequency was reported over 3 km away from the putative pollen source fields.

Volunteers can enhance gene flow potential. During an investigation of the causes of unexpected herbicide resistance in a fallow field in Alberta, glyphosate/imidazolinone resistant volunteers were identified over 500 meters away from the pollen source. Cross pollination of those volunteers with glyphosate/glufosinate resistant volunteers produced three progeny resistant to all three herbicides, glyphosate, glufosinate and imidazolinones (Hall et al. 2000). Pollen flow between canola fields, in conjunction with long term volunteer survival suggests that multiple resistant volunteers are present in measurable frequencies in many commercial fields.

Agronomic Consequences of Multiple Resistant Canola

Both conventional and herbicide resistant canola are approved by Health Canada and are routinely mixed before crushing. Therefore, there are no Canadian health and safety regulatory concerns associated with genetic mixtures of canola seed from pollen flow.

Genetic contamination of conventional canola with transgenes via pollen flow prohibits organic production of canola except in isolated areas due to a current zero tolerance for transgenes. Additionally, Canadian seed regulations allow only 0.25% off-types, including genetic contamination. Pollen flow and longevity of canola volunteers necessitates strict adherence to requirements for isolation distances between canola fields and years between canola crops. The ability to rapidly screen large numbers of seeds for specific transgenes using immunocytochemical techniques may enable both organic and seed producers to quantify seed purity.

When producers choose herbicides for volunteer canola control, they consider the type(s) of herbicide resistant canola grown in the field and adjacent fields. Glufosinate is only used in canola and therefore this transgene is effectively selection neutral. However, glyphosate is used routinely pre-seeding and pre- and post-harvest in all crops. ALS (acetolactate synthase) inhibitors, including imidazolinones, are used extensively in the other major Canadian crops, wheat, barley and peas. Imidazolinone resistant canola has partial or complete cross-resistance to other ALS inhibitors and therefore cannot be controlled in-crop when these herbicides are used alone. Uncontrolled resistant or multiple resistant volunteers increase in the population, preserving herbicide resistant traits and facilitating gene stacking.

All types of canola can be controlled by tillage or inexpensive auxinic herbicides such as 2, 4-D and MCPA, used alone or in mixtures with other products when applied at the appropriate stage. Glyphosate is now routinely mixed with an auxinic herbicide for glyphosate resistant volunteer canola control. While necessary for weed control, the mixture delays the seeding of crops sensitive to auxinic herbicides. Similarly, pre-harvest applications of glyphosate cannot be used to control glyphosate or cross-resistant volunteer canola. A more expensive option, diquat, must be substituted.

As most herbicide products used in cereals contain auxinic herbicides, minimal adjustments need to be made to in-crop application in cereals. However, in pulse crops, imidazolinones are the herbicides of choice, and imidazolinone tolerant volunteer canola cannot be easily or inexpensively removed. In summary, to control resistant and multiple resistant weeds, more herbicides mixtures are required. There is a greater dependence on record keeping and understanding herbicide mode of action.

While many weeds have become less of a concern in canola because of better and more reliable weed control measures in herbicide resistant systems, resistant and multiple resistant canola has become a weed that requires more consideration and planning.

Multiple herbicide resistant canola has not had dire consequences for Canadian canola producers. Most of the agronomic problems have been resolved in the first years and a list of strategies for control of herbicide resistant varieties developed (Table 2). Most producers rely on the use of herbicide mixtures to control all types of volunteer canola.

Table 2. Integrated weed management strategies to control, or reduce the impact of, herbicide resistant and multiple herbicide resistant volunteers (Hartman 2002).

1	For pre-seeding or chem-fallow weed control, add an auxinic herbicide to glyphosate or use the photosynthetic inhibitor diquat
2	Use herbicide mixtures when using glyphosate or ALS inhibitors in crops following canola
3	Rotate herbicides in subsequent crops

4	After harvest, leave canola seeds on or near the soil surface as long as possible
5	Use tillage immediately prior to seeding to control volunteer canola
6	Remove crop as silage or use as a green manure to control volunteer canola
7	Isolate fields with different herbicide systems.
8	Rotate canola with cereal, pea and forage crops
9	Scout fields for volunteer canola plants not controlled by herbicide application
10	Grow competitive cereal and pulse crops to ensure that fewer volunteer canola seeds are returned to the seed bank
11	Reduce canola seed loss at harvests by correctly setting harvesting equipment
12	Use certified seed to reduce contamination by herbicide resistant volunteers

In the absence of herbicide, resistant or multiple resistant canola are not more fit than their conventional counterparts and there is no evidence that these plants will invade natural areas (Crawley et al. 2001, Duke 1999, reviewed in Warwick et al. 1999). Resistant and multiple resistant volunteers extend over space and time the potential for gene flow, between crop plants, volunteers and between wild and weedy species. The longevity of canola seed and its proclivity to cross pollinate precludes the elimination of genes once they are released into the environment. Therefore, it is critical that we have a understanding of the environmental impact of a new trait on both volunteer and crop plants and be aware of the potential for gene stacking. Traits conferring enhanced fitness, such as tolerance to stress, should be carefully evaluated prior to release.

All decisions to release novel traits must be crop, trait and environment specific. While multiple resistant canola volunteers are an undesirable consequence of the release of herbicide resistant canola, their impact must be weighed relative to the potential positive environmental effects, including the reduced use of herbicides, reduced tillage and altered selection for herbicide resistant weeds (Beckie et al. 2001a,b).

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Preventing, Delaying and Mitigating Gene Flow from Crops – Rice as an Example

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Abstract

The shifting agronomy to direct-seeded rice, necessitated by the unavailability of labor for transplanting, has exacerbated weed problems, such as *Echinochloa* spp., the sedges and red and weedy rices. Engineered herbicide resistance allows selective control of all these weeds in rice but is confounded by a higher rate of introgression of resistance genes into red rice than had been expected, severely limiting the utility of the technology. Gene flow can be prevented or delayed (in theory) by single generation transformation, terminator (GURT), or inducible-promoter technologies that provide transient transgene expression, or by plastome or chondriome transformations where there is maternal inheritance. In some cases (e.g. plastome transformation) it is known that the protection is incomplete. Mitigation technologies are suggested as an adjunct, where the transgene of choice is flanked by mitigating genes (e.g. anti shattering, anti dormancy, or dwarfing genes) deleterious to weedy types. These would result in uncompetitive progeny. Transgenic rice bearing properties that might have a fitness advantage for weeds should not be released unless it bears introgression-delaying and mitigating systems, preferably stacked to minimize the risks.

Key words: *Oryza* spp., wild rices, red rice, gene flow, introgression, fitness, mitigation

Introduction

We have been warnedThere have been dire warnings that the cultivation of biotechnologically-derived herbicide resistant crops (BD-HRC) can lead to the evolution of "superweeds" that will inherit the earth.   So far, there are many documented cases of introgression of herbicide resistance and other genes from weeds into crops and far fewer vice versa [32]. The rapid commercial release of such crops transgenic crops that are related to weeds has often been without broad-based scientific scrutiny with the most competent experts being involved. This leads to a certain degree of skepticism among scientists about the rosy pictures often painted by industry that introgression is insignificant. This in turn contributes to the public questioning the needs, utility, risks, and values associated with the use of transgenic crops. The severe pressures by detracting groups on policy makers seems to have made it politically incorrect for pursuing public-sector research in this area, which prejudices the ability to perform experiments to obtain accurate information about the risks. Indeed, in some of the countries requiring field data, one the data cannot be legally obtained in those countries, as required. These pressures also prevent generating crops needing resistance to herbicides, or other traits available only via transgenics.

Herbicide-resistant rice (used as an example herein, abridged from ref. [32] can be of great benefit, but only if used with care to prevent or mitigate gene transfer to widely distributed related feral and wild rices, introgression that occurs more rapidly in the field than predicted. More sophistication will be needed than is presently being used with transgenic rice to mitigate such introgression, which can provide fitness advantages to hybrids and their progeny.

Rice needs transgenes to solve agronomic problems

According to the Food and Agriculture Organization (FAO) of the United Nations, rice is clearly a crop with problems:

(a) Rice culture has demographic problems due to the continuously increasing average age of rice farmers. Younger people flock to slightly higher paying, less arduous employment. The older farmers are tired, and unless agronomic strategies change, paddies will become weedier and rice yields will drop; herbicide resistant *transgenics are needed to replace labor.*

(b) Rice has geographic problems; areas are being taken out of production faster than are being brought into cultivation due to urban encroachment, competing crops, soil degradation and salinization, and lack of water; *transgenics are needed to increase yield on the remaining land.*

(c) Rice culture has agronomic problems. Direct seeding has alleviated the labor problems of transplanting while exacerbating the weed problems; *transgenics are needed to overcome weed*

problems.

When surveyed, farmers claim that the major constraint to rice production is weeds [41]; insects and pathogens together account for only 12% percent of losses. Much of the loss due to insects was later attributed to uncontrolled weeds that act as insect hosts. The weed issues do not get the attention due them and are rarely addressed by the international rice community, whose members are typically trained to deal with other subjects.

Herbicide use in rice facilitated weed control in low stature, high harvest index "green revolution" rices, allowing fewer farmers to harvest far more rice. Herbicides also facilitated the ecological and evolutionary changes that are just beginning to appear that tell us that chemical answers that allowed cultivation of direct-seeded short varieties are not forever. There can be no return to the labor-intensive, herbicide-free, back breaking, transplanted and later hand weeded rice. Japan uses cocktails containing a slew of herbicides to affect complete chemical control of weeds, much more than anywhere else. There are vast rice growing areas where one or two herbicides are often used, a grass killer and sometimes a broad leaf killer. The nature and economics of farming in this vast middle realm necessitates the use of cheap generic herbicides; 2,4-D for broad leaf weed and either propanil, butachlor, or thiobencarb for grass weed control. The latter three compounds are not rotated; each is typically solely used in a given region. This use of single compounds has led to resistance problems [32]. Broadleaf weeds have not escaped the sufficient control of 2,4-D, except in isolated instances [33].

Millennial weeds in rice There are three weed groups that somewhat arbitrarily fit the designation as millennial weeds, i.e. are globally distributed, pernicious, hard to control weeds that have become acute problems due to recently instituted cultural practices:

(a) *Echinochloa* spp. – always problem weeds, but are now evolving resistance to the rice herbicides used for their control;

(b) The sedges (*Cyperus* and other) that were never well controlled by any herbicide chemistry, and the areas infested are expanding; and

(c) The red, weedy, and wild *Oryza* spp. that were never selectively controlled in rice by herbicides [6; 64; 65]. Their control is especially amenable to biotech solutions that confer intra-generic and intra-specific selectivity. The same solutions are frightening and futile if the crop transgenes for herbicide resistance (or other traits) introgress into the weedy rices enhancing their competitive ability.

The *Echinochloa* spp. are major weeds wherever rice is grown [43]. Their distribution is truly global from temperate to tropics in a wide variety of crops. The *Echinochloa* spp. were major targets for graminicide development, and *Echinochloa* spp. were excellently controlled for a very long duration. The excellent control was the key to resistance problems. If there is excellent kill through all weed germination flushes, the only survivors are individuals that are totally resistant to huge herbicide doses, i.e. the selection pressure for evolution has been immense. Propanil provided excellent control throughout the Americas and in Europe until resistance evolved [33; 35; 86; 87]. Propanil resistance in *Echinochloa* was not at the photosystem II target site in the chloroplasts. Two *Echinochloa* spp. evolved elevated levels of the same acylamidase enzyme system that rice uses to metabolite the herbicide propanil to non-phytotoxic compounds [11; 56].

Large scale butachlor/thiobencarb resistances in southern China [28; 44] and the intergroup metabolic cross resistances of two *Echinochloa spp.* to a variety of herbicides in California [25; 26]. There has been a steady increase in the number of sites as well as area infested with ALS inhibitor resistant weeds of rice. Over half of the 24 herbicide-resistant biotypes in rice are resistant to ALS inhibitors [38].

The sedges The newer direct seeding cropping systems for rice favor sedges. The excellent control of grass weeds left an ecological vacuum, which nature abhors. The sedges *Alisma plantago-aquatica* in Italy and Portugal, *Cyperus difformis* and *Sagittaria montevidensis* in Australia and the USA, *Scirpus mucronatus* in Italy and the USA, and *Lindernia* spp. in Asia are of particular concern. The lack of good alternatives for control of some of these species in rice heightens the concern of growers. The only good chemical way to kill sedges is with systemic herbicides that will penetrate to the storage organs of these pests, preventing their regrowth. There are genes available to confer resistance in rice to a few systemically-translocated herbicides that kill sedges.

The weedy rices. Weedy con-specific red rice and other *Oryza* spp. have also filled a vacuum and are much harder to deal with in direct seeded rice where the cultivated rice does not have a head start due to transplanting from nurseries [6; 42; 64; 65]. Their genetic, morphological, and

phenological similarities to domestic rice kept them as minor camp followers, until cultivated rice was dwarfed to increase harvest index. The taller red and wild rices now have a competitive advantage. Two red rice plants per meter square can give a measurable yield loss [52] and the numbers that can be found will reduce rice yields by as much as 85% [21; 22; 24; 53].

In addition to U.S., Spain and Italy, where they grow only direct-seeded rice, red rice is now becoming an acute problem in Malaysia, Thailand, Vietnam, where direct seeding is becoming popular due to labor costs. In the Philippines red rice is increasing in rainfed direct-seeded rice areas. It had not been a problem in South Korea and Japan where transplanting rice is mechanized and rice heavily subsidized. South Korea is now getting red rice problems as they are increasing their direct-seeded rice areas. There is a move towards direct-seeded rice in China; the area increased nearly 6 fold from 1995 to 1999 (to >200,000 ha) in Zhejiang alone. The trend is expected to continue, and red and wild rice species could become a problem as they have everywhere else where direct seeding is used [91]. Red-rice may be less of a problem in China as it increases areas of direct seeded rice if the farmers continue to use hybrids, and red-rice is carefully kept from hybrid seed production areas.

Some of the conspecific red rice and other *O. sativa* types can be considered as progenitors to, or as recently evolved feral forms of domestic rice. There are other *Oryza* spp. that have weedy characters, as well as wild species that are not competitive at present in agroecosystems [89]. The various weedy rices shatter most of their seeds before cultivated rice is harvested, so the farmer loses rice yield while filling the soil seedbank with weeds [6]. Enough weed seed is left in the harvested crop to further sow farmers' fields with this problem. This weedy rice seed mimics rice seed; and it is nigh impossible to mechanically separate it from rice seed.

Another feral aspect of red and weedy rices is their prolonged dormancy, germinating over a number of years, resulting in a prolonged problem. Without these two qualities, shattering and dormancy the weedy rices would almost be domestic rice. The weedy rice species have become greater problems since farmers became more reliant on chemical means to control other weeds in rice. It should be no surprise to the geneticist or biochemist that the domestic and weedy rices are generally naturally-resistant to the same herbicides as cultivated rice [64].

Presently, the best farmers can do is to delay seeding rice until after the weedy rices have germinated and have been controlled with non residual graminicides to which they are still susceptible, or return to transplanted rice, which has far fewer weed problems. Delayed planting shortens the season, reducing the yield. Rotating rice with other crops and instituting strong control measures to reduce the seedbank of red rice also can be effective where there is no permanent paddy[3; 27]. Italian farmers have had to resort to ancient herbicides such as dalapon to control red rice before delayed planting of rice (A. Ferrero, pers comm.).

The easiest way to obtain selectivity among closely-related species such as rice and weedy rices is to engineer resistance into the crop. It has already been shown that red rice is easily controlled by glufosinate in transgenic rice bearing the *bar* gene conferring resistance to this herbicide [67; 68].

Molecular differentiation of rice strains: **historical evidence for gene flow**

Molecular techniques for distinguishing rice genotypes have been widely published, but predominantly related to their utility for breeding of cultivated types. Only recently have there been a few studies relating to the weedy rices – mainly those thought to be *O. sativa,* but there have been some surprises (Table 1).

Table 1. Molecular genetic characterizations of red and other weedy rices found

Molecular method	Locale	Relatedness	Ref.
Allozymes and morphometry	Asia	Indica-like mimics, indica-like self propagating, and japonica-like self propagating types	[57]
RFLP	Korea	Both indica- and japonica-like forms found	[12]
RAPD	Asia	Forms of indica and japonica and intermediates	[80]
SSLP	USA	Mainly indica but japonica-like and *O. nivara* and *O. rufipogon* found	[90]
SSR	USA	Distinguish domestic from groups of red rice, and hybrids of domestic and red rice	[30]
SSR/AFLP	France	Map weediness genes related to *O. rufipogon*	[10]

Based on RAPD analyses of a large number of weedy rice accessions as well as morphometry and isozyme analyses Suh et al. [80] present a most comprehensive story. They concluded that one group of weedy rices probably originated from gene flow between japonica and indica, another between wild and cultivated indica, one group is comprised of old rice varieties gone feral, and one group arose due to gene flow between wild and cultivated japonica types. Thus, gene flow has been rampant in the eight millennia of rice cultivation, and one can expect it to remain so. The gene flow is related to proximity; in Korea the short grain weedy rices were related only to japonica types and the southern longer grain weedy types similar to indica types grown in the south [80]. Many of the wild relatives of rice are weedy [89], but it was a surprise that some weedy rices in the USA, far from the center of rice origin, were found to be related to *O. rufipogon* and *O. nivara* [90]. How they arrived in the USA is an open question. Further analysis will be needed to ascertain whether they have introgressed genes from cultivated rice, information needed as part of risk analysis.

Most importantly by using controlled crosses between cultivated rice and red rice it was possible to distinguish hybrids using molecular techniques [30]. This is of utmost importance as it allows both the establishment of a baseline frequency of hybridization before transgenic rice is cultivated and before there is a strong selection pressure, that could favor hybrids carrying the new traits of rice. Of course multitudes of laboratory studies with transgenics have reinvented the wheel, they show that hybrids can occur, but they do not allow accurate prediction of the frequency at which they will occur in the field. Most studies have said "slowly", and regulators have permitted commercial release, as will be discussed in the section on introgression of herbicide resistance genes.

Adoption of BD-HR rice Varietal name recognition is considered a key reason for slow acceptance of new rice varieties [63], especially in epicurean countries such as Japan. Genetic engineering of single traits such as herbicide resistance does not substantially change a variety, (except in the particular engineered trait), which is a distinct advantage, so transgenics cannot be a problem in that respect. There are many political and socio-economic reasons why transgenic rices are late in coming. The major stated ones are the varietal nature of rice production (no single variety covers large areas) and the fear of insufficient profit from selling only herbicide (and not seed), and the fact that rice is not a big crop in the parts of the developed world that accept transgenics.

Maize, cotton, and soybeans usually have no close, interbreeding, weedy relatives where they are cultivated; rice does. There is a need to deal with the possibility of introgression of traits into these weed species. In the US, where herbicide resistant rice varieties have been released, these issues were only dealt with on paper with the transgenic varieties. Statements were required by USA regulatory authorities about the possibility of introgression during the registration process. The authorities believe that they have no legal authority to deny registration based on the possibility of introgression, nor do they have the ability to demand that monitoring for such introgression be instated nor do they have the ability to require that failsafe mechanisms to prevent or delay introgression be tested or instituted. Presumably the regulating authorities will obtain the moral or legal authority to do so after the first introgression of herbicide resistance into weedy rice is widespread and they are forced to remember that their responsibility is to farmers and the common weal, not to the marketers of herbicide resistant rice, or to the producers of herbicides. Resistance may evolve on a large scale in the USA, well before such rice is released elsewhere. The USA provides a large-scale testing laboratory to determine the rate of introgression, so that authorities and farmers elsewhere can learn.

Weedy vs. wild species; implications and risk analysis

Discussions of transgenic crops have rarely dealt with the risks from a weed biology perspective. The main risk stated by the detractors is claimed to be that of the transgenics becoming 'volunteer' weeds (in following crops), or their introgressing traits into a wild relative rendering it weedier. An attempt at such an assessment based on weed science was recently made using a defined set of uniform criteria in a decision tree format [37]. Decision trees, by requiring discrete answers to sequential, stepped questions, lower the bias in arriving at conclusions vis a vis the relative risks deriving from a given hazard. The use of the decision tree requires a committee of experts to answer the questions, as a detailed understanding of the crop/weed ecology, population

dynamics, genetics, physiology, agronomy as well as economics are needed to answer the questions. The decision tree delineates places where risk can be lowered by changing agronomic practices. For the purposes of this discussion we will assume a high risk of introgression, balanced with a great agronomic need for herbicide resistance rice. The question to be addressed is how to reduce the risk posed by weedy rice strains introgressing herbicide resistance. These issues as well as the remote likelihood of horizontal gene transfer are discussed at length for all weeds and wild species in ref. [32].

Risks of introgression of transgenes from rice to related weeds

It has already been shown that transgenic glufosinate resistance can be transferred genetically from rice to con-specific red rice [67; 73; 74], how easily this will occur in the field was unclear, as rice is predominately self-pollinated, before flower opening. Rice is a member of genus where there is far too little field information on natural introgression with other weedy and wild *Oryza* spp., despite considerable information in the breeders and cytogeneticists' laboratories. Cultivated rice *Oryza sativa* has an AA genome as does the red and many feral forms of weedy rice that are also *Oryza sativa* [1; 49; 63]. Genes readily move between the cultivated and feral forms, despite rice being predominantly self-pollinating and cleistogamous (most pollen sheds before the anthers protrude from the flowers) [Aswidinnoor, 1995 #17; Brar, 1997 #19 [47; 55; 59; 60]. There are many wild and weedy rice species, one of which, *O. rufipogon* has an AA genome, and is considered a major weed of rice [42]. Another major weedy rice, *O. officinalis* has a CC genome. The ease of homeologous gene transfer from the AA to genome is unknown. There are many other diploid and tetraploid wild (but not weedy) rices bearing genomes through the alphabet from AA to HHJJ. Breeders have transferred genes new traits from many of these wild and weedy species to rice, despite the chromosomal incompatibilities [9; 49]. This often requires embryo rescue and/or intermediate crosses through bridge species. The significance of this to field problems is unclear. The taxonomic differentiation among these species is also rather unclear. In many places where weedy rices are problems it is not known whether the weeds are the con-specific red and feral forms or the other species [6; 14]. Indeed, recent molecular studies have shown that the material in genetic resource depositories has been mis-classified.

Assaying introgression in the field Few contemporary studies dare to comparatively estimate how long it will take to have resistance introgress and predominate in field weed populations vs. how long it would take resistance to evolve by natural selection, vs. the expected commercial lifetime of the herbicide. This can be done with marker genes that require that each plant be analyzed for its presence. It is far more representative as well as allowing large scale field epidemiology to simply insert a gene conferring resistance to a rarely used herbicide as a selectable marker [37]. There would be little consequence of the herbicide becoming unusable due to the resistance disseminating into the wild, as herbicides are not used on wild populations. If weeds introgressed the resistance gene, the herbicide would be returned to the unused category.

Two recent papers from a collaborating group dispelled the myth that the breeding systems of rice would delay gene transfer. The researchers used acetolactate synthase (ALS) level mutant (non-transgenic) rice that had been released for use with imidazolinone herbicides. In the first year following imidazolinone herbicide use the researchers gathered seed from many plants that withstood the herbicide that second year. The ALS target site is highly mutable (typically 10^{-6}) and many of the weedy rice plants were ALS resistant without any cultivated rice morphological traits or molecular markers [20; 29]. Ten times more resistant plants had morphological and molecular signs of being hybrids at an outcrossing rate of only 0.01% [20; 29]. The hybrids found were most probably the result of crossing the resistant trait into wild rice, as wild rice shatters leaving behind seed and hybrids with cultivated rice would have been harvested Previously it was only possible to detect hybrids morphologically in a sea of non-hybrids, and the ability to control most non-hybrids with herbicide made detection simple. Never before had there been such a strong selectable markers for discerning introgression. The hybrids may be unfit in the Haldanian sense (when the selector is not used), and had the imidazolinone herbicide not been used in the second year, the resistant hybrids, might have succumbed due to competition from their susceptible siblings. Their Darwinian fitness (resistance to the herbicide selector) predominated because of the continued selection by repeated use of herbicide.

There will be far greater possibilities of introgression where hybrid rice is grown. This crop is much on the rise in China and its use will surely spread because of the much higher yields,

even when accounting for inputs. The male lines used to obtain the hybrids were bred to overcome cleistogamy. Their anthers protrude, shedding vast amounts of pollen increasing the likelihood of pollinating red rice. Thus, introgressional risks will be greatly elevated where hybrid rice is cultivated and red or other weedy rices are prevalent.

There has been a single attempt to model what might happen by computer simulation [58]. The simulations suggest that hybrids between rice and weedy rice would be a major problem after 2-4 years of herbicide use if there were 5% outcrossing resulting in hybrid formation (the variation dependent on the rate of predators devouring seed). At 1% outcrossing, the problems from hybrids would only be acute after 4-7 years [58]. Alas, the actual field data, with a far lower rate of outcrossing (0.01%), has suggested that hybrids would be an acute problem after 2 years [20; 29], as described earlier. No attempt was made to ascertain the effect of rotating two different herbicide resistant cultivated rice varieties, where the rotated herbicide would kill hybrids, resistant individuals and volunteer rice. Crop rotation has been previously been found to be a better tool for delaying resistance than even the most optimistic models had predicted [70]. The effectiveness of rotations would be predicated on hybrids not having weedy long-term secondary dormancy, i.e. having a seedbank of hybrids that will emerge two reasons hence.

Thus transgenic, herbicide resistant rices may be a very temporary answer to the red rice problems, but cannot be envisaged as sustainable single gene products. Herbicide-resistant rice can be of great benefit if used with care; farmers must delay or counter the further inevitable evolution of resistant weeds. They must also prevent the introgression of genes into con-specific (same species) red rice as well as into related weedy rice species. More sophistication will be needed than the glufosinate, imidazolinone, and glyphosate resistant rices presently being extensively field tested for commercialization, because of the Darwinian fitness advantage of hybrids over their susceptible cohorts. Transgenic insect and disease resistance can also provide a fitness advantage to weedy rices.

More resistances are needed. One can even choose bacterial genes for inexpensive old herbicides such as dalapon, modify them for plant codon usage, and transform them into grains [34]. Even if the dalapon dehalogenase does not confer total resistance, it can lessen the delay in planting time discussed above with non-resistant rice. Such genes have the advantage that it is harder (but not impossible) for the weeds to mimic bacteria than plants in evolving resistance.

Conversely, perhaps herbicide resistance is not needed in rice. In a long review on the needs for transgenic rice varieties from the International Rice Research Institute (IRRI) there is no mention of weeds being a constraint to rice production [18]. The only (passing) mention of herbicides is their use as selectable markers. Or perhaps IRRI is missing out on dealing with the major needs of the aging rice farmer, abrogating them to industry, which will not generate BD-HR rice with resistance to inexpensive generic herbicides, with failsafe mechanisms to prevent introgression.

Preventing and mitigating introgression

It is best to assume that if there has been a proven field movement or unassisted laboratory movement of any genes (transgenes or others) between crop and related weed in the past, then it will occur at some time in the future. Thus, if herbicide resistance in the weed will be a problem, then it is best to consider ways to delay the transfer from crop to related species and to mitigate the effects of transfer. In some cases it is clear that introgression will happen, sooner probably than later; i.e. transgenes from rice to red rice. In these cases, the consequences are great where the weeds exist. There are various failsafe mechanisms that can be used to prevent or mitigate the risk of introgression, when and if it does occur [36]. These vary from management practices (weed free zones around transgenic crops) to techniques that involve breeding, or more biotechnology. Those using a transgenic approach are discussed below, but other approaches such as using apomyxis are also conceivable [37].

Gene placement failsafes

Chromosomal Some crop species such as wheat and oilseed rape are composed of multiple genomes derived from different wild progenitors and often only one of the genomes of the crop is identical to that of a related weed, allowing easy "homologous" gene transfer. Cytogenetic localization could allow one to assure that the transgene is on the incompatible genomes and then ascertain if there is no homoeologous introgression, i.e. crossing over between the non-homologous

chromosomes. There is evidence though for a considerable extent of homeologous introgression between oilseed rape and *B. campestris* [62; 84], so there is little utility in this type of failsafe in oilseed rape. The utility in other crops must be examined. Rice has but the AA genome, as does the red and feral rice, so there is little chance of protection there. The level of homeologous introgression must be determined for other weedy, non homologous rice species, to ascertain whether lack of homology is a barrier..

Hybrids A simple failsafe mechanism can be found with hybrid crops. If a dominant transgene for herbicide resistance is placed in the male sterile line in close linkage with the male sterility gene, there will be no possibility of introgression in crop-production areas. This failsafe mechanism will not really be possible until methods are developed for position-specific transformation, and the position of a major nuclear male sterility gene is known. The hybrid rice now used is all a result of mechanical pollination and then separate harvest of seed from the female parent, i.e. does not use cytoplasmic male sterility. Present transformation technologies gave give rise to random insertion on chromosomes. There would be little value to have the herbicide resistance trait segregate from male sterility.   Much male sterility is cytoplasmic, inherited on the chondriome; chondriome engineering is yet unknown.   If one is engineering male sterility [94] by one of the newer technologies for nuclear male sterility, then the herbicide resistance could be coupled in tandem with the male sterility. Care will only have to be taken in the seed production areas when the male sterile line is restored. Such areas must be kept free of related weeds, a typical precaution in seed production, generally practiced before the advent of transgenics.

Plastome or chondriome maternally inherited traits If the transgene for herbicide resistance is placed on the mitochondrial or plastid genomes, as has been done in tobacco plastomes [16; 48], there should be limited but finite possibility of gene flow, due to the maternal inheritance of these genomes. Species such as tobacco that are often claimed to have no paternal inheritance often have about 0.1-0.5% pollen transfer of traits [4; 17]. The risk of transgenes being established in related populations is far large with herbicide resistant traits where the herbicide exerts selection pressure.

*T*rans-splicing to prevent movement   A system has been proposed and partly demonstrated [81] that was designed for the generation of herbicide-resistant hybrids, where only part of the segregating F2 generation would be resistant. Enzyme splicing in trans was demonstrated using the DnaE intein, which reconstituted functional DnaE protein. The gene for herbicide-resistant ALS was fused in frame to DnaE intein segments capable of promoting protein splicing in trans and was expressed as two unlinked fragments. Cotransformation with the two plasmids led to production of a functional enzyme by protein splicing in trans that conferred herbicide resistance [81]. If each plasmid integrates into a different chromosome, introgressing into a totally-crossing weed will give 25% of the weeds resistant, which is hardly a failsafe. If one of the genes is on a nuclear chromosome, and the other in the plastome, the rate of introgression will theoretically be half that of a whole gene being on the plastome. The rate of introgression will be near zero if one half of the gene could be placed on the plastome and the other half on the chondriome.

Transient transgenics It would be conceivable to insert certain metabolic traits encoding transgenes (e.g. catabolic herbicide resistance) on RNA viruses or in endomycorrhizae that are expressed in the plant, but are not carried through meiosis into reproductive cells. Attempts had been made to use endophytes to carry useful genes into plants (e.g. Bt genes) by pressure-infiltrating the endophytes into seeds [23; 83]. The advantage of the technology was that it was not variety specific and that the endophytes would not be transmitted via seed to the next generation. The technology as developed caused a yield reduction, probably due to the endophyte load. The same concept could be used to carry genes conferring metabolic herbicide resistance, if potent highly expressed genes are used with unobtrusive sparsely growing endophytes are used as vectors.

The same or other infection procedures could be used to introduce herbicide resistance genes into crop seeds using a systemic, but not seed transmissible, disarmed plant disease virus as the vector [37]. This would allow a central seed treatment facility to infect seeds with the genes for herbicide resistance into any rice variety susceptible to the virus. The gene for resistance could not move to other species, and the following generation of crop seed would not be herbicide resistant. Additionally, the transgenes would not be found in crop seed, an advantage in today's scientifically-irrational market place.

The possibility that such a procedure might work was borne out in many cases with dicots showing that they express encoded genes, e.g. [50]. It was possible to infect *Arabidopsis* with tobacco etch virus carrying the *bar* gene; the plants were resistant to glufosinate [93]. Cucurbits

artificially were infected with an attenuated zucchini yellow mosaic potyvirus containing the same transgene and the plants were herbicide resistant in the field [77]. Recent experiments demonstrating that an NTPII carrying wheat streak mosaic virus could be used to infect various grains [13]. They showed that the NPTII was expressed (immunologically) but not that the plants had antibiotic resistance. The virus carrying the genes was expressed in the roots following leaf infection, though not in all tissues [13]. Considerable technological obstacles of seed infection will have to be worked out. There are safety issues about the mode of disarming to be considered, and that there is a total lack of gene introgression from the virus to the plant chromosome, and total non transmission of the virus through ovules or pollen. Still, there are many crops, especially those with related, introgressing weeds (e.g. sorghum, barley, rice, sunflowers), where such a technology could be very worthwhile in safely solving weed problems. One would have to transfect the crop every generation, but the transgenes could not spread sexually.

Clearly the vilified 'terminator' technology [15; 69] recently renamed as GURT (genetic use restriction technology), would be an effective failsafe if it is foolproof. A chemically induced promoter that irreversibly turns off the transgene for (e.g. herbicide resistance) in the reproductive tissue is used. The chemical inducer is applied to seed just before planting in the farmers' fields, allowing the farmer to use the herbicide, but the following generation of crop, of volunteer weeds and of crop–weed hybrids will not possess the trait, and will be controlled by the herbicide. The treatment would have to provide 100% shut down of the transgene and could not segregate or become silenced. Whether this is the case is not yet known.

Terminator technology Chemically induced promoters If a herbicide resistance transgene is placed behind a strong chemically-induced promoter, there will only be resistance when the chemical inducer is used. Such a promoter was patented for use with a glyphosate-resistant EPSP synthase gene [46]. As glyphosate kills slowly, and inducers supply products within hours, the chemical inducer can be treated along with glyphosate. The herbicide can be used without the inducer as a pre-plant treatment, or in a naturally resistant crop to control the volunteer weed and weed crop hybrids the following year. If the herbicide resistant gene were to introgress to a wild species that does not inhabit agro-ecosystems where herbicides are not used, it would be of little value and would probably have enough of a fitness penalty and will not become established. A system that turns on transgenes, such as this may be preferable to one that turns them off, such as terminator (GURT). If the terminator gene is silenced, there is a possibility for stable expression of the transgene. If the inducer gene is silenced, then those individuals possessing the mutant are killed, and the germ-line not continued. Still, there is the possibility of an inducible promoter mutating to become a constitutive promoter, rendering progeny resistant.

Transgenetic mitigation (TM)

Genetic engineering can be used to mitigate any positive survival traits transgenes may confer after introgression from crop to weed. If the herbicide resistance gene engineered into the crop were flanked on either side by a transgenetic mitigation (TM) gene in a tandem construct, the overall effect would be deleterious to weeds introgressing the construct from a crop [36]. This is based on three premises: (a) Tandem constructs of genes genetically act genetically as tightly-linked genes and their segregation from each other is exceedingly rare; (b) There are traits that are either neutral or positive for a crop that would be deleterious to a typical or volunteer weed, or to a wild species; and (c) Because weeds are strongly competitive amongst themselves, and have large seed outputs, individuals bearing even mildly deleterious traits are quickly eliminated from populations. Even if one of the TM alleles mutates, is deleted, or crosses over, the other flanking TM gene will remain, providing mitigation.

TM traits that could be used are best visualized when observing the differences between crops and weeds. This is best illustrated with the potentially high-risk rice, and its weedy relatives, as summarized below.

Traits for transgenetic mitigation

Seed dormancy Weed seeds typically have secondary dormancy, with seeds from one harvest germinating throughout the following season, and over a number of years. This evolutionary trait is considered to be a risk-spreading strategy that maximizes fitness while reducing losses due to sib competition [45]. Staggered secondary dormancy prevents all the weeds from being controlled by a single agronomic procedure. Crops have lost secondary dormancy as a result of domestication. In places where rice has been continuously cultivated, there is a selective advantage for red rice not to

have secondary dormancy, and indeed there are a few populations from such areas with very low secondary dormancy, while most red rice has varying amounts of secondary dormancy [57]. Are these differences due to introgression from rice or to internal evolution? DNA relatedness studies might answer this question.

Genetically abolishing secondary dormancy would be neutral to both rice, but deleterious to red rice in most cases. Tillage, crop rotation, and preplant use of herbicides, all standard practices would control the uniformly-germinating TM weed seeds lacking secondary dormancy in rotational crops.

Ripening and shattering   Weedy rice species disperse their seed over a period of time and much of the ripe seed "shatters" to the ground, insuring continuity. A proportion of the weed seed is harvested with crop seed, contaminating the crop seed and facilitating weed dispersal to wherever the crop seed will be grown. Weeds have evolved morphological and phenological "mimicries" to the crop seed [7; 31], necessitating continual evolution and refinement of techniques to remove the contaminating weed seed.

Uniform ripening as well as anti-shattering genes would be detrimental to weeds, but neutral for crops that ripen uniformly and not shatter, and positive for recently domesticated crop species such as oilseed rape, which still have a shattering problem.

Dwarfing   Crops had been selected for height, to outgrow weed for millennia. Weed evolution kept apace, selecting for taller weeds. The advent of selective herbicides to kill weeds allowed for genetic dwarfing of these crops, with more seed harvest, and less straw. Various new systems of genetically engineered height reduction are being introduced. These include genes relating to hormone production [5; 71; 76], as well as those dealing with shade avoidance [72]. Shade avoidance is advantageous when competing with other species, but not in a weed-free crop stand where only siblings are competing by shading. The overexpression of specific phytochrome genes prevents recognition of shading and thus the plant remains short [72]. This is advantageous for a crop and could also be used where the native dwarfing genes presently available to breeders prevent obtaining the highest yields because they are closely linked to deleterious genes. It has recently been shown that the mutant dwarfing gene laboriously crossed into rice resulting in the green revolution varieties is a mutation in the gene encoding gibberellic acid$_{20}$ oxidase [75]. Dwarfing would be disadvantageous for a weed that must compete with the crops; it would be shaded over by the crop and weeds. Transgenic dwarfing in tandem with herbicide resistance would be ideal for the high value aromatic (perfumed) tall basmati type rices, which cannot be dwarfed by genetic "green revolution" technologies, without losing the aroma. Transgenically dwarfed perfumed rices should have aroma as well as high harvest index, as well as the herbicide resistance (with failsafe) needed for their cultivation.

Susceptibility to herbicides   It is possible to envisage using genes that would render a weedy or wild species that introgresses the primary transgene of herbicide resistance susceptible to other herbicides. This could even be to sensitivity to a specialty herbicide, used just for the purpose of disposing of crop-weed hybrids and their offspring. Such herbicides could also be used to control the transgenic crop when it is a volunteer weed in following crops.

Balancing primary and TM traits

If the primary transgenic trait confers an advantage to a weed or wild species; how much will TM traits actually counter-balance that advantage? Weeds are not only highly competitive with crops, they are competitive with weeds of other species as well as within their own species. Weeds typically produce thousands of seeds in steady state conditions, to replace a single plant, suggesting extreme competition to be the replacement; the selection for the highest competitive fitness is intense. The rare individuals that introgress the tandem constructs should be rapidly competed away, as demonstrated in a model system with tobacco [2].

Repercussions of introgressing TM

After a weed introgresses a transgene and then stabilizes (eliminates cytogenetic incompatibilities), the trait could quickly spread through a population, even if it has a marginally positive fitness advantage [82]. Conversely, one can balance the disadvantage of TM traits against the advantage of the primary trait. This must be done in both in the presence and absence of the herbicide, as herbicide resistance only provides an advantage to a weed when the herbicide is used. Indeed, when the herbicide is not present, the transgenic resistance trait can be disadvantageous; as has been demonstrated with an ALS resistance transgene [8]. Each TM trait should work in a

balance with the primary trait, and it might be necessary to have more than one TM trait in a construct to obtain negative balance. Combining TM traits with a cytogenetic failsafe, where these are available, can further decrease the risks of introgression.

Even if one or two TM genes were to confer some unforeseen and unforeseeable advantage in the future, this would be akin to evolving resistance to a herbicide. The first case would be reviewed and a decision could be made whether the situation warrants removing the TM transgenic crop from market to prevent spread and the occurrence of further cases. The only aspect that is predictable is the segregation of tightly linked genes, and that is why flanking the primary gene on both sides should be used. The segregation of a gene would visible (especially in the case of dwarfing TM genes), allowing any such material to be removed from the breeding population.

TM genes are available to mitigate movement of resistance

Some possible traits discussed above for TM constructs just exist as named genes that are inherited, others are also mapped to positions on various chromosomes, and a few are actually characterized as sequenced genes. Thus, not all TM traits have genes that are immediately available for insertion in tandem constructs. Still, there can be many different ways to confer a TM trait, and thus, more than one TM gene is available.

Secondary dormancy. Arabidopsis, the typical source for genes, has already been sufficiently domesticated that it is unlike cruciferous weeds; the laboratory strains no longer have strong secondary dormancy [88]. A mutant that is insensitive to abscissic acid and lacks secondary dormancy was found in a wild, undomesticated *Arabidopsis* strain [79]. Such a gene might be useful.

Shattering Physiologically, one way to avoid premature seed shattering is to have uniform ripening. The hormonology of the abscission zone controls whether shattering will occur and it is possible that if cytokinins are overproduced, then shattering will be delayed. The cytokinin pathway is well documented and there are genes that could be put it constructs of cytokinin overproduction [51; 61]. A SHATTERPROOF gene has recently isolated from *Arabidopsis* that prevents seed shatter by preventing seed dehiscence. What the phenotype of this gene would be in rice is an open question.

Dwarfing Many of the genes used for breeding dwarfism seem to have an unknown function. Still many genes are known, that control height.

Gibberellins Preventing the biosyntheses of gibberellins reduces height [92], which is the basis of many chemical dwarfing agents used commercially in grains. The three enzymes and genes controlling various steps in gibberellin biosyntheses are known and cloned [39; 40; 54; 95]. *Arabidopsis* mutations bearing mutations in any one of them are dwarfed, and the dwarfing is reversible by gibberellin treatment. Overexpression of a gene coding for *ent*-kaurene synthase, causing co-suppression also mimicked the mutant phenotype. Additionally, a defective GA receptor gene has recently been isolated that confers gibberellin insensitivity when transformed into rice (*GAI*) by competing with the native receptor; thereby inducing dwarfing [71].

Shade avoidance Various forms of the pigment phytochrome interact to detect whether a plant is being shaded [19; 78; 85]. The engineering of suppressive overexpression constructs of one of these phytochromes led to plants that did not elongate in response to shading [72].

Genes for susceptibility to herbicides and other toxicants At least one gene and the chemical pair is already available; a bacterial P450 that activates an experimental sulfonylurea pro-herbicide [66]. It has been used under a tapetum-specific promoter to prevent pollen formation, but could be used under a general promoter that would allow the use of the pro-herbicide to cull crop-weed hybrids, as well as volunteer crop weeds.

Other such pro-herbicide, exogenous activating gene could also be envisaged, for use as mitigating failsafes, should the primary transgene escape. Other chemically inducible suicide (*kev*) genes that could be considered for such purposes are discussed in a different context [32], but could also be considered here.

Concluding Remarks

There is a conundrum with rice: transgenics are direly needed, especially because of otherwise intractable weed problems; the transgenes are direly risky as they can introgress into the most intractable weed. There are failsafe mechanisms that can be tested to ascertain their utility in preventing, delaying such introgression or mitigating its effects. It would be unwise to release transgenic rice bearing traits that might enhance weedy rice fitness, unless effective failsafe mechanisms are utilized.

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Transgenes in maize landraces in Oaxaca: Official report on the extent and implications

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In November 2001 a report was published indicating that transgenes had been found in landraces of corn grown in the sierra of Oaxaca, Mexico (Quist, D. & Chapela, I.H., 2001 Nature 414:541-543). This report initiated a long debate on three main subjects: 1) The technical qualification of the report; 2) The validity of the results presented and 3) The possible consequences and implications that such an event could have.

As a consequence of such report the Mexican Government initiated a preliminary sampling and analysis through the National Institute of Ecology which indicated the presence of transgenes in corn in two states. Based on these findings, the Secretary of Agriculture, requested an investigation into the subject. An "ad hoc" committee was formed which included experts from different areas of expertise. The first step was to devise an approach to obtain representative samples from the State of Oaxaca and the neighboring State of Puebla. Once the sampling strategy had been planned it had to be implemented ensuring the "chain of custody" and that all relevant information was obtained for each sample at each location. The samples where then processed and distributed to the institutions that were going to carry out the testing. Tests performed on the samples included PCR for general transgenic traits such as the 35S promoter, NOS terminator or cry genes; protein analysis using "strip tests" and ELISAs for specific proteins such as PAT, CP4, Cry1A and Cry9C; sequence and Southern blot analysis to confirm the findings and identity of some of the genes found.

Up to this moment, the results presented by the Mexican Government have shown that transgenes such as cry1A can be found extensively in land races throughout the State of Oaxaca. The presence of cry9C has not been detected in any of the samples tested. As for any apparent consequences to the landraces themselves, this so far has not been the case. The small growers have not reported any phenotypic changes in their crops that could suggest that a major modification could take place. The changes observed are those expected when the farmers use a hybrid to "enhance" or improve their landraces, a practice that is very common among small growers in this area.

Farmers management of maize landrace diversity. A case study in Oaxaca and beyond

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Abstract

To assess the impact of farmer management on maize landrace diversity in the Central Valleys of Oaxaca, Mexico, where landraces comprise most of the maize grown, we interviewed farmers in six villages and collected and evaluated samples of seed. Among other things, we found that

- the level of deleterious and lethal mutations is high in the landraces;
- frequent seed and pollen exchanges, i.e., extensive migration and gene flow among landraces, result in a low differentiation between landraces; and
- these same landraces show a strong differentiation for traits under selection by farmers (mainly ear traits).

We also report on prior studies in Cuzalapa village, southwest Mexico and in Burkina Faso, which also examine the impact of migration and geneflow in open genetic systems. Because of high levels of recombination, most genes introduced from exotic varieties will behave independently and their diffusion is favored by seed and pollen exchanges. At the same time, these exchanges are components of the mechanisms that maintain and enhance the genetic diversity and viability of these landraces. Strategies that would restrict them will erode geneflow and result in genetic erosion.

Keywords: maize landraces, farmers' management, genetic diversity, Mexico

Introduction

Modern or industrialized agriculture is organized in a series of well-defined and specialized activities from the creation of new varieties to the utilization of products by consumers. Farmers are one link of this long chain; their activity being limited to production. Genetic diversity management and genes are under the custody of seed companies. Most of the transgenic varieties produced so far have been created for and released in this environment. Consequently, most of the biosafety rules related to geneflow have been established for this targeted environment.

However, another type of agriculture—traditional agriculture—is found in many parts of the world. In these agricultural systems, farmers create and conserve new varieties, the preponderance of which are landraces. In many cases, the farmers or producers are also the consumers of their products. They decide which materials to conserve or discard and they recycle seed on their farms, which are very often quite small. Such systems are very dynamic in terms of genetic content and adaptation of the landraces. Not surprisingly, regions known as centers of diversity are generally areas where farmers have been and are still actively cultivating local landraces, often for a combination of ecological, agricultural, and cultural reasons (Berthaud et al., 2001). Clearly, the consequences of introducing new and foreign varieties into such systems will differ significantly from similar introductions into modern agricultural systems. These circumstances must be considered if we want to establish effective biosafety rules related to release of transgenes in a

"new" environment.

In this paper, we describe the impact of farmers' management of landraces on their genetic diversity, their viability, and stability. To do this, we present a case study on maize landraces from the Central Valleys of Oaxaca (Mexico) and discuss some cases from other areas and countries to broaden the scope of our conclusions. Our results could be useful in establishing biosafety rules related to geneflow that are adapted to the specific situation of crop centers of diversity.

Case Study in Oaxaca and Beyond

Our case study is part of a broader project on conservation of maize diversity. The project was based in six communities (Fig. 1) of the Central Valleys of Oaxaca (Mexico), where almost no modern maize varieties have yet been planted. The project compared different participatory interventions to support farmers' efforts to conserve maize landraces on farm. One of these interventions consisted of identifying a subset of landraces that captured the diversity present in the region while at the same time were of interest to farmers (Bellon 2002a). This subset was selected from a larger collection that is representative of the regional diversity of maize landraces, collected as part of the project. For two years (1999 and 2000), the "elite" landraces were planted in demonstration plots in the project communities. At harvest, farmers were invited to visit these plots and could purchase seed of any of the "elite" landraces. The objective of this intervention was to foster experimentation among farmers and to enhance their access to the regional diversity. The project also included a detailed monitoring of participant and non-participant farmers to assess impact on farmers' livelihoods (Smale et al., 2002). For a description of the project and its main results, see Bellon et al. (2002b). The project also included a component that focused on the population genetics of these landraces and links to farmers' management. In this paper, our analysis is limited to assessing the dynamics of this genetic diversity and the impact of farmers' management of this diversity, based on information gathered from a sample of farmers in the study area.

Fig. 1. Map of the Central Valleys of Oaxaca (Mexico) with the six studied villages. 1. Huitzo, 1730 masl; 2. Mazaltepec, 1700 masl; 3. San Lorenzo, 1830 masl; 4. Amatengo, 1310 masl; 5. Valdeflores, 1447 masl; 6. Santa Ana, 1520 masl.

Material and Methods

We selected two samples of farmers from the project's six villages: (1) a random sample of farmers, for a description of diversity, from which we analyzed 31 seed lots: and (2) a sample of 59 farmers that participated in the project by purchasing the project's elite landraces, allowing a

comparison of management of local landraces and the introduced elite landraces. Information was obtained through interviews with farmers in January 2000 for the random sample and January 2001 for the sample of farmers who bought seeds from the project in 1999 and cultivated them, for one or two seasons.

Questions addressed were related to the history of the landraces, including number of years of cultivation, cultivated area, and total or partial replacement of the landraces. Total replacement occurs when a farmer decides to stop growing a particular landrace and subsequently sows another one. Partial replacement or seed mixture occurs when a farmer needs seeds to complement his or her own stock and gets seed from outside the farm.

When possible, all the different varieties cultivated by each farmer were collected. Forty ears were requested per accession. Field experiments were established to evaluate the agromorphological traits of the landraces, based on quantitative genetic tools. To conduct the quantitative trait analysis, we planted 12 seeds/ear from 18 ears taken from each of the 31 seed lots. The resulting plants were evaluated for plant and ear development and kernel traits. For neutral marker analysis, we characterized 20 plants per seed lot for twelve nuclear microsatellite markers and one chloroplastic marker.

The deleterious and lethal mutant frequency was estimated by detecting the presence of these mutants in self-pollinated progenies produced from the collected accessions and elite landraces.

Results

Dynamics of the management

The 59 interviewed farmers had a total of 101 landraces and 4 materials of hybrid origin (*acriollados*); and purchased 108 materials from the project. The data on the farmers' 101 landraces shows that their management is dynamic. Seventy-two materials had been maintained without any known changes or mixing (Table 1), 23 of those materials (32%) had been grown by the farmer for less than five years; equal to the number and percentage that had been grown without change for 20 years or more. The remaining 19 of those landraces (26%) had been added during a 5–19 year period. Nineteen materials had been mixed with other materials, most within the last five years. Of the total 101 materials, only 23% had been with the farmer for an extended time (20+ years) and without change.

Table 1. History and dynamics of a sample of maize landraces, Central Valleys of Oaxaca, Mexico

Years without change/since last partial replacement	Landraces without change		Landraces with seed mixing	
	All materials	**White grain**	**All materials**	**White grain**
< 5 years	23	13	11	9
5 to 9 years	8	5	3	2
10 to 19 years	11	6	3	2
Over or equal to 20 years	23	14	0	0
Total	72	38	19	15

Note: Not all varieties had a well-dated origin or information on when they were mixed.

Seeds of the landraces had several geographic origins. While most had a within village origin (87%), others may have been obtained from another village (5%), or from a local or regional market (8%).

Of the 108 elite landrace materials purchased from the project, 66 were planted in 1999 and 2000. Farmers who planted 44 of these materials said in January 2001 that they had seed and

Oaxaca maize landraces
Quantitative data on ear morphology
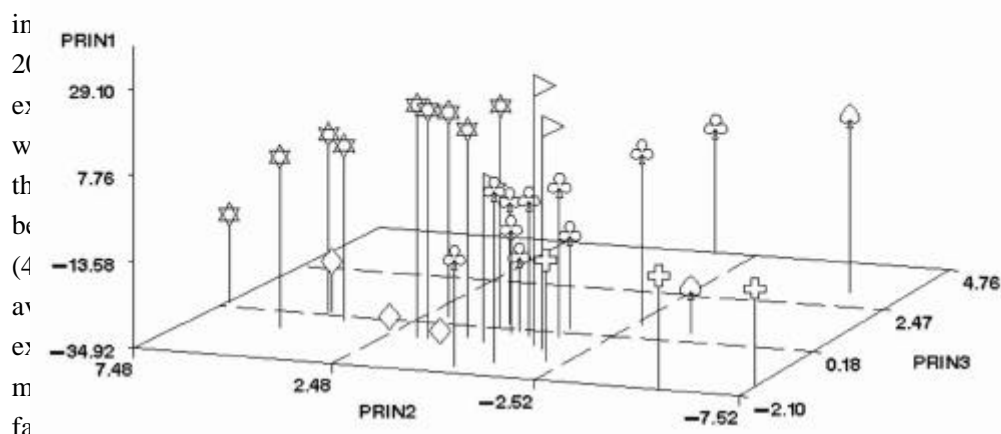
in ... summer of 2(... ore seed to e) ... said that w ... uld obtain th ... ted. If we b ... d of 61% (4 ... ds on the a ... e able to e) ... y plant. In m ... es. Not all fa ... g. In some cases, the farmers ran out of seed even though they wanted to continue planting that particular landrace.

Farmers'selection and population size

Generally, farmers select seed for the next planting season at home, when husking and shelling ears. They chose the best ears based on an explicit or implicit ideotype for traits related to ear and kernel traits. In the study area, selection was not conducted on plants in the field, but on ears after harvest.

Typically, farmers use a planting density of around 50,000 plants/ha. With an ear bearing 200 seeds, 250 ears are needed for planting a hectare. Plots smaller than a hectare are frequent, so in many cases farmers are managing relatively small populations in terms of effective genetic size. This effective population size can become very small when farmers experience unfavorable planting conditions, leading to a very low harvest and a small quantity of seed, which translates into genetic bottlenecks for the maize populations.

Replacement and adoption

When it comes to adoption of new landraces, farmers are quite cautious and are reluctant to take risks with their crop. They favor stability, but at the same time are very willing to experiment on a small-scale basis. Landrace purchases from the project by farmers ranged from 1 to 4 kg. We concluded from this that the first planting was an evaluation and for seed increase. Decisions about adopting or abandoning the new landraces were made after at least one experiment. Adoption could be undertaken as a new landrace per se or by mixing seeds with a landrace already in the farmer's possession. This shows that diffusion of any new landrace or variety is a slow and careful process that involves only small quantities of seeds.

Genetic structure

The results of the quantitative trait analysis show that based on quantitative ear traits there is a strong grouping of farmers' landraces by village (Fig. 2). This means that landraces from the same village are more similar than comparisons made with those from other villages, however, there is still a lot of diversity within a village. Since ear characteristics are the main traits farmers select for, this result illustrates the role of farmer selection in structuring maize diversity in this region.

Fig. 2. Principal Component Analysis for ear and kernel traits of maize populations from the Central Valleys of Oaxaca. Populations from six different villages.

The results of the analysis of nuclear and cytoplasmic markers (neutral markers) show that there is very low differentiation among landraces. This means that most genetic neutral diversity may be found within the seed lots. This result and our surveys (Table 1) suggest that a large amount of genetic migration among farmers' landraces takes place in this region. All populations, even those separated by up to 100 km, were found to share chloroplastic DNA haplotypes. This can be explained by long distance geneflow (i.e., seed exchange between villages). Within-village differentiation for both classes of markers is compatible with farmers' practice of selecting a reduced number of ears for planting the following season. This practice results in a limited but nevertheless significant level of local drift. These maize varieties have high levels of diversity maintained by high levels of migration among varieties.

Taken together, these results indicate that these two classes of markers provide very different data about maize population dynamics. While there is little differentiation for neutral markers due to significant geneflow among farmers' landraces, there is a strong differentiation for ear traits due to farmer selection. This means that parts of the genome are under strong selection by farmers, while others are not.

Mutations

An initial experiment conducted to measure the lethal and deleterious mutations present in these landraces detected high rates of deleterious mutations. On average, in the 17 elite landraces studied by the project, 53% of the plants showed a defect. The remaining landraces are being studied in this ongoing experiment, but preliminary results show a similar rate of accumulated mutations.

Acriollamiento or management of modern varieties in traditional agriculture

In another project (Bellon et al., 2002c), management of modern varieties within traditional systems has been studied on the coast of the state of Oaxaca and in Chiapas (Mexico). In these areas, traditional farmers have access to improved modern varieties derived from the tropical maize race Tuxpeño. This research shows that farmers apply the same management to the modern varieties as that given to the local landraces, and that in many instances, they favor mixing the two types. This process is called "acriollamiento" or local adaptation.

Case study in Cuzalapa, Jalisco (Mexico)

In a study published by Louette et al. (1997) on research conducted in Cuzalapa, it was again shown that seed exchange between farmers and partial replacement were quite high. Of 484 fields in the study, planted with 25 local landraces, it was observed that farmers used their own seeds in only

53% of the fields. In the other fields, seeds were obtained either from the same village (36%) or neighboring villages (11%).

Case study in Burkina Faso

In Burkina Faso, West Africa, maize cultivation may be classified into two very compartmentalized types. Early, yellow material is planted by women in their backyards; late, white maize is planted by men in larger plots, away from the village. Sanou et al. (1996) have shown that geneflow (genes from an improved modern variety distributed recently in this region) takes places between the two distinct types; genes from a modern variety, consistent with the second type of cultivation, were found in the landraces of the first type. We can conclude that this physical and cultural isolation is not effective in avoiding the exchange of genes between maize varieties.

Discussion

A metapopulation model

We have seen that very few farmers practicing traditional agriculture in the study area maintain their landraces and seeds over generations without change. Seed management by farmers in the study area stands in strong contrast to that of modern agricultural systems in which the commercial varieties comply with the three criteria of distinctness, uniformity, and stability. Indeed, these three criteria have no bearing at all on the management of diversity by these farmers. In fact, the criteria used in traditional agriculture emanate from the farmers who must react to many constraints: agroecological, economic, cultural, etc., and who modify (adapt) their varieties accordingly, using a common genetic background. Each landrace has a distinct history, which makes the definition of variety in this context very difficult. Ultimately, what matters is the evolution and resilience of the whole set of landraces cultivated in an area, the extinction of a particular landrace can be compensated for by the cultivation of a new landrace. These populations follow a metapopulation dynamic (Hanski and Gilpin, 1996), in which the metapopulation is maintained so long as there are more colonization than extinction events. Stability is also gained through exchanges. A farmer will receive a new landrace from one of his/her neighbors and pass it on to others. This neighbor can then lose the landrace, but will be able to start again with a new seed lot provided by those who were previously given the seeds. Partial replacement is also a factor of stability because it allows for slow changes and adoption of new landraces through local experimentation.

A genetic rescue hypothesis

Farmers' management relies on metapopulation organization with strong geneflow (migration rate). We have seen that this geneflow is mediated, in large part, by seed exchange and seed mixture and not only by pollen geneflow. The fact that geneflow is tolerated and in some cases favored by most of the farmers can also be seen as a strategy of genetic rescue (Keller and Waller, 2002). Given that mutations tend to accumulate in the absence of selection (Higgins and Lynch, 2001), and, as shown before in these landraces, many parts of the genome are not under selection, local landraces are prone to mutation accumulation. Deleterious mutations are expressed in homozygous plants. Geneflow promotes heterozygous plants, thus mitigating the expression of the deleterious mutations. Our hypothesis is that geneflow plays an important role in preserving the viability of these landraces for farmers. The logical consequence is that geneflow can be seen as a part of an integrated genetic system (the farmers' genetic system). Geneflow is not only a source of new diversity but may also be a "repair tool" for those landraces that have accumulated mutations, i.e., that are "getting tired"

according to an expression used by the Oaxacan farmers.

In this traditional system, limiting the existing geneflow for biosafety or other reasons without changing other components of the farmers'management would lead to a loss of viability of the local landraces and their abandonment by farmers.

Issues for a biosafety policy in traditional agriculture

Due to permanent geneflow between different landraces, the probability is high that in these traditional agricultural systems, genes from introduced varieties will find their way into the local landraces. We foresee at least two implications in terms of biosafety;

1) One could be tempted to establish strict rules and genetic barriers to restrict geneflow from the introduced varieties, in order to keep the landraces free of their genes. However, before establishing such rules and policy, one should carefully study the impact of such measures on the flow of other genes, and on the viability of the current landraces. In effect, if we consider our hypothesis that geneflow is one element of the farmers'genetic system, modifying it will have consequences on the adaptability and acceptability of the currently cultivated landraces.

2) What if a gene diffusing from a variety that complies with all the biosafety requirements is later found to be harmful long after the initiation of the diffusion process? How can we return to the pre-diffusion situation? i.e., How can this system be made reversible? Could this be accomplished by avoiding any new geneflow, or through more geneflow from landraces and varieties that are free of the offending gene? Are other options available? Overall biosafety will increase when rules and strategies are defined to establish when reversibility is needed and how it should be implemented in traditional agricultural systems.

Traditional farmers'management of diversity and traditional agriculture are not static. This implies that if we want to have a framework of effective biosafety rules in these traditional systems, we must consider all of the relevant variables and components of these systems. It is a challenging task.

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Concerns about the effect of transgene introgression in maize landraces and teosinte

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Mesoamerica is a region where plant domestication occurred about 10,000 years ago. Most scientists currently agree that maize was domesticated in Mexico and descended from an annual species of teosinte (*Zea mays* ssp. *parviglumis*). Costs and benefits of transgenic crops for Mexican agriculture have been the subject in several forums for the past ten years. Today, an intense debate continues, and this issue has been raised questions about the extent of the knowledge available about the long-term effects of this technology on biodiversity in centers of origin of cultivated plants, already threatened by habitat alteration. This paper presents data related to the importance of *Zea* species in Mexico and information about ongoing research that may help to conduct a scientific risk assessment for transgenic maize technology adoption. Great advances have been made in knowledge of the natural distribution of teosinte in Mexico, more gene-flow studies between maize and teosinte have been completed and more knowledge about genetic diversity, genetic incompatibility systems, and about insects that affect teosinte and maize are available. The most important concerns that have influenced the debate about the eventual release of transgenic maize in Mexico have been questions about the potential of transgenic maize to modify genetic diversity of landraces and their quality as food. Another set of concerns is related to the risks associated to transgene escape and its dispersal into teosinte species and potentially enhancing their ability to survive or compete with another species. As a result of several national and international conferences, consensus exists indicating that current knowledge in Mexico is insufficient for assessing risks and benefits of transgenic maize. It is critical to develop a system for risk assessment within the context of current practices and threats to understand the impact, if any, from modern varieties (conventional and transgenic) on genetic diversity of landraces and teosinte.

Possible effects of transgenes on genetic diversity

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Abstract

In this presentation, I will assess a number of issues related to the potential effect of transgenes on genetic diversity. Firstly, I will consider what is genetic diversity and how do we characterize it? Secondly, I will examine how whether diversity might be threatened, mainly in the wild gene pools. Thirdly, I will ask whether transgenes or transgenic cultivars play a special role, distinct from that of genes incorporated into cultivars by classical plant breeding, in threatening genetic diversity. Throughout my talk, I will discuss published data as well as data from my own research program on gene flow between wild and domesticated beans in Mexico, one of the centers of domestication of this crop.

Keywords: Genetic diversity, gene flow, domestication, wild progenitors

Genetic diversity and its characterization

The importance of genetic diversity of crops can be examined from two different perspectives. From one of them, genetic diversity may be a necessary condition to achieve high productivity and yield stability. From the other perspective, genetic diversity is the raw material used by plant breeders to develop improved plant varieties.

In an ecological sense, there is a general consensus that increased complexity is usually associated with greater productivity or stability of ecosystems (McCann 2000; Tilman *et al.* 2001). This relationship has recently come to the fore again because of increasing concerns with biological invasions. However, there is no unanimity on a simple relationship between diversity and stability or resilience (Loreau and Behera 1999; Pfisterer and Schmid 2002). This complexity, however, is usually described as a species complexity but does not address directly intraspecific genetic diversity (*e.g.*, Ives and Hughes 2002). Of interest in this discussion is obviously diversity present within species. Does genetic diversity increase the productivity of a crop grown in monoculture, *i.e.*, does growing a mixture of genotypes lead to higher yields than genetically pure stands? Here again, the results are mixed as illustrated by the results of (Schultheis *et al.* 1997) in cucumber, showing that only a very specific pair of cultivars yielded higher than either of the components. In developing countries, nevertheless, may grow mixtures of genotypes (*e.g.*, Martin and Adams 1987a, b) not only to maximize yield, but also to satisfy different needs such as different dishes and to minimize risk.

The second perspective deals with the utilitarian aspect of genetic resources in a breeding perspective. Until the advent of plant transformation technologies, access to genetic diversity in breeding programs was limited by sexual incompatibility. Plant breeders recognized three major gene pools based on the degree of sexual compatibility (Fig. 1; Harlan and de Wet 1971). Crosses within the primary gene pool, which includes the crop and its wild progenitor, do not encounter any reproductive isolation, in contrast to crosses between the primary gene pool, on one hand, and the secondary and tertiary gene pools, on the other. Plant breeders have traditionally emphasized closely related, well-adapted domesticated materials within the primary gene pool as sources of diversity (*e.g.*, Kelly *et al.* 1998). More recently, however, plant transformation and genomics have led to a fourth gene pool. Transgenesis allows us to bypass sexual incompatibility barriers altogether and introduce new genes into existing cultivars. It should be emphasized here that the major function of transgenic technologies is the creation of new cultivars but the generation of new gene combinations that can be used in breeding programs (Gepts 2002). Comparative genomics provides the means to identify sequences in a crop of agronomic interest based on homology for DNA sequence, transcription patterns, etc. with similar data in model systems such as Arabidopsis and rice (Gepts 1999).

In the last decades, awareness of the rich diversity of exotic or wild germplasm has increased leading to more intensive use of this germplasm in breeding (Frey 1975; Stalker 1980; Rick 1982). The use of molecular markers has facilitated the identification of genes of agronomic interest in wild germplasm through the dissection of quantitative traits using linkage-map-based approaches (Tanksley and McCouch 1997). The same technology helps transfer these genes into superior

varieties and accelerates the whole breeding process. Thus, genetic diversity of crop relatives is an increasingly accessible resource that needs to be protected for current and future use.

A necessary condition for any genetic diversity study is the availability of adequate plant material samples. Large germplasm collections exist both in the U.S.A. and internationally. However, these can only provide the materials for certain types of experiments or hypotheses such as germplasm-wide analyses of diversity. For other experiments, such as comparisons of genetic diversity in relation to specific spatial distribution patterns, special surveys and collections have to be conducted.

Characterization of genetic diversity of organisms can be achieved with phenotypic traits and molecular markers. Both traits have their advantages and disadvantages and they may not always be correlated. Phenotypic traits have the advantage that they may be directly related to the fitness of the populations and the usefulness for plant breeding. A thorough evaluation of these traits, however, requires multi-location, multi-year trials to account for environmental effects and genotype x environment interactions.

There currently exists a wide range of molecular markers that can be used to characterize genetic diversity, each with its own set of advantages and disadvantages. Many of these classes of markers, such as RAPDs and AFLPs require little prior genetic knowledge of the species of interest. Microsatellite markers are vary attractive for gene flow studies given the high level of polymorphism and their co-dominance (Rafalski and Tingey 1993). It is becoming increasingly easy to develop these types of markers (Zane *et al.* 2002). Furthermore, genomics efforts in major crops provide additional markers, such as microsatellites (*e.g.*, Marek *et al.* 2001)and candidate genes for specific phenotypic traits (*e.g.*, maize: (Wang *et al.* 1999, 2001); common bean: (Geffroy *et al.* 1999; Geffroy *et al.* 2000). Assessing diversity with actual genes responsible for evolutionarily important traits affecting fitness, such as reproduction, growth habit, resistance to diseases, and tolerance to abiotic stresses, may assist in improving the correlation between molecular and phenotypic analyses of genetic diversity.

Joint analyses of molecular and phenotypic diversity, as well as attempts at predicting the breeding value for different phenotypic traits depending on the molecular marker diversity or genotype of the parents, generally show a poor correlation between the two types of data (Reed and Frankham 2001). This situation can be attributed to a variety of reasons, principally the lack of tight linkage between molecular markers and genes coding for phenotypic traits. Other possible reasons include the lack of correspondence in gene action between phenotypic traits (additive, dominance, or epistatic actions) and molecular markers (indirect measure of additive gene action), differences in heritability (low to high for phenotypic traits vs. high for molecular markers), and mutational load (high for phenotypic traits vs. low for molecular markers with the possible exception of microsatellite markers).

Finally, an additional aspect of genetic diversity that is not often discussed at least among biologists, is the appreciation of biodiversity for other than biological utilitarian reasons, such as esthetic, moral or spiritual reasons (Pagiola *et al.* 1998).

The effect of gene flow from domesticated types on the genetic diversity of relatives (in the absence of transgenes)

As shown in Figure 1, crops belong to the same biological species as their wild progenitor (gene pool I). Thus, they can cross easily with these progenitors. Moreover, their progenies are viable and fertile. Gene flow can then take place within the domesticated gene pool among cultivars. From the perspective of this discussion, the gene flow from transgenic cultivars to landraces in centers of domestication is of particular importance. A recent article by (Quist and Chapela 2001; Quist and Chapela 2002) raises this important issue for maize in Mexico (see also A. Alvarez-Morales, this conference). Clearly, these issues can be extended to all crops in their respective centers of domestication (for a list of crops and their centers of domestication, see (Gepts 2001, n.d.). Another "target" of gene flow are the wild relatives of the crop, especially the immediate progenitors who also belong to the primary gene pool. With regard to genetic diversity, these are potentially more sensitive because it is now well established that they contain more diversity than their respective crops. Domestication has induced marked bottlenecks in genetic diversity in most if not all crops analyzed (Doebley 1992; Gepts 1993). Therefore, there is un untapped reservoir of genetic diversity among the wild progenitors of crop plants. It this reservoir that may be potentially more threatened by gene flow with domesticated types, whether transgenic or not.

Fig. 2 illustrates the complexity of experimentation to be conducted to determine whether gene flow from a transgenic crop to its wild relative will lead to long-term transgene escape (*i.e.*, whether the transgene will provide a sizable benefit in fitness to the wild population and thus be positively selected). Gene flow can be conceived of a series of successive steps each of which is necessary for the next step to occur, until the last one which is the end result mentioned (green box). Each of these steps can be investigated with a series of experiments. The flow of experiments illustrated here is an over simplification for several reasons. Each step actually consists of several experiments that may take several years. Also, the outcome is most likely not a yes or no as pictured here but rather a quantitative response such as a frequency: how often? In what circumstances?

The different steps to consider are the following:
1) Wild relatives: To have transgene escape to wild relatives, these have to grow within pollen dispersal range of the transgenic crops. Many crops in most regions of the world have been imported from their respective centers of origin (for examples, see http://agronomy.ucdavis.edu/ gepts/pb143/lec10/pb143l10.htm), hence they will generally have close wild relatives in most of their areas of cultivation). For example, maize, cotton, and soybean have no close wild relatives in the U.S. because the former two were domesticated in Mexico or South America and the latter in China. On the other hand, sunflower and strawberries have wild relatives in the U.S. For these crops potential escape of transgenes may become an issue, assuming that viable and fertile hybrids can appear (see next step). Likewise, the government of Mexico has instituted a moratorium on the deployment of transgenic maize cultivars because of uncertainties associated with the ecological effects of transgenes as these make their way not only into local maize cultivars but also into native wild maize (teosinte) populations (see also J. J. Sánchez-González, this conference). In addition, such factors as the mating system (*e.g*, autogamy) and the frequency of pollinators will also affect the possibility of transgene escape. Empirical data show, however, that these are not significant barriers especially when considered over large areas and multiple years (P. Gepts, A. González, and R. Papa, unpubl. results in common bean; squash: Montes-Hernandez and Eguiarte 2002).
2) Crosses yield viable and fertile progeny: For transgenes to escape, transgenic crops have to be able to mate with their wild relatives and these matings have to yield viable and fertile progenies. This may sound self-evident but needs to be verified. One element is whether the transgenic crop and the wild relative flower at the same time. If they do, it remains to be determined what the success rate is of the cross and what the degree of viability and fertility of the progeny. There has to be a minimum of fertility so that the progeny can backcross to the wild progenitor to maintain the transgenes in the gene pool of the wild relative.
3) In field or natural environments, a necessary, but not sufficient, condition for gene flow is the synchrony or at least a partial overlap between crops and their wild relatives for their flowering times. It is only when these flowering time coincide that pollen from one can make its way to the pistil of the other and potentially effect fertilization.
4) Assuming then that pollen is in a position to effect fertilization, the progeny will have to have a certain degree of viability and fertility. Even a partial viability and fertility will allow transgenes to be transmitted as long as full viability and fertility is restored in subsequent generations. This could be achieved for example by spontaneous backcrossing of the hybrid progeny to the wild relative populations.

Research into the previous four steps have shown that whenever these conditions are satisfied there will be almost certainly gene flow (Ellstrand *et al.* 1999).
5) The last and most critical, and certainly most difficult, step to ascertain is the potential selective advantage conferred by the transgene in wild populations. The advantage conferred by, for example, virus or pest resistances in domesticated populations may not necessarily exist to the same degree in wild populations. Other mechanisms exist to maintain disease or pest pressure at a lower level in wild populations compared to those found in the standard monocultures. Therefore, hybrids that carry additional resistance conferred by transgenes, or resistance genes introduced by classical breeding, may not show increased fitness compared truly wild populations. On the other hand, escape of herbicide resistance gene to populations of wild relatives, some of which are among the most noxious weeds of our major crops, such as red rice for rice and shattercane for sorghum, may mean the loss of effective herbicides, an important tool in the control of these weeds.

Finally, it should be mentioned that there is an additional form of gene flow, namely the dispersal of seeds. This dispersal can take place by a variety of ways including mechanical (transportation, farm equipment), wind, and animals. In summary, gene flow can lead to the escape of transgenes to wild populations of relatives. Whether or not this actually happens and it actually has an ecologically significant effect needs to be determined carefully and not just assumed or dismissed with a slight of hand.

There are remarkably few data on this important topic, especially in crop species with regard to gene flow between domesticated and wild progenitor types. Crops and their wild progenitors exchange genes (Ellstrand et al. 1999), consistent with the fact that they generally belong to the same biological species. However, there are few studied that compare introgression of genes from domesticated types between sympatric and allopatric populations. The few crops with information published in refereed journals include beets (Bartsch and Ellstrand 1999; Bartsch *et al.* 1999) and sunflower (Linder et al. 1998). A review of the methodology of these experiments and the conclusion that can be inferred from them is presented in Table 1.

An extreme consequence of hybridization is genetic extinction by displacement of the native allelic diversity. Examples are provided primarily by non crop plants, including wild walnut in California (http://sandiego.sierraclub.org/rareplants/130.html; http://www.savemountwashington.org/welcome/FFWALNUT.HTM) and Catalina mountain mahogany (*Cercocarpus traskiae*: http://www.mobot.org/CPC/27801484.html) on Catalina Island off the coast of California. Among crop plants some Mediterranean and tropical trees, including date palm, olive, and coconut have few remaining truly wild populations (*e.g.*, olive: (Bronzini de Caraffa *et al.* 2002). Many supposedly wild populations are either escapes from cultivation, remnants of ancient groves, or hybrids between wild and domesticated types.

The effect of gene flow in the presence of transgenes

Two aspects deserve discussion. Firstly, will the transgene be subject to (positive or negative) selection in wild populations? Secondly, if it is subject to selection what is the effect on the rest of the genome of wild populations?

Whether or not a transgene will spread into wild populations depends on a number of factors including the level of gene flow in any given growing season and in successive seasons and the selective effect of the transgene. As pointed out for the previous section, information on the year-to-year and location-to-location variation of gene flow is rare. In addition, the selective value of a transgene in wild populations may or may not be similar to that in domesticated populations. Further considerations include the degree of dominance, the presence of epistatic interactions, and the existence of genotype x environment interactions. Depending on the magnitude of these different evolutionary factors, the situation faced by transgenes may amount to a migration-drift or migration-selection balance.

If the transgene is subject to selection, then it will affect the rest of the genome as well. If the selection is positive (*i.e.*, individuals containing the transgene will be favored), the genome will be subject to a selective sweep. If the selection is negative, the genome will be subject to background selection. In both cases, selection will lead to reduced diversity. Both phenomena can, however, be distinguished on the basis of the frequency spectrum of DNA sequence variants around the gene under selection (Charlesworth *et al.* 1995; Cummings and Clegg 1998). Recurring gene flow, however, can increase genetic diversity through repeated introductions of genes.

The proportion of the genome subject to this reduction will vary according the level of recombination around the genes. For outcrossing individuals, characterized by high levels of heterozygosity and therefore effective recombination, the region of the genome that remains linked around the transgene is very small (of the order of 500-100kb in maize: Wang et al. 1999, 2001; Remington *et al.* 2001). In predominantly selfing organisms, the linked region (said to be in "linkage disequilibrium" with the transgene) will be much larger, although data are definitely lacking for plants with this type of mating system. For vegetatively propagated organisms, the entire genome is in linkage disequilibrium, regardless of whether the marker loci are located on the same chromosome as the transgene or different chromosomes.

Other organisms can provide insights into this issues because selective sweeps or background selection appear to have operated in them. Insecticide resistance in insects is potentially analogous to the situation of a transgene introduced in a wild population, in that resistance gene can have a strong selective value in the presence of the insecticide. The prediction that genetic diversity would be

reduced around insecticide resistance genes was verified in *Aedes aegypti*. Populations of this insect had been subjected to DDT and, subsequent to the interdiction of DDT, to organophosphate (OP) insecticides. Reduced genetic diversity and increased population differentiation was found around an OP resistance locus but not a DDT resistance locus. The latter was presumably due to re-equilibration of the population following the discontinuation of the application of DDT (Yan *et al.* 1998).

In Drosophila, the pattern of genetic variation across the genome reflects the occurrence of multiple selective sweeps. An example is provided by the locus coding for a sperm-specific axonemal dynein protein (Nurminsky *et al.* 1998), around which diversity is markedly reduced. Finally, it may be appropriate to consider a limited number of crop plant-wild progenitor pairs that could constitute model systems for the type of experiments considered here.

Effects of transgenic cultivars on genetic diversity in a socio-economic context.

Transgenic technology did not appear in a vacuum but has been part of significant changes in the seed industry in the last twenty years. There has been a shift away from public institutions, largely land grant universities, in the area of plant breeding as documented by Frey (1996). This shift has largely been correlated with the advent of biotechnology, *i.e.*, the ability to isolate, modify, and transfer genes by recombinant DNA and plant transformation technologies. Concurrently, the intellectual property regime changed as a consequence of the U.S. Supreme Court Diamond vs. Chakrabarty (1980) decision affirming the validity of a patent for a genetically modified Pseudomonas bacterium. This was a momentous decision because it created a precedent allowing the patenting of novel life forms.

The combination of the molecular technology and the capability to protect molecular inventions led to significant activities in the private sector in the area of genetic engineering of crop plants. To make the products available to farmers, however, private companies involved in genetic engineering had to acquire capabilities in classical plant breeding to develop cultivars as a vehicle to deliver the results of their genetic engineering technology such as herbicide or insect resistance. This was achieved by buying smaller seed companies, which had neither the financial or technological wherewithal to survive in this new environment. This has led to a situation where only five major firms now sell genetically modified seeds: Monsanto, DuPont/Pioneer, Aventis, Syngenta and Dow. These same companies account for about a quarter of total seed sales (Fulton and Giannakas 2001). For example, in 1998, Monsanto and Pioneer-HiBred controlled 15% and 39% of the US seed corn market, respectively. For soybean seed, these companies controlled around 24% and 17%, respectively. market. For US cotton, Delta & Pine Land and Stoneville, had 71% and 16%, respectively, of the seed market (Kalaitzandonakes and Hayenga 2000).

The effect of this increased concentration on the genetic diversity of the domesticated gene pool of crops such as soybean, maize, and cotton remains to be determined, especially with regard to the diversity of the cultivars currently grown by farmers. Transgenic cultivars of some field crops now occupy a significant proportion of the acreage (Fig. 3), raising questions about the overall level of genetic diversity in these crops. Because the domesticated gene pool represents only a fraction of the genetic diversity contained in the wild relatives (Gepts 1993, 1995), it remains to be determined how much genetic diversity is left in the current U.S. domesticated gene pool prior to the introduction of transgenic varieties but also whether this introduction would further decrease genetic diversity. The U.S. soybean germplasm can be traced back to a few ancestral lines imported within the last two centuries (Kisha *et al.* 1998; Li *et al.* 2001). The hybrid maize germplasm of the U.S. is based on a single heterotic combination - involving the Lancaster Sure Crop (flint) and Reid's Yellow Dent complexes (Doebley *et al.* 1988; Smith 1995).

If and when these maize hybrids are imported into Mexico, gene flow between these hybrids and native materials such as open-pollinated varieties and wild, teosinte populations could presumably introduce some genetic diversity depending on the gene flow levels and selection regime. At this stage, no comprehensive data are available in the refereed literature on the effect of gene flow between introduced and local maize germplasm in the center of origin of the crop. It is clear, however, that U.S. maize has been imported into Mexico in recent years (Fig. 4). This maize is destined for food, feed, and industrial purposes but it is not clear to what extent it is transgenic. It is also not clear how much of this maize is being planted in spite of the moratorium imposed by the Mexican government.

U.S. maize is produced at roughly 40% of the cost of production in Mexico, and average yields

vary from 1.8 tons per hectare in Mexico to 8 tons per hectare in the U.S. (Nadal 2000). The reduction in price fetched by maize in Mexico (50% between January 1994 and August 1996) as a consequence of cheap imports has led to disruptions of the farming sector in that country, including migration to urban areas and the U.S. The abandonment of farm employment may have deleterious effects on the genetic diversity of maize in Mexico. Sixty per cent of Mexican producers (1.8 million) use locally adapted corn varieties, covering 80 per cent of the total area under corn cultivation (Nadal 2000).

This discussion shows that, although transgenes cannot be directly implicated in potential losses of genetic diversity, they are part of a new socio-economic system that may have such an effect.

Additional areas for inquiry

There are several gaps in our knowledge. These include the following:

- What is the value of genetic diversity, not only from a biological but also a social and esthetic standpoint?
- What is the long-term effect of gene flow on the genetic diversity of relatives, both landraces and wild populations? This will require both empirical data and modeling studies.
- Are there regions of the genome that are less "susceptible to invasion" by transgenes, especially with regard to genes for adaptation?
- What may be some unexpected effects of transgenes introduced into new genetic background, such as the increased lignification observed in Bt corn (Saxena and Stotzky 2001)?
- How have the changed socio-economic conditions (consolidation of seed companies involved in cultivar development, international trade, patenting of life forms) affected genetic diversity of crops and their landraces and wild relatives?

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Table 1. Summary of experiments comparing from crops to sympatric and allopatric wild populations

| Source	Crop	Sympatric/ allopatric populations?	Markers	Method	Results
Bartsch et al. 1999	Beet	26 D; 65 W[a]	Allozymes: 12 loci	Unique alleles	a) Gene flow from D to W b) Slight increase in diversity c) Maintained morphological differences between W and D
Linder et al. 1998	Sunflower	3 W, sympatric; 4 W, allopatric	RAPD: 18 (absent in allopatric)	Unique alleles	High level of crop-specific markers in sympatric W: 0.32-0.38

[a] D: domesticated; W: wild
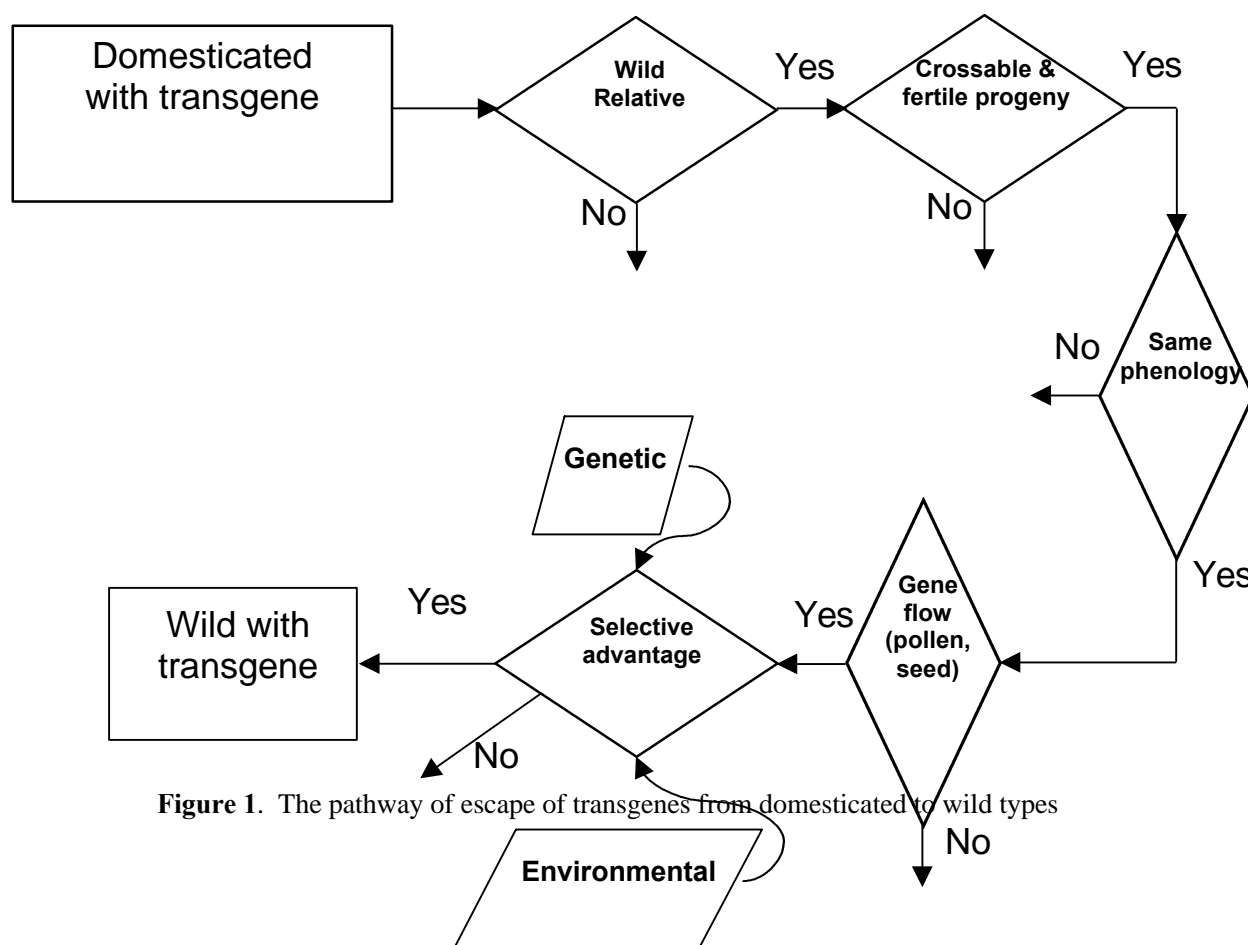
Figure 1. The pathway of escape of transgenes from domesticated to wild types

Biosafety Regulation in China

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Abstract

Genetically modified organisms (GMOs) including GM crops have been developed for a relatively short time. Just like many other major changes in human history, modern agricultural biotechnology has involved some concerns and mistrust over the world. The issues elevated include the environmental safety and human health (food safety). In this paper, the Chinese experience as a developing country on why and how to regulate GMO biosafety at a national level is briefly introduced. The authors consider that it is very important to internationally cooperate in the area of GMO biosafety regulation in order to facilitate the realization of the potential benefits of modern biotechnology for agriculture and food production.

Key words GMO, Biosafety regulation, Agricultural biotechnology, China

Introduction

Modern biotechnology is of critical importance to increasing agricultural productivity and improving natural environment. Research in agricultural biotechnology dates to the early 1980s in China. According to a nation-wide survey made in 1996 by the Ministry of Agriculture (MOA), there were over 100 organisms under research by scientists in the public sector (including scientists at both agricultural and non-agricultural systems) and over 190 genes were used to transform those organisms. Among these data, there were 47 plants in research and 103 genes used for transformation; 22 animals under study and 32 genes used for transformation; and 31 species of microorganisms under study and 56 genes in use for transformation. These figures have further been expanded since a significant progress has been taken place in agricultural biotechnology during the last six years. In this period, one of the most successful achievements made by the scientists is the development and commercial production and application of transgenic insect-resistant cotton (Bt cotton). Field testing of the Bt cotton lines first began in 1995. Demonstration trials in 1996 and 1997 showed a good ability for insect resistance and reduced insecticide use in the farmers' fields. Commercial planting of the new cotton variety was approved by MOA in late 1997. Since then, several other transgenic crop varieties have been approved as well for commercial uses by the GMO Biosafety Committee after safety evaluation. However, as of today, no transgenic staple food and oil crops have been approved for commercial use in China yet.

How to Regulation and Why

As with any powerful new technology, modern biotechnology (transgene technology) needs to be employed carefully. In modern societies, technological decision is often made by three factors, namely: society, government and technology. Each factor interacts with the others through specific actions.

It is a real challenge for the government agencies to develop working regulatory systems that neither over-regulate nor under-regulate. To ensure that agricultural biotechnology is used effectively and appropriately in China, the Ministry of Agriculture (MOA) has developed and implemented biosafety systems that cover laboratory and greenhouse experimentation, field evaluation and commercialization since 1996. By the end of 2000, MOA received a total of 443 cases of GMO applications for biosafety review, of which 322 were approved. Among these, 189 cases were approved for small-scale field trials, 93 cases were for experimental field releases and 40 for commercial productions. Most of these cases were transgenic crops, especially transgenic cotton.

As agricultural biotechnology is expanding at an unexpected speed that biosafety legislation, regulatory requirements, review process and decision-making process need to be reviewed and updated from time to time.

On 23 May 2001, the State Council issued the Regulations on Safety of Agricultural

Genetically Modified Organisms (the GMO Regulations). In accordance with the GMO Regulations, the MOA published a series of three implementing regulations on 5 January 2002. They are:

(1) Implementation Regulations on Safety Assessment of Agricultural GMOs;

(2) Implementation Regulations on the Safety of Import of Agricultural GMOs; and

(3) Implementation Regulations on Labeling of Agricultural GMOs.

The purposes of these regulations are set to strengthen safety administration of agricultural GMOs, to safeguard human health and safety of animals, plants and microorganisms, to protect the environment, and to promote the research on agricultural GMOs.

Some of the general principles and regulatory approaches considered in these new regulations are as follows:

a. Biosafety (and risks) associated with genetically modified (GM) crops and other GMO products should be assessed on a case-by-case (e.g. variety by variety, line by line) basis and in a stepwise manner.

b. Decision-making should be based on demonstrated risks (bioharzards).

c. Biosafety reviews should focus on the scientific questions and data.

d. The expert panel should play an important role in the decision-making process.

e. Regulatory requirements should be consistent, dynamic and transparent.

The safety of GM crop application is achieved by carrying out certain sequential steps: hazard identification, safety assessment, safety management and safety communication.

In general, an assessment of the safety (or risks) to the environment and human health associated with the use of GM crops is based on a consideration of the following key parameters, when appropriate:

a. The characteristics of the organism: the recipient plant; the center of origin.

b. The introduced trait: the relevant information on the donor organism and the vector used; the insert and the encoded trait.

c. The characteristics of the intended use: the specific application of the field trial or field release or commercial production and placing on the market, including the intended scale and any management procedures and waste treatment.

d. The potential receiving environment and the experiment design; and

e. The interaction between the above factors.

Safety assessment requires a range of expertise. Knowledge of and experience with any/all of these provide familiarity, which plays an important role in risk assessment. A relatively low degree of familiarity may be compensated for by appropriate safety management practices.

Safety management strategies should be commensurate with the results of the safety assessment. Management strategies include designing procedures and methods to minimize risks and their consequences, or deciding not to proceed. Development of safer gene technologies such as marker-free technology, use of containment measures and monitoring strategies will present options for risk management to enable safe use of GMO products. Different safety-management practices may be applied, depending on the scale of the proposed release and its duration. The type of safety management to be applied depends on the GMO product and its particular application. For contained uses, the degree of containment achieved depends primarily on the type of physical barriers and the application of appropriate work procedures. For controlled field releases, different types of barriers, such as biological, chemical, physical or temporal barriers can be used to limit the dissemination and impacts of a GMO and/or to provide genetic isolation, if required.

The effective communication of risks and benefits is a prerequisite to informed decision-making and should result in an overall advantageous outcome for society. Some indicators of a good decision may be increased safety, food and economic security, improved human health and welfare, and agricultural and environmental sustainability.

In the case in which an organism with novel traits are to be transferred to its centre of origin, such as rice and soybean in China, there is a need to pay particular attention to safety assessment and management, because of possible negative impacts on related species that are present, to ensure adequate protection of genetic resources and agricultural biodiversity.

Future Challenges

Genetically modified organisms (GMOs) including GM crops have been developed for only a

short time. Just like many other major changes in human history, biotechnology has involved some concerns and mistrust. The issues elevated concerning agricultural biotechnology include environmental safety and biodiversity, human health and food safety, trade and economic impacts, social and ethical considerations and much more. Many of the approaches and policies dealing with these issues are of recent origin, still evolving and largely unresolved. As a developing country, China considers that it is very important to have international cooperation and exchange in the regulation of GMO biosafety. Currently, some of the major challenges facing us are: an appropriate regulatory approach, a science-based safety assessment, capacity building, transparency, communication and information exchange. We believe a good environment is necessary to facilitate the realization of the potential benefits of modern biotechnology.

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Criteria for Evaluating Biosafety Frameworks:
Objectives and Standards

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Globally the acres planted with biotechnology derived crops (agriculture biotechnology) have grown exponential over the last 7 years. Adoption of new technologies within the U.S. agricultural sector has resulted in sustained increases in agricultural productivity, contributed to economic growth, and ensured an abundance of food (Economic Research Service, USDA, AER 810 – May 2002). Rapid developments in plant genomics will enable even greater expansion. Critical to this greater expansion are appropriate national biosafety oversight systems. The challenge for these regulatory systems is to ensure that the agriculture biotechnology products meet appropriate safety standards but not unduly inhibit technology innovation.

To meet this challenge, national authorities are establishing regulatory oversight systems based upon the end product and use; novelty of the enhanced trait; and method by which the product was developed . These different approaches reflect the variety of national authorities used as a basis for the biosafety systems. Although the national approaches are different, they should all share common objectives and appropriate safety. The national approach should seek to be science-based regulatory systems that are comprehensive, commensurate, transparent, inclusive and predictable. This paper will explore some of the common objectives and standards and offer recommendations about "model" biosafety frameworks.

Strategic approaches to biosafety studies in China

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Abstract

Modern biotechnology offers a great potential for food security and poverty alleviation in China. Biosafety research over the last decade has played a key role in accompanying the development and commercialization of genetically modified organisms (GMOs). A particular feature of the GMO biosafety research projects in China is that they are carried out on a joint basis by the State-owned research institutions and National Laboratories. The overall aim of the GMO biosafety research is to promote the safe development and commercial application of GMO products, while some projects may aim at enhancing knowledge on possible biological impacts of GMOs on the environment and human health by corresponding (basic science) research and the others may aim at providing a sound scientific basis for biosafety assessment, biosafety management and regulation.

Key words: GMO, Biosafety, safety research, transgenic crops, China

New developments in modern biotechnology and transgenics (or transgene technology) in particular, offer the hope of crops with high yields, pest resistance, salt tolerance, drought- and cold-resistance, and superior nutritional characteristics – especially for small-scale farmers in developing counties such as China. In China, transgenic (genetically modified, GM) cotton with gene(s) for insect resistance from bacteria and/or other plant species allows pesticide spraying to be reduced by 70% to 80% (from 15 to 2 times) in one-third of the total cotton areas in 2001, and GM rice testing lines for insect and disease resistance in demonstration and experimental trials offer 15% higher yields without the need for increases in other farm inputs. The transgenic approaches to plant improvement arise from a lack of suitable conventional approaches to dealing with a particular agronomic problem or need (e.g. rice bacterial leaf blight, rice sheath blight, insect pests, etc.). There are inherent limitations in conventional breeding such as lack of practical access to useful germplasm due to sexual incompatibility barriers or undesirable linkage block. Transgenic approaches have considerably broadened the range of genepools which are now accessible for plant improvement purposes. For crops, improved agronomic traits include: yield, pest and pathogen resistance, herbicide tolerance; tolerance to abiotic stresses such as acid soils, drought, salinity, and cold; shade and high density planting tolerance; water and nutrient use efficiency; reduced mycotoxin contamination; crop reproductive biology; and enhanced nutritional and product quality.

Biosafety research over the last decade has played a key role in accompanying the development and commercialization of genetically modified organisms (GMOs) in China. Since the early 1980s, an increasing investment has been input in the research and development of modern biotechnology including transgenic crops in food and agriculture by the government. According to an unpublished statistic data of the author's laboratory in 2001, there were over 60 plant species under research and 121 genes (not including selection and marker genes) used for transformation. Commercial uses of the transgenic delayed-ripening tomato and insect-resistant cotton have been approved by the GMO Biosafety Committee and the Ministry of Agriculture (MOA) in 1997. Commercial approval has also

been granted by the GMO Biosafety Committee to other GMOs, such as virus-resistant sweet pepper, virus-resistant tomato, virus-resistant chili pepper, color-altered petunia, nitrogen-fixing bacteria, and vaccines for animal use. Biosafety research results have been taken into consideration for safety assessment and provided appropriate reasons for releasing these GMOs.

Early biosafety studies has been done by the GMO product producers. The GMO producers have allocated more funding to safety research since 1997, when the MOA implemented its first GMO regulations. In the mid- 1990s, government-funded programs (for example, the National High-Tech Research & Development Program generally referred to as the 863 Program) supported many GMO safety studies as an integrated part of the GMO product development. Since then, funding for GMO biosafety research has further been expanded from both the governmental and private sources. The first national biosafety initiative is the National Transgenic Plant Program in 2000. The new 863 High-Tech Program, 973 Basic Science Program and the National Key Science & Technology Program have also supported biosafety research projects directly targeting GMO safety assessment and safety management. These projects have been conducted by There are many others which focus on some other subject but may contain important elements or implications for GMO safety.

The biosafety projects vary in research area. Some research focuses on a single narrow topic, others may cover a much broader area. In summary, the GMO biosafety research projects have concentrated on food and environment safety, GMO detection methodology and technical standards.

Particular features of the GMO biosafety research projects described in this paper are that they are carried out on a joint basis by the State-owned research institutions and National Laboratories. We understand that genetic engineering is an important and powerful tool in the development of strategies for the sustainable food and agricultural production. The overall aim of the GMO biosafety research is to promote the safe development and commercial application of GMO products, while some of the projects may aim at enhancing knowledge on possible biological impacts of GMOs on the environment and human health by corresponding research and the others may aim at providing a sound basis for biosafety assessment, biosafety management and science-based regulation. The research results should help to understand the genetically modified (GM) technology, GM product and its impacts, and enable the National GMO Biosafety Committee to improve its ability to make safety evaluation and recommend safety conditions for the release of GMOs. Some results should lead to the establishment of best practice for GMO production as well.

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Studies on Gene Flow in China – A Review

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Transgenic crops have been widely grown worldwide in recent years and a great deal of social, economic and environmental benefit has been achieved. The Chinese government has considered the recombinant DNA technology, closely combined with the conventional techniques, as a key factor for its sustainable agriculture development to ensure food security. In past decades, more and more investments were allocated to promote the R & D in this field. The philosophy bind this is that we should be aware of the most severe risk for a developing country like China, is sit idle and do nothing in developing own biotechnology.

Meanwhile, for safe use of genetically modified organisms (GMOs) to ensure environment safety and human health, both government and private sector-funded research on safety of GM crops have been significantly increased during recent 5 years. For example, there are government-funded 4 projects in the National Transgenic Crop Program, 6 projects in the 863 National High-Tech Program, 7 projects in the 973 National Fundamental Science Program, and 3 projects in the National Key Program for Tenth Five-Year-Planning. Gene flow from transgenic crops is one of the major concerns regarding biosafety of GM crops. In this article the results obtained to date in China are reviewed.

1. Rice (*Oryza sativa*)

Transgenic rice with insect-resistance (*Bt Cry* IA, *CpTI* gene), disease-resistance (*Xa* 21 gene) and herbicide-tolerance (*bar*, *EPSPs* gene) has been developed. The products are now in the pipeline readily for commercialization if it is approved by the regulatory agency. In addition, other transgenic rice products with characteristics of tolerance to biotic and abiotic stresses and improved qualities are also being developed. Therefore, study on the possible environmental impacts of these products is urgently needed, especially in the Southeast Asia where is the origin and center of diversity of rice. The major concerns are as following: (1) Stability of the inserted genes; (2) Gene flow through pollen dispersal from crop-to-crop and from crop-to-wild relatives; (3) Possible impacts on non-target organisms; (4) Other environmental aspects. Here we only discuss the gene flow on crop-to-crop and crop-to-wild relatives.

Since rice is a self-pollinated species in nature and pollen dispersal is mainly through wind, following factors affecting gene flow of crop-to-crop and of crop-to-wild relatives are considered: (1) Compatibility of sexual crossing and fertility of offspring derived from cross; (2) Wind speed and directions; (3) Distances from pollen donor plants; (4) Amount of pollen produced from different pollen donor plants; for example, *indica* rice produces more pollens than that of *japonica* rice; (5) Pollen and stigma viability under different weather conditions including temperature, humidity and rain etc.

As for gene flow, in our opinion, firstly, particular concern should be diverted to the pollen transfer from transgenic rice to the male sterile (ms) lines used for hybrid rice production. Hybrid rice of *indica* and *japonica* produced via either three-lines methodology (ms line, maintainer and restorer lines) or two-lines methodology (photoperiod-sensitive or temperature-sensitive ms line and restorer line) are now widely used in rice production in China. Since ms lines generally possess higher percentage of stretched stigma for increasing the efficiency of hybrid seed production during flowering time, its outcrossing rate is much higher than that of the common rice cultivars. Preliminary data showed that the percentage of stretched stigma in *indica* and *japonica* male sterile lines (10 and 3 lines investigated respectively) was 12.2 ~ 79.0%, which was much higher than that of *indica* or *japonica* restorer lines (0 ~ 25.0%), common cultivars (3.0 ~ 27.2%) and hybrids (13.0 ~ 35.0%) (Qian et al., unpublished, personal communication). Accordingly, the gene flow frequency from transgenic rice (use of *bar* gene or Basta[R] as a marker or indicator) to male sterile lines is much higher than that to common rice cultivars. In the case of side-by-side plantation of transgenic rice together with male sterile lines of *indica* (Zhong 9A) or *japonica* (Ning 67A) rice, the gene flow frequency is 56.45% and 67.13% respectively, while the gene flow frequency to same maintainer lines (Zhong 9B and Ning 67B) is only 4.08% and 1.82% respectively (Jia et al., unpublished). It

suggests that the gene flow from transgenic rice to ms lines should be considered at a rank of high-risk, while gene flow to common cultivars is at low-risk. The gene flow frequency is also affected by wind speed, directions and distances from pollen donor plants. The frequency is dramatically reduced with distance increased. For example, when wind is mainly from Southeast (4d), Southwest (3d) and South (2d) at a speed of 2.5 ~ 6.2 m/sec during flowering time (total 11d), the outcrossing rate for Zhong 9A at 20 meters apart (North) is 0.38% only (Table 1, Jia et al., unpublished).

Rice production in China is divided into different ecological zones, including South-China, Yangtze River and Yellow River Valley, Southwest, and Northeast, where the climate conditions are quite different. During flowering time the wind direction and speed are different in these zones. Therefore, it is needed to investigate the factors affecting gene flow case by case under these conditions. It is particularly important to note that during the winter season, thousands of rice materials from different provinces are brought to and planted in Hainan Island for speed up seed production or breeding process. We are now conducting experiments in Jiangsu and Zhejiang (Yangtze Rive Valley) and Guangdong province (South China) this summer and will continue to do experiment in Hainan in coming winter to generate data in determining the maximum distance of gene flow and the effective isolation distance for ensuring seed purity in hybrid rice production. At the same time, we will investigate how is a practical measure to avoid hybridization between transgenic and non-transgenic isolated by flowering time not to meet each other. More data will be available until the end of this year.

Secondary, much attention should be paid to the gene flow from transgenic cultivated rice (AA genome) to the wild relatives that share same AA genome, such as *O. rufipogon*, *O. perennis, O. nivara*, since the compatibility between them is expected to be higher than that with wild relatives containing other genomes such as CC (*O. officinalis*), GG (*O. granulata, O. meyeriana*), CCDD (*O. latifolia, O. alta, O. grandiglumis*), BB or BBCC (*O. punctata*), and FF (*O. brachyantha*) etc. In the later case, there is no available data showing that outcrossing may occur in natural conditions. Generally, successful men-made crossing between cultivated rice and wild rice with CC or GG genome should be accompanied by application of embryo rescue techniques.

By emasculation of anthers of *O. officinalis* (with CC genome) and pollination with pollen derived from the two transgenic *japonica* rice cultivars, Y0003 and 99-t, in which a *bar* gene was inserted, Song et al. (2002) reported the incompatibility between *O. sativa* and *O. officinalis*. Pollen growth of transgenic rice on stigma of *O. officinalis* was investigated by using fluorescence microscope. It was found that although pollen grains could germinate and pollen tubes might penetrate into stigma, the further growth was ceased before entering embryo sac and failed to carry on fertilization. As a result, the cross did not set any seeds. Therefore, the incompatibility was occurred at a relatively late stage rather than early stage of pollen germination and penetration.

In China, there are three wild species of rice naturally existed, including *O. rufipogon*, *O. officinalis* and *O. granulata*. Considering their compatibility with cultivated rice, we will concentrated our self on the study of risk assessment of gene flow from transgenic rice to common wild rice *O. rufipogon*.

Thirdly, the possibility of gene flow from transgenic rice to weed species is also considered. The barnyard grass (*Echinochloa crusgalli* var. *mitis*) is a common weed occurred in rice fields. Dr. Qiang's laboratory (personal communication), in the Nanjing Agriculture University, has studied the possibility of gene flow from two varieties of transgenic rice with *bar* gene (Y0003 and 99-t as a male parent) to barnyard grass (as female). They investigated germination and growth of rice pollen grains on barnyard grass stigma under microscope at 30 min, 1-4 h after crossing by hands. The results were compared with the germination and growth of barnyard grass pollen grains at the corresponding time after selfing. Results showed that the germination and growth of rice pollen grains on barnyard grass stigma are differed significantly from self-pollinated pollen grains of barnyard grass. In the later case the pollen grains germinated and pollen tubes penetrated into stigma normally, and the number of pollen grains being condensed or releasing their inclusions increased with the time after self-pollination. Pollen grains of transgenic rice on the stigma of barnyard grass did not germinate or did not grow normally and the pollen tubes could not penetrate into the stigma. Emasculated barnyard grass pollinated with rice pollen grains did not set seeds further confirmed the incompatibility between these two species. In natural conditions there is no any evidence showing they will cross each other. Therefore, it is negligible in terms of gene flow between them.

2. Wheat (*Triticum aestivum*)

Wheat is a self-pollinated species and any outcrossing that does occur is facilitated by wind pollen dispersal. For following reasons, the outcrossing rate for wheat under field conditions is low: (1) Wheat pollen is relatively heavy, a characteristic associated with the high ploidy level of wheat [6x for common wheat (*T. aestivum*) and *T. compactum*] (de Vries, 1971). (2) Wheat pollen is produced in a relatively small amount and has a limited viability period (Treu & Embelin, 2000). Under field conditions the wheat pollen lost its fertilizing capacity after 15~20 min. Wiese (1991) reported that cross-pollination under field conditions normally involves less than 2% of all florets.

Wheat has little potential for hybridization under field conditions with any other crops belong to Graminae species, since it is highly autogamous. Although an artificial hybrid crop, *Triticale*, has been produced between wheat and rye, and is now commonly grown, there is little or no evidence that hybrid between cultivated wheat and rye or barley existed naturally.

In the Mediterranean area and in Southwest Asia, 27 wild *Triticum* species are found. It is known that wheat may hybridize spontaneously with *T. turgidum* and *Aegilops* species. However, all hybrids are highly sterile. This hybrid sterility may explain why hybridization generally appears to be restricted to the first cross with little evidence for subsequent introgression. For example, jointed goatgrass (*Aegilops cylindrica*) shares same D genome with wheat that allows hybrids between these two species to be produced in the field. However, the resulting interspecific hybrids showed only 2% of female fertility that allowed for backcrossing either with *Ae. cylindrica* or wheat (Zemetra et al. 1988).

According to above description, the European Science Foundation (ESF) and the European Environment Agency (EEA) considered wheat as a low risk in terms of gene flow from crop to crop and from crop to wild relatives (Eastham & Sweet, 2002). The same is true for the data generated in China.

By using different experimental design, Lu et al. (2002) conducted two types of gene flow studies in wheat:

1. A 10 m × 10 m plot of pollen donor was planted in the center and an emasculated pollen recipient of same wheat variety was grown in the pots placed at different distances in 8 directions. Results indicated that maximum distance for wheat pollen dispersal was 10 ~ 80 m depending on wind directions, that was 10 m and 80 m for head-wind and tail-wind directions respectively (Table 2). Since there were no wheat plants grown in between pollen donor and recipient and the recipient was emasculated, the competition of pollen grains for fertilization was not considered in this experiment. Therefore, the above figure only indicated the maximum distances for wheat pollen dispersal.

2. A 10 m × 10 m plot of blue-kernel wheat (*T. aestivum*) variety U4235-1 as a pollen donor was center-planted and surrounded by a white-kernel common wheat variety Shandong 1455 as a recipient. At harvesting time, seed samples were collected at different distances of 8 directions for further analysis. The number of seeds with blue kernel vs total No. of seeds harvested was calculated. Results shown that the maximum distance of gene flow, in the case of pollen competition existed, was only 20 m. The highest outcrossing rate was only 0.24 % within 0 ~ 2 m. Outcrossing rate was 0.091 %, 0.018 %, 0.001 % and 0 % for 0 ~ 1 m, 4 ~ 5 m, 19 ~ 20 m and 24 ~ 40 m respectively (Table 3).

It is known that there are 27 species in genus of *Aegilops*, including diploid, tetraploid and hexaploid species, of which only *Ae. tauschii* Cross (2n = 2x =14, DD genome) is naturally existed in wheat fields as weeds in Henan, Shanxi and Xinjiang provinces in China. Therefore, it was examined for possible gene flow from transgenic wheat to *Ae. tauschii*. Lu et al. (2002) obtained hybrids between *Ae. tauschii* × *T. aestivum* via artificial cross combined with embryo rescue technique. Self-pollination of hybrids did not give rise seed setting (0/3302). When hybrid was backcrossed with male parent of common wheat or *Ae. tauschii*, the rate of seed setting was 0.33 % (3/916) and 0% (0/198) respectively. To date, there is no report on successful outcrossing between *T. aestivum* and *Ae. tauschii* occurred in natural conditions. Therefore, it is of little concern on gene flow between these two species. However, the experimental data shown that in natural condition the outcrossing rate between common wheat (as a pollen donor) and *Ae. cylindrica* (2n = 4x = 28, CCDD) was 0.25 % (8/3200), while that between *T. aestivum* and *Ae. ovata* (2n = 4x = 28, $C^U C^U M^O M^O$) was 0 % (Lu et al., 2002). It suggests that in the region where *Ae. cylindrica* is naturally existed the gene flow should be studied further in more detail.

3. Foxtail millet (*Setaria italica*)

Green foxtail (*S. viridis*) is a common weed in foxtail millet field. Wang et al. (2001) used a dominant homozygous sethoxydim-resistant line SR3522 derived from conventional breeding as a pollen donor and a population of green foxtail POP26 as a recipient to test the gene flow from cultivated foxtail millet to its wild relatives. A 10-m^2 plot of SR3522 was planted in the center and samples were collected from POP26 at different directions and distances at harvesting time. Results show that although it is well known that these two species are autogamous, the pollen from foxtail millet can fertilize the green foxtail up to 60 m (0.003%). The highest frequency of gene flow on average was 1.14% at 0.03 m. The frequency decreased rapidly with distance increased. A sharp decrease was occurred at 0.5, 1 and 4 m where the outcrossing rate was 0.458%, 0.267% and 0.085% respectively. Generally, the frequency of gene flow was 0% at 20 m, but it was possible that pollen dispersed up to 60 m in some cases. The wind direction was the major factor influencing the gene flow frequency. In addition, 60% of pollens of the hybrid derived from cross was not viable that give rise only 25% seed setting on main stems. The seed setting on tillers was much lower than that of the main stems. Therefore, it is concluded that the risk of gene flow between these two species is low.

4. Cotton (*Gossypium hirsutum*)

Upland cotton (*G. hirsutum*, 4x) is a frequent outcrossing species mainly mediated by insect pollination. The outcrossing level is much higher than that of the self-pollinated species. In our laboratory, we used a transgenic cotton line containing *tfd*A gene that conferred resistance to herbicide 2,4-D, as a pollen donor grown in the center of the field surrounded by non-transgenic cotton plants. At harvesting stage seeds were collected from different distances in 8 directions. The seeds were sown in field and sprayed with 300 ppm of 2,4-D at the 7~8 leaf stage. Results shown that the outcrossing rate was as high as 11.20% on average at 1 meter apart but it was dramatically reduced with distance increased. Gene flow frequency at 5, 10, 20 and 50 m was 0.61, 0.16, 0.09 and 0.03% respectively. At 100 and 150 m no outcrossing was detected (Zhang et al., 1997). Therefore, the effective isolation buffer area should be at least 100 m in a small-scale field test, which was in accordance with the requirement for cotton pure seed production.

Shen et al. (2001) also studied gene flow from upland transgenic Bt cotton to non-transgenic cotton of same species and to *G. barbadense*. At varying distances from transgenic cotton, seeds produced by the non-transgenic cotton were collected and screened for marker gene (Kan[R], Dot-ELISA) and Bt gene (PCR). Results indicated that gene flow between cultivars of same species (*G. hirsutum*) was up to 36 m, higher at 0~6 m (1.85~11.05%). Gene flow between species of *G. hirsutum* and *G. barbadense* was up to 72 m, but lower at 0~6 m (1.42~8.67%). The authors proposed at least 72 m buffer zone would serve to limit gene flow from transgenic cotton in small-scale field test.

In China, there is no cotton wild species naturally existed. Thus it is of little concern in terms of gene flow from transgenic cotton to wild relatives. However, some semi-wild cotton species such as *G. arboreum* (2x, with A_2 genome) still grown in a small area. The incompatibility between *G. arboreum* with *G. hirsutum* [4x, $(AD)_1$] or *G. barbadense* [4x, $(AD)_2$] is well documented because of differences in ploidy level and genome constitution that they cannot cross each other. In Yunnan and Guizhou plateau there are some perennial semi-wild woody cotton species (4x) that may cross with cultivated cotton and set seeds, but with reduced fertility in subsequent generations. Since its growing area is not the cotton cultivation area in China, the gene flow to these semi-wild species is out of consideration.

In conclusion, although we have obtained preliminary data on gene flow of major crops in China, some are still missing such as for soybean, corn and rapeseed. Therefore, further study is urgently needed for keeping up with the rapid development of transgenic crops in our country.

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Table 1 Outcrossing rate for male sterile line Zhong 9A at different distances and directions

Directions	Distances (m)	Outcrossing rate (%)
	0	56.45
SE	7	0.08
SE	50	0.06
NE	10	0.71
NE	40	0.06
N	15	0.68
N	20	0.38

Table 2 Effect of wind direction on wheat pollen dispersal

Wind direction	Times	Maximum speed (m/sec.)	Direction of sampling	% Seed setting within 10 ~ 90 m	Maximum distance (m) with seed setting
E	4	1.7	W	0.417 (6/1438)	60 (1/164)
SE	0	-	NW	0.316 (4/1265)	30 (1/138)
S	8	5.7	N	0.591 (8/1354)	80 (1/174)
SW	1	0.7	NE	0.264 (3/1137)	40 /(1/142)
W	0	-	E	0.135 (2/1480)	10 (2/176)
NW	1	0.3	SE	0.519 (6/1154)	40 (1/152)
N	1	0.3	S	0.407 (5/1230)	40 (1/104)
NE	4	1.7	SW	0.319 (4/1254)	40 (1/136)

* Figures in parentheses represent: No. of seeds/No. of florets

Table 3 Outcrossing rate between blue- and white-kernel wheat variety at different distances

Distances (m)	Outcrossing rate (%)
0 ~ 1	0.091 (98/107829)
1 ~ 2	0.040 (47/118552)
2 ~ 3	0.027 (29/106531)
3 ~ 4	0.021 (22/107262)
4 ~ 5	0.018 (18/99811)
5 ~ 6	0.004 (4/103935)
6 ~ 7	0.010 (10/101507)
7 ~ 8	0.008 (8/99775)
8 ~ 9	0.003 (3/107619)
9 ~ 10	0.003 (3/118306)
14 ~ 15	0.004 (5/130184)
19 ~ 20	0.001 (2/134239)
24 ~ 25	0 (0/112085)
29 ~ 30	0 (0/88999)
34 ~ 35	0 (0/72017)
39 ~ 40	0 (0/72110)

* Figures in parentheses represent: No. of seeds with blue-kernel / Total No. of seeds investigated.

Research And Development of Recombinant Microbial Agents And Biosafety Consideration in China

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Abstract   Since 1997, Chinese scientists have identified and cloned 36 *cry* and *cyt* genes from *Bacillus thuringiensis*, which accounted for nearly one-third of total Bt gene numbers nominated worldwide in the same period. One recombinant Bt agent was approved for commercial production and 3 agents with higher toxicity or broader spectrum to insect pests to be released on field trials. Also, on the basis of biosafety assessment, AC1541 from *Alcaligenes faecalis*, a new type of recombinant microbial inoculant with traits of nitrogen fixation and plant growth promotion was approved to limited commercial production. Poor spread and low survival potential capability of the recombinant implied its safety to ecological environment

Key words. Recombinant microbial agents, Biosafety, *Bacillus thuringiensis, Alcaligenes faecalis*

The R&D of agricultural recombinant microorganism in China started in 1980's, when the molecular biology and gene manipulation of nitrogen fixing bacteria were focused. Since " the National Program for Development of High Technology"(863) was carried out in 1986, microbial biotechnology has been rapidly developed and made remarkable progress.

Major Areas

The major research areas of agricultural recombinant microorganism include:
1) Crop production:
   - Microbial biocontrol agents: e.g. entomopathogenic bacteria, virus and fungus, antagonistic bacteria to plant pathogen, antibiotic producing bacteria (especially *streptomyces*).
   - Microbial nitrogen fixing agents: e.g. rhizobia, associative diazotrophus.
2) Feed additive:
   e.g. phytase, glucanase, xylanase etc.
3) Control of environmental pollution:
   e.g. microbial degradation for pesticide, cellulose, manure, plastics, and aromatics etc.

This topic just focuses on some microbial agents used for crop production, which were more studied and relatively fast developed.

According to statistics by the Chinese Ministry of Agriculture, there are 2 recombinant microbial agents approved for a limited commercial production, 11 microbial agents approved to be released in large scale on field trials (in a open system, the maximum area is about several ha) and 15 microbial agents approved into pilot experiment (in a control system).

Progress

1. Recombinant *Bacillus thuringiensis* (Bt)

Bt is a Gram-positve bacterium producing insecticidal crystal protein (ICP) during its sporulation. The specific toxicity of these ICPs against insect pests provides the basis for the use of this microorganism as the most valuable biocontrol agent. Much is known about the molecular mechanisms of Bt toxin activity and specificity. The ICP gene recombination is the basic strategy for

Bt improvement, which makes Bt more effective or with broader insecticidal spectrum.

Since 1997, Chinese scientists have identified and cloned 36 Bt *cry* and *cyt* genes from local Bt resource, which accounted for nearly one-third of the total gene numbers world wide named by the International Bt Nomenclature Committee.

On the basis of research of Bt molecular biology, numbers of genetically engineered insecticidal microbes have been constructed. WG001, one of the crecombinant bio-insecticide which is highly toxic to *Lepidopteran* (cotton bollworm, diamond back moth etc.), has been approved to industrial production. Three of others which are toxic both to *Lepidoptera* and *Coleoptera*, or effective both to insect pests and plant pathogens, or highly toxic to beet armyworm (*Spodoptera exigua*), respectively are approved to release in field trail.

2. Recombinant *Alcaligenes faecalis*

Alcaligenes faecalis is a species of associative diazotrophus, but actually a PGPR (Plant Growth- Promoting Rhizobacterium) with function of nitrogen fixation. A positve regulatory gene of nitrogenase, *nifA* and *ntrC*, a regulatory gene for general nitrogen metabolism were co-transferred into strain A1501, a wild type strain isolated from the paddy field. The recombinant strain with high NH^+_4 tolerance (10 mm, 65%) is named AC1541. It was shown that AC 1541, as an effective inoculant, can increase rice production by 8%, tomato or cucumber by 11-33%, or decrease the dose of chemical fertilizer by 15-50%. AC1541 has become a new type of bio-fertilizer and got approval for limited commercialization in Northern China.

Biosafety Consideration

The general principles of biosafety assessment for recombinant microorganism, such as safety classification, scope and procedure of evaluation, control measures and management etc, are the same as other GMOS. When doing evaluation, the safety grade of recipient microorganism should be firstly determined, then the impact of genetic manipulation be decided, finally a comprehensive assessment of the recombinant microbial agent be made.

For microbial agents, the following content should be more concerned than that in other GMO products.

1) Vector name, source, function, recombinant DNA structure, constructive method, replicate characteristics, inserted or deleted DNA fragment (including antibiotic resistant marker)

2) In comparison with the recipient microorganism, the following characteristics of recombinant are changed or not: capability of colonization, survival, spread and transmission, genetic variation, ecological relation with plants and other microorganisms toxicity or pathogenicity, identifying and monitoring methods.

3) Possibility for other organisms in environment to obtain the target gene from recombinant microorganism.

Case Analysis

1) *Bacilus thuringiensis* **WG001 (P19®YBT1520)**
   - The transferred P19 as a type of chaperone protein comes from Bt itself. ICP gene recombination in Bt naturally occurred very often.
   - New resolution vector (pBMB8017) based on Tnp I-mediated site-specific recombination system of Bt transposon Tn4430 was developed. Only target gene was maintained, while antibiotic resistance genes and other non-Bt DNA were eliminated.

2) *Alcaligenes faecalis* **AC1541 (*nifA+ntrC*)**

- Vectors and target genes were safely used for a long time. No harmful marker gene in vectors.
- Stable chromosome integration of target genes without risk of plasmid transmission.
- Poor spread capability. No detection from soil or water over 10 meters apart from spraying site in rice paddy field.
- Low survival potential capability for recombinants. The number of recombinants quickly decreased (nearly 10 fold reduction on average each month). Changed to minor population 60 days after inoculation. No detection in next spring.

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Economic Impacts of Bt Cotton in China[*]

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Abstract:

Bt cotton is spreading very rapidly in China pulled by farmers demand for technology that will reduce their costs of pesticide, exposure to pesticide, and allow them to do other things with the time they would have been spraying pesticides. Based on surveys of hundreds of farmers in the Yellow River cotton growing region in northern China in 1999, 2000, and 2001, over 4 million small holders have been able to increase their yield per ha, reduce pesticide costs, reduce the time that they spend spraying dangerous pesticides, and reduce the number of times that they are sick from pesticide poisoning. The expansion of this cost saving technology is increasing the supply of cotton and pushing down the price of cotton, but they are still sufficiently high that adopters of Bt cotton as making substantial gains in net income from Bt cotton.

Keywords: Bt cotton, Economic impacts, China

Despite growing evidence that Bacillus thuringiensis (Bt) cotton is reducing the use of insecticides, reducing farmers costs of production and increasing yields in the U.S. (The Plant Journal 2001), China (Pray et al., 2001; Huang et al., forthcoimg-a), S. Africa (Ismael et al., 2001), and Mexico (Traxler et al., 2001), the critics of biotech continue to doubt its usefulness particularly for small farmers in developing countries. A recent article in the journal of the Genetic Resources Action International (GRAIN, 2001) argues that Bt cotton does not have any positive impact on yields and implies that bollworms that are resistant to Bt are already becoming a problem in China.

This article documents the impact of Bt cotton in China using three years of farm level surveys. In our earlier work, we examined the impact of Bt cotton in China using data from a study of 283 farmers in Hebei and Shandong Provinces in 1999 (Pray et al., 2001; Huang et al., forthcoming-a; Huang et al., forthcoming-b). These articles demonstrated that Bt cotton adoption led to positive and significant economic and health benefits for poor, small farmers.

However, China's rural economy is evolving fast and it may be that the environment has changed so much in the past several years that the benefits and costs from Bt cotton to farmers in China has also changed. Although the commercialization of cotton markets began in the late 1990s, most cotton was still purchased by the state Cotton and Jute Corporation in 1999 at a price fixed by the government. Since 2000, the government has allowed the price of cotton to fluctuate with market conditions. Cotton mills are now allowed to buy cotton directly from growers. On the input side, the New Seed Law passed in 2000 gave legitimacy to private seed companies and allowed them to operate in many provinces. These changes led to sharp changes in the price of cotton, increased Bt cotton seed availability, and changes in pricing strategy of Bt cotton seed.

In the context of China's changing agricultural economy, the overall goal of this paper is to review the findings of our earlier papers that analyzed the effect of Bt cotton adoption in 1999 and the results of 2 follow-up surveys that were conducted in 2000 and 2001. Reports from government officials indicate that Bt cotton is spreading rapidly in the major cotton growing regions of China. Our survey data on yields indicate that the adoption of Bt cotton continues to increase output per hectare in 2000 and 2001 and that the yield gains extend to all provinces in our sample. More importantly, Bt cotton farmers also increased their incomes by being able to reduce the use of pesticides and labor. However, Bt cotton's success has attenuated its benefits. Rising yields and

expanding area has begun to push cotton prices down. As a result, some of the gains that accrued previously to producers are now being enjoyed by consumers. Finally, data from the survey shows that Bt cotton continues to positive environmental impacts by reducing pesticide use. We provide evidence that farmers have less health problems because of reduced pesticide use. We conclude with evidence that China is not unique and that there are lessons for other developing countries in their experience.

History of development and adoption of Bt cotton in China

China has made a major investment in biotechnology research (Huang et al., 2002). These investments started in the mid-1980s and were accelerated in the late 1980s by the Ministry of Science and Technologies' 863 Project.[1] Unlike biotechnology research in most other countries of the world, the private sector has not played a major role in biotech research in China.

Insect pests, particularly the cotton bollworm (*Helicoverpa armigera*), have been a major problem for cotton production in northern China. China's farmers have learned to combat these pests using pesticides. Initially, farmers used chlorinated hydrocarbons (e.g., DDT) until they were banned for environmental and health reasons in the early 1980s (Stone, 1988). In the mid-1980s, farmers began to use organo-phosphates but in the case of cotton, pests developed resistance. In the early 1990s, farmers began to use pyrethroids, which were more effective and safer than organo-phosphates. However, as in the case of other pesticides, China's bollworms began to rapidly develop resistance to pyrethroids in the mid 1990s. At this time, farmers resorted to cocktails of organo-phosphates, pyrethroids and whatever else they could get (including DDT, although use of choloinate hydrocarbons is illegal) with less and less impact on the pests.

With rising pest pressure and increasingly ineffective pesticides, the use of pesticides by cotton farmers in China has risen sharply. Farmers use more pesticide per hectare on cotton than on any other field crop in China (Huang et al., forthcoming-b). In aggregate, cotton farmers use more pesticide than farmers of any other crop except rice (since the sown area of rice is many times more than that of cotton). Per hectare pesticide cost reached US$ 101 in 1995 for cotton, much higher than that for rice, wheat or maize, and many times more than the level applied by most other farmers in the world. Cotton production consumes nearly US$ 500 million in pesticides annually (a et al, forthcoming-b).

China's pest problems have led the nation's scientists to seek new pesticides, to breed cotton varieties for resistance to pests, and develop integrated pest management (IPM) programs to control the pests. Consequently, when the possibility of incorporating genes for resistance to the pests came closer to reality, China's scientists started working on the problem. With funding primarily from government research sources, a group of public research institutes led by the Chinese Academy of Agricultural Sciences developed Bt cotton varieties using a modified Bt fusion gene (*Cry1ab* and *Cry 1Ac)*. The gene was transformed into major Chinese cotton varieties using China's own methods (pollen tube pathways). . Researchers tested the varieties for their impact on the environment and then released for commercial use in 1997 (Pray et al., 2001).

Monsanto, in collaboration with the cotton seed company Delta and Pineland, developed Bt cotton varieties for the U.S. which were approved for commercial use in the U.S. in 1996. They began to collaborate with the Chinese National Cotton Research Institute of CAAS at Anyang, Henan in the mid 1990s. Several of their varieties worked quite well in China. In 1997, several varieties were tested and approved by the Chinese Biosafety Committee for commercialization. At the same time, scientists in the Cotton Research Institute were working on their own varieties. The research team began to release their varieties in the late 1990s.

As Bt cotton has spread, government research institutes at the province and prefecture levels have also produced new Bt varieties by backcrossing the Monsanto and CAAS varieties into their own local varieties. These varieties are now spreading in Henan, Shandong and elsewhere.

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[1] The "863" Plan, also called High-Tech Plan, was initiated in March 1986 to promote high technology R&D in China. Biotechnology is one of 7 supporting areas of the "863" Plan.

Interviews with officials from local seed companies and officials in July 2001 confirmed that such practices were widespread in almost every province in North China.

At present, CAAS has permission from the Biosafety committee to sell 22 Bt cotton varieties in all provinces of China. The Biosafety Committee has approved the sale of five Delta and Pineland Bt varieties in four provinces.[2] Many other varieties are from national institutes like the Cotton Research Institute, Anyang, and provincial institutes are being grown, but some of these local varieties do not go through the official approval procedure set by the Chinese biosafety committee.

In the wake of the commercialization of these approved and non-approved varieties, the spread of Bt cotton has been very rapid. From nothing in 1996, provincial officials, research administrators and seed company managers estimate that farmers plant nearly 1.5 million hectares of Bt cotton (Figure 1). This means that approximately 31 percent of China's cotton area was planted to Bt cotton in 2001.

While the spread of Bt cotton has relied on the varieties introduced by the public research system and seeds sold (at least initially) by the state-run seed network, the adoption of Bt varieties has been the result of decisions by millions of small farmers. Our survey indicates that there were more than 4 million farms had adopted Bt cotton in 2001.

Figure 2 shows the spread of varieties by province. A few thousand hectares were planted in Hebei for seed production in 1997. Commercial production by farmers started in 1998 in the Yellow River cotton region of Hebei, Shandong and Henan. It spread rapidly to 97 percent of the cotton area in Hebei by 2000 and 80 percent of Shandong by 2001. In Henan it appears to be leveling off at about a third of the cotton area. Bt cotton started a year later than the other provinces in the southern provinces of Anhui and Jiangsu. It is spreading fairly rapidly in Anhui. There are small amounts of Bt cotton planted elsewhere including Xinjiang in the West.

Data and methods

To assess the impact of biotech in China we have conducted a series of surveys in the years 1999, 2000, and 2001. In each successive year, we increased the size of our sample and the provinces covered as Bt cotton spread into them. In 1999, we began with a sample of 283 farmers in Hebei and Shandong. The counties where the survey was conducted were selected so that we could compare Monsanto's Bt cotton variety, CAAS Bt varieties and conventional cotton. Hebei had to be included because it is the only province in which Monsanto varieties have been approved for commercial use. Within Hebei province Xinji county was chosen because that is the only place where newest CAAS genetically engineered variety is grown. We chose the counties in Shandong Province because the CAAS Bt cotton variety GK-12 and some non-Bt cotton varieties were grown there. After the counties were selected, the villages were chosen randomly. Within the selected villages the farmers were randomly selected from the villages' list of farmer and then these farmers were interviewed.

In the second year, we included Henan province so that we could assess the efficiency of Bt cotton by comparing them to the conventional cotton varieties that were still being grown there. Henan is in the same Yellow River cotton growing region as Hebei and Shandong and as such has agronomic and climatic characteristics that are similar to them. In 2001, we added Anhui and Jiangsu provinces because Bt cotton had now spread further south. As we did in 1999 counties were selected so that they would contain both Bt and non-Bt cotton producers. In the second phase of sample selection, villages and farmers were randomly selected. In 2000 and 2001, we also continued to survey the same villages in Hebei and Shandong, which we surveyed in the 1999. The total number of farmers interviewed increased to about 400 in 2000 and 366 in 2001.

Impact on yield, pesticide use and costs of production

In China Bt cotton was developed in order to provide more effective protection against pests.

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[2] Hebei, Shandong, Henan, and Anhui Provinces. Len Hawkins, Delta and Pineland, Beijing, email April 22, 2002.

Scientists expected that farmers who grew Bt cotton would be able to substantially reduce the amount of pesticide and have a better control of bollworm, which would reduce costs of production and increase yield. Scientists expected that Bt cotton would yield more per hectare because it would reduce the damage that bollworms did to cotton even with the best available chemical pesticides. By reducing these losses, which farmers might not even realize them, Bt cotton would increase yields.

In the provinces that still grew some non-Bt in 2001, the mean yield of the Bt cotton varieties are 5 to 6 percent higher than the yields of the non-Bt varieties (Table 1). For all of the farms in the sample, Bt varieties were about 10 percent higher yielding in 2001. This is consistent with the 8 percent yield increase due to Bt cotton in 1999 that what we found using the econometric techniques, which examined the impact of Bt adoption on yields after accounting for other inputs (Huang et al., forthcoming-a).

Yields of Bt cotton in the provinces that have used them for several years also have increased. Thus, according to our data, there is no obvious deterioration of the effectiveness of Bt varieties over time. The increasing yields also counter suggestions that bollworms are becoming resistant to Bt cotton. Instead, the trends in our over sample suggest that farmers may be learning to manage the Bt varieties better and are obtaining higher yields by better using the advantages that Bt varieties offer.

Our data also demonstrates that Bt cotton varieties continue to lead to reduce total pesticide use. Table 2 shows that pesticide use has remained low in the states that adopted Bt cotton first – Hebei and Shandong. In the provinces Henan and Anhui where Bt cotton was recently introduced commercially the mean application of pesticides was reduced by 24 to 63 kilogram per hectare. Only in Jiangsu, where red spider mite rather than bollworm is the main pest (Hsu and Gale, 2001), was the reduction in pesticide small, only seven kilograms per hectare. This suggest that the spread of Bt cotton may slow down as it moves away from the center of the region in which bollworms have historically been the major pest (Hebei and Shandong). The reasons for the slowdown in Jiangsu appear to be that bollworm is not as much of a pest problem. As a consequence, the economic benefits from Bt are not great – especially at the higher prices of Bt seed in this region. In Henan, the bollworm problems are as important as they were in Hebei, but the problem appears to be that farmers can only buy inferior varieties of Bt cotton. There is a virtual monopoly on seed production and sales by the Provincial Seed Company supplying varieties from the local research institutes. In addition, for some reason, China's Biosafety Committee has refused to allow 33B or 90B to be grown in the province. Thus, farmers have to grow illegal "33B" and CAAS varieties supplied by private seed traders or local Bt varieties that have not approved by the Biosafety committee. Part of the problem of the Henan varieties is that the level of Bt expression of those varieties falls by midseason (Wu, 2002).

However, our sample does appear to show some increase in pesticide use per hectare on Bt cotton in 2000/2001 over 1999 when we examine the entire sample (Table 2). Most of this increase, however, is due to the addition of high pesticide use provinces in the south – Anhui and Jiangsu – where red spider mites rather than bollworm are the main pests. In those provinces in which we have data over time, the record of pesticide use per hectare is mixed. In Hebei province, for example, it increased between 1999 and 2001. In Shandong, however, after increasing between 1999 and 2000, it decreased in 2001. Between 2000 and 2001 pesticide use per hectare fell.

While it is not possible to definitively say why the increased pesticide uses in some locations 2000 have occurred, there are several possibilities. One explanation could be that the higher use is just due to differences in naturally occurring fluctuation in pest pressure, so the effect would be expected to disappear over time. The changes also could be due to the fact that farmers have begun to save their seed instead of buying new seed, an act that could reduce the effectiveness of the Bt protection since saved seed is lower quality. It could also be that bollworms are beginning to develop resistance. However, there is evidence that this increase is not the case. The Institute of Plant Protection has been collecting bollworms moths and testing them for resistance to Bt since 1997. In 2001, the latest year for which data is available they had not found any evidence of bollworm resistance to Bt cotton (Wu, 2002).

The impact of these changes on cost of production and net income are shown in Table 3.

The costs of seeds were always greater for Bt varieties. However this was offset by a much greater reduction in pesticide use and a reduction in labor use because Bt cotton farmers did not have to spend as much time spraying pesticide. The total cost per hectare of producing Bt cotton was much less than non-Bt each in 1999 and 2001, but slightly higher in 2000 mainly due to higher fertilizer inputs in Bt cotton (Table 3).

Because higher yield of Bt cotton, and as shown in our earlier work, the prices of Bt and non-Bt cotton were virtually identical, the output revenues of Bt are higher than the revenues of non-Bt cotton (the first row, Table 3). After deduction of total production costs from output revenues, Table 3 shows that net income (last row) from producing Bt varieties was higher than non-Bt.

Impact on farmer health and the environment

The reduction of pesticide use due to Bt cotton has been substantial (Table 2). In China since pesticide is primarily applied with small back-pack sprayers, which are either hand-pumped or have a small engine, and since farmers typically do not use any protective clothing, applying pesticide is a hazardous enterprise because they almost always end up completely covered with pesticide. Hence, it is important to know if the reduction in pesticide use can be linked to improved health. In the past, large numbers of farmers become sick from pesticide applications each year (Qiao et al., 2000).

According to our data, by reducing the use of insecticides Bt cotton has also reduced the number of farmers who are poisoned by pesticides each year. Table 4 divides our sample farmers into three groups: those who exclusively use non-Bt varieties, those who use non-Bt, and those who plant only Bt varieties of cotton. In the first group a higher percentage of farmers reported poisoning in each year. The percentages were particularly high – 22 percent and 29 percent in the first two years. In contrast between 5 and 8 percent of farmers who used only Bt cotton reported that they had become sick from spraying pesticides.

Perhaps most importantly, the total decline in pesticide use has been impressive. Using the differences in average pesticide use in Table 2 and the area reported in Figure 1, the declines can be calculated. In 1999 the reduction in pesticide use was approximately 20 thousand tons of formulated pesticide while in 2001 due to increased area under Bt and increased savings per hectare was 78 thousand tons or about one quarter of all the pesticide sprayed in China in the mid 1990s.

Impact on total cotton production and location of production

Bt cotton has rejuvenated cotton production in the Yellow River area of China (North China). Cotton production was at its highest level in 1991 when the nation produced more than 3 million tons. Production in the Yellow River region then plunged again to 1.4 million tons two years later in 1993. This was largely due to a severe bollworm infestation, as well as increased labor costs in the region and changes in relative crop returns (Hsu and Gale, 2001: p.19). When Bt cotton started to spread extensively in the region in 1999, cotton area rebounded. In Hebei and Shandong Provinces cotton area went from 729,700 ha in 1998 to 876.100 ha in 2000 (NSBC, 1999-2001). Farmers were responding to the pest-resistant characteristics of the Bt which allowed them successfully grow cotton despite the bollworms, reduced their costs of production, and saved labor.

At the same time cotton production in the Yangtze region (South China) has remained steady while cotton production has risen gradually in the Northwest. The Northwest cotton region is basically irrigated desert. As a result they have less pest problems, higher yields, and higher fiber quality than other regions of the country. Their major problem is being so far away from markets for cotton, which are primarily in the Yangtze region and to a lesser extent in the Yellow River region. To offset the cost of transportation and encourage more production in this region, the Chinese government provides subsidies for important inputs like irrigation and mechanized tillage, planting, and harvesting.

Other things held equal, the recent increases in production due to lower costs should have led to lower prices of raw cotton, which would have passed some of the gains from Bt cotton along to consumers of cotton. Instead the prices of cotton went up between 1999 and 2000. They did not decline until 2001. Farmers in our sample received 3.4 yuan per kilogram for Bt cotton and 3.32

yuan per kilogram for conventional cotton in 1999. Prices of Bt cotton and non-Bt cotton then went up to 4.45 and 4.42 in 2000, an increase of about 30 percent. In 2001, price declined sharply to 3.02 and 3.07 for Bt and conventional cotton, a level approximately 10 percent below 1999 prices.

These fluctuations in prices – particularly the increase from 1999 to 2000 - are primarily due to the changes in the structure of cotton markets and other supply and demand factors. They most likely have little to do with the introduction of Bt cotton. However, the decline between 2000 and 2001 may be partially due to Bt cotton. The Foreign Agricultural Service of USDA (U.S. Embassy, 2002: p.1) reports that "Improved yields over the past two years likely reflect the growing use of genetically modified Bt cotton..." and "… driven by domestic production, cotton procurement prices hit record lows in early 2001."

The implications of the price trends are that unlike 1999 when all of the gains went to producers, in the past several years, some of the gains from the adoption of Bt cotton are starting to be passed along to consumers. In this case the first set of consumers are the large cotton mills that produce yarn and cloth. Reports by the USDA (U.S. Embassy, 2001) suggest that yarn and cotton cloth prices, like raw cotton prices, are subject to considerable downward pressure. Thus, some of the gains due to Bt cotton are probably being passed along to consumers in both China and in the international market.

Despite the decrease in prices in 2001, farmers were still able to obtain increased net incomes of about $500/ha by growing Bt cotton instead of non-Bt cotton (Table 3).

Is China different from other developing countries?

Many of critics of biotechnology have argued that the benefits from Bt cotton, which have been shared by over 4 million small farmers in China, can not be gained by producers in other developing countries. They argue that China's farmers are forced to grow Bt cotton. However, according to our survey results and fieldwork, these critics are wrong and do not understand China's agriculture. For more than two decades, and increasingly in the past 10 years, most of China's farmers make their own decisions about what to plant and what technology to use. In this way, China's farmers are like those of other countries. However, it is true that there are important differences between China and other developing countries that other countries need to consider when drawing lessons from the China's experience.

First, China's farmers are no longer forced by the government to grow cotton. In fact, in recent years the opposite has been the case. In 1999 while pre-testing our questionnaire we explicitly asked farmers in Hebei Province, if they were required to grow a certain amount of cotton. They reported that in the past the government did put pressure on them to grow cotton by requiring that each farmer sell a fixed quantity of cotton to the government. By the mid 1990s, although these quotas were still in place, in fact, they were no longer effectively enforced. Moreover, nearly every farmer in the sample stated that by 1998 cotton quotas were gone entirely. Since then the market for cotton has been further liberalized and they face even less pressure for cotton production – in fact in recent years the government has been trying to discourage them from expanding cotton production with little or no success (U.S. Embassy, 2001).

Moreover, we found no evidence of pressure to buy Bt cotton. Government agencies have been providing conflicting messages about Bt cotton. Commercialized government seed companies and private seed companies encouraged farmers to buy Bt cotton seed. At the same time, however, Plant Protection Stations and government-owned pesticide companies tried to discourage them from growing Bt cotton so that they could sell more pesticides.

Like Indian, Pakistani, or Indonesian cotton growers, producer in China are primarily small holders. On average, China's cotton farmers have even smaller farms than farmers in other countries. Since they buy their seed in competitive markets and sell their output in a competitive market, they differ little in these respects from their counterparts in other countries.

The main difference from other countries, however, is the major role of the public sector in providing GM technology. A large share of the Bt cotton varieties that farmers cultivate was

developed by scientists working in public research institutes and sold by government seed companies. Political support from these scientists to allow commercialization of GM technology is one of the reasons that China approved the commercialization of GM crops earlier than most other developing countries (Paarlberg, 2001). In addition the competition between local government firms and foreign firms in providing Bt cotton varieties is undoubtedly one of the reasons that the prices of Chinese GM cotton seed is so low.

Conclusions

Bt cotton is spreading very rapidly in China pulled by farmers demand for technology that will reduce their costs of pesticide, exposure to pesticide, and allow them to do other things with the time they would have been spraying pesticides. The evidence from five years of experience with Bt cotton is that this technology is extremely valuable to over 4 million small holders in China. They have been able to increase their yield per ha, reduce pesticide costs, reduce the time that they spend spraying dangerous pesticides, and reduce the number of times that they are sick from pesticide poisoning.

As predicted by economic theory, the expansion of this cost saving technology is increasing the supply of cotton and pushing down the price of cotton. We have not yet done the modeling required to estimate how much of the decline in prices is due to Bt cotton, but the good news is that prices are still sufficiently high that adopters of Bt cotton as making substantial gains in net income from Bt cotton.

The last part of the paper argues that China is similar to other developing countries in that farmers are making the decisions to adopt Bt cotton based on their assessment of the costs and benefits. They find it profitable and so we would expect cotton growers on small farms in many other developing countries to achieve similar gains. Especially in countries, such as India, where cotton growers face the same bollworm pressures, and where the bollworm has become resistant to many of the most common pesticides, farmers are likely to benefit greatly from this technology.

The other lesson from China is the importance of local research on biotechnology. The fact that Bt cotton was developed by government researchers at about the same time that international companies were introducing it into China, clearly made it more palatable to the government and ensured that there was a strong lobby in favor of the technology.

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Table 1. Yield of Bt and non-Bt cotton in sampled provinces, 1999-2001

	Number of plots			Yield (kg/ha)		
	1999	2000	2001	1999	2000	2001
Hebei						
Bt	124	120	91	3197	3244	3510
Non-Bt	0	0	0	Na	na	na
Shandong						
Bt	213	238	114	3472	3191	3842
Non-Bt	45	0	0	3186	na	na
Henan						
Bt		136	116		2237	2811
Non-Bt		122	42		1901	2634
Anhui						
Bt			130			3380
Non-Bt			105			3151
Jiangsu						
Bt			91			4051
Non-Bt			29			3820
All samples						
Bt	337	494	542	3371	2941	3481
Non-Bt	45	122	176	3186	1901	3138

Note: Cotton production in Henan was serious affected by flood in 2000, which lowered the yield. Counties included in the surveys are: Xinji (1999-2001) and Shenzhou (1999-2000) of Hebei province, Lingshan (1999-2001), Xiajin (1999-2000) and Lingxian (1999-2000) of Shandong province, Taikang and Fugou of Henan province (2000-2001), Dongzhi, Wangjiang and Susong of Anhui province (2001), and Sheyang and Rudong of Jiangsu province (2001).

Source: Authors' surveys.

Table 2. Pesticides application (kh/ha) on Bt and non-Bt cotton, 1999-2001.

Year	Location	Bt cotton	Non-Bt cotton
1999	All samples	11.8	60.7
	Hebei	5.7	
	Shandong	15.3	60.7
2000	All samples	20.5	48.5
	Hebei	15.5	
	Shandong	24.5	
	Henan	18.0	48.5
2001			
	All samples	32.9	87.5
	Hebei	19.6	
	Shandong	21.2	
	Henan	15.2	35.9
	Anhui	62.6	119.0
	Jiangsu	41.0	47.9

Note: Red spider mite is the most serious problem in Anhui and Jiangsu in 2001, while bollworm is less serious.

Source: Authors' survey.

Table 3. Average per hectare costs and returns (U.S. $) for all surveyed farmers, 1999-2001.

	2001		2000		1999	
	Bt	Non-Bt	Bt	Non-Bt	Bt	Non-Bt
Output revenue	1277	1154	1578	1013	1362	1265
Non-labor costs						
Seed	78	18	59	21	62	63[a]
Pesticide	78	186	52	118	31	177
Chemical fert.	162	211	132	128	154	154
Organic fert.	44	53	41	18	28	34
Other cost	82	65	86	70	120	88
Labor	557	846	840	841	616	756
Total costs	1000	1379	1211	1196	1011	1271
Net revenue	277	-225	367	-183	351	-6

[a]. Seed prices for conventional cotton were so high because 9 farmers reported growing a new variety "Bu Xiu Cotton" which was supposed to have fewer labor and management inputs and had seed costs of $155/ha. $1=8.3 Yuan.
Source: Authors' surveys.

Table 4. Impact of Bt on farmer poisoning, 1999-2001.

		Farmers planting non-Bt cotton only	Farmers planting both Bt and non-Bt cotton	Farmers planting Bt cotton only
1999	Farmers	9	37	236
	Number of poisonings[a]	2	4	11
	Poisonings as % of farmers	22	11	5
2000	Farmers	31	58	318
	Number of poisonings[a]	9	11	23
	Poisonings as % of farmers	29	19	7
2001	Farmers	49	96	221
	Number of poisonings[a]	6	10	19
	Poisonings a % of farmers	12	10	8

a: Farmers asked if they had headache, nausea, skin pain, or digestive problems when they applied pesticides.

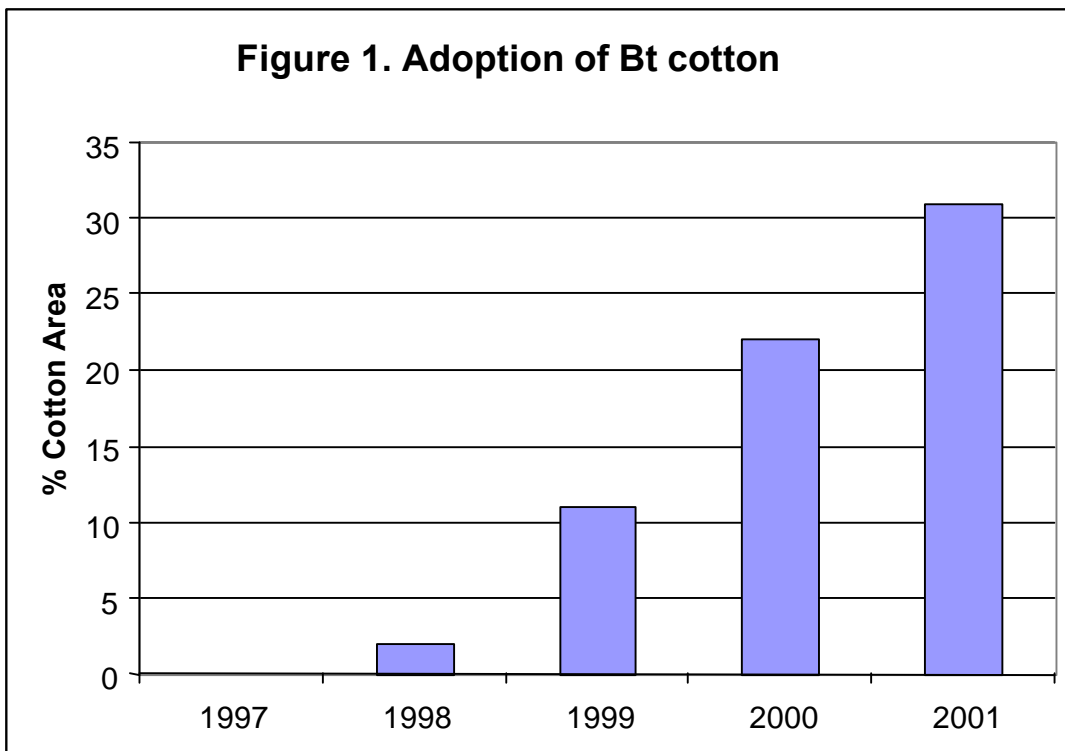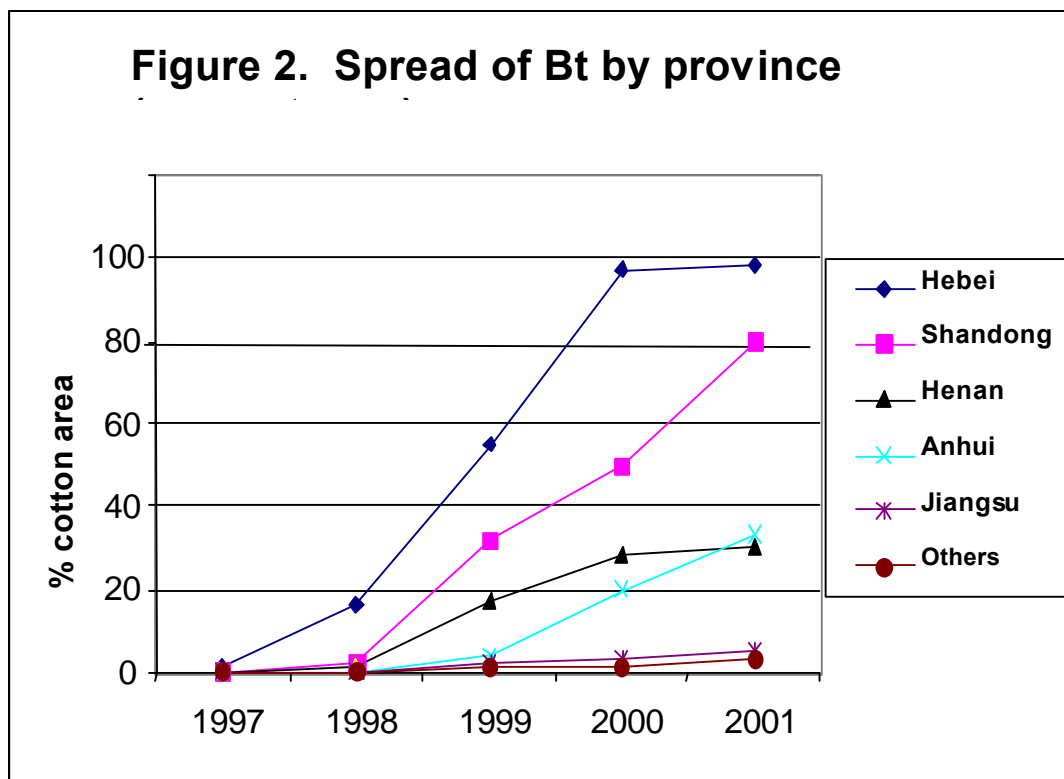Source: Authors' surveys.

Figure 1. Adoption of Bt cotton

Figure 2. Spread of Bt by province

Environmental impact of Bt cotton: a case study from China

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Abstract A series of studies on ecological safety of Bt cotton have been conducted since 1995, which include efficacy of Bt cotton against *Helicoverpa armigera*, impacts on non-target insects and arthropod biodiversity, baseline for *H. armigera* resistance to Bt cotton and its resistance monitoring, mechanisms of *H. armigera* resistance to Bt cotton, evaluation of natural refugia function, and the biology of *H. armigera* in relation to resistance evolution. The present results indicated that the use of Bt cotton in production agriculture of northern China is presenting the advantages of reducing the use of chemical insecticides for control of two key insect pests, *H. armigera* and cotton aphid. This is resulting in increased the biodiversity of arthropod, decreased environmental pollution and in lower cost for insect control in cotton. Additionally, by reducing the area sprayed with insecticides and the frequency of sprays, the rate of insecticide resistance evolution is decreasing. However, although field populations of cotton bollworm are still susceptible to Bt cotton after several year commercialization, the studies for risk evaluation of the pest to Bt cotton in both the laboratory and field shows that the large-scale use of Bt cotton is likely to cause evolution of Bt toxin resistance from the pest. A crop-planting system consisted of wheat, Bt cotton, soybean (or peanut) and corn which could supply natural refugia for different generations of *H. armigera,* is suggested to use as a low risk crop-planting mode to delay evolution speed for the pest resistance to Bt cotton.

Key words: Bt transgenic cotton, *Helicoverpa armigera*, environmental impact, biodiversity, resistance management

Since the mid-1980s, *Helicoverpa armigera* (Hübner), a most destructive insect pest to cotton *Gossypium hirsutum* L., maize *Zea mays* L., sorghum *Sorghum bicolor* L. Moench and many other crops, has become increasingly resistant to insecticides used in China, presenting a major threat to cotton production. Natural resistance of cotton to the insect has been used in the development of germplasm lines and cultivars. However, no single germplasm line or cultivar released to date exhibits a level of resistance high enough to preclude the use of insecticides, especially under high insect infestations. The availability of genetically modified cotton plant that carry gene for insect resistance from other organisms may increase the chance of developing germplasm lines with higher levels of resistance to the insect. Both Monsanto Co. and Biotechnology Research Institute, Chinese Academy of Agricultural Sciences (CAAS), have successfully developed transgenic cotton varieties expressing the *Bacillus thuringiensis* Berliner (Bt) Cry1A δ-endotoxin, encoded by the gene *cry1A*. These Bt cotton varieties have been planted widely in northern China. In order to understand the impact of Bt cotton planting on environment, a series of studies on ecological safety of Bt cotton have been conducted by the Center for Biosafety Research, Institute of Plant Protection, CAAS since 1995, which include efficacy of Bt cotton against *Helicoverpa armigera*, field abundances of natural enemies, impacts on non-target insect pests, arthropod community structure in the Bt cotton ecosystem, baseline for *H. armigera* resistance to *Cry1Ac* protein, resistance monitoring, selection of resistant strains of *H. armigera* and resistance inheritance, resistance mechanisms, evaluation of natural refugia function, and the biology of *H. armigera* in relation to resistance evolution. In this paper we report on the major results as follows.

1 Efficacy of Bt cotton against *Helicoverpa armigera*

Several Bt cotton varieties from Monsanto Co. and Biotechnology Research Institute (CAAS) were evaluated for resistance to *Helicoverpa armigera* during in recent years in both northern and southern China. The results showed that there were no significant differences in egg densities between Bt cotton and their parental varieties during the seasons, but the survival of larvae on Bt appeared significantly reduced. High larval densities observed on non-Bt cotton appeared in great contrast to the low larval populations observed on Bt cotton plants during the seasons. In an environment of no insecticide sprays, the annual ginned cotton yields in Bt plots were about 4 times from the non-Bt cotton fields. It was also demonstrated, however, that under severe egg densities, potentially damaging bollworm larval densities may develop in transgenic cotton fields. A density-independent reason for increased survival, especially in later bollworm generations, may be associated with reduced levels of available toxin in plant tissues as they age. Insecticide application is still an essential alternative for late-season bollworm control in Bt cotton field in some years. It is concluded that the high level of field-efficacy for Bt cotton against *H. armigera* in China may pave the way for reduced pesticide applications and an expansion of alternative pest-control strategies.

2 Impacts of Bt cotton planting on status of insect pests

While the Bt protein is directly toxic to only a narrow spectrum of Lepidopteran species, the dynamics of other species may be indirectly affected. As a result of Bt cotton deployment, the amount of insecticide use has been drastically decreased in cotton field. Effects on non-target predatory and parasitic species may be positive due to the removal of disruptive pesticides, or negative due to the effective removal of lepidopteran prey.

Field surveys indicates that the densities of major natural enemies in Bt cotton fields are significantly higher than those on non Bt cotton due to decrease of insecticide application for control of *H. armigera* on Bt cotton, which include in lady beetles (*Coccinella septempunctata* Linnaeus, *Leis axyridis* (Pallas) and *Propylaea japonica* (Thunberg)), lacewing (*Chrysopa sinica* Tjeder, *Chrysopa septempunctata* Wesmael, *Chrysopa shansiensis* Kawa and *Chrysopa formosa* Brauer), spiders (*Erigonidium graminicolum* (Sundevall) and *Misumenopos tricuspidata* (Fabricius)) and *Orius similis* Zheng.

Cotton aphid, *Aphis gossypii* Glover, is an important insect pest of cotton in northern China. Insecticide use for *H. armigera* control disrupts aphid natural enemies and is responsible for aphid resurgence in mid season. The influence of Bt cotton on population dynamics of cotton aphid, *Aphis gossypii* Glover, was investigated during 1998-2001 in northern China. The field experiments were conducted in plots of Bt cotton and conventional cotton that received no insecticide applications, and in plots of conventional cotton where pyrethroid and organophosphate insecticides were used regularly for control of *Helicoverpa armigera*. The results indicated that resistance of cotton aphids to majority of insecticides used for control of *H. armigera*, and lower densities of predators in late June and early July caused by insecticide use, caused population densities of cotton aphids to become significantly higher in plots of insecticide-treated conventional cotton than in Bt cotton plots. These results suggest that Bt cotton planting not only played an important role in control of *H. armigera*, but also efficiently prevented cotton aphid resurgence in response to insecticide use.

Lygus lucorum Meyer-Dür, *Adelphocoris fasciaticollis* Reuter and *Adelphocoris lineolatus* (Goeze) (Hemiptera: Miridae) are important secondary insect pests in cotton fields. The seasonal dynamics of their mixed populations on a transgenic variety expressing the insecticidal Bt protein *Cry1A*, and a cotton line expressing proteins of *Cry1A* and *CpTI* (cowpea trypsin inhibitor gene) were compared to seasonal dynamics on similar but non-transgenic varieties from 1998 to 2001. The results indicated that there were no significant differences between population densities of these bugs on unsprayed normal cotton and unsprayed transgenic cotton. However, mirid density on unsprayed transgenic cotton was significantly higher due to a reduced number of insecticide sprays against *Helicoverpa armigera* compared with the number of sprays in the normal cotton. This suggests that the mirids have become key insect pests in transgenic cotton fields, and that their damage to cotton could increase further with the expansion of the area planted to transgenic cotton if

no additional control measures are adopted.

3 Effects of Bt cottons on arthropod biodiversity

The effects of transgenic cotton on the structures and composition of pest and beneficial arthropod communities in the cotton fields were investigated during 2000-2001 in northern China. The field experiments were conducted in non-insecticide application plots of transgenic cottons (SGK321 – transgenic cotton variety containing *Cry1A+CpTI* genes, GK12 -- transgenic cotton variety carrying *Cry1Ac* gene) and conventional cotton (Shiyuan 321 and Simian 3), and in plots of conventional cotton (Shiyuan 321 and Simian 3) where pyrethroid insecticides were used regularly against cotton bollworm. All the arthropods were sucked up using a portable suction device and identified to species wherever possible. They were later sorted into guilds (phytophages, predators, parasitoids, scavengers/decomposers, and tourists) for diversity analysis using the method from Shannon's index. The results showed that the species number of pests and beneficial arthropods in transgenic cotton plots were almost the same as those in conventional cotton field, but the species composition and dominancy were significantly different. For the structure and composition of pest community, the following results were obtained: 19 species of 15 families in average were observed in transgenic cotton filed whereas 17.5 species of 14 families in average obtained from untreated conventional cotton plots and 17.5 species of 13.5 families in average from insecticide-treated conventional cotton filed; more rich pest species occurred in transgenic cotton plots than in conventional cotton field where the insecticide-treated plots were almost dominant by two species, *Bemisia tabaci* Gennadius and *Aphis gossypii* Glover population of *Lygus lucorum* Mayer-Dür in transgenic cotton plots was much higher than that in conventional cotton field; *Helicoverpa armigera* was the common species on conventional cotton but absent on transgenic cotton. For the structure and composition of beneficial arthropod community, the following results were observed: 39.3 species of 24.3 families in average were collected from transgenic cotton filed whereas 43.5 species of 24 families in average obtained from untreated conventional cotton plots and 38 species of 25 families in average from insecticide-treated conventional cotton filed; *Orius similes* Zheng, *Campylomma diversicornis* Reut and species of Euhephidae were dominant in the transgenic and untreated conventional fields, species of Araneida increased and became the rich species in the transgenic and untreated conventional plots; *Trioxys rietscheli* Mackauer and *Propylaea japonica* (Thunberg) was dominant, and those parasitoids on *Aphis gossypii* Glover, such as species of Eucyrtidae, Aphelinidae and Aphidii were the rich species in the insecticide-treated conventional plots. the diversity of arthropod community in transgenic cotton plots was similar to that in conventional cotton field without spray, but Shannon's index for total arthropod community and neutral insect sub-community in Bt cotton fields were significantly higher than those in sprayed plots in the mid and late growing stages of cotton. It could be concluded that biodiversity could be effectively maintained in transgenic cotton plots, and this could be greatly favorable for the sustainable management of cotton pests.

To exactly define the biosafety of pollens from Bt cotton on non target species of some important economic insects, the effects on the growth and development as well as cocoon quality of the silkworm, *Bombyx mori* Linnaeus caused by feeding on the pollens from transgenic cotton containing *cry*1Ac / *cry*1Ac+*CpTI* genes were evaluated, compared with pollens from the non-transgenic normal cotton as well as the non-pollen treatment. In contrast to the latter ones, pollens from Bt cotton showed no marked adverse effects on larval mortality, cocoon weight, pupa weight, cocoon shell weight, pupation rate, emergence rate and fecundity of the silkworm as its neonates were fed with the pollens for 72 hours, and also no dosage-response effects of their pollens were found.. Though the first instar larvae duration was prolonged as compared with those of the non-pollen treatment, they were not significantly different from those fed with the pollens of non-transgenic cotton. Meanwhile, the weights of the third molters fed with transgenic pollens were obviously different from those of non-pollen treatment, but they were all significantly heavier than those of the checks. Consequently, it could be considered that the adverse effect of transgenic insect-resistant cotton on the growth and development of the silkworm is negligible.

4 Analysis for the secondary resistant metabolites in Bt cotton

The tendency and the relationship between the content of Bt insecticidal protein (BtIP) in Bt transgenic cotton and the morality of *H. armigera* were studied by applying ELISA and laboratory bioassay. The results showed that there were obvious spatio—temporal changes of the content of

BtIP along the developmental process of Bt transgenic cotton. The dynamics of larval moralities in the bioassay were consistent with that of BtIP contents in different growing periods/organs, i.e. high morality occurred in the growing period /organ possessed high BtIP content.

The contents of terpenoids chemicals in Bt transgenic cotton and their parental varieties were analyzed by HPLC method. The results indicated that Bt-ICP expression had no negative effect on synthesis of gossypol, total heliocide and total resistant terpenoids. The results of the combined dosage test of Bt-ICP and gossypol in vitro showed that there was no interaction between gossypol and Bt-ICP on the mortality of cotton bollworm larvae. This means the actions of Bt-ICP and gossypol on cotton bollworm are additive. HPLC analysis showed that Bt toxin in Bt transgenic cotton had no negative effect on synthesis of flavonoids (mainly rutin isoquercitrin and quercetin).The result of combined dosage test in vitro showed that rutin had significantly enhanced the resistant effect of Bt-ICP on the cotton bollworm larvae, and also rutin had very significantly resistant effect on the cotton bollworm larvae.

5 Influences of Bt cotton planting on resistance evolution of *H. armigera* to insecticides

Insecticide resistance is a key factor resulting in outbreak of *H. armigera* in China. In order to assess potential impact of Bt cotton that is rapidly expanding in China on the insect resistance to insecticides, the susceptible changes of a field population of *H. armigera* to cyhalothrin, phoxim and endosulfan, three major insecticides which were sprayed for control of *H. armigera* in China, were monitored while it is selected with *Cry1Ac* toxin. After selection for 32 generations, in comparison to the original baselines its resistance to *Cry1Ac* toxin increased 29 fold, but the resistance levels to cyhalothrin, phoxim and endosulfan dropped 36, 70 and 4 fold, respectively. It is suggested that there is no significant cross resistance between Cry1Ac toxin and these insecticides, and a large scale planting of Bt cotton in continuous years could exist resistance risk of the pest to Bt cotton, but decrease of insecticide use in Bt cotton fields would increase its susceptibilities to insecticides. The resistance monitoring confirms that resistance level to insecticides from the field populations of *H. armigera* has showed a decrease tendency.

6 Mechanism of *H. armigera* resistance to Bt cotton

Laboratory screening was conducted for obtaining the resistant populations of cotton bollworm respective to Bt pesticide, Bt protoxin and Bt transgenic cotton. After selected for 46 generations, the relative resistance ratios of *H. armigera* to Bt pesticide, Bt protoxin and Bt cotton were up to 1083.33, 417.00 and 48.67 times respectively as many as that of susceptible strains. It was found from the results of cross-resistance tests that when cotton bollworm had resistance to certain Bt preparation, it possessed positive cross-resistance to other preparations contained the similar Bt gene. When fed with fresh leaves of Bt transgenic cotton, the mortality of the larvae form the population already resistant to Bt transgenic cotton decreased significantly. Furthermore, the resistance of cotton bollworm to Bt transgenic cotton was determined as an incomplete recessive inheritance controlled by single autosomal allele. In comparison with susceptible strain, the larval survival and body-weight of *H. armigera* feeding on the diets containing Bt insecticides that having high level resistance to Bt increased significantly, while its mating rate and fecundity of adults, and hatching rate of eggs were decreased obviously, and the oviposition stage was shortened markedly, and those parameters of the crosses between resistant and susceptible strains were evidently higher than self crosses of resistant lines, and the larval survivals of the former were obviously increased than that of susceptible strains.

The activities of three detoxification (the   -naphthylacetate esterase, acetyl- cholinesterase and glutathione-S-transferase) and midgut proteases in susceptible and Bt resistant cotton bollworm populations were compared. The K_m and V_{max} values of   -naphthylacetate esterase and acetylcholinesterase were also tested. The specific inhibitors were used in enzyme identifications. As compared with the susceptibl population, the activities, K_m and V_{max} of acetylcholinesterase in Bt resistant populations increased, and the inhibitions of paraoxon methyl to acetylcholinesterase also obviously enhanced. It was found that either the differences of the activity of the total protease, or the weak alkaline trypsin-like enzyme and the chymotrypsin-like enzyme in the midgut between the larvae of susceptible and resistant populations were not significant. However, the activities of the active alkaline trypsin-like enzymes in third instar larvae of the Bt resistant populations were higher than those in the sensitive population.

Under the transmission electron-microscopy, it was found that the midgut tissues of the third

instar larvae were physiologically and pathologically changed after they fed separately on the diets containing three kinds of Bt preparations, in which the action of Bt pesticide on the midgut of the larvae was the most rapid, that of Bt protoxin was the second, and that of the Bt transgenic cotton was rather slow. The changes were more clearly along with time going, and after 7 days, the amounts of mitochondria and endoplasmic reticulum reduced, and cristae mitochondriae were not clearly. Compared with the susceptible population, a series of pathological changes also took place in the midgut structures of the Bt resistant populations fed on Bt preparations. The main changes of the midgut structures in resistant larvae to Bt pesticide were in microvilli, and there were no significant changes in the midgut cell, but there were obvious concentric circle markings in the cell. In the resistant larvae to the Bt protoxin, the variations of midgut structures occurred in the cell, the mitochondria hollow, cristae mitochondriale absent, but the change of microvilli negligible. There were some changes in the cell and the microvilli in the midgut structures of the resistant larvae to the Bt transgenic cotton, but the latter was more obvious.

By using Mg/EGTA method, to centrifuge with different speeds, the BBMV in midgut of *H. armigera* could be successfully separated, and most of the APN activities in BBMV were preserved. The CHAPS can enhance the dissolution of BBMV, and PI-PLC can cleave the APN from midgut membrane, the combination of Mono-Q and FPLC methods can purify partial APN. The results of Western blotting showed that the 120 and 170kDa proteins were the main ones that bound Cry1Ac in the BBMV, and the protein at 120kDa was the APN anchored by GPI. By using ligand blotting test, a 120kDa protein in BBMV was recognized binding with ^{125}I-Cry1Ac, it was reconfirmed that the 120kDa APN is the receptor for Cry1Ac.

By using ^{125}I-labeled Bt toxin, the differences of binding kinetics among Cry1Aa, Cry1Ab, Cry1Ac and BBMV in larval midgut of resistant and susceptible *H. armigera* were tested. After the saturation test and homologous/heterologous competition and dissociation assays, it was found that the combination of BBMV and Bt toxin protein was related in a certain degree to Bt toxicity, while there was no direct relationship between the activity of APN and Bt resistance. The occurrence of resistance to Cry1Ac in *H. armigera* is probably related to the decrease of binding-site number of BBMV and Cry1Ac. Nevertheless, it looks as if there will be not only the receptor protein, but also the other factors are related to Bt resistance. It was presumed that there is a common binding-site for Cry1Aa, Cry1Ab and Cry1Ac, and other one for Cry1Aa only. By designing the degenerate primers and using RT-PCR and RACE methods, 5 genes encoding APN proteins of *H. armigera* were cloned. The amino acid sequences of these APN possessed the common character of APN family. The results of homology analysis, by neighbor-joining (NJ) tree method, showed that the 5 genes encoding APN mentioned above belonged to 3 types. In comparison with APN1 in susceptible strain, three nucleotide mutations were observed in the APN1 of resistant strain to Bt cotton that translated into two amino acid differences in the putative protein sequences, and eight nucleotide mutations were observed in that of resistant strain to Bt insecticide that translated into 5 amino acid difference.

7 Susceptible baseline and resistance monitoring for *H. armigera* to Bt cotton

The value of Bt cotton could be seriously diminished by widespread development of insect resistance to Bt toxins. Numerous resistance management strategies have been proposed and debated in an attempt to address concerns over the potential for resistance development and to preserve the utility of Bt crops. Regardless of the strategy chosen, resistance monitoring, or susceptibility monitoring, is an activity generally accepted as an integral tool to measure success. Monitoring must be conducted regularly to follow changes in the susceptibility of pest populations. Geographical variations in sensitivity of cotton bollworm to the Bt protein *Cry1Ac* was studied in 1997 to establish a geographical baseline for comparing future population responses to increased use of Bt products in agriculture in China. More than 20 bollworm populations were collected from 5 cotton-growing regions of China, and the dose responses to *Cry1Ac* protein in terms of mortality and growth inhibition were evaluated. On the basis of the baseline study, sensitivities of field populations of *Helicoverpa armigera* to *Cry1Ac* were monitored during 1998 – 2002. A total of 65 strains were sampled, and most of them were collected from Bt cotton planting regions. It was determined that the field populations sampled during the 5 year's study were susceptible to *Cry1Ac* protein, and no development of resistance was apparent.

8 Risk evaluation and management strategy of *H. armigera* resistance to Bt cotton

Continuous production of Bt toxin in transgenic plants leads to season-long selection for insect resistance to the Bt insecticidal protein, and the large-scale use of Bt crops is likely to cause evolution of Bt toxin resistance in the insect pest. The rate of resistance evolution in an insect population to a Bt crop depends on a number of factors, including pest bionomics, initial frequency of resistance alleles in the pest population, genetic mode and stability of resistance, fitness of resistant individuals, temporal and spatial distribution of the insect pest on different host plants, and gene flow among different geographical populations.

In China, the cropping system is quit different from large-scale cotton farming in USA and Australia. There are many small-scale mixed planting of cotton, corn, soybean and peanut, etc., planted by individual farmers. Most of these crops are host plants of *H. armigera,* and chemical control of this insect is only used on cotton. Under such cropping patterns, host plants other than Bt cotton can provide various natural refugia for *H. armigera* and reduce the potential for resistance development to Bt cotton. For *H. armigera*, a desirable refuge should have two characteristics' it must produce sufficient susceptible individuals, and the emergence time of adults from refugia and Bt cotton fields must coincide to allow mating between the populations. Reported here are the evaluation of a natural refuge function for *H. armigera* within Bt cotton growing areas in north China.

The density of *Helicoverpa armigera* populations on Bt transgenic cotton, corn, peanut and soybean, differences in its development on Bt cotton and common (non-transgenic) cotton, and the potential for mating among populations from Bt cotton fields and other crop fields were investigated in the suburbs of Xinxiang City (Henan Province), and Langfang City (Hebei Province) in the southern and northern parts of north China, respectively. Although development of *H. armigera* on Bt cotton was much slower than on common cotton, there was a still high probability of mating between populations from Bt cotton and other sources due to the scattered emergence pattern of *H. armigera* adults, and overlap of the 2nd and 3rd generations.

In north China, where wheat is the main host of 1st generation *H. armigera* larvae, there is little risk of resistance evolution to the Bt toxin because of the absence of Bt use on this crop. The major host plants of 2nd to 4th generation *H. armigera* are cotton, corn, peanut, soybean and other plants, is comprise approximately 14 million ha in the proportions of 5, 55, 15, 15 and 10%, respectively. The refuge function of various crops for *H. armigera* is quite different. The largest refuge crop, early and late planted corn, could be a vast refuge for 3rd and 4th generation *H. armigera*, but is not for 2nd generation *H. armigera*. Although peanut and soybean could serve as a refuge for various generations of *H. armigera*, their value is limited because of smaller areas planted to these crops and lower larval densities. In view of the various composition and proportions of these crops, their utility as refugia for *H. armigera* would vary in different areas.

In most cases, the cotton/corn system is a major planting pattern in northern China, and there is no non-cotton refuge for 2nd generation *H. armigera*. A large number of 3rd generation *H. armigera* in corn may come from Bt cotton fields, where the pest is selected for resistance to the Bt protein. Therefore, the refuge function in this type of planting is imperfect, which could delay development of *H. armigera* resistance to Bt cotton but be not sufficient to prevent resistance evolution. In cotton and soybean/peanut mixed areas, non-cotton hosts provide natural refugia for 2nd to 4th generation *H. armigera*, but the positive or negative function of the refuge is dependent on the proportion of Bt cotton. Consequently, it is likely necessary to compensate the original mixed cropping patterns and standardize the management system in different areas to delay *H. armigera* evolution to Bt cotton. Considered the different refugia function from corn, soybean and peanut, it is concluded that a mixture planting system that consists of wheat, corn and soybean (or peanut) could be a low risk mode for management of the pest resistance to Bt cotton.

In addition to current resistance management theory of non-transgenic refuges (crop or appropriate non-crop) for the preservation of susceptible alleles, it is also important to consider the prudent use of insecticides, especially late in the season, to reduce overall larval densities in transgenic fields. If late-season survivors in Bt fields (under direct selection by the toxin) are selectively reduced by insecticide use, the total number of resistance alleles in a region could be reduced. Therefore, it is important that late-season larval density on Bt cotton plants be carefully monitored and controlled with effective insecticides.

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Analysis of Risks of Transgenic Insects for Pest Management: Past and Future Guidelines

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Abstract

Genetic modification using recombinant DNA methods can now be used, almost routinely, to transform pest and beneficial insects that could be used to improve pest management programs. Goals include modifying mosquitoes, and other insects that transmit human and animal diseases, so that they are unable to transmit the causal pathogens. Transgenic methods could improve genetic control programs by producing sterile males or producing only females. Other goals include producing honey bees and silk moths that are disease resistant or have other desirable traits. Natural enemies used in biological control programs could be modified to enhance their effectiveness in several ways.

Risk assessments must be conducted prior to releasing transgenic insects into the environment for either short term experiments or permanent establishment. Potential risk issues to be resolved prior to releases include whether: the inserted gene(s) (trait) is stable; the traits (especially pesticide or antibiotic resistance genes) can be horizontally transferred to other populations or species; released insects will perform as expected with regard to their geographic distribution, host or prey specificity and other biological attributes; released insects will have unintended environmental effects; and, in the case of short term releases, the released insects can be recovered from field sites. Risk assessments of fitness and host specificity are relatively easy to assess in the laboratory, but horizontal gene transfer and unintended effects on ecosystem function are more challenging.

Permission to release a transgenic insect will have to be obtained from (several ?) regulatory agencies. Initial releases initially are being made into small plots, perhaps in cages and, in the USA, are intended to be short term experiments. Current regulations of the U.S. Department of Agriculture require the researcher to retrieve all transgenic insects from the environment at the end of the experiment. If transgenic insect strain(s) perform well and risk assessments are completed satisfactorily, permanent releases into the environment may be allowed, but guidelines for such releases are lacking. Many pest management programs, especially those involving *replacement* of pest populations by a transgenic population, will require permanent establishment in the environment. Several drive mechanisms have been proposed to insert genes into populations but analyses of the potential risks of such drive mechanisms have not been carried out. International guidelines are needed for risk analyses of transgenic insects because insects are highly mobile and could move beyond individual countries' boundaries.

Key words

transgenic insects, risk assessment, containment, horizontal transfer, dispersal

Introduction

Genetic modification using recombinant DNA methods can now be used, almost routinely, to transform pest and beneficial insects (Ashburner et al. 1998, Handler and James 2000). Such genetically engineered insects and mites could be used to improve future pest management programs.

Why Genetically Modify Insects?

Beneficial insects Domesticated and semidomesticated insects have been modified by traditional breeding methods for hundreds of years. Genetic manipulation has improved disease resistance and silk production in silk moths (Yokoyama 1979, Gopanathan 1992) and disease resistance and pollination attributes in honey bees (Rothenbuhler 1979).

More recently, natural enemies of insects and mites used in biological control programs in agriculture and forestry have been modified by traditional breeding methods and by hybridization of different strains to achieve hybrid vigor (Hoy 1976, 1990a, 1993, Whitten and Hoy 1999). A pesticide-resistant predatory mite, developed with traditional breeding methods, was incorporated into a integrated mite management program in almonds in California (Hoy 1985). These predators provided effective control of spider mites, reduced the need for costly pesticides, reduced production costs, and saved almond growers approximately $22 million per year, most of which was due to fewer applications of pesticides to control the spider mites (Headley and Hoy 1987).

Pest insects During the past 40 years, a number of pest insects have been sterilized by irradiation or chemicals for use in genetic control programs (Wright and Pal 1967, Pal and Whitten 1974, Curtis 1979, LaChance 1979, Whitten 1979, Tan 2000). The sterile insect release method (SIRM), in which male insects are sterilized by irradiation, has been used to control a number of serious pests, including the Mediterranean and Caribbean fruit flies, mosquitoes, and the New World screwworm *Cochliomyia hominivorax.* The SIRM is used to eradicate pests or reduce populations of pests having a significant economic impact on agriculture and human health.

For example, the SIRM initially was used to eradicate the New World screwworm from the USA. Later the program was expanded to eliminate this pest from Central America (Belize, Guatemala, Honduras, El Salvador, Nicaragua and Panama) in order to provide a buffer zone to preclude its reintroduction into the USA. Benefits of the SIRM program in 1996 to US, Mexican and Central American cattle producers were estimated to be $796 million, $292 million, and $77.9 million, respectively (Wyss 2000). The benefit to cost ratios for the eradication programs ranged from an average of 12.2 to 1 for Central America to 18 to 1 for the US and Mexican programs (Wyss 2000). In addition, screwworm eradication has a significant human and wildlife health component that was not been included in these calculations.

Why Use Transgenic Methods to Modify Insects?

Traditional genetic methods have limitations and recombinant DNA methods offer new opportunities for improving pest management programs. For example, significant benefits could accrue if recombinant DNA methods reduced the negative effects of irradiation on sterilized insects to be released in SIRM programs. During the irradiation process, the insect's whole body is irradiated, which results in damage to all tissues. As a result, the SIRM requires rearing very large numbers of males for release into the environment. Commonly, pest populations are first reduced by pesticide applications or through natural seasonal (winter) mortality to reduce the number of insects that have to be released. The number of males released is usually a multiple of the estimated number of wild males, with a 100:1 ratio of sterile to wild males commonly necessary. Rearing such huge numbers of insects is costly and difficult.

Recombinant DNA methods also could allow molecular markers to be inserted into the sterile insects which would allow SIRM program managers to immediately discriminate between released sterile males and wild males. Current marking methods using fluorescent dusts are not satisfactory.

Recombinant DNA methods may make it possible to control the sex of insects being reared in SIRM programs, introduce lethal genes or genetic loads into pest populations, produce vectors of human and animal diseases that are unable to transmit diseases such as malaria, dengue, yellow fever, and sleeping sickness (Heinrich and Scott 2000, Robinson and Franz 2000, Thomas et al. 2000).

Recombinant DNA techniques could make genetic improvement of beneficial insects, such as silkworms (*Bombyx mori*), honey bees (*Apis mellifera*) or natural enemies, more efficient and less expensive (Beckendorf and Hoy 1985, Walker 1989, Heilmann et al. 1994, Walker et al. 1995, Beckage 1998). Once a useful gene has been cloned, it could be inserted into a number of beneficial species in a relatively short time. Furthermore, recombinant DNA methods broaden the number and type of genes potentially available for use; no longer is a project dependent upon the intrinsic genetic variability of the species under study.

Genetic improvement of natural enemies for biological control of pest insects and mites by traditional genetic methods has involved selecting for resistance to pesticides, lack of diapause, and increased tolerance to temperature extremes (Hoy 1990, 1992, 1993), although modification of other traits theoretically could result in improved biological control.

Traits primarily determined by single major genes are most appropriate for manipulating insects by recombinant DNA techniques because methods for manipulating and stabilizing traits that are determined by complex genetic mechanisms are not yet feasible with insects.

Genetic manipulation with recombinant DNA methods requires methods for efficient and stable insertion of foreign genes into the genome of the insect, the availability of useful genes, and appropriate promoters and other regulatory elements to obtain effective expression of the inserted gene in both space and time. Transgenic insects should be contained in the laboratory with effective procedures until permits have been obtained for their release into the environment.

Many have speculated about the role that recombinant DNA methods could play in the genetic control of insects that serve as the vectors of human and animal diseases or pests of agricultural crops. Some have proposed transgenic technology as a new and vitally important pest management tool for the control of serious pests that cannot be controlled by any other means. Others have expressed reservations about the goals and methods suggested. For examples of various viewpoints see: Whitten 1985, Walker 1989, Crampton et al. 1990, Meredith and James 1990, Eggleston 1991, Fallon 1991, Handler and O'Brochta 1991, Besansky and Collins 1992, Kidwell and Ribeiro 1992, Collins 1994, Collins and Besansky 1994, Curtis 1994, Hoy 1995, Spielman 1994, Durvasala et al. 1997, Ashburner et al. 1998, Curtis and Townson 1998, Beaty 2000, Blair et al. 2000, Collins et al. 2000, Curtis 2000, 2001, Hoy 2000, James 2000, 2001, Robinson and Franz 2000, Enserink 2002, Spielman et al. 2002.

A number of steps are involved in a program designed to control pest insects through transgenic methods (Table 1). First, the target species must be identified as a significant pest for which conventional control tactics are ineffective, because genetic manipulation is usually more expensive and difficult than other pest management approaches. Furthermore, genetic manipulation with recombinant DNA techniques may generate concerns about risk, requiring additional time and resources.

How best might our knowledge about the pest species' physiology, ecology, or behavior be used against it? How will the transgenic strain can be deployed in a pest management program?

Once a target trait has been identified, it must be genetically altered using appropriate genes and genetic regulatory sequences to ensure the new trait is expressed at the appropriate time and in appropriate tissues. After a modified strain has been developed, it must be evaluated in the laboratory for fitness and stability. If ultimate deployment requires mass rearing of very large numbers of high quality insects, mass rearing and release models will need to be developed. Eventually the manipulated strain must be released into greenhouses or small field plots in the field for evaluation.

Table 1. Questions To Answer When Developing a Genetic Manipulation Project If It Is To Be Deployed Successfully

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PHASE I. Defining the Problem and Planning the Project

- What genetic trait(s) limit effectiveness of beneficial species or might reduce damage caused by the pest?

  - Do we know enough about the biology, behavior, genetics and
    ecology of the target species to answer this question?
  - Is the potential trait determined by single or multiple genes?

- Can alternative control tactics be made to work more effectively and inexpensively than genetic manipulation projects, and are they more environmentally friendly?

    - The costs of genetic manipulation projects are high and the time to
        develop a functional program can be quite long.
    - Transgenic technology may not be appropriate if traditional genetic or
        other control methods can be used because issues surrounding
        risk assessment of releasing transgenic arthropods into the
        environment for permanent establishment have not been
        resolved.

- How will the genetically-manipulated strain be deployed?

    - Will releases be inoculative and some type of selection or drive
        system used to replace the wild strain?
    - Will the desired genes be introgressed (introduced)into the wild
        population? What selection mechanism will be used?
    - Will augmentative releases of very large numbers be required?
    - Will multiple releases be required over many years?

- What risk issues, especially of transgenic strains, should be considered in planning?

    - If pesticide resistance genes are used as a selectable marker for
        beneficial species is there a possibility of the resistance gene
        moving to a pest?
    - What is known about the potential for horizontal gene transfer?
    - If transposable element or viral vectors are used in the transformation
        process, what risks might they pose if the transgenic strain is
        released into the environment?
    - What health or other hazards might be imposed on human subjects if
        the transgenic strain were released?

- What advice do the relevant regulatory authorities give regarding your plans to develop a transgenic strain?

    - Which agencies are relevent to consult for your project?

**PHASE II. Developing the Genetically-Manipulated Strain and Evaluating It In
        The Laboratory**

- Where will you get your gene(s)?

    - Should the transgene(s) sequence be modified to optimize expression
        in the target species if it is from a species with a different codon
        bias?

- Is it important to obtain a high level of expression in particular tissues or life stages?

    - Where can you get the appropriate regulatory sequences?

- How can you maintain or restore genetic variability in your selection or transgenesis program?

    - Because both artificial selection and transgenic methods typically
        involve substantial inbreeding to obtain pure lines, how will

you outcross the manipulated strain with a field population to improve its adaptation to the field or otherwise increase genetic variability?

- What methods can you use to evaluate "fitness" in artificial laboratory conditions that will best predict effectiveness in the field?

    - Have life table analyses and laboratory studies of the stability of the trait under no selection been correlated with efficacy in the field?
    - Is it possible to carry out competitive population cage studies?

- Do you have adequate containment methods to prevent premature release of the transgenic strain into the environment?

    - Have these containment methods been reviewed by appropriate regulatory authorities?

- Do you have adequate rearing methods developed for carrying out field tests?

    - Are artificial diets available to reduce rearing costs?
    - Are quality control methods available to maintain quality during mass rearing?

- What release rate will be required to obtain the goals you have set?

    - Do you have an estimate of the absolute population density of the target species in your field test?
    - What release model are you applying: inundative, inoculative, introgressioin, complete population replacement?

- Have you tested for mating biases, partial reproductive incompatibilities or other population genetic problems?

- If the strain is transgenic, have you obtained approval from the appropriate regulatory authorities to release the strain into the greenhouse or small plot?

    - Can you contain it in the release site?
    - Can you retrieve it from the release site at the end of the experiment?
    - Can you mitigate if unexpected problems arise?

- How will you measure effectiveness of the modified strain in the field trials?

PHASE III. Field Evaluation and Eventual Deployment in Practical Pest Management Project

- If the small scale field trial results were promising, what questions remain to be asked prior to the deployment of the manipulated strain?

    - Are mass rearing methods adequate?
    - Is the quality control program in place?
    - Is the release model feasible?
    - Were there unexpected reproductive incompatibilities between the released and wild populations?

- If permanent releases are planned, have all the risk issues been evaluated?

- How will the program be evaluated for effectiveness?

- Will the program be implemented by the public or private sector?

- What did the program cost and what are the benefits?

- What inputs will be required to maintain the effectiveness of the program over time?

(Modified from Hoy 2000)

Regulatory Issues

Containment Transgenic insects are being produced in many laboratories around the world. Fruit fly workers, for example, may have hundreds of transgenic *Drosophila* colonies in their laboratories that are used in experiments to investigate a variety of basic research questions. These scientists do not have any intent to release these transgenic *Drosophila* into the environment, although accidental escapes must occur because containment procedures usually are relatively limited. These transgenic *Drosophila* could, in theory, establish in the environment or interbreed with wild populations of *Drosophila*. In warmer geographic regions, such as California wineries, *Drosophila* can be an agricultural pest. However, no one appears to have looked for establishment of transgenic *Drosophila* or gene flow from them to wild populations.

Scientists in university and government laboratories in the USA working with other transgenic insects have approached containment in two different ways. Some have argued that the containment facilities and procedures should be tailored to the specific risks posed to the environment by each GM insect and the genes that have been inserted; they have proposed several levels of containment for insects that are vectors of human and animal diseases (http://klab.agsci.colostate.edu/~mbenedic/ACGdocuments/Nov2000mtgsummary.pdf). A second option is to apply to transgenic insects the guidelines and facilities developed by APHIS for the containment of insects that are plant pests. This approach would result in a single level of containment, with standardized containment facilities and procedures, designed to prevent accidental release of any transgenic insects (Hoy et al. 1997, OECD 1998). This single standard has the value of uniformity and clarity; when someone indicates they are using these containment guidelines, the regulator knows what the facilities are like and what the containment procedures should be. At present, however, each laboratory appears to be developing their own containment facilities and procedures on an *ad hoc* basis and there is no standardization. Containment guidelines for "factories" rearing insects for the purpose of producing drugs or vaccines have not, as far as I am aware, been considered. Accidental escape of such insects might have detrimental effects on predators feeding on them or result in transfer of the transgenes into wild populations.

Containment of transgenic insects is important because:
- Insects are highly mobile and capable of colonizing new environments.
- Effective containment procedures allow regulatory authorities to consider risk issues before purposeful releases are made for pest management programs.
- Effective containment procedures could restrict escape of transgenic insects being reared for production of vaccines or drugs.

Some argue that containment is expensive and time consuming and detrimental to research programs. However, the accidental and premature release of transgenic insects into the environment would have detrimental effects on the public's perceptions of this new technology, as well as

negative effects on the environment (Hoy 2000).

Current US regulations Permission to release a transgenic insect will have to be obtained from (several ?) regulatory agencies, including federal agencies, state agencies and institutional oversight committees. Short term releases initially will be made into small plots, perhaps in cages. To date, all releases of transgenic insects into the environment in the USA are intended to be short term experiments, and current regulation of such releases by the U.S. Department of Agriculture require the researcher to retrieve all transgenic insects from the environment at the end of the experiment (Young et al. 2000).

Examples of releases that have been permitted include (USDA 2001):

- Release of a predatory mite (*Metaseiulus occidentalis*) that had a marker gene (lacZ with a *Drosophila* heatshock 70 promoter) inserted without using a specific vector (integration occurring through some form of illegitimate recombination after 'maternal microinjection'?), March 1996.
- Release of a pink bollworm with a GFP marker gene for a genetic control program in 2001.

It is unknown what information will be required to allow transgenic insects to be released into the environment permanently. Many pest management programs, especially those involving *replacement* of pest populations by the transgenic insect population, will require permanent establishment in the environment. The use of several 'drive mechanisms', including the release of insects containing active transposable elements or *Wolbachia*, have been proposed for replacement, but analyses of the potential risks of such drive mechanisms have not been carried out. Clearly, the driver and the gene to be driven should be strongly linked if the combined system is to spread through a population and it is unclear whether such tight linkage is feasible (Curtis 2000, Boete and Koella 2002).

Researchers in the USA are sometimes confused as to which regulatory agency to go to. Currently, the USDA-APHIS regulates releases of potential agricultural plant or animal pests, but it is unclear who would regulate transgenic insects producing medicinal proteins or vaccines; several agencies might be relevant, including the U.S. Food and Drug Administration, the Environmental Protection Agency and the US Department of Agriculture. Who will regulate transgenic insects that are vectors of human diseases? Will the fact that humans could be affected by the transgenic insect vectors require the involvement of the Department of Health and Human Services?

Potential Risks and Research Needs

Vector sequences and antibiotic resistance genes Concerns raised from risk evaluations of transgenic crops could be applied to transgenic insects. For example, consideration should be given to eliminating vector sequences or antibiotic resistance genes from transgenic insects, as is being considered in transgenic plants.

Gene silencing Another problem that requires research and affects risk assessments is the potential loss of efficacy due to gene silencing. Transgenic plants and mammals are known to inactivate multiple copies of transgenes that overexpress proteins or are otherwise abnormal (Dorer and Henikoff 1994, Wolffe 1997, Henikoff 1998). This phenomenon is thought to be due to systems that have evolved as a means to prevent high levels of expression of transposable elements or viruses that can cause genetic damage to their hosts. Multiple mechanisms of transgene silencing occurs in the fruitfly *D. melanogaster* (Dorer and Henikoff 1994, 1997, Pal-Bhadra et al. 1999, Jensen et al. 1999). Thus, methods may have to be developed to eliminate transgene silencing effects in insects or it could reduce the effectiveness of transgenic insects after their release in pest management programs.

Horizontal gene transfer One risk issue that is unusually difficult to quantify is the risk of horizontal transfer of transgenes to other organisms (Droge et al. 1998). Our knowledge of horizontal transfer and transposable elements only began in the 1950s when Barbara McClintock discovered transposable elements in maize. Horizontal gene transfer could occur from one insect

population to another of the same species, from one insect species to another, or to other organisms in the environment. It is difficult to quantify this risk because we lack fundamental information on the frequencies and mechanisms of horizontal gene transfer. The whole topic of horizontal gene transfer in insects has received limited scientific attention until relatively recently.

We do know that horizontal transfer of genes may occur between insect species by movement of naturally occurring transposable elements (Houck et al. 1991, Plasterk 1993). Horizontal transfer is thought to be rare, yet we have observed more than one such transfer within historical times in *D. melanogaster* and may have missed other examples because we were not looking. The *P* transposable element appears to have invaded *D. melanogaster* populations within the last 50 years, perhaps from a species in the *D. willistoni* group, by unknown mechanism(s). Controversy exists as to whether *P* elements were transferred between *Drosophila* species by a semiparasitic mite *Proctolaelaps regalis* (Houck et al. 1991). Another transposable element, *hobo*, appears to have invaded natural populations of *D. melanogaster* around the 1960s (Bonnivard et al. 2000), representing the second invasion of this well-studied insect in the past 40 to 50 years. Horizontal transfer of *P* and *mariner* elements across species of *Drosophila* provide some of the best data for horizontal movement in insects. Recently, Jordan et al. (1999) showed that a long terminal repeat retrotransposon (a different class of element than the *P* and *mariner* elements) also could move horizontally. Their data suggest a recent movement from the *D. melanogaster* group species to *D. willistoni*, perhaps within the last 100 to 200 years.

Interspecific (horizontal) transfer of transposable element vectors could move from insects to other organisms, including humans, although these movements are expected to occur very rarely. For example, the transposable element *mariner* is found in many insects and other organisms (Lampe et al. 2000). The diversity of species containing *mariner* elements suggests that: 1) *mariner* elements have been present in insects for a long time, although some lineages have lost them, and 2) horizontal transfer has occurred between different insect families and orders although some transfers occurred so long ago that many of the *mariners* are degraded and inactive, probably due to a successful defense against the damage they cause by the insect's genome. Lampe et al. (2000) noted the "most recent events occurr[ed] at least 100,000 years ago". The two *mariner*s in the human genome probably invaded in the "past 100 million years" (Lampe et al. 2000).

We are still discovering new aspects of the biology and ecology of transposable elements and this lack of knowledge makes it difficult to predict precisely what would happen if insects were released that contained either active or inactive transposable elements. One possibility is that some transposable elements will mobilize inactive transposable elements from totally different 'classes'. Petrov et al. (1995) found that transposable elements of several types (*Ulysses, Penelope, Paris* and *Helena*) can be mobilized in crosses between strains of *Drosophila virilis*. These elements represent the major types of transposable elements found in higher organisms. Petrov et al. (1995) suggested that they were mobilized by a sort of 'genomic stress' due to the breakage of double-stranded DNA, which can be caused by exposure to UV light and other agents. These triggers have been shown previously to increase mobilization of some retroelements. Horizontal transfer of DNA could be mediated by insect viruses. The *piggyBac* transposable element was discovered embedded within the genome of a baculovirus (Fraser 2000). If horizontal transmission of transgenes by viruses were to occur in the field, there is no guarantee that genes inserted into an insect species using a transposable element vector would remain within that species.

These data suggest that the safest course might be to remove any introduced transposable element vector sequences from a transgenic insect strain prior to its permanent release into the environment to reduce the probability that the transgene will move, either within the strain or horizontally between different populations or species. Whether insects can, or should, be released with active transposable elements as drivers remains to be resolved.

Whether horizontal gene transfer will cause harm would certainly depend on the gene(s) transferred and its location. The most serious harm might occur if the transposable element or viral vector inserted into germ line tissues so it could be transmitted to succeeding generations. However, damage might also occur if the elements damaged somatic tissues; for example, the movement of the

mariner element in the soma reduced the lifespan of *Drosophila simulans* males (Nikitin and Woodruff 1995). The movement of retroelements into human breast, colon and testicular tissues can induce cancer or Duchene muscular dystrophy (Capy et al. 1996).

The role of transposable elements in the evolution of genomes is undergoing reevaluation and it is clear that naturally occurring horizontal gene transfer between species has provided some of the variability upon which evolution has acted (Plasterk 1993, Krishnapillai 1996, Britten 1997). It is unlikely that the presence of a transgene in an insect will increase the small probability that the transgene will be transferred to another species by horizontal transfer *if* the transposable element or viral vector sequences used to insert the transgene could be removed prior to release into the field. Even then, however, the probability of horizontal transfer will not be zero.

Disabled transposable element vectors probably pose a low risk of horizontal gene transfer of the transgene to other organisms. However, it is possible for an inactivated vector to become active through a process called 'conversion'. Peronnet et al. (2000) showed that 'conversion' can transform an inactive *P* element into an active one through the interaction of three different *P* elements in the genome in a three-step process (Peronnet et al. 2000). Conversion could make a transgene unstable within the transgenic insect's genome and could, in theory, pose a risk for horizontal gene transfer.

Finally, the ability of transposable element vectors to serve as 'drivers' for inserting useful genes into insect populations should be evaluated on a case-by-case basis (Hastings 1994, Kiszewski and Spielman 1998). As noted by Kidwell and Evgen'ev 1999), "special care needs to be taken in generalizing the results from model organisms to other species." Kidwell and Evgen'ev (1999) argued that "the transposability of mobile elements, their potential for rapid, and sometimes massive, amplification in copy number, their ability to change genomic locations, as well as their propensity for horizontal transfer, makes the generalization of results from model organisms far less reliable. Extrapolation of results from one species to another must therefore be made with caution."

Gut symbionts Genetic engineering of insect gut symbionts could allow the movement of the inserted genes between the many types of microorganisms found within the insect gut (Armstrong et al. 1990). For example, *Enterobacter cloacae*, a bacterium found in the guts of insects, and *Erwinia herbicola*, a bacterium that grows on the surface of plants, were found to grow in the guts of silk moth larvae and exchange genetic information via plasmids at very high rates (Watanabe and Sato 1998, Watanabe et al. 1998). The bacteria containing the new genetic information were found in the feces of the insects, suggesting that this method of horizontal gene transfer is a frequent event in nature. If gut symbionts of pest insects are transformed with antibiotic resistance genes, these genes could move horizontally to other bacteria within the insect, perhaps leading to antibiotic resistance in pathogens. Antibiotic resistance has led to a serious medical crisis because some human pathogens are now resistant to almost all available antibiotics (Witte 1998).

Disruption of ecosystem services The potential effects of transgenic insects on ecosystems is a big topic and difficult to evaluate using laboratory tests (Hoy 2000, Spielman et al. 2002). Questions need to be asked about any potential negative effects of transgenic insects on threatened and endangered species. Unfortunately, ecologists often are surprised by the intimate and surprising relationships between insects and other organisms in their environment. Insects serve as pollinators, as prey for predators including other insects, fish, birds, bats, lizards and frogs, as vectors of human, plant and animal diseases, as plant feeders, and as natural enemies of pest insects.

Risks Assessment Methods
Levels of risk? Releases of transgenic insects into the environment fall into at least three categories that probably represent increasing levels of risk:

- Releases of transgenic insects for evaluation in field cages or other contained environments that allow the researcher to eliminate or retrieve all released insects.
- Release of transgenic sterile insects as part of a genetic or biological control program; because the sterile insects cannot reproduce they should pose less risk than insects released for permanent establishment.

- Releases that require the transgenic insects to establish permanently and may require that the released insects interbreed with wild populations to insert genes and/or drive elements (active transposable elements or *Wolbachia*) associated with genes into wild populations.

Risk equals the potential for damage *and* the likelihood of its occurrence. Risks may be different for pest and beneficial insects and may depend on whether the insect is expected to persist in the environment or is unable to reproduce and thus cannot persist. Risks also will vary with the transgene(s) inserted. It is easier to propose types of harm than to quantify the likelihood of their occurrence at this time.

Relative risks The least risky transgenic insects could be the domesticated silk moth (*B. mori*), which is unable to survive on its own in the wild; most transgenic *B. mori* are unlikely to have a negative effect on the environment because they should not be able to persist, although waste product disposal could pose an environmental risk if silk moths are grown in factories to produce drugs or vaccines.

Transgenic pest or beneficial insects that are sterile and unable to reproduce should pose lower risk than insects that are able to reproduce and persist in the environment, although horizontal transfer of the transgenes could, in theory, occur at a very low rate. Transgenic pest or beneficial insects that are unable to persist because the environment is unsuitable during a portion of the year also are likely to pose a lower risk.

Honey bees, *Apis mellifera,* are only semi-domesticated and can escape human management to survive in the wild, so transgenic honey bees could pose a greater risk than the domesticated silk moth.

General risk issues Evaluating the risks associated with releasing insects and mites that have been manipulated with recombinant DNA techniques will likely include, as a minimum, the questions or principles outlined in Tables 1 and 2, but other issues may become important as we learn more. Current concerns can be summarized as:

- Is the transgenic population stable?
- Has its host or prey range has been altered?
- Does it have the potential to persist in the environment?
- Will the transgenic strain will have unintended effects on other species or environmental processes?

Table 2. Some Risk Issues Relevant to Short Term Releases of Transgenic Insects Into the Environment

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A. Attributes of the Unmodified Organism
- What is the origin of the transgenic organism (indigenous or nonindigenous) in the accessible environment?
- What is the insect's trophic level (predator, parasite, plant feeder) and host range?
- What other ecological relationships does it have?
- How easy is it to monitor and control it?
- How does it survive during periods of environmental stress?
- What is the potential for gene exchange with other populations?
- Is the insect involved in basic ecosystem processes?

B. Attributes of the Genetic Alteration
- What is the intent of the genetic alteration?
- What is the nature and function of the genetic alteration?
- How well characterized is the genetic modification?

- How stable is the genetic alteration?

C. Phenotype of Modified Organism Compared to Unmodified Organism
- What is the host/prey range?
- How fit and effective is the transgenic strain?
- What is the expression level of the trait?
- Has the alteration changed the organism's susceptibility to control by natural or artificial means?
- What are the environmental limits to growth or reproduction (habitat, microhabitat)?
- How similar is the transgenic strain being tested to populations previously evaluated in field tests?

D. Attributes of the Accessible Environment
- Describe the accessible environment, whether there are alternate hosts or prey, wild relatives within dispersal capability of the organisms, and the relationship of the site to the potential geographic range of the transgenic strain.
- Are there endangered/threatened species present that could be affected?
- Are there agents that could move the transgenic strain present in the release environment?
- Do the test conditions provide a realistic simulation to nature?
- How effective are the monitoring and mitigation plans?

(Modified from Tiedje et al. 1989 and from a discussion held at a conference on "Risks of Releasing Transgenic Arthropod Natural Enemies", held November 13-16, 1993 in Gainesville, Florida.)

The first three questions are relatively straight forward to answer with a variety of laboratory experiments (Li and Hoy 1996, McDermott and Hoy 1997, Presnail et al. 1997). The last issue is much more difficult to answer.

A question risk assessment officials may need to ask is how far and how quickly can the transgenic strain disperse from the experimental release site? Less is known about dispersal behavior of many insects than might be needed. For example, Raymond et al. (1991) suggest that there has been a worldwide migration of *Culex pipiens* mosquitoes carrying amplified organophosphorus resistance genes, perhaps aided by accidental human transport. If migration is the basis for these widespread genes, then dispersal of some transgenic strains could be far more rapid and extensive than anticipated.

Transgenic arthropods could pose somewhat increased risks over those posed by invasive species because they are likely to be released in very large numbers; presumably most invasive species invade the new environment in low numbers. Discussions of risk should include questions about survival, reproduction, and dispersal of transgenic populations and their effects on other species in the community.

At present, releases of transgenic arthropods in the U.S.A. are evaluated by regulatory agencies on a case-by-case basis. Initial permits for releases will be for short term releases in controlled situations so that unexpected outcomes might be mitigated more readily (Young et al. 2000, USDA 2001). Currently, there are no guidelines for evaluating the risks of releasing transgenic arthropods for *permanent* establishment in the environment. In the USA, both state and federal regulatory agencies, including state departments of agriculture and the U.S. Department of Agriculture, Animal and Plant Health Inspection Service (APHIS), have to be consulted for permission to release transgenic agricultural pests and natural enemies of agricultural pests. Questions about the impact of the transgenic arthropods on threatened and endangered species will be asked by state and federal agencies, including the US Department of Interior, Fish and Wildlife Service (Young et al. 2000).

Models as risk assessment tools? Can we use models to predict the outcome of releases of transgenic insects in pest management programs? Many types of population and genetic models could be used in attempts to predict what will happen when genetically-modified insects are released into the environment in pest management programs. We do not know, however, which model types

are most likely to be predictive of the actual outcome of field releases because few models have been evaluated with empirical data.

Some current population models may lack key ingredients, such as partial reproductive isolation. For example, Caprio and Hoy (1995) developed a stochastic simulation model that varied the degree of mating bias between pesticide-resistant and susceptible strains, diploidy state (diplo-or haplo-diploid), degree of dominance of the resistance allele, and degree to which mating biases extended to the hybrid progeny. The results obtained were counterintuitive and illustrated the point that models can offer insights into the complexities of population genetics and dynamics that might be overlooked. The common assumption made in models is that all genotypes of a species mate at random, but this assumption may mask a considerable number of important interactions. The efficacy of transgenic insect release programs could be jeopardized if mating biases exist between released and wild populations.

Boete and Koella (2002) produced a theoretical model to predict the success of genetic manipulation of malaria mosquitoes in malaria control and concluded that if refractoriness (inability to transmit the protozoan causing malaria) "is less than 100% effective, control programs based on genetic manipulation of mosquitoes will have very little impact on the epidemiology of malaria, at least in areas with intense transmission." This type of model could be useful in allowing regulators to ask questions about efficacy and risk.

Unfortunately, empirical data generally are lacking to compare the relative usefulness of different model types in predicting insect population dynamics. Theoretical ecologists usually assume homogeneous and continuous populations. Metapopulation models, by contrast, assume that populations exist in patches varying in area, degree of isolation, and quality. Metapopulation biology increasingly is being recognized as relevant to our understanding of population ecology, genetics, and evolution (Hanski 1998). Recent data, and a variety of metapopulation models, indicate that spatial structure affects populations as much as birth and death rates, competition, and predation (Caprio and Hoy 1994).

Conclusions

The issue is not *if* transgenic insects should be released, but when and how? The debate over evaluation methods and interpretations should include a variety of viewpoints. Much of the debate will be parallel to the debate on introducing natural enemies for classical biological control programs (Follett and Duan 2000). Most introduced insect natural enemies have provided large benefits, with only a few examples of potential or demonstrated harm to the environment. Ewel et al. (1999) reported the conclusions of a workshop on the risks of deliberate introductions of species into new environments. The participants did not discriminate between the potential risks of genetically modified organisms and unmodified organisms introduced into new enviroments. Research questions were put under four headings: Guarding against risks without sacrificing benefits, Alternatives to introductions, Purposeful introductions, and Reducing negative impacts. They recommended a single framework for evaluating all types of introductions, a need for retrospective analyses of introductions; a holistic view of the invasion process; and fewer, more effective introductions. Ewel et al. (1999) concluded:

At the extremes, these views [of risks] range from a handful of advocates of no introductions, or of such rigorous pre-introduction proof of benignness that all introductions are effectively prohibited, to an equally small group that advocates a freewheeling global eco-mix of species....most proponents of purposeful introductions understand the risks (but believe that technology can deal with them), and most conservation biologists recognize the potential benefits to be derived from carefully controlled introductions. Clearly, there is a need to bring all parties together on common ground that can lead to objective, science-based decisions by policymakers."

Unfortunately, there are no guidelines for evaluating the risks of permanent releases of nonsterile transgenic insects into the environment and it is difficult to predict what issues will be considered. Nor is it clear which regulatory agency(ies) in the USA will regulate some of the transgenic insects, such as mosquitoes that vector human diseases. The "Coordinated Framework"

that regulates transgenic organisms appears to have gaps in it, making regulation of transgenic mosquitoes problematic. Uniform national regulations regarding appropriate facilities and procedures for containing transgenic arthropods prior to their release into the environment also are lacking (Hoy et al. 1997).

A beginning was made toward an international evaluation of potential risk assessment issues associated with transgenic insects in April 2002. A meeting was convened by the Food and Agriculture Organization (FAO) and the International Atomic Energy Association (IAEA) that began the process of identifying potential hazards associated with releases of transgenic insects for pest management programs. Much additional work remains, however, to develop consensus protocols of assessing risks.

Recommendations

- National-level scientific discussion and study is needed to develop risk assessment protocols for evaluating potential risk assessment methods in the USA prior to the permanent releases of transgenic arthropods into the environment. Such a study group ideally would include ecologists, evolutionary biologists, conservationists, molecular biologists, sociologists/psychologists, regulatory officials, ethicists, economists, consumers, and others.

- International risk assessment protocols are needed because transgenic insects can readily move out of release sites. Some programs would take place in other countries, which may not have adequate resources or expertise to conduct evaluations of risk. Ideally, international assessment protocols will be compatible so that researchers and others know what is expected of them.

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Possible ways of using transgenic mosquitoes for malaria or dengue control and risk assessment

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1. The burdens due to malaria and dengue

In tropical Africa, and also in Papua New Guinea, the rate of malaria transmission by *Anopheles* mosquitoes is intense. This is because the *Anopheles* species in those areas selectively bite humans, are less attracted to animals, and are able to breed at high density within villages. In the tropical African lowlands over all seasons each person receives an average of more than one *Plasmodium* infective bite per night. Consequently, in these regions the disease burden due to malaria is immense – about 400 million clinical cases and 1-2 million deaths. This is comparable to that due to each of the other major killers among infectious diseases: pneumonia, diarrhoeal diseases, HIV/AIDS and TB. In South and South East Asia and the Amazon region of Latin America the burden due to malaria is not so great but is still significant.

Dengue, due to four distinct but related viruses transmitted by *Aedes aegypti* mosquitoes (and in some areas by *Aedes albopictus* also), is of local importance. This is especially the case in urban areas of tropical Latin America and South East Asia. However, on a world scale the burden (as measured by Disability Adjusted Life Years lost) due to dengue is only about 1.3% of that due to malaria (Curtis & Davies, 2001). Thus in the relatively affluent tropical areas where dengue is perceived as a threat it is reasonable to devote considerable local resources to this problem, but it would not deserve international donor funding on a scale comparable to that of malaria in Africa.

2. Current control methods

Currently control of malaria transmitting *Anopheles* is attempted by:

(a) indoor residual spraying with insecticides, such as DDT or pyrethroids, aimed at reducing the number of mosquitoes which survive long enough for *Plasmodium* to mature to the stage at which it can be transmitted to another victim;

(b) insecticide-treated bednets which aim to:

    (i) provide personal protection from biting *Anopheles* which mostly bite late at night when most people are in bed;

    (ii) when used on a community-wide basis to kill mosquitoes attracted to the nets by the odour of the sleepers and hence to reduce the population of the mosquitoes which survive long enough to become dangerous, in approximately the same way as with house spraying;

(c) in appropriate conditions, especially in urban, mining, industrial and arid areas control of larvae by

    (i) drainage or screening of sites;

    (ii) use of appropriate insecticides, especially temephos;

    (iii) biopesticides such as the bacterial toxin Bti, which selectively kills mosquito larvae, or juvenile hormone mimics which interfere with metamorphosis;

    (iv) use of biological agents, especially larvivorous fish.

Control of *Aedes* vectors of dengue is attempted mainly by control of domestic breeding sites by clearing garbage which can retain rain water, screening water storage pots or treatment of tanks with temephos, Bti or the larvivorous copepod *Mesocyclops*. In the event of epidemics, insecticidal space spraying is sometimes attempted with the aim of killing virus-infected mosquitoes in flight.

3. Successes and failures of conventional control

In the 1950s and 60s there was, in many malarious areas, well organised indoor residual spraying with DDT against fully susceptible vector populations. This successfully eradicated malaria in southern Europe and Taiwan and reduced malaria cases in India from about 75 million to about 105,000 per year (i.e. by 99.8%). Even in tropical Africa, with its intense transmission, DDT house spraying reduced malaria prevalence to near eradication in limited areas, for example Zanzibar (Curtis & Lines, 2000).

Regrettably, more recent results with DDT house spraying have not been so good in India or

Zanzibar, due to evolution of DDT resistance in some of the vectors and/or to poor percentage coverage of houses in communities. In South Africa, resistance to pyrethroids in *An.funestus* (Hargreaves et al., 2000) led to a switch back to DDT spraying and this was followed by a turn-round in the rising trend of malaria cases. In the Madagascan highlands restoration, after a gap of 35 years, of house spraying terminated an epidemic which was estimated to have killed 40,000 (Curtis, 2002). Unfortunately tropical Africa, which needs malaria vector control more than anywhere else, has very little of it. Significant reductions in child mortality have been recorded in African trials of insecticide-treated nets in which virtually 100% coverage of all the beds in each village had been assured (Lengeler, 1998). However, the results were not as good as in house-spraying trials in Africa in the 1950s, 60s and 70s (Curtis & Mnzava, 2000). Attempts are being made to introduce insecticide treated nets more widely in Africa, but mostly on a semi-commercial basis and, understandably, poor rural people find it difficult to pay for the insecticide required for protection of their children. In Vietnam organised free provision of insecticide treatment for the nets of 11 million people during the 1990s has been associated with a remarkable national decline in malaria (www.mekong-malaria.org/mcis/mmf6) which is held up by WHO as an example of what can be achieved when the political will is present. In Sri Lanka there has been resurgence of malaria, after near eradication in the 1960s. One of the problem areas is where vector breeding is predominantly in hundreds of hand-dug gem pits. Because the location of all these pits is well known to the villagers it was feasible to treat each pit in four villages twice a year with a handful of a sand-granule formulation of a synthetic juvenile hormone mimic. This highly significantly reduced adult vector densities and malaria incidence and prevalence, compared with untreated villages (Yapapandara et al, 2001).

In the 1950s *Aedes aegypti* eradication was certified by WHO for the whole of Brazil. This remarkable achievement was based on rigorously enforced searching for all potential breeding sites. More recently re-infestation has occurred, probably as a result of transport of drought-resistant eggs in imported second-hand tyres. More recent community based attempts at control of breeding sites have been far less effective than the rigorous enforcement in the 1950s. In certain Vietnamese villages *Ae. aegypti* has been eradicated by the efforts of village-based teams in ensuring that there are predatory *Mesocyclops* in every water tank in each village (Vu Sinh Nam et al, 1998). In Singapore, legally enforced control of domestic breeding has greatly reduced the percentage of *Aedes*-infested premises since the 1970s. However the recorded incidence of dengue has increased over the same period. It is suggested that this paradoxical result is because babies are no longer regularly becoming infected and thus are not building up immunity. Furthermore there is evidence that non-immune people becoming infected later in life are suffering worse symptoms than when infection was acquired early in life (Coleman et al, 2002). This suggests that the only satisfactory solution would be sustainable vector eradication, after which lack of immunity would no longer be dangerous. A malaria "rebound" due to fading immunity, where malaria vectors are controlled but not eradicated and where symptoms are worse in older people, has also been suggested as a possibility (Trape & Rogier, 1996).

In summary:-
(a) there are problems of insecticide resistance in some, but not all, vector populations;
(b) the intensity of searching operations for houses needing treatment or for breeding sites is often insufficient to ensure acceptable levels of disease control;
(c) in some cases, vector eradication may need to be the target.

4. The possibility of genetic control of mosquitoes

In view of all the above problems, it is worth considering genetic control (the sterile male technique and related ideas) as an alternative control method. Instead of relying wholly on conscientious humans to seek out all houses or breeding places needing treatment, genetic control aims for universal coverage by harnessing the highly efficient mate-seeking behaviour of male insects. Such behaviour is presumably one of the principle characteristics that has been selected for in their evolutionary history. Insecticide resistance is irrelevant to success of this method, but one has to consider the possibility that wild females will avoid mating with artificially reared and sterilised males. Nevertheless, there is a real possibility of sterile male releases eradicating isolated target populations because, as one begins to suppress the density of the wild population, the ratio of this density to the output from one's sterile male production facility becomes more and more

favourable. Examples of eradications of pests/ vectors of diseases of domestic animals by sterile male releases are the eradication of the Screw Worm Fly from Florida and from the whole area from Texas to Costa Rica (Wyss, 2000) and eradication of the tsetse vector of cattle trypanosomiasis in Zanzibar, *Glossina austeni* (Msangi et al, 2000). Where there is continued immigration of females which have already mated with fertile males, eradication would be permanently prevented. This is because most dipteran females tend towards monogamy and therefore refuse to re-mate with sterile males after arrival in the target area of the releases. It is a curious fact that monogamy was at one time thought to be a necessary requirement for success of genetic control, but actually monogamy is a major obstacle to success (Klassen & Curtis, in prep.),.

Where continued immigration is inevitable (e.g. across the huge, virtually continuous, zone of rural tropical African malaria), it is hoped that one could introduce mosquitoes carrying genes which block the development of *Plasmodium* or dengue virus, i.e. "refractoriness" genes. It has long been hoped that such genes could be linked to a "driving" factor which would raise the frequency of the refractoriness allele to a high level and would, furthermore, counteract the effects of immigration by selecting against the normal pathogen susceptibility alleles brought in by immigrants (Curtis, 1968). There seems to be no point in trying to introduce a refractoriness gene by mass release without a driving factor because such a system would offer little "resistance" to immigration. Furthermore, sterile males would be expected to do a more efficient job because of the above described phenomenon of improving released : wild ratio, which applies only where the releases cause a reduction in the wild population, not merely a conversion of the same numbers to a genotype which is harmless to humans.

It might be argued that refractoriness genes would be favoured by natural selection because they would protect mosquitoes from damage due to human pathogens (Boëtte & Koella, 2002). However, the proportion of mosquitoes carrying infectious agents (and therefore subject to such selection) is always low and would become lower if one began to succeed in lowering prevalence of infection. Also, if there were an appreciable net natural selection pressure in favour of refractoriness it seems most probable that a natural mutation would have arisen in evolutionary history and would already have become the wild type.

5. Sterile male mosquitoes and how transgenesis could improve them

In the late 1960s and early 1970s there was much research on sterile male mosquitoes in the USA, India and El Salvador. It was realised that mass release of male mosquitoes (which do not bite) would only be acceptable if their biting and disease- transmitting sisters were separated off and not released. With *Culex* and *Aedes* (culicine) mosquitoes accurate sex separation was achievable on the basis of pupal size and, in India, hundreds of thousands of *Culex* or *Aedes* males per day were regularly produced with an admixture of only 0.2% females, by use of a pupal sieving device which the small male pupae could penetrate but the larger females could not (Sharma et al., 1972; Ansari et al., 1977).

In *Anopheles,* the pupal size method is not effective for sex separation. Instead, genetic systems based on the translocation of semi-dominant genes for insecticide resistance on to the Y chromosome were developed in several important malaria vector species (e.g. in *An. gambiae* by Curtis et al., 1976; and in *An. albimanus* by Seawright et al., 1978). In *An. albimanus* the continued linkage of the resistance gene to the Y chromosome was reinforced by an inversion, and the system was used for routine production of a million sterile males per day (Dame et al., 1981). This seems very encouraging, but one must not overlook the problems that were encountered on trying to scale up genetic sex separating systems in the Medfly (*Ceratitis capitata*) to the scale of many millions of males per day. Even the most unlikely forms of genetic recombination could occur and set in train a process of decay of the perfection of the sex separation. Therefore, an elaborate process for continuous purging of the breeding stock in the "fly factory" has been devised to overcome this problem (Caceres, in press). In mosquitoes, unlike higher flies, genetic crossing over is a recognised phenomenon in males and a sexing system based on an entirely different transgenic principle, which could apparently not be disrupted by crossing-over, is highly desirable.

The RIDL system proposed by Thomas et al (2000) may provide such a system. It consists of a dominant lethal associated with a female-specific factor such as a vitellogenin gene promoter in such a way that expression of the dominant lethal is switched off so long as tetracycline is provided to the breeding stock. However, for batches being prepared for release this substance is removed, thus causing the death of all the females. This system has been made to work in *Drosophila* and is

being actively pursued in *Ae. aegypti,* with *Anopheles* on the agenda in the near future (Alphey and Andreasen, 2002). If successful, it could be used instead of the above mentioned sexing methods, or in addition to one of them as a supplementary safety precaution against any release of biting insects.

Gamma radiation has been used to induce dominant lethal mutations in virtually 100% of the sperms of various species of fly. These dominant lethals consist of chromosome breakages which kill embryos after fertilisation. These have been used successfully to eradicate many pest populations by mass release of irradiated males. Gamma-irradiated insects are not radioactive and no safety hazard has ever been claimed to be associated with them, even by the most critical environmental activists. The males will only be effective if the radiation does not harm their survival and competitive behaviour. One can irradiate pupal flies with a fully sterilising dose with little or no harm to the emerging adults but, for unknown reasons, this is not the case with mosquitoes – adults emerging from irradiated pupae are distinctly sub-normal in survival and mating competitiveness (Smittle & Patterson.1974; M.Andreasen, in prep.). Results are better if adult mosquitoes are inactivated by chilling and irradiated, but doing this on a scale of many millions would be difficult. For this reason, in the trials in the 1970s in India and El Salvador, reared pupae were immersed in solutions of alkylating chemosterilisants such as thiotepa or bisazir, the pupae were carefully rinsed and adults allowed to emerge. They carried dominant lethals in virtually 100% of their sperms and studies of their competitiveness in the field showed quite good performance (Grover et al, 1976a, 1976b; Lofgren et al 1974). Chemical analysis of these chemosterilised males showed extremely low residues (LaBreque et al, 1972). However, from an unreplicated study it was reported that spiders fed an diet of nothing but chemosterilised mosquitoes themselves became sterile (Bracken & Dondale, 1972). This study should be repeated but it seems unlikely that, in the present day climate of concern about pollution by bio-active substances, releases of mosquitoes, with even the most minute residues of mutagenic alkylating agents, would be allowed.

In view of these problems with use of either radiation or chemosterilants for mosquitoes it is fortunate that the above mentioned RIDL system could provide, not only elimination of females from batches of males for release, but also non-production of any viable daughters from matings by RIDL males with wild females. This is the case because their progeny would grow up in an outdoor environment with very low tetracycline concentrations. For mosquitoes the only sterility that is required is non-production of biting daughters. Sons produced by released RIDL males would, to some extent, propagate the elimination of female progeny into later generations. Variants of the RIDL system have been hypothesised which would also kill sons of released males (R.J.Wood and L.Alphey, pers.comm.). Such sterility would be required for species where male larvae or adults are as harmful as females

6. Risk assessment of RIDL releases

If RIDL male mosquitoes stand up to rigorous testing of the reliability of the construct in killing all females not provided with the dietary supplement, one could give an assurance that released mosquitoes of this type would be all males which would therefore not bite or make other contact with humans or domestic animals. Rare revertants in a RIDL breeding stock could presumably be dealt with by a purging process similar to that for Medflies mentioned above. Because male mosquitoes do not bite there need be almost no concern about horizontal gene transfer of RIDL constructs to humans or mammals. Male mosquitoes are subject to predation by spiders etc and, by analogy with the chemosterilant residues mentioned above, a check for horizontal gene transfer to spiders should be considered a necessary precaution before initiating mass releases.

7. Targets for RIDL releases

In the sterile male releases in India and El Salvador in the 1970s, it was found that, despite reasonably good mating competitiveness, very high percentages of egg sterility were not obtained in rural areas, apparently because of immigration of already mated fertile females across barrier zones 3-4 km wide created by careful anti-larval measures in these zones (Yasuno et al., 1978; Dame et al., 1981). In the Screw Worm sterile male operation immigration was overwhelmed by creating a rolling front of releases of millions of sterile males. Ideally this would be possible with mosquitoes but unfortunately, in the real world, far more resources can be obtained for dealing with pests of cash crops, such as ranched cattle, than for control of the vectors of diseases which kill the children of the poor. A more feasible type of target vector population for eradication would seem to be populations on actual islands or in ecological "islands". Actual islands might be appropriate for trials and for

certain cases where a substantial human population on an island has a disease problem for which vector eradication seems to be the only real solution, and where the governments concerned could afford to fund such an operation. One example would seem to be Singapore, with its persistent dengue transmitted by very intensively controlled, but not eradicated, *Ae. aegypti* and *Ae. albopictus*. Separate RIDL-based eradications for these two species would be required and these would probably have to be extended to southern Malaysian cities to avoid re-infestation of Singapore via traffic crossing the 1 km long causeway. Another example might be Réunion which now has no malaria but it has *An. arabiensis* as a potential vector. Its government (that of metropolitan France) spends considerable sums on precautions against re-infestation with malaria parasites via imported malaria cases. Thus eradication of the vector could be seen as a worthwhile investment.

Also considered as targets for eradication have been ecological islands such as the narrow strip of humid land beside the Nile in northern Sudan where *An. arabiensis* can survive, isolated by desert on either side. However, a type of ecological "island" with a much denser human population consists of urban areas in which a vector species exists which differs from that in the surrounding rural areas. It appears that *An. stephensi stephensi* in southern Indian cities may be such a case, though checks for gene flow between this form and the more rural *An. stephensi mysorensis* would seem to be a necessary stage before considering embarkation on a large programme. The *An. arabiensis* populations which exist in southern Nigerian cities are another apparently suitable target because they are surrounded in rural areas by the non-interbreeding sibling species *An. gambiae s.s.* (Coluzzi et al.,1979; Kristan et al, 2002). It appears that the latter species cannot tolerate urban conditions so that successful eradication of urban *An. arabiensis* would not give an opportunity for re-population by rural *An. gambiae s.s* . However, as a precaution, ecological studies should be undertaken on survival of the larvae of these two species in urban breeding sites.

It must be appreciated that plans for extensive mosquito eradication attempts in cities would be examined critically by the local population. It would be essential that the all-male nature of the releases and solid data on the lack of hazard from horizontal gene transfer of the RIDL construct were available and were presented with complete openness. Such presentations would have to be tailored so as to be understandable to members of the population with different educational levels. It must also be made clear that the target of the releases was the malaria vector population, not urban nuisance *Culex* which, however, should be simultaneously attacked by more conventional means. Limiting the target to urban populations leaves out of account African villages where the worst malaria problems are. However release of sterile males there seems almost bound to be defeated by inter-village migration of mosquitoes. It should be recalled that a larger and larger fraction of Africa's human population are moving to cities. If malaria vector eradication could be achieved in an aggregate of only a few tens of square km of urban area by a combination of conventional targeting of breeding sites (Trape et al., 2002) with "mopping up" of the surviving vector populations by RIDL releases, one of the many burdens on the health of many millions of people would have been lifted.

8. The production of Plasmodium-refractory strains of Anopheles

It is an attractive idea that one could deal with the rural malaria problem by "seeding" with limited releases of mosquitoes carrying refractoriness genes tightly linked to a driving system. There have been studies reported over the last several decades involving conventional genetic selection for *Plasmodium* refractoriness (e.g. Collins et al., 1986). However, for it to be feasible to link such a genotype to a driving factor, control of refractoriness by one, or at most two, major genes seems essential. Furthermore, genetic dominance of the refractoriness trait is highly desirable if the driving process is to be efficient. The conventionally selected examples of refractoriness do not meet these criteria. However, recently Ito et al. (2002) reported production of a transgenic strain of *An. stephensi* which was about 80% refractory to a rodent *Plasmodium* species. Boëtte and Koella (1992) have pointed out that, for a reasonable prospect of success in an area of intense malaria transmission, a gene causing virtually 100% refractoriness would be needed. Such is the amount of effort now being put into transgenic mosquitoes it seems probable that such high levels of refractoriness to relevant strains of *P.falciparum* will soon be achieved in relevant vector species.

If one succeeded in driving a refractoriness gene to a high frequency there would be intense selection pressure on the local *Plasmodium* to evolve a genotype which evaded the action of the refractoriness gene. On the same principle as multi-drug therapy, it would seem wise to incorporate two or more independently acting genes for refractoriness into the construct, but this would add to

the difficulties of ensuring unbreakable linkage of the complete refractoriness genotype to the driving factor.

9. Testing for susceptibility to pathogens other than *Plasmodium*

In contrast to attempts to eradicate by sterile or RIDL males, use of genes for refractoriness inevitably involves humans being bitten by transgenic mosquitoes. It does not seem at all likely that engineering refractoriness to *Plasmodium* would have the side effect of making the strain more susceptible to some other human pathogen. However, before making any irrevocable release of such constructs linked to systems which are intended to drive them onward indefinitely in space and time, it would be necessary to carry out relevant feeding tests. These should include filariae (of which *Anopheles* are already a vector) and a wide range of viruses. This should include HIV, of which mosquitoes are almost certainly not a vector now. If, however, engineering *Plasmodium* refractoriness had the effect of making them HIV vectors the effects of releases of them would be disastrous. Even if well-informed arbovirologists consider this possibility as absolutely inconceivable, the public does not, and this is a question that people are constantly raising. Therefore solid data proving that there is absolutely no HIV risk must be available before releases are considered.

Survival time is a major component in determining vectorial capacity. It is almost inconceivable that engineering refractoriness would enhance mosquito survival above what natural selection has been able to achieve over evolutionary history. However, a careful check to prove the obvious could, and should, be easily done.

10. Driving systems

Several types of factor have been proposed as driving factors over recent decades, but the most hopeful ones now seem to be:

(i) Transposable elements which can be expected to make multiple copies of themselves and spread as *P* elements did in the world's wild *Drosophila melanogaster* (Kidwell & Ribeiro, 1992) . *P* elements are not functional in mosquitoes but other transposable elements are. They are also functional in a wide range of other insects and the general expert opinion seems to be that in the present state of knowledge the results of release of transposable elements would be too unpredictable, and potentially likely to lead to horizontal gene transfer, for such releases to be allowed.

(ii) *Wolbachia* symbionts are inherited though the maternal cytoplasm and cause sterility in matings of *Wolbachia* infected males to uninfected females. Infected females are therefore at no risk of being sterilised whereas uninfected females are at such a risk. Therefore the infected state has a tendency to spread, as observed in wild *Drosophila* (Turelli & Hoffmann, 1991). *Wolbachia* occur in many insects, including *Ae. albopictus,* and they can be moved in the laboratory from species to species (Sinkins, et al., 1997). They have not been found in *Anopheles,* which makes these mosquitoes potentially vulnerable to a release campaign with members of the same species artificially infected with *Wolbachia* carrying a construct causing *Plasmodium* refractoriness. However, so far artificial infection of *Anopheles* with *Wolbachia* has not been achieved.

(iii) The idea of negatively heterotic systems for genetic control of pest/vector insects was proposed by Serebrovskii (1940) and Curtis (1968) and has now been brought up to date by Davis et al (2001). They propose an engineered transgenic system of two unlinked lethal genes, each linked to a suppressor of the other lethal. Thus either chromosome on its own is lethal but, when the two are present together, both lethals are suppressed and the insect survives. Under these conditions there is a threshold frequency and, if releases exceed this, selection would favour the artificial construct. It is assumed that one or more refractoriness genes are linked into the system. It would seem that this system is more akin to well understood classical genetic systems of lethals and suppressors. For that reason it would raise less concern about unpredictable hazards of release into a wild population than would transposable elements or *Wolbachia*.

11. The linkage problem

It would be very difficult or impossible to ensure enduring linkage between refractoriness genes and a driving system.. It seems to the present author that this problem of the inevitable lack of total linkage is unfortunately a fatal objection to the idea of engineering refractoriness and introducing it into wild vector populations with the aim of interrupting disease transmission. As found with the Medfly sexing systems, some recombination would inevitably occur between any two genes when one scales up the population to the millions. It should be noted that the method of purging rare recombinants from a captive colony is inapplicable to the situation where a released genotype is intended to maintain itself in the wild for an indefinite time after release. In the field, selection would almost certainly favour the recombinant driving system relieved of the "load" of the refractoriness gene. Once this unloaded driving system had swept through the wild population, that population would no longer be susceptible to re-use of a re-purified version of the same driving system linked to the refractoriness gene.

12. Summary

1. One of the reasons for the generally poor control of vector mosquitoes in recent decades is lack of ability or willingness of vector control teams to search adequately for female pest/vector insects or their habitats. However, genetic control aims to harness the mate seeking behaviour of male insects in the hope that this will do a more efficient job of searching for female insects.

2. With mosquito disease vectors it is essential that, if mass releases of sterile males are made, their biting sisters are not released with them.

3. It may be possible to improve on existing methods of sex separation and sterilisation by the RIDL system in which a transgenic construct puts female lethality under the control of a dietary supplement, which is made available at high concentration to the breeding stock, but not to batches of insects being prepared for release or to the progeny of release insects.

4. It would seem that the best chance of successful use of such a system would be against urban vector populations surrounded by a different species in rural areas.

5. By eliminating all female progeny, the RIDL transgene system imposes a severe fitness penalty on any recipient, so would be eliminated rapidly from any population which it invaded, unless maintained by continued release. Therefore concerns about horizontal gene transfer are much less with such a system than with the currently more popular idea of engineering into mosquitoes refractoriness to human pathogens and causing such systems to spread in both sexes of wild vector populations.

6. Before any releases of such refractory strains, they should be checked for the unlikely possibility that refractoriness to one pathogen causes enhanced susceptibility to another. For RIDL strains in which no transgenic biting females should exist in the field these issues are not a significant concern.

7. The difficulty or impossibility of ensuring unbreakable linkage of a refractoriness construct to a system for driving it through wild populations would seem to be a fatal objection to the attractive idea of rendering vector populations genetically harmless.

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Field trials, the permitting process, comments and risks.

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Abstract:

We detail experiences in the process of designing and preparing experiments, obtaining permits and the perceived and potential risks associated with trial releases of genetically modified/transformed arthropods. What was first proposed as a free release was modified to an environmental release in very large field cages. The insects to be released were transformed with a *piggyBac* transposable element encoding the fluorescent protein EGFP. Biological characteristics of the transformed strain are detailed. Descriptions and responses to the comments and criticisms received during the public commentary and comment period associated with obtaining the release permit are detailed and analyzed. Certain of the critiques provided during the permitting process are summarized and discussed, as are our responses to those critiques. Discussion is made of some perceived hazards and estimates of risk associated with such a release along with a discussion of statistical analysis.

Introduction and rationale:

Lepidoptera are key pests on food and fiber crops worldwide. Increasing insect resistance to pesticides, along with heightened public distaste and ecological concerns driving a movement away from chemical control methods presents an increasing challenge to pest control. Our ability to manipulate insect species at the molecular level will help advance our understanding of insect biology and we hope promote the development of novel field applications consistent with reduction of ecologically undesirable pesticide use.

An immediate use of transgenesis technology is indelible genetic marking of insects produced for Sterile Insect Technique (SIT) programs. SIT strategy is summarized as follows. Target insects are colonized and mass reared. A proportion of the colony is retained for the next generation and the remainder are exposed to a dose of a mutagen, most often ionizing irradiation, to the point where every gamete carries at least one induced dominant developmentally lethal mutations. Mutagenized colony insects are then released to mate with wild population. Because each offspring of matings with colony insects receives dominant developmentally lethal genes, embryos from such unions will not develop. Alternative strategies relying on introducing conditional dominant developmentally lethal mutations have been proposed as an extension of SIT (Fryxell & Miller 1995, Thomas et al 2000).

Pink Bollworm as a study subject for Biological Risk Assessments:

Significant questions about biological safety of transgenic insects can be effectively addressed by studies on model organisms such as transgenic PBW (Pink BollWorm), *Pectinophora gossypiella*(Ashburner et al 1998, Peloquin et al 2000). PBW has many characteristics that enhance the safety of transgenic studies with this insect in North America compared to other organisms. Close sympatric non-target relatives, especially if related closely enough to allow hybridization and fertile offspring pose a special risk as the potential for the transgene to become introduced into a non-target population would be significant. This resembles the controversial assertion, henceforth corroborated by new work, that transgenic maize interbred with untransformed Mexican landraces and thereby introduced transgenes into the latter's populations (Quist & Chapela 2001). PBW has no

known indigenous co generic relatives in North America. Therefore, transgene escape from PBW into non-target insect populations can be considered extremely unlikely. Additionally, PBW have restricted geographic and host ranges. In North America they are incapable of surviving harsh northern winters. They can feed only on cotton and Malvacea relatives. Mass rearing of this insect is well developed enough to produce billions of insects on a regular basis. PBW adults, unlike biting flies *etc*. have minimal or no potential to directly injure humans or animals, much less transfer their transgenes to them. Finally, genetic marking of SIT insects was proposed as a crucial first step in the use of genetically modified arthropods and as an important low risk means for hazard identification and risk assessment in transgenic arthropods (Ashburner et al 1998). The biology of PBW, especially referring to the factors discussed above, provides substantial safeguards for uses associated with transgenetic manipulation.

Transgenic PBW, their production:

The utility of transposable elements to manipulate an insect genome has been clearly demonstrated in *Drosophila* with the narrow host range P element (O'Brochta et al 1991, O'Brochta et al 1994, Rubin & Spradling 1983, Spradling & Rubin 1982). Broader host range elements like *mariner* (Coates et al 1995), *Minos* (Loukeris et al 1995), *Hermes* (O'Brochta & Atkinson 1996, Sarkar et al 1997a, Sarkar et al 1997b)and *piggyBac* (Elick et al 1996, Elick et al 1997, Fraser et al 1996) led to vector systems for transformation of more insects including the medfly, *Ceratitis capitata* (Handler & McCombs 2000, Handler et al 1998), the mosquito *Aedes aegypti* (Coates et al 1998, Coates et al 1999, Jasinskiene et al 1998) and now Silkworm and Pink Bollworm(Peloquin et al 2000, Tamura et al 2000). Successful germline transformations of insects rely on rescue of a mutant phenotype or on dominant marker genes such as fluorescent proteins [Peloquin, 2000 #2044;Toshiki, 2000 #2607;Loukeris, 1995 #1562;Jasinskiene, 1998 #946;Handler, 1998 #1961;Handler, 2000 #2602;Handler, 1999 #1966;Coates, 1998 #1167;Coates, 1999 #1942].

We constructed a *piggyBac*-derived vector containing Enhanced Green Fluorescent Protein (EGFP) as a marker for transformation after piggyBac was shown to integrate into the PBW genome [Thibault, 1999 #1636]. Green Fluorescent Protein's (GFP), its derivatives', and other fluorescent protein's (*e. g*. DsRed) utility as dominant, visible, non-destructive markers in a variety of insect (Brand 1995), mammalian (Pines 1995), and plant systems (Haseloff et al 1997) made us confident of its utility in PBW, which was borne out in our successful transformation of PBW (Peloquin et al 2000).

We transferred strain #35 of transgenic strains produced in our work to Phoenix as described in our USDA/APHIS permit for movement of transformed insects between labs in Riverside and Phoenix. This strain of several generated was chosen because of its clearly recognizable phenotype. The integrated constructs have thus far proven stable under laboratory rearing conditions, though further investigation under mass-rearing conditions is continuing. Tests of mating competence of the transgenic strain will also be performed with attention to containment, release and biosafety issues (Caprio & Hoy 1995, Hoy 1998).

Permits and Initial release experiments:

Although transgenic technology for insect control is promising and beginning to show concrete results (Kokoza et al 2001), the biological safety and attendant risks of this approach are a topic of considerable debate. Addressing some of those concerns will require well-controlled and contained release experiments. Initially such study must be restricted to low risk transgenes in low risk insects. We contributed to this field of research with a series of experiments employing a benign

marker gene in a cage contained field release of genetically modified Pink Bollworms. This was not the experiment we envisioned initially as we first pursued a permit for free release of EGFP marked transgenic pink bollworms. Applications for the originally requested permit was first submitted to the Arizona State Department of Agriculture in which plans for the planned release in Arizona were described along with the purpose for the release. We obtained comments on the proposed experiment from within that department. After initial inspection and go-ahead from the state secretary of agriculture, we entered the permitting process at the federal level. This involved a public comment period and a solicitation of concerns and objections to the proposed experiments followed by our replies. We redesigned the proposed experiments and containment protocols after stakeholder suggestions to minimize the risk of transgene escape into the wild. Subsequent to this redesign and the necessary permit, we performed the first series of releases and are now analyzing the data in Phoenix.

Issues important to the people who volunteered their remarks were not entirely the concerns we expected. We take these unexpected observations as validation of the public comment portion of the permitting process. Selected reviewer's technical questions suggested that we should have more clearly stated aspects of the release and pertinent issues. Reviewers at all levels wanted specific details of transforming constructs. We replied to this by providing DNA sequence information from the plasmids used. Although this information was readily obtainable and we anticipate it to be in the future, there may be situations in which details of such information are either unavailable, or a matter of commercial or trade secret. We feel that there should be some mechanism in the permitting process to allow for retention of trade or commercial secrets and the protection of proprietary interests, while allowing effective stakeholder scrutiny of the proposed experiments and releases.

Certain technical questions were posed that were difficult to address. At the state level of release-permit, the reviewers wanted to know if there was "read-through expression" and wanted to know the exact transcription termination. Other than the unusual cases of certain viruses and viral derived elements, eukaryotic genes do not typically function in this manner. Though this question would be of importance in a prokaryotic system, the post-transcriptional processing of mRNA in eukaryotes suggests that this information would neither be pertinent nor available.

Questions were raised regarding the rate for transgene movement. Since we didn't see any indications for transgene movement, and have not after 20 + generations, we can only say that this rate must be very low, and thus we could not provide any estimate for this rate. The state level review asked "Can intragenic movement be masked by lethal gene disruptions?". We read this question to be "may the reason for not seeing any transgene movement be that, for some reason transgene movement is lethal to any insects in which this happens". If this hypothesized phenomenon occurred, then transgene movement would result in a dead end without any biological risk, since the animal in which transposition occurred would die. The net effect would be that no such movement could take place due to lethal effects.

The question of potential insertional inactivation or enhancement of expression of PBW genes was brought up by the reviewer's question, "Is the transgene inserted in a gene?" Our reply was that no genes were seen in a homology search of the databases. The flanking DNA sequences we obtained through inverse PCR did not align with anything using Blast comparisons with GenBank. The reviewer responded with the critique that our search sequence must have been too short to find a match in the database. We further replied that the Blast documentation (Altschul et al 1990, Madden et al 1996) suggests that sequences of only 25 bases or less may very well provide negative results. However our query sequence was the entire sequence available from the PBW DNA flanking the transgene, 516 base pairs. This should be clearly quite long enough for finding matches in the event

they exist in the database. Of course this does not preclude there being an accession to the database subsequent to the time we did our search that does indeed represent a potential gene sequence in PBW.

The reviewers addressed concerns about mobilization of transgenes by endogenous transposase activity with their question, "Are there other mobile elements in PBW?". We assumed that this question referred to possible interactions of the transgene with endogenous elements. We replied that we did not detect *piggyBac* homologs in PBW with low stringency Southern blots nor was any endogenous *piggyBac* transposase activity detected in transposition assays performed without an added source of *piggyBac* transposase (Thibault et al 1999). However, this work did not satisfy at least one of the reviewers at the federal level. Frustratingly, this reviewer did not address nor reference the appropriate peer-reviewed literature dealing specifically with this finding (Thibault et al 1999), nor did they address the important and very pertinent recent work on mariner that suggests interactions between transposase and transposon are sensitive to slight changes in the sequences of the transposon believed to interact with transposase(Lampe et al 2001). This reviewer, for unknown reasons referred to work done with *Bombyx* rather than the readily available literature on PBW. Even in referring to work in Bombyx, this reviewer referred to data from the published *Bombyx* transformation(Tamura et al 2000) claiming that this data indicated instability in the transgenic silkworms. This reviewer's conclusion was inconsistent with the conclusions drawn by the authors reporting transformation and *piggyBac* stability in *Bombyx*. This last oversight by the reviewer was rather disconcerting to us, as we expected that the review process would engage reviewers sufficiently familiar with the extant literature or at least the reviewers would choose to refer to the most pertinent literature in making their conclusions.

Public statements during the public comment phase were against any release of a transgenic insect. However, at least one of these negative commentators accepted that both GFP and the EGFP transgenes were benign. The major criticism of transgenic insect technology centered on the nature of the transposable element used to make the transformation vector and the fact the original autonomous *piggyBac* element was found by insertional mutagenesis of a baculovirus in cell culture. Because of this observation that an autonomous *piggyBac* element could insert into a virus, claims were made that this indicated a significant biological hazard pertaining to *piggyBac*. Nevertheless, it is known that *piggyBac* is not alone in this behavior and that a variety of transposable elements will insert into DNA of viruses, including relatively unrelated Tc1-like elements(Jehle et al 1998).

Significant Time Points on Timeline to release experiments:

March 1998 Transgenic PBW made

Sept 1998, applied for transfer permit to move transformants to Phoenix

March 1999, transfer permit issued

Insects transferred to Phoenix USDA/APHIS

Jan. 2000 Release permit draft submitted

May 2000 Solicitation of Public comments for permit request

July 2000 revision to confinement protocols- in light of critiques and comments

Permit resubmitted for a confined field cage release

Feb-April 2001 E. A. (Environmental Assessment) of proposed experiment/permit written

May-June 2001, Review of E.A., APHIS counsel waives review

June-July 2001, E. A. made available to public and comments on E. A. solicited

 July-Aug. 2001 Public comments analyzed; with a finding of no significant impact, the E. A. is again rewritten.

Sept. 2001 rewritten E. A. & analysis of comments are reviewed by APHIS OGC prior to publication in the US Federal Register

Releases commence Oct. 5 through Oct. 16, 2001

More releases planned for Summer 2002

An evaluation of results underway

The release was performed to gain information and to compare the following biological responses:

Compare EGFP and non-EGFP male response to pheromone in the field

Compare EGFP and non-EGFP male longevity in the field

Compare EGFP and non-EGFP female's ability to solicit and mate with EGFP and non-EGFP males in the field

The releases were performed in a 3-acre cotton field located 1 km from the PBW rearing facility. It is important to note that his release was not made freely into the environment. Containment precautions were made that included:

- Chain Link fence surrounding field to limit access by humans and animals
- The location was guarded to prevent vandalism etc.
- The releases took place in large mesh field cages that excluded insects and retained the PBW released within the cage.
- Pheromone traps were placed at edges of field to capture escaped insects.
- The field was treated with 100 Sterile PBW/acre 3 times a week.
- EGFP female wings were clipped.
- EGFP females kept in "mating stations" within the cages that prevented them from moving from their locations
- At the end of the release, cotton bolls from release field were destroyed to preclude development of PBW from any source.
- Only irradiated transgenic males were released

Public Comment Requested on GM Bollworm Release:

USDA's APHIS announced its intention to prepare an Environmental Impact Statement (EIS) on the proposed release of genetically modified (GM) pink bollworms. The public is invited to comment on what issues should be addressed in the EIS.

In addition to the scientific goals of initial release these releases were made to assess public commentary, particularly with a benign transgene. This experiment was inherently low risk, and was the type of study recommended as a "first release" experiment. Perhaps most important of all, it allowed proof of effective containment protocols and would, we hoped, assuage the public's fears of GMOs in general as we hope to be seen to be making good faith efforts to accommodate concerns of the interested public related to this matter.

Public comment, Horizontal Transmission:

Horizontal transmission of the transgene, that is transmission by means other than sexual, was of considerable importance to the critics of this work. We operated on the assumption that horizontal transmission of the *piggyBac* transgene was rare or possibly non-existent. Therefore, how does one estimate frequency of such an extremely rare event. Without unlimited resources it may indeed be impossible to obtain reasonable estimates of such rare frequencies. Perhaps, the risk associated with

horizontal transmission should be assessed in terms of the possible damage that could result from this event. Some questions that come to the fore resemble questions asked about exotic pests. These include the following:

1. Would horizontal transfer of a given transgene result in a more dangerous or virulent pest due to the nature of the transgene?
2. Would such a transgene negatively impact natural enemies?
3. Would alterations of pest biology due to the transgenic process result in a more virulent pest?
4. Would the transferred transgene negatively impact natural enemies?

Statistical analysis estimation of frequency of rare events and the importance of confidence limit levels[i].

Of course to make estimates of risk of horizontal transfer, some estimate of the likelihood of such a transfer would be helpful. Such an estimate requires quantification of the frequency of such transfer. Examples from studies of horizontal transfer from prokaryotes and some GMO crops suggest the horizontal transmission rate is low, but these examples may not be good models for insects. The flow of transgenes among prokaryotes and between GM crops and their weed relatives is most often by sexual or conjugative mechanisms, unlike the horizontal transmission scenarios proposed for transfer of transgenes in insects. However, a considerable body of work exists on vertebrates particularly mammals (Doerfler et al 2001a, Doerfler et al 2001b, Doerfler et al 1995a, Doerfler et al 2001c, Doerfler & Schubbert 1998, Doerfler et al 1998, Doerfler et al 1996a, Doerfler et al 1997, Doerfler et al 1995b, Doerfler et al 1996b, Hohlweg & Doerfler 2000, Hohlweg & Doerfler 2001, Hohlweg et al 1999, Mueller & Doerfler 2000, Mueller et al 2001, Remus et al 1999, Schubbert et al 1997, Schubbert et al 1998, Schubbert et al 1994, Schubbert et al 1996) that suggests that horizontal transmission of any foreign DNA, not just transposable element DNA, is very common at least to the somatic cells. This discovery then begs the question of why do mammals and other organisms have recognizable genomes at all given that DNA from their food can integrate quite commonly into the consuming organism's DNA. Presumably, there is an effective mechanism (gene conversion?) that prevents these common integrations of foreign DNA from entering into the germline.

No matter how common or uncommon horizontal transmission may be, to predict horizontal transfer levels from transformed PBW, it will be necessary to assay for movement of transgenes from PBW. Essentially, one would look for the transgene in organisms where we would not otherwise expect it through some sort of assay. Of course, no test is perfect. Thus, one must consider various, and possibly *apriori* unknown levels of test error before one can get an estimate of the numbers of animals to be screened.

The appropriate statistics to use for this analysis are an important issue. The error rate of the test in examining a particular species for the transgene can be determined by testing known positives and known negatives for the transgene and comparing the results from the test to the known and expected values. This would provide the error rate for this particular insect and transgene combination. However, such an analysis requires that there be available *bone fide* examples of transformed insects. Of course this will not be available for the majority of the likely candidates for HT from transgenic insects including PBW. Investigating transfer to other organisms will require methods to deal with the test reliability that do not rely on known transgene positive and negative insects. In the case where multiple tests from each single individual can be performed, test reliability may be estimated by means of probabilistic mixture distribution modeling. This "mixture problem" is analogous to a series of coin flips with two types of differently biased coins (e.g. high probability

of heads versus low probability of heads) where one wants to estimate the probabilities of heads (or tails) in each coin type, and additionally estimate how many flips were done with each type of coin. The appendix outlines the mathematics for such an approach. In the case at hand, where an organism either has the transgene or does not, the problem approximates to a mixture of two binomial distributions with the associated statistics.

Figure 1 is based on data resulting from a hypothetical experiment with two treatments: 1) organisms which have never been exposed to a transgene and where we assume none of them has the transgene. 2) organisms that have been exposed to conditions of possible horizontal transgene transmission (e.g. predators having fed on transgenic prey). A sample of specified size of each treatment is tested for the presence of the transgene. The test is imperfect with a certain rate of false positives and a certain rate of missed positives. The test results are such that in the treatment that has been exposed to potential transgene transmission, no more positives have been detected than in the treatment without exposure. The question to be asked is thus "How sure can we be that the rate of transgene transmission is below a certain threshold?" Graphs in Figure 1 suggest several things. Most importantly, huge numbers of insects must be assayed for even 95% confidence that the transgene was not present above a given level. With increased sample size we gain certainty, but there are diminishing returns. The desired confidence level has perhaps the most significant influence on the necessary number of assays. If 95% confidence is acceptable; an upper limit on the event (HT in this case) can be placed at a much lower value. As more restrictive confidence levels are employed, the upper limits of frequency are considerably higher. Missed positives (false negatives) in the range below 5% are not as important as one might intuitively expect; as increasing the incidence of missed positives from 0.5% to 5% decreased the discriminatory power only slightly. False positives, however, have a more severe effect on increasing the upper limit of HT. False positives basically add noise to the data, and thus add uncertainty. Hence optimization of test procedures should primarily focus on reducing the rate of false positives.

Although test reliability can be empirically established for PBW itself, given the above, whether test reliability data can be extended to other insects is open to debate. The end point for numbers of organisms to be assayed for transfer of the transgene is difficult to determine. Perhaps the best that can be done is to put a ceiling on HT frequency within the traditional 95 % confidence limits. If the true rate of horizontal transmission is very low, say 10^{-8}, or less, then the resources needed to detect such a rare event would make this a very difficult and expensive study indeed.

An additional complication is that horizontal transfer to prokaryotic symbionts associated with the animal host is possible. Gut bacteria are nearly ubiquitous in metazoans, including insects. These prokaryotes could potentially pick up a transgene, multiply and increase the effective concentration of the transgene and thereby serve as conduit to gene transfer between PBW and other organisms, including natural enemies. As prokaryotes are easier to assay in great numbers than are metazoans, it may be possible to exclude this phenomenon to a low level of probability. However, the analysis for HT will be heavily reliant on PCR with attendant problems. In figure 1, graphing confidence in results versus the necessary number of observations to have a given level of confidence in the results again demonstrates that huge numbers of assays must be done.

Assessing HT (Horizontal Transmission) from PBW, an ecological perspective:
First, is important to assay biological risk in ecologically sensible systems. For example, the hazard of transgenes in PBW being incorporated into an infecting virus has been noted. What risk this represents is open to debate, not in the least because of the specific biology and behavior of the transformed insect, PBW. Baculoviruses are not a significant source of morbidity of PBW in the

field. Thus the likelihood of any of these viruses infecting PBW may actually be very low. Secondly, the original discovery of *piggyBac* was as the result of an insertional mutation. This mutation rendered the host baculovirus plaque-defective, and thereby a less competitive virus, perhaps even less infective- working against the virus's potential as a vector for a transposable element. In general, the ecology and the host range of baculoviruses, particularly with respect to PBW, suggest that this would be ecologically unimportant pathway for HT of a transgene.

As HT, if it occurs at all, may be rare, looking for evidence of such transfer should be concentrated on organisms that have an ecological, relationship with the transgenic insect, particularly trophic interactions. Good examples of this are PBW natural enemies, scavengers and saprophytes on the corpses of the insects. As movement of the transgene is largely if not entirely dependent on a source of transposase, these ecologically sensible organisms should be examined for the presence of autonomous related elements as a source of transposase. It is important to note that in *Mariner*, the best studied of the wide host range DNA mediated transposable elements, it is necessary for the transposon's sequence to be closely matched, if not identical, to a given canonical transposase recognition sequences at the termini for excision and integration to occur at all (Lampe et al 2001). So, though *piggyBac* biology is still not completely understood, the evidence from *Mariner* suggests that distantly related elements may not provide transposase activity necessary for movement. PBW DNA when ingested may quickly be broken down into oligonucleotides through the digestive process of the natural enemies etc., thus lessening the likelihood of HT. Therefore, an important ecological consideration would be transgene persistence in predators etc. The same would pertain to transgene persistence in PBW corpses etc. These phenomena are accessible to analysis.

Subsequent to the determination of ingested transgene survival, tests for HT can be made in the suspected recipients of the transgenic DNA. Though transgene survival in natural enemies of PBW is not expected, there is a body of work from mammalian studies on the fate of ingested DNA. According to these studies, DNA does indeed survive ingestion and furthermore integrates as fragments of less than about 1000 bases into the somatic cell genomes of the animal fed the heterologous DNA (Doerfler 1996, Doerfler et al 2001a, Doerfler et al 2001b, Doerfler et al 1995a, Doerfler et al 2001c, Doerfler & Schubbert 1998, Doerfler et al 1998, Doerfler et al 1996a, Doerfler et al 1997, Doerfler et al 1995b, Doerfler et al 1996b, Schubbert et al 1997, Schubbert et al 1998, Schubbert et al 1994, Schubbert et al 1996)Whether similar processes take place in non-mammalian organisms is unknown, though this should be an avenue of investigation in determining biological risk on transgenic insects.

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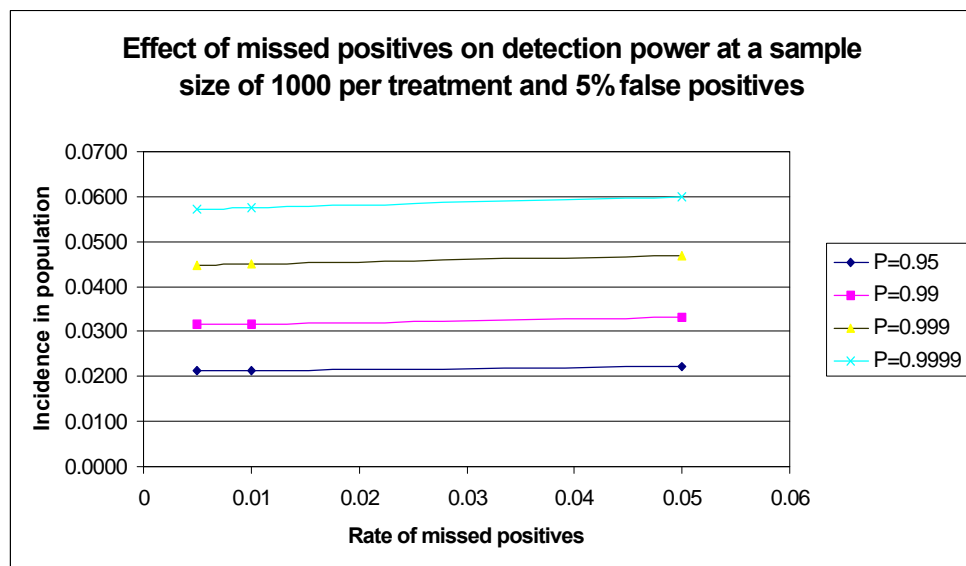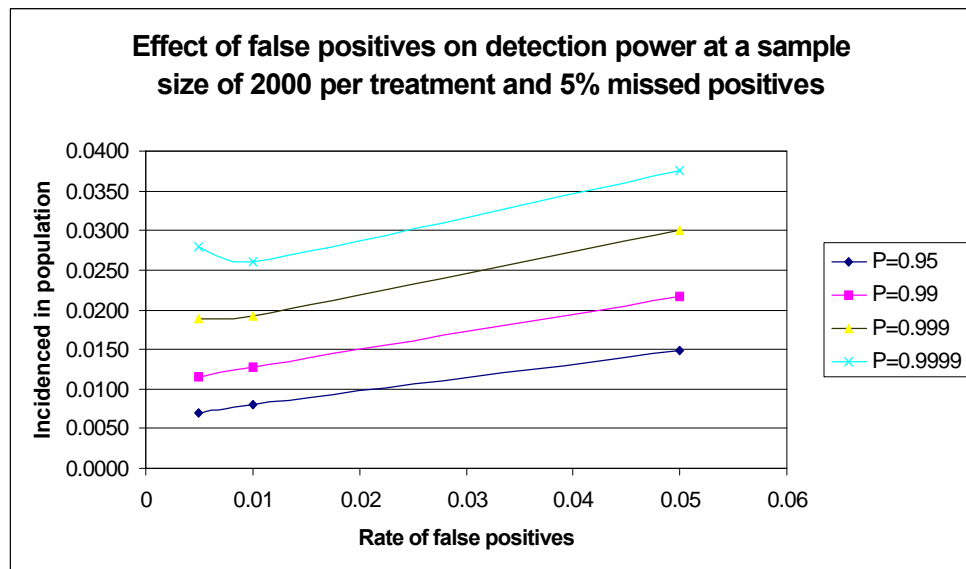
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positives and 1% false positives

i The s... ssion is taken ... n Trials and T... LMO arthro...

Figure

Effect of false positives on detection power at a sample size of 2000 per treatment and 5% missed positives

Effect of missed positives on detection power at a sample size of 1000 per treatment and 5% false positives
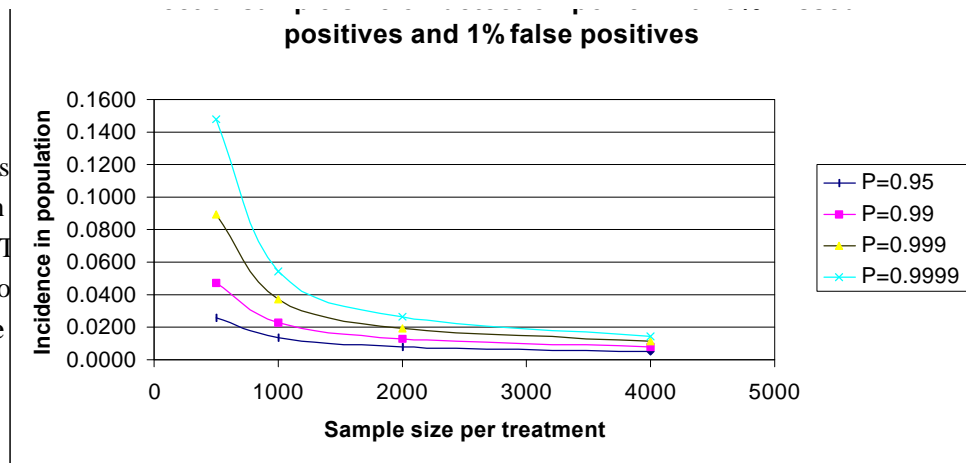
Appendix

Obtaining confidence limits for true incidence of transgene in organisms exposed to potential horizontal transfer (Fig. 1).

Experimental design and hypothetical data:

The experiment consists of two treatments: 1) Organism which have been exposed to the potential horizontal transmission of the transgene. 2) Organisms which have not been exposed to any transgene at all. From each treatment a sample of size $N = n_{1+} = n_{+1}$ is tested for the presence of the transgene. From external sources we have an estimate of the rate of missed positives $(1-b_1)$ of the test.

Results: a) In treatment 2 a certain number n_{12} of the organism tests positive for the presence of the transgene. Assuming that none of the organisms of treatment 2 are truly positive $n_{12}/ n_{1+} = b_2$, the rate of false positives. b) Assuming (for the hypothetical experiment) horizontal transfer did not occur in treatment 1 the same number organisms as in treatment test positive (they are all false positives.

Procedure to determine the upper confidence limit of the rate of horizontal transfer:

The actual frequency data from the experiment can be organized in a 2 by 2 contingency table. From this arrangement of the frequency data exact exact confidence limits for the odds ratio were calculated (Proc Freq, SAS 1999) according to an iterative algorithm based on that presented by Thomas (1971). Since only upper confidence limits were desired one tailed probabilities were employed (the significance level was set at

$1 - 2$).

From the upper confidence level of the odds ratio we calculated threshold observable probabilities for the 2 by 2 table that we then converted into true probabilities according to Fleiss (1981)

Determination of exact confidence limits for odds ratio

Table frequencies observed:

Transgene	Treatment		Totals
	exposed: A_1	not exposed: A_2	Totals
present: B_1	n_{11}	n_{21}	n_{+1}
absent: B_2	n_{12}	n_{22}	n_{+2}
Totals	n_{1+}	n_{2+}	n_{++}

Expected frequencies are: $m_{ij} = E(n_{ij})$

and thus the expected odds ratio:

$$q = \frac{m_{11}m_{22}}{m_{12}m_{21}}$$

The conditional distribution of n_{11} given n_{++}, n_{1+}, and n_{+1} is

$$f(n_{11} \mid n_{++}, n_{+1}, n_{+1}; q) = \frac{\binom{n_{1+}}{n_{11}}\binom{n_{++} - n_{1+}}{n_{+1} - n_{11}} q^{n_{11}}}{\sum_u \binom{n1+}{u}\binom{n_{++} - n_{1+}}{n_{+1} - u} q^u}$$

where the index of summation ranges from $\max(0, n_{1+} + n_{+1} - n)$ to $\min(n_{1+}, n_{+1})$.

For testing

$H_0 : \boldsymbol{q} = \boldsymbol{q}_0$ against $H_1 : \boldsymbol{q} > \boldsymbol{q}_0$
the p - value is $P = \sum_S f(n_{11} \mid n_{++}, n_{+1}, n_{+1}; \boldsymbol{q}_0)$, where $S = \{t : t \geq n_{11} \}$

"Exact confidence limits for $\boldsymbol{q}$ can be obtained by inverting the test (Thomas 1971).
For testing against $H_1 : \boldsymbol{q} < \boldsymbol{q}_0$
$S = \{t : t \leq n_{11} \}$

Conversion of observable into true probabilities

True (without classification error) probabilities:

Transgene	Treatment	
	exposed: A_1	not exposed: A_2
present: B_1	$P(A_1 B_1)$	$P(A_2 B_1)$
absent: B_2	$P(A_1 B_2)$	$P(A_2 B_2)$

Observed (with classification error) probabilities:

Transgene	Treatment	
	exposed: A_1	not exposed: A_2
present: B_1	$p(A_1 B_1)$	$p(A_2 B_1)$
absent: B_2	$p(A_1 B_2)$	$p(A_2 B_2)$

Probabilities of correct and incorrect classification:

True status	Classified status	
	B_1	B_2
B_1	b_1	$1 - b_1$
B_2	b_2	$1 - b_2$

$1 - b_1$: missed positives (false negatives) in test
b_2:   false positives in test

Thus from the observe probabilities the true probabilities can be calculated as follows:

$$P(A_1 B_1) = \frac{p(A_1 B_1) - b_2 p(A_1)}{b_1 - b_2},$$

$$P(A_2 B_1) = \frac{-p(A_1 B_1) - b_2 + p(B_1) + b_2 p(A_1)}{-p(A_1 B_1) + b_1 p(A_2)},$$
$$P(A_1 B_2) = \frac{-p(A_1 B_1) + b_1 p(A_2)}{b_1 - b_2},$$

$$P(A_2 B_2) = \frac{p(A_1 B_1) - b_{1+} - p(B_1) - b_1 p(A_1)}{b_1 - b_2}$$

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Transgenic Mediterranean Fruit Flies for the Sterile Insect Technique

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Abstract

The Sterile Insect Technique (SIT) is now an accepted component of various integrated approaches to suppress, eradicate or prevent the establishment of Mediterranean fruit fly, *Ceratitis capitata* (medfly) populations. It relies on the areawide release of sterilized insects into a field population resulting in the induction of sterility in that population. Repeated releases lead to population decline and if the target population is isolated then the population collapses to extinction. Following the successful development of genetic transformation technology for many pest insects it has been suggested that this technology may be utilized to produce strains that can increase the efficiency of medfly SIT. The fact the only sterile insects are released removes the risk of the vertical transmission of any transgene, a major advantage for regulatory approval. There are three areas where transgenic technology may make a contribution to increasing the efficiency of medfly SIT. Firstly, it may be possible to produce improved male only strains for release. Such strains, based on conventional genetics, are now being used successfully in almost all medfly SIT programmes and there is a demand to produce these strains in other species. Secondly, transgenesis can be used to introduce a phenotypic marker into medfly that would replace the use of fluorescent dye for the external marking of released insects. Thirdly, research is underway to develop transgenic strains that lead to dominant lethality in the field following release. The paper will discuss these options and the technical problems that may need to be solved before they can be introduced into an operational SIT programme. In addition, reference will be made to the regulatory and bio-safety issues related to the use transgenic insects in SIT

Key words: medfly, transgenesis, genetic sexing, sterile insect technique, sterilization.

Introduction

Insect pests continue to be threat to man, his animals and his crops notwithstanding the considerable efforts of the scientific community to develop effective management strategies that meet the requirements of all interested parties. Using toxins to kill insect pests was the obvious first approach and this is likely to remain the main line of defence in the near future. There have been continuous improvements in the efficacy of the different toxins both in their species specificity and in the mode of delivery culminating in the widespread use in agriculture of transgenic plants that produce an insect toxin, a major area of interest at this symposium. Other environmentally benign approaches to pest insect management include biological control (Bellows & Fisher 1999) or manipulation of insect behaviour using semio-chemicals (Suckling & Karg 1998). However, classical biological control is now not escaping criticism due to the sometimes unpredictable consequences of introducing a natural enemy into a new ecosystem (Howarth 1991).

Insect pest control has been traditionally applied to directly protect the crop, animal or human. This seems a logical approach until it is realized that a large proportion of the pest population never comes into contact with the crop, animal of human and as such is never exposed to control. This leads to a very unsatisfactory situation in which the control intervention has to be applied frequently and on a continuous basis. To deal with this paradox, the use of the areawide approach is now being promoted as it recognizes the importance of taking into account the spatial and temporal distribution of the total pest population when implementing insect management field programmes. In certain situations this approach can lead to the eradication of a pest population over a wide geographic area (Wyss 2000).

One way to exploit this areawide principle is the use of the sterile insect technique (SIT) where mass reared, sexually sterile males, following their release over large geographic areas, mate with and sterilize wild females. The insects are sterilized by exposure of adults or pupae to ionising radiation that induces dominant lethal mutations in sperm (for review see Robinson 2002, Alphey & Andreasen 2002). The SIT is now an increasingly important component of integrated approaches for the management of key insect pests (Tan 2000).

Following the successful germ-line transformation of *Drosophila melanogaster* (Rubin & Spradling 1982), many ideas were developed as to how this technology could be utilized to improve insect control if effective means were developed to transform pest insects. These ideas included the creation of mosquito strains that would be refractory to the development of the malaria parasite (Collins 1994), the improvement of biological control agents by introducing insecticide resistance genes (Hoy 1992) and increasing the efficiency of the SIT by developing male producing strains (Robinson & Franz 2000).

This paper will update the situation as regards transformation in pest insects, describe the essential components of an operational SIT, identify and describe the possible impact that transgenic technology could have on the efficiency of the SIT and indicate where the biosafety concerns are likely to be most important. The paper will focus on the Mediterranean fruit fly (medfly), *Ceratitis capitata* as there are large operational SIT programmes for this pest and much work has been done on developing transformation technology, in fact the medfly was the first pest insect to be transformed (Loukeris et al 1995).

Status of transformation in pest insects

Genetic transformation of non-drosophilid insects is now routine (see Atkinson et al. 2001, and Handler 2001, for reviews) and four different transposon-based gene vector systems have been used. The Table below gives an overview of the current status.

Table 1. Transformation in non-Drosophilid insects		
Species	**Element**	**Reference**
Ceratitis capitata	*Minos*	Loukeris et al 1995
Ceratitis capitata	*Piggy Bac*	Handler et al 1998
Ceratitis capitata	*Hermes*	Michel et al 2001
Bactrocera dorsalis	*piggyBac*	Handler & McCombs 2000
Anastrepha suspensa	*piggyBac*	Handler & Harrell, 2001
Musca domestica	*piggyBac*	Hediger et al 2001
Musca domestica	*Mos1*	Yoshiyama et al 2000
Lucilia cuprina	*piggyBac*	Heinrich et al 2002
Stomoxys calcitrans	*Hermes*	O'Brochta et al 2000
Aedes aegypti	*Hermes*	Jasinskiene et al 1998
Aedes aegypti	*Mos1*	Coates et al 1998
Aedes aegypti	*piggyBac*	Lobo et al 2002
Anopheles gambiae	*piggyBac*	Grossman et al 2001
Anopheles stephensi	*Minos*	Catteruccia et al 2000
Anopheles stephensi	*piggyBac*	Ito et al 2002
Anopheles albimanus	*piggyBac*	Perera et al 2002
Culex quinquefasciatus	*hermes*	Allen et al 2001
Bombyx mori	*piggyBac*	Tamura et al 2000
Pectinophoa gossypiella	*piggyBac*	Peloquin et al 2000
Tribolium castaneum	*piggyBac*	Berghammer et al 1999
Tribolium castaneum	*Hermes*	Berghammer et al 1999

These vector systems, constructed from the *mariner*, *Minos, Hermes* and *piggyBac* transposable elements, have been used to transform 14 species of insects including Diptera, Lepidoptera and Coleoptera and there is no technical reason why most insect species should not be amenable to this technology. SIT programmes are currently being carried out on several of the pest species listed above. A major improvement in the efficiency by which transgenic insect strains could be isolated was the availability of robust genetic marker systems for recognizing transgenic insects (Chalfie et al 1994, Horn et al 2002). These dominant marker systems, based on a variety of fluorescent proteins, combined with highly conserved gene regulatory sequences, provide reliable methods for detecting, maintaining and recognizing transgenic insects. In Drosophila, transgenic strains carrying constructs of relevance to improving the SIT have already been tested successfully; for example, conditional gene expression systems have been used to achieve female-lethality for genetic-sexing and paternal transmission of embryonic lethal genes (Thomas et al 2000, Heinrich & Scott 2000). Other conditional lethal systems can result in death of all offspring from released males in response to either low or high ambient temperatures (Fryxell & Miller 1995).

It is clear that the laboratory tools to transform most insect species are now available. The next step will be to try to transfer some of the potentially useful systems, which have been developed in Drosophila, to pest species. This transfer may not be straightforward, as some of the regulatory elements used in their construction may not be sufficiently conserved across species lines. Even if this can be done, it still remains to be seen if the systems are robust enough to be incorporated into operational programmes to increase their effectiveness. The more complex a system is required to be, the more susceptible it will be to perturbation in an operational programme.

Some Essential Components of a Successful medfly SIT Operational Programme

The medfly is an important agricultural pest in many parts of the sub-tropical world where it attacks a wide variety of fruits and vegetables (Robinson & Hooper 1989). In the 1970s there was a large successful programme for the eradication of this pest from large parts of Mexico that catalysed the development of similar programmes elsewhere in the world. All these programmes use essentially the same technology and any improvements using trangenesis could be transferred to them all, as demonstrated by the recent adoption of genetic sexing strains in most medfly mass rearing facilities (Robinson et al 1999). Current thinking suggests that transgenic technology may be used in SIT programmes in the three areas outlined below (Handler 2002).

Male only strains

As described above, the SIT relies on the release of sterilized insects into a wild population followed by mating of the released sterile males with the wild females. These matings produce no offspring and repeated releases lead to a gradual reduction in the size of the wild population. It is therefore required that only sterile males need to be released for the technique to be successful and if a way could be found to do this then programme costs would be reduced and the biological efficiency increased in many different ways (Hendrichs et al 1995). With these points in mind, considerable efforts have been made to develop genetic sexing strains (GSS) in medfly and these have resulted in the transfer of this technology to almost all SIT programmes for the pest (see Robinson et al 1999). The current GSS are based on Mendelian genetics and use a recessive temperature sensitive lethal mutation that is homozygous in females and heterozygous in males. The dominant wild type allele has been linked to the male determining chromosome using a translocation and eggs are heat treated to kill females. By selecting translocations with breakpoints close to the wild type allele it has been possible to produce strains that are extremely stable over many generations. Nevertheless there are some associated disadvantages. First, as the strains are based on a translocation, the colony has a 50% reduction in fertility, secondly the females in the colony are homozygous for the mutation and they show reduced viability, thirdly, the systems are not transferable to other species.

Genetic sexing using molecular approaches can either be targeted towards killing females or transforming putative female zygotes into males but both systems are required to be conditional for colony maintenance. If female medflies are targeted for killing then lethality should be induced at an early stage, ideally in the egg, as very large numbers of zygotes can be treated in relatively small volumes of water. This requires that early acting female specific promoters are identified and placed under conditional control as described below.

Transforming female medflies into males requires a detailed knowledge of sex determination in this species. It is known that the X:A balance system as described in Drosophila is not used by the medfly (Zapater & Robinson 1986) and it has been shown that the primary signal for sex resides on the Y chromosome where a male determining factor has been genetically mapped (Wilhoeft & Franz 1996). Using Drosophila genes as probes, *Sex-lethal* (Sxl) and *doublesex* (*dsx*) and homologues have been cloned from medfly to try to identify sex-specific splicing sites. *Sxl* produces the same protein in males and females whereas *dsx* is sex specifically spliced. This differential splicing mechanism can be used to obtain female specific expression of lethality. This knowledge of sex determination in medfly has enabled advantage to be taken of the RNAi technique to transform female medfly embryos into males. The RNAi technique relies on inhibition of gene function using double stranded RNA (dsRNA). By injecting dsRNA from the *doublesex* gene, into medfly embryos it has been

possible to transform female embryos into fertile males (Saccone pers. comm.). It remains to be seen if this effect can be duplicated using a transgenic approach.

Marking

In order to monitor the progress of a medfly SIT programme it is important that the released flies can be differentiated from the wild flies following trapping in the field. The data from the traps are used to calculate the ratio of released to wild flies and to directly monitor the relative size of the wild population. In order to accomplish this, all the pupae destined for release as sterile flies are mixed with fluorescent dye and during their emergence the insects trap particles of the fluorescent dye behind the ptilinum (Hagler & Jackson 2001). When trapped flies are brought in from the field their origin is determined by squashing the heads and examining under a UV light for the presence of the fluorescent particles. This process is very labour intensive, expensive and subject to serious errors of interpretation. Transgenic techniques could enable insects to be marked either with a specific DNA sequence or with a fluorescent protein provided that the penetrance of the marker is complete.

Using a genetic marker for released flies requires that the marker be dominant and that it can be monitored in dead adults, as flies are usually dead when removed from the traps. There are currently two fluorescent protein markers available to accomplish this i.e. green fluorescent protein (GFP) (Prasher et al 1992) and red fluorescent protein (DsRed) (Matz et al 1999). Generalized expression of the proteins can be obtained by using polyubiquitin and actin promoters (Handler & Harrell 2001, Peloquin et al 2000) and the protein can be observed in dead flies (Handler pers. comm. and Franz pers. comm.). Particular strains can show very strong levels of expression but the fitness cost to the fly for the production of this exogenous protein is unknown. No data is available on the effect of this marker on the behaviour of the fly in the field or indeed of the response of conspecifics or predators to marked flies.

Sterilization

The use of ionizing radiation has proven to be an extremely effective way to sterilize insects for release into the field with the procedure being carried out at as late a developmental stage as possible so as to reduce somatic damage. This physical process is a fairly fail-safe procedure when the correct protocols are followed; it also is not subject to the development of resistance, can be used on any strain and does not interfere with the mass rearing process. As with all the procedures that the released flies have to be subjected to, radiation does have some effect on the fitness of the irradiated individuals. However, the detrimental effects of radiation have sometimes been exaggerated as the overall competitiveness of the released insect is determined by a whole combination of different factors related to factory adaptation, selection, and handling and release procedures etc.

It is proposed that transgenic strains may be developed that will induce embryonic lethality in eggs fertilized by released transgenic males and so remove the need for radiation. Any system to induce sterility in the field through dominant lethality must be conditional in some way so that mass rearing can be carried out. This conditionality can be achieved by using transcriptional activation or suppression systems based on the presence or absence of antibiotics in the diet (Heinrich & Scott 2000, Thomas et al 2000). The permissive condition in the facility will require the presence of an antibiotic or its analogue during the mass rearing but following release, the female progeny of any wild female mated with a transgenic male would die in the absence of the antibiotic. However, more recent experiments have shown that it is possible to engineer a Drosophila strain so that both males and females die as embryos in the field (Horn & Wimmer 2002). A specific concern with these types

of systems that require the addition of a bioactive compound to very large volumes of larval diet is the disposal of the diet; the medfly facility in Guatemala produces about 25 tons of diet/day. In addition, maintaining the appropriate concentration throughout the diet will not be easy. A second concern is the effect of the diet on the level of antibiotic. As the diet is a microcosm of bacterial and fungal growth, it will not be easy to standardize the exposure of the larvae to the antibiotic. It will also be important to choose the appropriate type of dominant lethality with which to kill the progeny in the field. Any genes involved in cell death mechanisms or which show general cell toxicity would probably not be suitable and/or would raise environmental concerns. Effective sterilization of the wild females remains the key to a successful SIT programme, there is no room for error in the sterilization procedure and any proposed biological system must be extremely robust, controllable and accurate.

Potential Problems
Mass rearing

The ability to produce very large numbers of high quality insects in a predictable fashion is probably the major technical challenge in SIT. Mass rearing systems confront the insect population with a totally artificial biotic and abiotic environment that impacts drastically on important components of the quality of the insect (Cayol 2000) and many of these changes have a genetic basis. Experience with conventional GSS during mass rearing has revealed that unexpected and very rare events can have a major impact on usefulness of the strains for SIT. There are in fact two processes operating in a mass rearing environment, the occurrence of extremely rare events followed by stringent selection for the best adapted genotype (Franz 2002). For example with conventional GSS, a very rare recombination event can lead to the creation of a male that is fully fertile and individuals with this genotype have a huge selective advantage in mass rearing and they rapidly accumulate and totally destroy the sexing characteristics of the strain. It is impossible to completely eliminate the occurrence of these types of very rare event and rearing systems have to be out in place to deal with their consequences. For medfly, mass rearing of GSS a Filter Rearing System (FRS) has been developed and implemented to deal with this problem (Fisher & Caceres 2000). The FRS is essentially a small closed colony in which exceptional individuals are removed at every generation and from this colony eggs are harvested, and used to initiate 3-4 generations of mass rearing. In the generation before release, the eggs are heat treated, the males reared and sterilized and then released. No insects that have been through the mass rearing process are returned to the colony.

A major difficulty faced during the introduction of new strains into a mass rearing facility is the absence of an experimental model as the number of insects reared in a large facility is several orders of magnitude larger than can ever be reared in a normal laboratory. In addition the level of stress under which the strain is reared is much greater in the facility. Factors related to both genotypic and phenotypic stability will assume a very important role in mass rearing of transgenic strains and even extremely rare events influencing these attributes can have major consequences for the usefulness of the strains. It will be essential that a system similar to the FRS be developed in order to maintain the integrity of any transgenic strain under mass rearing conditions as it is unrealistic to expect that any system will work with 100% accuracy over long periods of time.

Genomic variability in field populations

There is the possibility that elements that are used for insect transformation may be mobilized either by related elements in genomes of wild flies or by novel molecular processes such as template dependent gap repair (Lohe et al 2000). These processes can lead to either a change in expression of

the effector gene or an increase or decrease in the copy number of the transgene construct. Any of these events would cause serious problems for the use of transgenic strains in SIT. A transgenic construct that is engineered to cause lethality in the embryos of wild insects will be confronted with the full range of genomic diversity once fertile insects carrying the transgene are released. Any interaction between the construct and the wild genome that leads to changes in the expression of the effector gene would be very detrimental and assuming that this interaction is genetic, it would quickly sweep through the wild population and nullify the effect of the released transgenic insects. There will be a very strong selective pressure on the wild population as the biological fitness of the matings with the original transgenic male is zero. Any mutation that compromises the expression of the "sterility" transgene will rapidly increase in frequency and render the strain useless. Transgenic constructs are introduced into highly inbred laboratory strains that are very unrepresentative of the target population in the field. Field testing of constructs which induce sterility will be extremely difficult as the natural variability modulating the effects of the transgene is unknown.

Backcrossing and strain replacement

Before a strain is introduced into an operational programme, the local manager will sometimes require that the strain be backcrossed into the local genetic background before it can be introduced into the mass rearing facility. This reason for this procedure is to try to ensure that the released strain does not pose any additional threat to the local agriculture should it become established. In addition it is generally thought that increasing genetic variability will lead to better performance of the strain in the field. This procedure presents another opportunity for interaction between the construct and the genomic variability of field populations. On several occasions, during backcrossing of a conventional genetic sexing strain to wild populations, great difficulties were experienced in re-isolating the strain (Franz et al 1996 and Franz pers. comm.). Backcrossing of a strain to a wild population for use in the SIT is also not just a one-off procedure at the time that the strain is introduced. Over time mass reared strains lose their effectiveness in the field and most programmes now have procedures in place to regularly replace or refresh their strains with material from the field.

Biological fitness

Knowledge of the biological fitness of transgenic strains will be crucial for any eventual use in medfly SIT and very little is known about the true effect of transgenesis itself on the overall phenotype. The more the fitness of the strain is compromised, the higher the selective advantage will be for any exceptional individuals that appear in the mass rearing. There is however accumulating evidence that most transgenic strains do in fact show various levels of fitness reduction, independent of the marker, species or element used (Franz pers comm., Chrisanti pers. comm. and Jacobs-Lorena pers. comm.) As the insertion process is essentially random, at least regarding the insertion position, it is impossible to predict laboratory fitness levels and these can only be obtained following detailed life table studies for each individual strain. As with all other strains for medfly SIT, the transgenic strain would then have to go through field cage evaluations of competitiveness and field assessment of dispersal and survival. The final evaluation would be the development of the quality control profile of the strain during mass rearing over extended periods of time. This would include detailed information on stability of the transgene.

This whole series of steps, as well as taking considerable time, is likely to uncover unexpected biological events that impact on the performance of the strains. The evaluation process will be based on feed-back loops that identify and solve the problems as strains move through the

system. Based on experience with the development of conventional genetic sexing strains, very few of the initially selected transgenic strains will make it through to the latter stages of the process. Considerable R and D will be required to deal with problems that arise during this testing phase and following introduction into operational programmes.

Regulatory Concerns

The issue of assessing risk in the use of transgenic strains in insect control is complex and has certainly not kept pace with the technical developments (Hoy 1995, Hoy 2000, Ashburner et al 1998). In SIT, where the insects are sterilized by radiation, the released insects cannot become established in the environment so that horizontal transfer of the transgene to other organisms remains the main problem. Horizontal gene transfer is a natural phenomenon and its impact on risk assessment in transgenic insects relates to impact of the transgene should it enter the genome in the wild insects. For these reasons antibiotic or generalized cell death genes should be avoided. The situation is however entirely different when fertile transgenic males are released which induce death in the embryos of wild females fertilized by these males. In this case if there is a change in the expression of the lethal system then the transgene could leak into wild population. More significantly if the wild population develops some form of genetically based resistance there would be an extremely rapid spread of this factor through the wild population resulting in failure of control.

Recently there have been numerous meetings organized by both scientists, regulatory bodies and organisations involved with transgenic insects, to try to develop concepts by which an appropriate regulatory framework for the use of transgenic insects can be developed. Whilst some progress has been made there remain serious concerns, most recently expressed in a review of the topic by the US National Academy of Sciences (NAS 2002). Although this review only focused attention on the release of fertile transgenic refractory mosquitoes it did identify some concerns that also apply to the use of transgenic insects in SIT. The concerns are centered on the mobility of the released insects and their ability to escape and become established.

Traditional SIT, relying on radiation to induce full sterility in the released males, prevents vertical transmission of the transgene and may provide the first possibility of using transgenesis in pest control programmes. The problem of horizontal transmission remains to be evaluated. A trivial but logistically important aspect for medfly SIT would be the disposal of used diet containing transgenic larvae.

Conclusions

Technical problems associated with the introduction of exogenous genes into most pest insect genomes have been essentially solved and have provided a very valuable experimental tool to study gene expression and regulation. Currently 14 species of insects have been transformed using one of four different vector systems currently available. The use of fluorescent dominant markers has made the task of identifying transgenic lines considerably easier than previously. However, considerable problems still remain to be solved before transgenic strains can be used in operational SIT programmes.

It is essential that the general mechanisms of transposition, transposon behavior and regulation of normal function and repression is thoroughly understood. This will include a detailed understanding of how transposons behave in caged populations and hence how they may behave in wild populations.

Research will be needed on transgene instability and how horizontal transmission could be minimized. This may be realized by engineering vectors that can be immobilized subsequent to

genomic integration by the deletion or rearrangement of sequences required for mobility. Studies of vector and host-genome interactions are urgently needed focusing on epigenetic interactions that might result in unintended or unexpected transgene activity or repression. New gene transfer systems are required that will include recombination based systems producing target or docking sites for integration, in this way the problem of random insertion will be eliminated.

Whilst the above studies will be essential to provide appropriate transformation vectors for applied use, concurrent studies are urgently needed on all aspects of fitness of already established transgenic lines. Currently, each transformed strain is unique and the insertion is associated with its own cost for the insect that can only be assessed following detailed life table and mating studies in field cages.

The response of any transformed strain to the exigencies of mass rearing is at present completely unknown. The effect of increased stress and the rearing of extremely large numbers of insects may have unknown effects on the behaviour of transgenic strains and additional R and D will be required to solve these problems. Major difficulties associated with carrying out these studies are the very few places where this can be carried out and the length of time (generations) needed to get meaningful results.

SIT operational programmes are conservative by nature and they are not in the business of taking risks and will need to be convinced that any new strain will really improve programme efficiency. The fact that a new transgenic strain is based on some very clever science will not be taken into account as long as there is no significant improvement for the end-user.

The major targets for transgenic approaches for medfly SIT remain the development of marker strains and genetic sexing strains to be used in the release of sterile insects. The use of molecular methods to sterilize wild populations, i.e. the release of fertile transgenic insects, is still some way off and it will need to deal with the unknowns of genomic variability in wild populations. The regulatory framework in which transgenic insects can be released is still poorly developed.

Acknowledgement

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Exploitation of genetically modified *Pseudomonas* for industrial ecology applications

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Rhizosphere competent fluorescent pseudomonads are ideal candidates for utilization as biocontrol and bioremediation inoculants. Direct links between biocontrol efficacy and production of anti-microbial compounds have emerged via utilization of recombinant DNA technology. Production of antimicrobial 2,4-diacetylphloroglucinol (Phl) is the central mechanism utilized by P. fluorescens F113 in biocontrol. Environmental and microbial signals modulate regulatory processes governing production of Phl at the functional genomics level. Innovative design strategies based on reprogramming regulatory mechanisms via manipulation of these signals can be employed to improve biocontrol efficacy of Pseudomonas inoculants. Further more P. fluorescens F113 can be used as an ideal carrier strain for polychlorinated biphenyl (PCB) degradative genes facilitating development of novel rhizoremediation bioinoculants for controlled degradation in contaminated biosystems. However, public concerns as to the biosafety of genetically modified bacterial strains in the environment must be considered. Developments in molecular microbial ecology have facilitated assessment as to the impact of bacterial inoculants on soil-borne non-target microbial communities. Plant root exudates together with microbial signals can regulate the composition of indigenous microbial communities in the soil. P. fluorescens F113 wild type strain had no significant impact on key microbiota ranging from arbuscular mycorrhizal fungi to fluorescent pseudomonads. Genetically modified derivatives of P. fluorescens F113 also had no significant influence on indigenous microbiota. An understanding of signalling mechanisms occurring in plant-microbial interactions using a functional genomic approach has the potential to improve biosafety of genetically modified inoculants and facilitate registration processes for utilization of plant microbial protection products in industrial ecological applications such as biocontrol and bioremediation

Monitoring the identity and survival of genetically modified or non-modified plant growth promoting bacteria and their impact on soil microbial communities

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Plant growth promoting rhizobacteria (PGPR) are of increasing importance as inoculants for bio-fertilisation, bio-stimulation and biological control of plant pathogens in sustainable agriculture. A substantial proportion of bacteria which can be isolated by traditional cultivation techniques from the rhizosphere of plants are beneficial for plant growth in a direct or indirect way. Bio-fertilisation, the ability of rhizobacteria to fix atmospheric nitrogen, accounts for a substantial nitrogen supply to crops (Bloemberg and Lugtenberg, 2001). A direct enhancement of plant growth can be achieved by plant hormones such as auxins secreted by beneficial rhizobacteria such as *Azospirillum*. Increased plant growth can also result from the suppression of deleterious micro-organisms by antagonistic bacteria (Berg et al. 2000, 2001; Chet et al. 1990; Emmert and Handelsman, 1999; Neiendam-Nielson et al., 1998; O'Sullivan and O'Gara, 1992; Whipps, 1997).

However, to fully exploit the huge resources and potentials of PGPRs, a better understanding of the biotic and abiotic factors influencing the fate of microbial inoculants, as well as of the dynamics of microbial communities in the rhizosphere is required (Van Veen et al., 1997).

The rhizosphere is defined as the narrow zone of soil adhering to the root (Sørensen, 1997). A variety of organic compounds, such as mono- and polysaccharides, amino acids, organic acids, fatty acids, enzymes, auxins or HCN, is released either actively or passively by the plant root (Neumann and Römheld, 2001). The release of root exudates is affected by a number of abiotic and biotic factors, the plant species and the physiological stage of the plant. Some bacterial or fungal populations might take more profit from the nutrients offered by the plant than others which might affect their numerical dominance and activity. In return, microbes enriched in the vicinity of the root can be beneficial to the plant or cause harm (Brimecombe et al., 2001). Thus, the interactions between rhizobacteria and plants are highly complex, making predictions on the fate and activity of microbial inoculants difficult (Van Veen et al., 1997). The introduction of reporter genes by means of gene technology has greatly facilitated the tracking of introduced GM bacteria in the environment (Jansson, 1995; Unge and Jansson, 2001). Our understanding of the composition and dynamics of

microbial communities in rhizosphere soil has been extremely biased due the fact that only a small fraction of rhizobacteria can be recovered from this system by traditional cultivation techniques (Staley and Konopka, 1985; Amann et al., 1995). Cultivation-based limitations can now be overcome by the analysis of nucleic acids extracted directly from rhizosphere or bulk soil samples (Van Elsas et al., 2000 a). On the one hand, this technology opened the chance to track introduced microbial inoculants independent from their ability to form colonies on plates. On the other hand, 16S or 18S rDNA fragments amplified from community DNA by PCR allow insights in the composition of the microbial community when analysed by molecular fingerprinting techniques.

Monitoring the identity, fate and metabolic activity of microbial inoculants as well as their impact on rhizosphere and soil microbial communities is needed to guarantee a safe and reliable application of these independent of whether they are genetically modified or not. While European regulations demand such a kind of monitoring for genetically modified micro-organisms (GMMs), no such requirements exist for non-modified PGPRs.

The intention of our contribution is to briefly assess the tools presently available to monitor the identity of microbial inocula and their fate and impacts on the soil microbiota, and to illustrate their use in two research projects on microbial inocula. One of these projects dealing with monitoring the fate and impact of genetically modified PGPRs used in the field in China was performed in collaboration with the Institute for Application of Atomic Energy, Beijing. In the second project, the fate and metabolic activity of the *Ralstonia solanacearum* antagonist *Pseudomonas chlororaphis* is analysed in the rhizosphere of tomato plants. Since the potato pathogen *R. solanacearum* is a quarantine organism, this study is performed under confined greenhouse conditions. For more information on the topic we refer the reader to the reviews by Van Veen et al. (1997), Van Elsas et al. (1998), and Bloemberg and Lugtenberg (2001).

Strain identity

The first and crucial prerequisite for the safe and successful use of PGPRs is that the strain identity and activity are continuously confirmed, in particular when PGPRs are grown in large-scale fermenters and used for large-scale applications in the field. The prevention of contamination during large-scale fermentation processes is an obvious requirement which is not new. An aspect which has received less attention in the past is the natural plasticity of an inoculant´s genome and the expression of genes involved in the beneficial effects. In particular when genetically modified bacterial inoculants are used, the stability of the introduced novel genes needs to be confirmed regularly. While in the past selective plating has been used primarily for strain confirmation, we nowadays know that these tools, albeit rapid and inexpensive, are not sufficiently reliable for inoculant strain confirmation since spontaneous mutants (e.g. antibiotic resistant) can easily occur. Molecular fingerprints generated by PCR with primers annealing to repetitive sequences (rep-PCR: REP, ERIC and BOX) and random DNA stretches, or ARDRA (amplified ribosomal DNA restriction fragment analyses) have proven to be suitable to recognise the identity of inoculant strains

and to detect genomic rearrangements (Rademaker et al., 1998; Marten et al., 2000). BOX fingerprints also served to characterise the genetically modified rhizobacteria *Alcaligenes faecalis* and *Klebsiella oxytoca* used on a large scale to improve plant growth of several important crops in China. *Alcaligenes faecalis* strain A1501, which was isolated from the rice rhizosphere in South China, is able to grow under high salt conditions, maintaining its IAA (indole acetic acid) production and N_2 fixing abilities (You and Zhou, 1991). A tight association between A1501 and rice roots could be shown. Dinitrogen fixation by A1501 resulted in an 8% increased N-content in the rice plants and a strong plant growth promoting effect. However, the associative diazotroph *Alcaligenes faecalis* is sensitive to high NH_4^+ concentrations in terms of its *nif* gene expression. To increase inoculant efficiency, *nifA* or *nifA/ntrC* located on Tn*5* were introduced into the genome of the A1501 strain. Recently, sequencing of the 16S rDNA showed that the sequence with the highest similarity was one of *Pseudomonas stutzeri* indicating that the strain had previously been misidentified, thus, it became necessary to re-name A1501. Comparing the BOX PCR fingerprints of the wild-type (A1501) and the genetically modified strain (A1523) it was easily found that the patterns of both strains were identical, while the patterns of the wild-type (NG13) and the genetically modified *Klebsiella oxytoca* (NG1390) strain only had very few bands in common, suggesting major genomic rearrangements or, which is more likely, that another strain was cultivated.

Since the genetic modification (NG1390: *nifA;* A1523: *ntrC nifA*) was introduced by transposon Tn*5* mutagenesis, PCR primer systems targeting the *nptII* gene or hybridisation of Southern-blotted genomic DNA should be an easy way to confirm the presence of the construct in the inoculant genomes. While PCR failed to detect the *nptII* gene in both GMM strains, Southern blot hybridisation showed at least signals for A1523 (unpublished data). However, the agarose gel electrophoresis indicated that the *nptII* homologous DNA was localised on a plasmid. It remains unclear why PCR primer systems, developed on the basis of the Tn*5* sequence, did not work.

Our experience shows that large-scale production of PGPR strains requires the availability of reliable tools such as PCR primer systems specific for the construct, reference material of the genomic DNA, as well as of a molecular fingerprint which helps to identify the strain. Although strain confirmation by molecular techniques is an obvious requirement, it might not always be easily implemented because modern molecular techniques for characterisation of inoculant strains are rather costly and thus mean that their use in developing countries is limited.

Monitoring the fate of the inoculant strain

Rhizosphere competence which describes the ability of inoculant strains to survive in, and efficiently colonise the rhizosphere is crucial for the efficiency of the PGPR (Van Veen et al.,1997; Lugtenberg and Dekkers, 1999). Investigations of several groups showed that microbial diversity is dependent on the plant species (Berg et al., 2002; Dalmastri et al., 1999; Grayston et al., 1998; Lemanceau et al., 1995; Miller et al., 1989; Smalla et al., 2001; Westover et al., 1997). These observations have implications for the successful colonisation of microbial inoculants. Furthermore,

the soil type was reported to affect the survival of inoculant strains (Horwath et al., 1998; Latour et al., 1996). Investigations on the fate of inoculant strains should be done in the phase of field testing before the actual commercialisation of the strains. To follow the fate of inoculant strains in the rhizosphere of crop plants and non-target plants, cultivation-dependent methods are most frequently applied. Most suitable for tracking inoculants by selective plating is the use of rifampicin resistant mutants (mutation of the ribosomal binding site) of the PGPR strains (Lin et al., 2000; Lottmann et al., 2000) as the background level of indigenous soil bacteria with resistance to rifampicin is low. On the other hand, levels of resistance to kanamycin, streptomycin or gentamycin is too high (in the order of 10^4 to 10^6 cfu per gram of soil) to allow for a sensitive detection of the inoculant when the numbers dropped below 10^5 cfu per gram. Ideal tools to perform monitoring of PGPRs are provided by the marker gene technology. Using marker or reporter genes such as those based on *luc*, *lux* (firefly and *Vibrio fischeri* luciferase) or *gfp* (green fluorescence protein of *Aequorea victoria*) from PGPRs can be tagged with traits which allow their rapid and unambiguous detection. Reporter genes have been successfully applied to monitor genetically modified PGPRs under field and microcosm conditions (Schwieger and Tebbe, 2000; Tebbe, 2000; Unge and Jansson 2001). Monitoring the persistence of genetically modified inoculants is, to our opinion, required since a long-term survival of the inoculant at relatively high cell densities might pose potential ecological risks such as shifts in the structural and functional diversity, dissemination into non-target areas or horizontal transfer of transgenic DNA. For a range of bacterial species it is documented that they can enter a state called VBNC (viable but nonculturable; Roszak and Colwell, 1987). The VBNC state has also been described for bacterial species such as *Pseudomonas fluorescens* or *Sinorhizobium meliloti* and can be induced by different environmentally induced stresses (e.g., high or low temperatures or nutrient deprivation) (Oliver, 2000). This is particularly relevant for monitoring GMM because plate counts might indicate that the GMM disappeared whereas the strain might be still present in a state which does not easily support the formation of colonies. Cultivation-independent methods such as in-situ microscopic techniques or detection of construct-specific DNA in total community DNA allow to circumvent the cultivation biases. While in-situ microscopy is extremely helpful to localise inoculant strains associated with the plant, to our opinion this technique is not suitable to monitor large numbers of samples. For a cultivation-independent monitoring of the fate of genetically modified inoculant strains we would recommend to use the PCR-based amplification with construct-specific primers from directly extracted DNA in combination with Southern blot hybridisation. This approach allows a sensitive and specific detection with estimated limits of detection of about 10^3 cfu per gram of soil. Following the fate of non-modified microbial inoculants independent from cultivation is more complicated since strain-specific PCR systems often are not available. The fate of a rifampicin resistant mutant of the PGPR strain A1501 inoculated into soil microcosms planted with rice was followed by a cultivation dependent (selective plating on a rifampicin medium) and a cultivation-independent approach (16S based DGGE fingerprints. A probe generated from the hypervariable region V6 of strain A1501 was employed which was shown to be rather specific for

strain A1501 although two soil isolates gave weak hybridisation signals. The plate counts of strain A1501R introduced into soil microcosms showed an initial increase of up to 10^8 cfu per gram of soil and then showed a gradual decline. The inoculant kept a roughly stable population size of between 10^6 and 10^7 per gram of soil (Lin et al., 2000). The DGGE patterns of 16S rDNA fragments amplified with eubacteria-specific primers showed a high reproducibility between the replicates taken at each time point. A dominant band co-migrating with the PCR product generated with strain A1501R, which was absent from the profiles of uninoculated soil, was consistently detectable in the profiles derived from the inoculated soils up to day 15 (Lin et al., 2000). These data show that a cultivation-independent detection of inoculant strains might be possible as long as their cell density is sufficiently high. In 1995, rice plants that were inoculated with the genetically modified strain A1523 (*nifA* localised on Tn*5*) were sampled in the Jiaxing and Guangzhou area. Kanamycin resistant isolates were analysed by *nptII*-specific colony hybridisation and by PCR. Furthermore, total community DNA isolated directly from rhizosphere and soil samples was analysed by *nptII* PCR in three different European laboratories. The results showed that in none of the approaches *nptII* was detected (van Elsas et al., 2000b). Today this result can be explained by the fact that also the genetically modified strains A1523 and NG1390 were *npt*II negative suggesting that the construct was possibly lost.

A polyphasic approach was used to analyse the fate of the *Ralstonia solanacearum* antagonist *Pseudomonas chlororaphis* 24_4 in the rhizosphere of tomato plants grown in soil seeded with high cell numbers of *R. solanacearum*, or in soil free of the pathogen. At each sampling time the survival of the biological control strain was assessed by selective plating on rifampicin containing medium. In addition three aliquots of the bacterial pellet recovered from the rhizosphere were used to directly extract total DNA or RNA, or after incubation with bromo deoxyuridine (Borneman, 1999) The latter two methods allow to study the metabolically active bacterial fraction. The 16S rDNA fragments amplified with three kinds of primer sets (eubacterial, β-proteobacterial and *Pseudomonas*-specific) were analysed by denaturing gradient gel electrophoresis. We could show that 24_4 had an excellent rhizosphere competence. The cfu numbers were around 10^7 cfu/g root material three weeks after inoculation. In the eubacterial and Pseudomonas-specific patterns a band co-migrating with 24_4 was visible indicating that the biocontrol strain belonged to the dominant bacterial populations. Furthermore, strain 24_4 remained metabolically active during the time course of the experiment as evidenced by fingerprints obtained from DNA extracted after BrdU incorporation. In addition to plating on a *Ralstonia* selective medium, the fate of the pathogen was followed by DGGE of β-proteobacterial 16S rDNA fragments amplified from DNA directly or after BrdU incorporation. The relative abundance of the pathogen and its metabolic activity was drastically reduced in the rhizosphere of tomato plants inoculated with the biocontrol strain 24_4.

Effects of the bacterial inoculant on soil microbial communities

To evaluate potential shifts of microbial rhizosphere communities as a result of the application

of microbial inoculants it is of utmost importance that baseline data are available to relate potential changes to natural fluctuations. The dilemma that only a small portion of bacteria are readily accessible by standard cultivation techniques (Staley and Konopka 1985; Amann et al. 1995) and that bacterial cells might lose the ability to grow on solid media in response to environmental stress (Roszak and Colwell, 1987; Oliver, 2000) complicates also the assessment of potential impacts of microbial inocula on rhizosphere microbial communities.

Nucleic acid-based analysis of bacterial communities allows us to overcome biases of cultivation-dependent methods (Van Elsas et al., 2000a). To study spatial and temporal variation of rhizosphere and soil bacterial communities, multiple sample analysis is essential (Van Elsas and Smalla 1996; Muyzer and Smalla 1998). For this purpose approaches based on cloning and sequencing of 16S rDNA fragments PCR-amplified from community DNA or on the characterisation of bacterial isolates are too labour intensive and time-consuming. Molecular fingerprinting techniques such as denaturing or temperature gradient gel electrophoresis (D/TGGE: Muyzer et al. 1993), single-stranded DNA conformation polymorphism (SSCP: Schwieger and Tebbe 1998) or terminal restriction fragment analysis (t-RFLP: Liu et al. 1997; Osborn et al., 2000) based on 16S rDNA fragments amplified from community DNA have opened a new dimension of studying bacterial rhizosphere communities. The advantage of the ribosomal RNA genes as target for community analysis is that not only fingerprints are generated but that the sequence information can be used as a phylogenetic marker (Hugenholtz et al., 1998). Profiles of multiple replicates can be run next to each other allowing to easily identify bands that occur in all replicates or populations that are detectable only in replicates of certain treatments. If such a block of replicates taken at one sampling time is followed by a block of replicates taken at a later stage of plant development, populations appearing or disappearing from this fingerprint are easily identified by eye. Similar as for traditional cultivation approaches where colonies are usually picked from plates of dilutions giving 10-100 colonies for further characterisation, the DGGE fingerprints of 16S rDNA fragments obtained with eubacteria specific primers are fingerprint of the most dominant populations (Muyzer et al. 1993; Heuer and Smalla 1997). However, a direct correlation between the intensity of a band and the cell number is not possible because bacteria harbour different numbers of 16S rDNA operons (Fogel et al. 1999; Klappenbach et al. 2000). The intensity of a DGGE band reflects the abundance of 16S rDNA fragments sharing an identical melting behaviour. The nested PCR approach of using taxon-specific primers in a first PCR followed by the TGGE-PCR with eubacterial primers does not only allow to reduce the complexity of the pattern but also to analyse minority populations (Gomes et al., 2001; Heuer et al., 2001). The potentials and limitations of ribosomal RNA gene-based analysis of total community DNA were discussed by von Wintzingerode et al., 1997 and Muyzer and Smalla, 1998.

Recently several studies were published where molecular fingerprints were used to analyse the dynamics in the rhizosphere during plant growth development and the effect of the plant species on the relative abundance of bacterial populations in the rhizosphere (Duineveld et al. 2001, Gomes

et al., 2001; Smalla et al. 2001; Heuer et al., 2002). Particularly strong shifts in the composition of bacterial communities could be observed for rhizosphere communities of maize grown in tropical soils (Gomes et al., 2001). Furthermore, a plant dependent bacterial diversity could be demonstrated using molecular fingerprints (Smalla et al., 2001; Wieland et al., 2001; Schmalenberger and Tebbe, 2002). It is supposed that differences in root exudation and morphology at different stages of plant development influence the composition of the microbial community (Jaeger et al., 1999; Yang and Crowley, 2000). Recently several studies were published in which the impact of microbial inoculants on the soil micro-biota was assessed using molecular fingerprinting methods (Lottmann et al., 2000; Schwieger and Tebbe, 2000; Glandorf et al. 2001; Tebbe, 2000).

Conclusion

Recent research on rhizosphere microbial communities by means of advanced molecular tools has clearly shown that the composition of microbial communities in rhizosphere soils is highly dynamic. The relative abundance of microbial populations in the rhizosphere was shown to be dependent on the plant species and the soil. The improved understanding of microbe-plant-environment interactions will improve future applications of PGPR strains. The tool set available to characterise the identity of microbial inoculants has been considerably improved making it possible to regularly confirm strain identity and the stability of the genetic modification. In particular for genetically modified strains, specific and sensitive methods to track the inoculant strain in the environment are available, either based on PCR amplification with construct specific primers or by means of reporter gene products. However, methods to follow the expression of genes, e.g. involved in biocontrol, are still in their infancy. While molecular fingerprinting techniques either based on the analysis of 16S or 18S rDNA amplified from directly extracted DNA allow to rapidly determine potential effects of microbial inoculants on the structural composition of microbial communities, monitoring methods which determine the potential impacts of inoculants on the soil functioning are not yet fully available.

While monitoring the fate of microbial inoculants and their potential impacts on soil microbial communities should be analysed in the phase of field testing, the regular testing of inoculants strain identity is particularly important when the strains are used in a larger scale. Thus capacity building in countries which use PGPR strains on a large scale is an important prerequisite for the safe and successful use of microbial inoculants.

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Microbial inocula, activity and impact on ecosystem function and soil microbial diversity

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Abstract

Pseudomonas fluorescens SBW25 (WT) and its genetically modified, phenazine-1-carboxylic acid (PCA) producing variant 23.10, are PGPR (plant growth promoting rhizobacteria) which protect a number of crop plant species from damping-off caused by *Pythium* species. The GMM, 23.10 carries a single copy of the *phzABCDEFG* genes, under the control of the P*tac* constitutive promoter, on its chromosome. Variant 23.10 has improved biological control activity when compared to wild type SBW25, effectively suppresses infestations of *Pythium sp.* at 100x normal back ground densities and has an improved spectrum of activity over several plant phytopathogens including *Fusarium* and *Rhizoctonia* spp. The inocula survive and persist in the rhizosphere of several crop plants, show plant specific tropism as they establish higher population densities in pea and wheat compared to sugar beet. However, the density of the total microbial communities in these different plants were not adversely affected by the addition of either WT or GMM inocula. Any observed changes in microbial diversity (bacteria, fungi and mycorrhizae) were negligible when assessed by either selective plate count methods (CFU/g), culture independent molecular assays (SSU rRNA based PCR-DGGE) or histology. Variation directly correlated to infection, plant type and plant age, not the presence of bacterial inocula in disease free plants. Inocula suppressed infection and promoted an increase in plant biomass. Rhizosphere community profiles in infected plants in the presence of inocula were highly similar to disease free systems. Histological assessment of impact of inocula on established mycorrhizae associations were conducted on cores collected from field margin grassland pasture. In all instances the plant species and presence of a phytopathogen has a greater recordable impact on diversity and function that the presence of inocula.

Introduction

There has been considerable debate on the relative merits in the use of biological control agents (BCAs) for the protection of crops from soil-borne plant diseases. Advantages include the potential to reduce the use of chemical pesticides while increasing crop productivity. One group of organisms, the fluorescent pseudomonads has been identified as ideal candidates. They are recognized plant growth promoting rhizobacteria (PGPR), where different isolates have the capacity to produce a number of secondary metabolites that are effective as antifiungal compounds (AFCs) in the suppression of a number of soil associated fungal phytopathogens. The efficacy of a number of isolates has also been improved by genetic modification by either introducing the genes responsible for the biosynthesis of these AFCs into other rhizo-competent bacteria, or by increasing their expression in the inocula. However, despite a considerable history in the safe and effective use of microbial-BCAs, concern has been expressed over the environmental release of genetically modified bacteria and the impact transgenic bacteria may have on the diversity and function of the soil ecosystem. Over the last decade, as our understanding of microbial ecology has improved considerably, appropriate methodologies have been developed to discern the detail of the complex interactions that take place within and between microbial communities and the plants and soils they colonize. These studies have shown that that the initial concerns, relating to the potential for gene transfer from GMMs to the resident microflora in the environment, were largely unfounded. The capacity for gene transfer is common to many bacteria, however it is a specialized trait that is usually highly regulated in response to local environmental signals. Where gene transfer is undesirable, or could pose a potential risk due to the known activity or toxicity of the introduced functional trait, transfer can be minimized and potentially eliminated by the appropriate design of the GMM. For example the introduction of the novel gene(s) in to the recipient bacterium's chromosome by the use of site directed homologous recombination (Bailey et al., 1995) or the use of disarmed transposons (De Lorenzo, 1990). Although careful studies of the genetic stability of released GMMs continue there has been a shift in emphasis when evaluating the impact and biosafety of bacterial inocula used in plant protection. This shift has been towards determining the impact that introduced bacteria have on the natural diversity of soil microbial communities (Glandorf et al., 2001; Moenne-Loccoz et al., 2001), particularly whether inocula perturb ecosystem function and the biogeochemical cycling of nutrients in soils, components in which bacteria and fungi play a central role.

We have demonstrated that introducing the *de novo* capacity for constitutive PCA biosynthesis significantly enhanced the ability of *P. fluorescens* SBW25 to persist in soils, suppress infestation and control pea pre-emergence disease caused by *P. ultimum* Trow (Timms-Wilson et al., 2000). The isolate was modified by the chromosomal insertion of a single copy of *phzABCDEFG* under the control of a P*tac* promoter, in the absence of the known regulatory components *phzI* and *phzR*. Previous investigations of complementation or heterologous expression of introduced genes for synthesis of antifungal metabolites typically describe the use of plasmid vectors or the over-expression of native antifungal metabolites (Fenton et al., 1992; Sarniguet et al., 1995; Schnider et al., 1995; Reimmann et al., 1997). As we already have extensive knowledge of the ecology and genetics of SBW25 *in situ* the PCA producing variant 23.10 has been further investigated not only for its commercial potential as a PGPR but also as an ideal organism to study the biology and potential impact of a GMM that carries a known ecologically functional trait (the production of an AFC; phenazine –1- carboxylic acid (PCA)). The objective of the investigations reported here has been to determine whether the expression of a trait common in rhizosphere pseudomonads, PCA biosynthesis, when expressed by another common rhizo-competent, fluorescent pseudomonad directly influences the ecosystem function and diversity of non-target, plant beneficial rhizosphere

bacteria and mycorrhizal fungi in different crop and field margin plants. Established methods for studying the diversity and community succession of the community were applied (Prosser, 1994; Grey and Head, 2001). These included quantitative methods based on colony isolation on selective agars (Thompson et al., 1995), molecular methods to assess the diversity of accessible operational taxonomic units (OTU) (as determined by the PCR amplification and DGGE analyses of 16S and 18S ribosomal RNA and rRNA gene diversity for bacteria and fungi respectively) (Myzer et al., 1993: Smalla et al 2001) and direct microscopic counting of root-mycorrhiza associations following histological staining.

Materials and methods

Bacterial Strains. To facilitate monitoring of bacteria we selected spontaneous rifampicin resistant mutants of *Pseudomonas fluorescens* SBW25 (Bailey et al., 1992: Rainey et al., 1996) and its variant, 23.10 which carried a single copy of the phenazine-1-carboxylic acid biosynthetic pathway in its chromosome (Timms-Wilson et al., 2000). The *phz*ABCDEFG genes from *P. fluorescens* 2-79 were inserted under the control of the P*tac* promoter into a miniTn*5* disarmed transposon. SBW25 has been shown to be a highly competent colonizer of the rhizosphere and phyllosphere of a number of plants and an effective PGPR in the suppression of damping-off disease (Ellis et al., 1999; Ellis et al., 2000). When modified to constitutively express PCA an ecologically functional GM-BCA was isolated. This variant, 23.10, was as able as the wild type SBW25 to colonize roots and persist in the rhizosphere, could suppress damping-off disease caused by *Pythium ultimum* var. Trow. at x100 normal levels of soil infestation, had an extended capacity to inhibit fungal development *in vitro* than SBW25 and produced greater quantities of PCA that *P. fluorescens* 2-79 (Timms-Wilson et al., 2000). All these attributes make 23.10R an ideal candidate with which to adequately address a number of ecological and environmental concerns relative to the impact of releasing GM-BCA (genetically modified biological control agents) on non-target species.

Bacterial inocula. *P. fluorescens* SBW25R (rifampicin resistant) and 23.10R (PCA+, kanamycin and rifampicin resistant) were grown on PSA-CFC to single colonies from stocks held at -70°C in glycerol saline. Single colonies were used to inoculate LB without antibiotics and grown over night 28°C, 180 rpm. After washing twice by centrifugation in sterile water bacteria were resuspended in sterile water to approximately 5 x 10^8 cfu/ml.

Pythium ultimum var. Trow inocula. *P. ultimum* inocula were stored in the dark at 15°C. Essentially, *P. ultimum* Trow. soil inoculum was prepared by mixing 1 l sterilized Mendip loam (Minster Brand Products, Heatherwood Nurseries, Ashington, Winborne, BH21 3DD, U.K.) with 6 g of oospore inoculum (Zeneca Agrochemical, Jealott's Hill research station, Bracknell) and 2g Beam natural wheat germ (The Vitamins Company, Brentford, Middlesex, UK). Infested soil (500 g) was placed in seed trays (16 cm x 21 cm x 5 cm), planted with 25 pea seeds, covered with compost and watered with 100 ml ddH$_2$O. Trays were incubated at 15°C in a growth cabinet and watered daily with 100 ml ddH$_2$O for 1 week. In the second week watering was gradually reduced to encourage the production of oospores. The aerial portion of the plants was removed after 2 weeks; the infested soil was then allowed to air dry at 15°C for 4 weeks, sieved (2 mm pore), sealed in airtight containers and stored in the dark with out loss of titre for several months.

Soil and plant inoculation. Commercially derived loam top soil typical of that used in the glasshouse production of plants (www.gemgardening.co.uk) was prepared in containers (22.5 x 16 x

7.5 cm). The soil in half of the available containers was mixed with *Pythium* inocula (1x 10^5 pfu/g soil) to provide a high density of infection. Trays were then surface drench with 150 ml of the suspensions of SBW25R or 23.10R (to provide an inocula of ca. 5 x 10^7 cfu/g soil), water alone was included as the untreated control. Soils were then left to acclimatize for 24 h in a plant growth room (21°C, 18 h photoperiod) before planting with seeds of pea (*Psium satvium* var. *quincy*), wheat (*Triticum aestivum* var. *pena wawa*), or sugar beet (*Beta vulgaris* var. *amythyst*). The relative soil moisture content was maintained throughout the experiment by regular application of water to the soil surface.

Sample preparation, and colony counts from the rhizosphere. A triplicate set of 1.0 g soil samples was collected from each tray prior to planting. These were evaluated immediately to confirm the background densities of soil bacteria and fungi and inocula in the control and treatment blocks. After planting and germination individual seedlings were collected periodically from each of the three replicates for each treatment, n=3. Loosely adhering soil was removed and collected, and the plants separated into aerial and root parts. Bacteria and fungi were suspended from these samples by vigorous mixing in the presence of sterile glass beads (2 mm diameter) and sterile distilled water (5 ml / g material). Samples were decimal diluted in sterile water for further analyses.

Population dynamics, fate and persistence of inocula. Data on total plate counts were collected by spreading 100 µl of the diluted suspensions on to Potato Dextrose Agar (PDA, Oxoid, Difco, UK) for fungi and Pythium, *Pseudomonas* selection agar containing CFC supplements (PSA, Oxoid, Difco., UK) for total pseudomonads, TSBA (Oxoid, Difco, UK) for total bacteria, PSA-rifampicin (100 g/ml) for SBW25R and PSA-Rif kanamycin (75 µg/ml) for 23.10R.

Plant biomass and disease index. Plants were visually assessed for signs of disease or wilting, and biomass determined by weighing collected plants at the time of sampling.

Preparation of total community DNA and DGGE analyses of 16S and 18S OTU diversity. Total nucleic acids (DNA and RNA) were extracted from 50% of the rhizosphere soil and plant homogenates using the bead-beating CTAB method described by Griffiths et al., 2000. Standard methods of PCR and RT-PCR analysis of 16 S rRNA genes and 16S rRNA were applied to prepared samples using universal eubacterial primers 338 containing the GC clamp and 530R to amplify the V3 region (Whiteley and Bailey, 2000). PCR products were separated by denaturing gradient gel electrophoresis (DGGE) in 10% acrylamide, 10-60% denaturant gradient at 60°C, 100V for 16h using the D-CODE system (BioRad, UK). The general primer pair NS1 and NS2+10-GC (as modified from Simon et al., 1992) were used to target fungi and a second primer pair, NS31 and AM1-GC to target AM fungi (as modified from Helgason et al, 1998). The GC clamped PCR products amplified from the fungal 18S rRNA gene targets were separated on 6% acrylamide DGGE gels as described above. DGGE gels were stained with Sybr Gold (Molecular Probes, Oregon), digital images were collected using GeneSnap image acquisition software (Syngene, UK) and analyzed using Phoretix 1D software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Bands were firstly automatically detected and then manually checked to add or remove incorrectly assigned bands. Lane profiles were corrected for differences in migration rates by manually assigning Rf lines to marker lane bands, which were constructed from amplimers generated from a mixture of identified culture isolates. Following band matching two data tables were produced, a binary matrix containing data on the presence or absence of bands, and a proportional matrix displaying the

percentage of each band based on relative pixel intensities for each lane. DGGE banding patterns were analyzed using simple cluster analysis on band presence or absence, which was performed from within the gel analyses software and utilized the unweighted pair-group method using mathematical averages (UPGMA). Data sets for each replicate were pooled and compared against treatments. Where appropriate bands were removed from the DGGE gels and resuspended in 200 µl ddH$_2$O and used as a template for PCR amplification using the 338F and 530R primers. PCR products were cloned into TOPO TA (InVitrogen, UK) following the manufactures instructions prior to sequence analysis on a Beckman CEQ2000XL capillary apparatus.

Data handling and statistical analyses of data. In order to assess the similarity among the communities PCA (principal components analysis) and cluster analysis was undertaken using MSVP (Multi-Variate Statistical Package *version* 3.1, Kovach Computing services, UK.).

Root histology and distribution of mycorrhizal fungi (MF) in established field margin plants. Replicate blocks (n=6) of a well established pasture on brown forest soil (pH 4.5-5.0) were collected from the Sourhope Field Experiment Site in the Scottish Borders (U.K.) to a depth of 20-25cm. Total bacterial plate counts as estimated on TSBA showed an average through the core of 1.4 x 10^7 cfu /g soil. Blocks (of approximately 350 g) were cut and placed in 10 cm diameter plant pots. Pots were incubated in plant growth cabinets at 20°C with a 16 h photo period. Cores were watered with 100 ml of distilled water every other day. Replicate, 1 cm cores (n=3) were cut with a cork borer from each of the three replicate pots to determine the initial extent of AM infection in the plant roots. After a further three weeks of acclimatization another set of samples were evaluated to establish that the levels of AM colonization were stable prior to the addition of bacterial inocula. Inocula were prepared as previously described and introduced as a drench. Approximately 1 x 10^7 cfu /g soil of SBW25R or 23.10R were added to independent cores. Sample 1 cm cores (n=3) were taken from each of 3 replicates at 4 and 20 days to determine whether inocula perturbed the established AM association in the roots in these grassland samples. A general measure was made of the AM distribution as it is not possible to specifically identify plants on the basis of their root morphology.

Root staining for arbuscular mycorrhiza. Soil cores were removed from microcosms or the field using a 1 cm diameter cork borer. Each sample was washed through a 700 µm sieve with distilled water to separate roots from the soil. Roots were cleared in 2.5% (w/v) KOH by autoclaving for 15 min. Samples were then bleached in freshly made bleaching solution (0.5 % H$_2$O$_2$, 0.2% NH$_3$) for up to 2 h until they appeared white, rinsed in water twice before acidifying in 1% HCL for 1 h and staining in 0.05% (w/v) trypan blue, 50% (v/v) glycerol, 0.2% (v/v) HCL by autoclaving for 3 min. After rinsing roots were kept in storage solution (50% (v/v) glycerol, 0.2% (v/v)HCL) before mounting on microscope slides in polyvinyl lactoglycerin (PVLG) under coverslips. PVLG was prepared as follows, 1.66g polyvinyl alcohol in 10 ml Lactic acid, 1 ml Glycerol, 10 ml ddH$_2$O. Three slides were counted for each sub-sample, but all 3 slides were treated as a single unit when estimating % root colonization by AM. Roots were aligned parallel to the long axis of the slides and observed at a x200 magnification. The field of view of the microscope was moved using the using the stage graticule to make a complete pass across each slide perpendicular to its long axis every 2mm. All intersections between roots and the vertical eyepiece crosshair were considered. The position on the root surface at which the center of the eyepiece crosshairs entered through the side of the root was taken as the point of intersection. Rotation of the vertical crosshair ensured each intersection was at right angles to the long axis of the root. To examine each

intersection, the plane of focus was moved completely through the root and a note made of whether the vertical crosshair corresponding to the presence of arbuscle, vesicle or hyphal structures. Intersections were then scored either positive or negative and a percentage colonization calculated from at least 100 intersections for each replicated sample.

Results and discussion

Three plant species have been investigated, pea (*Psium satvium* var. *quincy*), wheat (*Triticum aestivum* var. *pena wawa*), and sugar beet (*Beta vulgaris* var. *amythyst*). These represent typical hosts for damping-off disease, which results in considerable agronomic loss world wide, and are potential commercial targets for the use of PGPR as biological control agents. Each of these plants provide reliable models for the study of rhizosphere population biology, pea and wheat represent dicot and monocot species with well described mycorrhizal associations, sugar beet do not form mycorrhizal associations. The data reported here confirm that the *in situ* performance of an already effective biological control agent *P. fluorescens* SBW25 which survives well in soils and is competitive in low nutrient environments, can be enhanced significantly by the insertion of novel functional traits (Ellis et al., 2000; Timms-Wilson et al., 2000).

Plant growth and impact of inocula on disease. To assess the impact of the GM-BCA *P. fluorescens* 23.10R, microcosms were established to mimic conditions typical of field or glass house grown plants. Soils were planted with pea, wheat and sugar beet seeds, with and without *Pythium* and with and without inocula. Microcosms were sampled over several life stages of the different plant species up to 40 days post germination. Little or no effect was recorded on the density of background communities, as determined by plate count methods, following the introduction of inocula when treatments were compared with the appropriate controls (data not shown). Inocula established higher population densities in the roots of peas and wheat when compared to sugar beer seedling, although the densities of wild type when compared with 23.10R were not significantly different when a particular plant type was assessed over the 5-6 week duration of the experiment. Once established the inocula persisted as the plants developed. However, in all plant species infected with Pythium the densities of inocula, as determined on PSA-rif plates, and the indigenous rhizosphere bacteria, as determined on TSBA plates, were significantly greater than uninfected plants. As expected from previous investigations the PCA producing variant, 23.10, has improved biological control over the wild type *P. fluorescens* (Ellis et al., 2000; Timms-Wilson et al., 2000). Biological control assessments, based on observed plant health and biomass measures for individual plants, showed that plant yeild was considerably improved by the presence of the inocula. Plant protection was particularly enhanced in the presence of the PCA producing variant 23.10 in both infected plants and unifected plants (Table 1).

Table 1. Biomass (mg) of the roots and shoots of individual seedlings (n=9) were determined after of emergence of first true leaves. Plant growth promoting properties were compared for *P. fluorescens* WT and 23.10 inocula (5 x 10^7 cfu/g soil) in the absence or presence of high densities of *Pythium ultimum* Trow. (1 x 10^5 pfu/g soil).

	Root wt. (se)		Shoot wt. (se)	
Treatment	**Infected**	**Uninfected**	**Infected**	**Uninfected**
Wheat				
water	0.146 (0.013)	0.180 (0.021)	0.043 (0.003)	0.045 (0.005)
WT	0.185 (0.021)	0.183 (0.018)	0.046 (0.004)	0.044 (0.003)
23.10R	0.245 (0.017)	0.189 (0.023)	0.052 (0.006)	0.053 (0.006)

Pea				
water	1.974 (0.216)	2.306 (0.372)	0.1577 (0.014)	0.1703 (0.012)
WT	3.025 (0.327)	2.739 (0.406)	0.2056 (0.006)	0.2281 (0.017)
23.10R	3.096 (0.477)	3.092 (0.482)	0.1994 (0.021)	0.2346 (0.049)
Sugar beet				
water	0.087 (0.024)	0.113 (0.046)	0.049 (0.003)	0.041 (0.002)
WT	0.168 (0.038)	0.280 (0.053)	0.058 (0.006)	0.069 (0.008)
23.10R	0.301 (0.057)	0.307 (0.037)	0.058 (0.004)	0.072 (0.005)

Note. *Only 5 to 20% of seeds exposed to Pythium (**infected**) germinated and produced plants in the absence of bacterial inocula (water only control). These surviving plants illustrate the natural variation in the biological system studied.

Disease, plant type and age have a greater effect than inocula on the abundance, diversity and succession patterns of rhizosphere bacterial and fungal communities. Impacts on total poplation dynamics was measured using several complementary methods, including plate counts, molecular community diversity profiling, and direct physiological measures of perturbance to AM as key indicators of health in established plant standings. Generally, as observed by a number of other studies groups (De Leij et al., 1995; Thompson et al., 1995; Glandorf et al., 2001; Leeflang et al., 2002; Thirup et al., 2001) our data showed that impact was only transient and that changes in diversity and community succession were were more affected by the plant species and the growth of the plant (Schwieger and Tebbe 2000; Smalla et al., 2001). Molecular fingerprinting analysis allowed the estimation of changes in bacterial and fungal diversity (without culturing) to be measured over time following soil inoculation with either the wild type or 23.10R. Diversity changes were estimated by comparing banding pattern changess in the DGGE profiles (images of gles have not benn included) produced by PCR based on the assumtion that each band produced on the gel is representative of an individual OTU. Surprisingly few differences were observed in profiles produced by RT-PCR of 16S rRNA or PCR of 16S DNA. Additional detailed studies by us (Griffiths et al., 2000; 2002) have confirmed that only subtle differences in profiles are observed in the analysis of soil biota diversity, based on RNA or DNA. It is therefore assumed that the DNA and RNA targets represent the predominant, accessible, active, and presumably viable, OTUs present in any environmental sample, and that RNA may be long lived in soil bacteria. These data are consistent in the suitability of the method to record perturbation to diversity folowing impact or changes in diversity due to natural community succession that results from plant type and plant maturation.y. Principal component analysis of within gel diversity for each plant type were produced for the root (Fig 1) and shoot (data not shown) for both bacterial and fungal diversity. In all cases these profiles revealed no major impact on diversity due to incoula, and that diversity and community structure, successsion was directly determined by plant type and the presence or absence of disease.

Figure 1. Principal Component Analysis of DGGE profiles of the impact of wild type and phenazine producing *P. fluorescens* SBW25 variant 23.10R on the temporal changes in the diversity of bacterial (16S) and fungal (18S) microbial communities in the rhizosphere / root of wheat, pea and sugar beet in the presence and absense *Phythium* infection.
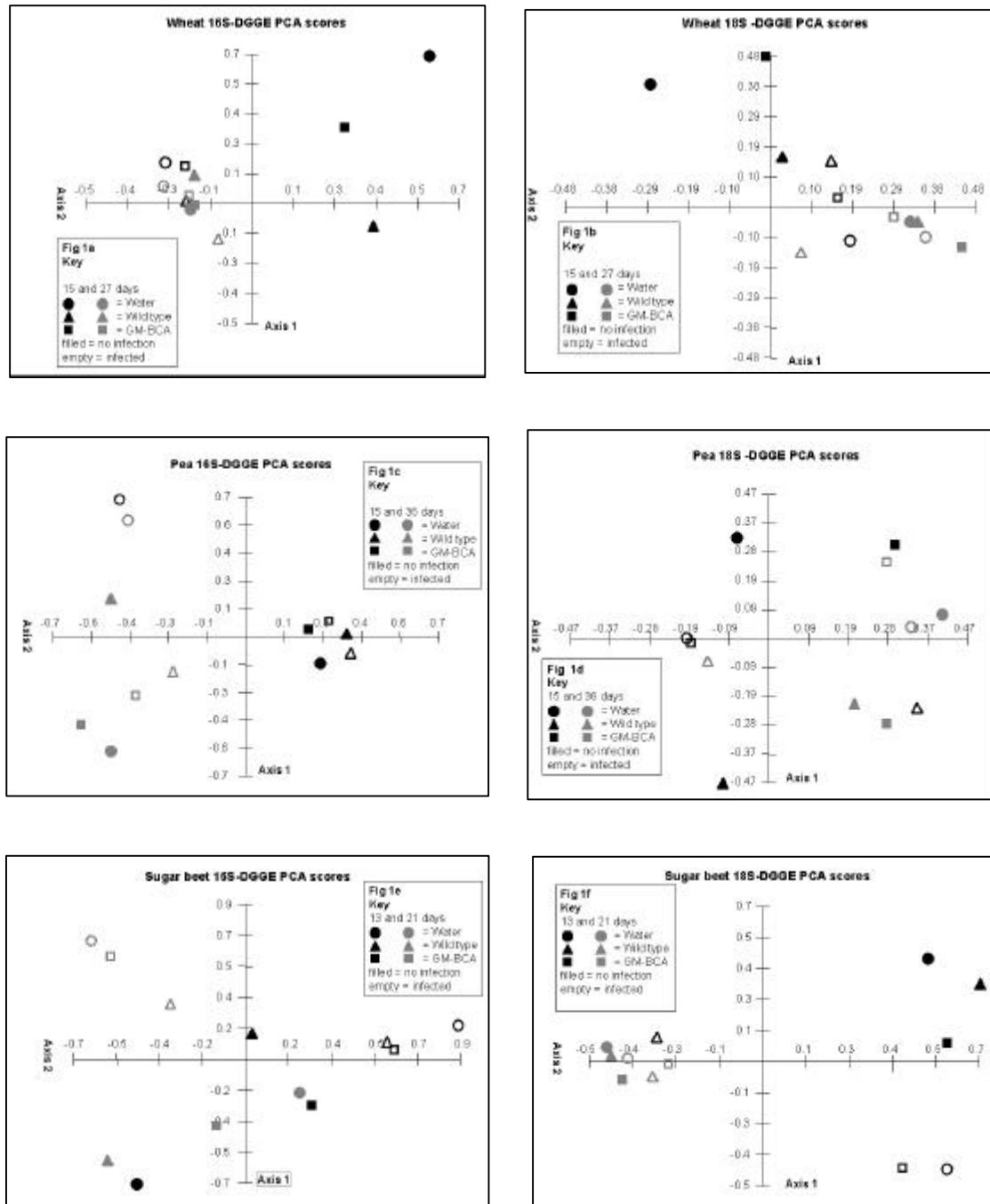
Legend: (Figure 1)

Total nucleic acids were extracted from the sample and target 18S and 16S ribosomal RNA genes were amplified using primers EUB GC-338/530R and NS1/NS2+10-GC respectively and the community profiles genrated by PCR-DGGE and staining. Data were digitised and shifts in relative diversity in respect of time and treatment were compared for each plant type. Data are presented as Principal Component Analaysis comparisons. A. eubacterial community, wheat; B. fungal community, wheat; C. eubacterial community, pea; D. fungal community, pea; E. eubacterial community, sugar beet; F. fungal community, sugar beet.

Note: 1. attempts to use 18S primers NS31/AM1-GC, to target AM 18S rRNA genes or 18S rRNA (by RT-PCR) were largely unsuccessful. 2. For illustrtaive purposes data for two time points only are presented, devlopoing and maturing plants. Each data point in the plots represents the combination of at lest three independent analyses of extracted nucelic acid (16S and 18S rRNA gene analyses shown). 3. Typically 80 to 90% of seeds infected with Pythium in the absense of bacterial incoula failed to germinate and produce plants.

The impact of disease on diversity and function is lessened in the presence of inocula. Although no significant shifts were recorded when wild type or 23.10R inoculated plants were compared. inocula and damping-off disease, did result in a discernible shift in community banding patterns when compared to untreated controls or bulk soil. Pea, wheat and sugar beet each influenced and were colonized by different communities selected from the soil. The impact of the plant type and infection was greater (Fig 1) in all assessments made of diversity and activity than the impact of inocula (wild-type or the genetically modified variant). Despite the thorough analysis of data from hundreds of samples in replicated microcosms we conclude that no adverse effects resulted from the *de novo* synthesis of the antifungal compound PCA by P. fluorescens SBW25. To illustrate the extent of diversity a number of individual bands common to the DGGE gels were removed to identify the key OTUs that were amplified by PCR. BLAST searches of the ca. 200bp fragment spanning the V3 region revealed typical plant associated bacteria such as *Pseudomonas* spp., flavobacterium, *Enterobacteriacea*, *Shingomonas* spp., Cytophagales and other previously reported "yet to be cultured" soil bacteria in the RDP data base.

Figure 2. Impact of wild type and PCA producing variant of *P. fluorescens* SBW25 on the extent of AM infection in the roots of natural field margin pasture plants. Direct counts were made on sampled roots cores (n=3) after staining.
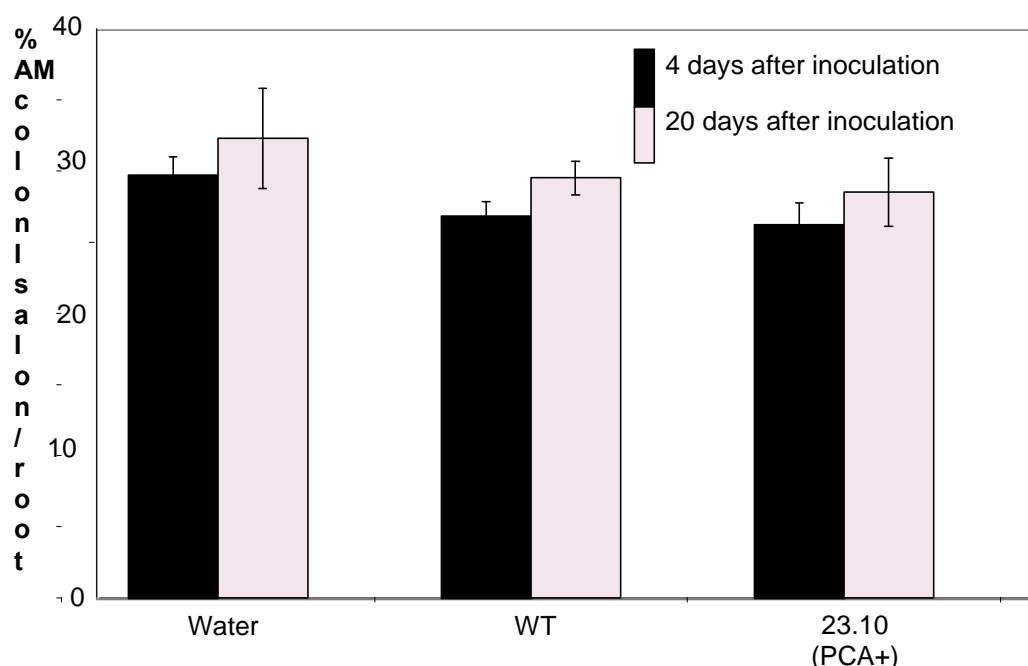
Inocula may influence the distribution of AM in the roots of field margin plants. Attempts to generate PCR products from rhizosphere samples to assess mycorrhizal diversity were unsuccessful, probably as our methods for extration were insufficient to lyse plant cell debris, or, as is more likely, that AM associations were not formed in the young seedlings studied. We have confirmed by molecular methods and histology (data not shown) that older crop plants grown under field conditions do have obvious AM signatures, these observations support those of Daniell et al. (Daniell et al., 2001). However we had anticipated the limitations of a seedling based assay and therefore extended our investigations to evaluate whether short term perturbations to established plant–AM associations could result following the accidental contamination of field margins and pasture with GMMs. To this end we collected blocks of a typical pasture soil colonised with a variety of plants. We successfully studied root AM infection/colonisation by use of histology based staining and microscopy and evaluated the impact of our wild type fluorescent pseudomona and and its PCA producing variant, 23.10R. Following root staining and microscopy we observed that AM infection was abundant in plant species typical of a grassland field margin pasture, and recorded a small decrease in mycorrhizal association when wild type or 23.10R were compared with the water control 4 days after inoculation (Fig 2). This perturbation was only transient as the relative amount of AM association with roots increased 2-3% 16 days later showing that mycorrhizal associations were able to grow normally.

It is apparent that he selection of appropriate strains, typical of the microflora present in the target habitat, is a recommended route for the safe development of beneficial organisms that will have a minimal or negligible impact to the environment. When considering the use of genetically modified, or indeed an exotic inocula of unknown ecological effect on the environment it is also important to consider the attributes of the selected strain in respect to the following biotic and abiotic factors that may reflect impact and change. Preferred plant type (intended target habitat) and habitat range; the rhizosphere effect; presence or absence of disease; soil structure; soil chemical properties;

disturbance (predicted and measured); soil microbial community diversity; ecosystem function in respect of impact and function, and decomposition rates. It is essential that we improve our knowledge of soil health, and apply that knowledge to define what we consider as "normal soil" so that valid representative comparisons can be made. The impact measures used in this and related studies have shown little or no lasting effect. This may be due to the careful selection of strains and traits used in the construction of GMMs. It is just as likely, that the microbiota, typical of the soil environment, is highly resilient and capable of responding to perturbations which are considerably greater than those likely to be caused from the accidental or deliberate e release of GMMs. However, as more data are collected we increase our understanding of plant microbe interactions and the extent to which nutrient cycling supplies a viable soil. Future work must incorporate sensitive methods that allow the key groups of functional organisms to be studied. Mychorrhizae for example, may represent suitable indicator groups for determining short-term impact to otherwise "pristine" or established habitats. Finally, most studies with GMM inocula have recorded only minor, transient perturbation to microbial communities, but as far as we are aware this is the first direct demonstration that a functional, AFC producing GMM also has only a transient impact on mycorrhizal associations in established plant communities.

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Fate of GM rhizobial inoculants: lessons from Europe and elsewhere

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Abstract

The nitrogen-fixing symbiosis between leguminous plants and bacteria of the Rhizobiaceae has been exploited in agriculture for millennia. Commercial rhizobial inoculants, applied to legume seed where insufficient compatible rhizobia are present in soil, have been available for over a century. Their agronomic importance, familiarity and the relatively good knowledge of their biology and genetics, has made rhizobia a target for improvement by genetic manipulation (GM). For example, *Sinorhizobium meliloti* genetically modified to increase nitrogen fixation was field-tested in the USA in 1994 and given commercial approval by the US Environmental Protection Agency in 1997.

In a programme of research funded by the EU, survival in the field of *Rhizobium leguminosarum* biovar *viciae* inoculants marked by GM has been monitored since 1987. At Rothamsted, populations declined sharply in the months following application but then stabilised although at two other sites the same inoculant could not be detected the year following application. Dispersal from the inoculation site was consistent with root growth and soil movement due to ploughing and no genetic interactions between the inoculant and native strains was detected in the field. There was evidence that the symbiotic plasmid of *R. leguminosarum* biovar *viciae* (which carries genes for host plant nodulation and nitrogen fixation) confers a survival advantage in the rhizosphere of both host and non-host plants. These observations are compared to results from other releases of GM rhizobia in Europe and the USA.

Keywords

Rhizobium leguminosarum, Sinorhizobium meliloti, genetic modification, field release, monitoring survival, symbiotic plasmid

Introduction

Because rhizobia have been the subject of a relatively large number of field release studies, compared to other groups of bacteria, they represent one of the few model systems for the release of GM bacteria. Prior to the development of the techniques of *in vitro* DNA manipulation, commonly referred to as "genetic modification", there had been many field experiments to determine the efficacy, competitive ability and survival of different rhizobial inoculants. These often involved strains with unusual phenotypic properties, or with spontaneous mutations to antibiotic resistance, to facilitate selection and identification on re-isolation from root nodules or field soil. However, using such methods, the limit of detection was insensitive and identification ambiguous. The advent of GM technology made more detailed studies of rhizobial ecology possible, in addition to offering the potential for improving the symbiotic performance of inoculants.

Most releases of GM rhizobia to date have aimed either at assessing inoculants for enhanced performance, or investigated aspects relevant to biological safety, although some provided information relevant to both topics. This paper reviews some of the release experiments that have taken place in both Europe and the USA over the past 15 years.

Field testing potentially improved inoculants

Several strains of *Sinorhizobium meliloti* and *Bradyrhizobium japonicum*, modified to increase nitrogen fixation by increasing the expression of genes for nitrogenase and dicarboxylic acid transfer into the cell (this provides energy for fixation), were field-tested in the USA in 1989 (Ronson *et al.,* 1990). No yield benefits were seen but overall the inoculant strains formed more than 50% of the nodules, in a soil where indigenous rhizobial numbers were low. The *S. meliloti* strains were tested at four different sites the following year. Where soil N and organic matter were lowest, one construct gave statistically significant increases in alfalfa biomass (Bosworth *et al.*, 1994). Where indigenous *S. meliloti* populations were low (< 70 cells per g dry soil), the GM inoculant strains were found in > 90% nodules in most cases, whereas where indigenous *S. meliloti* numbers were high (>10,000 cells per g dry soil), the inoculants occupied, on average, < 6 % nodules. After two to three years of alfalfa cultivation one these sites, it was apparent that locus in the *S. meliloti* chromosome where the transgenes were inserted was as (or more) important than the transgenes themselves in the yield improvements (Scupham *et al.*, 1996). The inoculant strains continued to form a significant proportion of nodules at many sites, often >50%. A subsequent field experiment revealed no apparent difference in the soil microflora in sites inoculated with the different strains, apart from a slightly higher population of aerobic spore-forming bacteria in sites inoculated with one of the strains with increased nitrogen fixation capability (Donegan *et al.,* 1999).

A plasmid maintenance system to facilitate introduction of transgenes, in which cells lacking the chromosomal genes for thymidylate synthetase remain viable only when they retain a plasmid carrying the *thy*A genes, was field tested in Ireland in 1991 (O'Flaherty et al 1995). Where alfalfa growing in soil with no indigenous *S. meliloti* and relatively high N was inoculated with the thy^- mutant carrying the *thy*A plasmid, >90% nodules isolates maintained the plasmid, but when a thy^+ strain was used, < 30% of nodule isolates had lost the plasmid.

Proline is thought to be an important energy source for *S. meliloti* in the alfalfa rhizosphere and may accumulate in roots under drought stress. In Spain in 1999, an *S. meliloti* control strain and derivatives with increased expression of the proline dehydrogenase gene *put*A, marked by chromosomal insertion of the *E. coli* glucuronidase gene *gus*A, were released in fields with indigenous *S. meliloti* populations (van Dillewijn *et al.*, 2001). In bulk soil, inoculant numbers dropped by five orders of magnitude in five months but in the rhizosphere and rhizoplane the population stabilised at around 10^3 colony-forming units (CFU) per g root for the strain over-expressing *put*A and 10-fold more for the control strain. No clear benefit was seen with increased *put*A expression, although it was concluded that it could be of benefit where water is limited.

These releases were concerned mainly with the potential yield benefits of GM strains, and examined the competitiveness for nodule formation, but did not investigate the persistence of inoculants.

Field testing for biological containment

In theory, bacteria impaired in their ability to repair DNA damage, e.g. lacking the DNA recombinase A gene *rec*A, should be at a survival disadvantage. In 1994 a potential biological containment system was tested in Germany when $recA^+$ and $recA^-$ *S. meliloti* strains, marked by insertion of the firefly luciferase gene, *luc*, were applied to lysimeters in the field prior to planting alfalfa (Schwieger *et al.,* 2000). Over two years, there was an overall decline in numbers from 10^6 to >10^4 cells per g soil and populations were similar except at two sampling times where $recA^+$ numbers exceeded those of $recA^-$. There was no evidence for leaching of the inoculant strains but horizontal spread to non-inoculated alfalfa in adjacent lysimeters was detected, possibly due to

dispersion during lysimeter set up. Because the soil did not contain detectable levels of indigenous *S. meliloti*, any misplaced bacteria would face no competition to colonise and nodulate alfalfa roots. Subsequent monitoring of the recA$^+$ and recA$^-$ strains, in different release experiments, showed no clear differences in survival. In 1997, in S. Germany, numbers of both strains declined from 10^6 CFU per g soil on application (as peat based inoculant to soil) to 10^4 after one month, remaining stable thereafter for 18 months (W. Lotz, personal communication).

In 1995 the compostion of the bacterial populations on roots of alfalfa and a weed, *Chenopodium album,* in soil inoculated with the recA$^+$ strain marked with *luc* was compared to non-inoculated soil (Schwieger & Tebbe, 2000). The alfalfa rhizosphere population size and composition appeared to be influenced by the presence of inoculant, but this was not the case for *C. album*. Some inoculant *S. meliloti* were detected in plants on non-inoculated plots, numbers on alfalfa approaching 30% of those on roots in inoculated plots 12 weeks after planting, numbers on *C. album* approaching 4% (although numbers present at start of growth were assumed to be very low). This was assumed to arise from cross-contamination at the time of soil inoculation.

Ecological studies

In 1987, as part of an EU-funded project to examine the biosafety of transgenic bacterial inoculants, RSM2004, a strain of *Rhizobium leguminosarum* biovar *viciae* marked with Tn*5* on its conjugative symbiotic plasmid (pSym), was released in the field at Rothamsted UK, Bayreuth in Germany and Dijon, France (Hirsch & Spokes, 1994). The strains was designated GM according to the 1978 UK Genetic Manipulation Regulations then in force although the updated 1989 GM regulations and the 1990 EC Council Directive 90/219/EC would not have considered it GM; neither was it defined as GM in France or Germany in 1987. At Rothamsted and in Bayreuth, it was applied as a seed-coating for peas; in Dijon it was applied as a liquid inoculant. At Rothamsted it was also broadcast as a granular inoculant on soil before planting cereals. Together with antibiotic resistance markers on the bacterial chromosome, the Tn*5* marker facilitated the monitoring of RSM2004, enabling sensitive detection in soil and unambiguous identification in root nodules. The limit of detection by selective plating was around 100 CFU per g soil, and development subsequently of sensitive PCR detection increased sensitivity to fewer than 20 cells per g soil (Cullen *et al.,* 1998). The inoculant was applied to give 10^4 - 10^5 CFU per g soil, equal to the indigenous population of *R. leguminosarum* biovar *viciae*: this declined in the UK by 100-fold after application then stabilised at around 100-1000 CFU per g soil, where numbers have remained at similar levels to date. However, in Bayreuth the strain could not be detected after 30 weeks (the first winter) and in Dijon it could not be detected after two weeks. This was possibly due to very heavy rain following the application, as subsequent experiments at Dijon showed that survival of *R. leguminosarum* biovar *viciae* inoculants was similar to that observed at Rothmasted (Amarger & Delgutte, 1994). The differential survival in the UK and Germany could be due to different soil and climatic conditions (Table 1). The inoculant had to compete for nodulation with the indigenous population and it was found to form 6% of the nodules at Rothamsted; three years after application, it formed 2% of nodules on peas. Where only cereals had been grown rather than peas RSM2004 numbers were approximately half those where peas had been grown, possibly demonstrating a small but significant advantage conferred by the host plant but also reflecting the different modes of inoculation. Nodules from peas and other legumes were screened for any evidence of Tn5 transfer to other rhizobia, but none was detected although in laboratory experiments, the pSym could transfer from RSM2004 to three out of four field isolates tested (Hirsch & Spokes, 1994). The spread of RSM2004 from the release site was investigated after three years, and some horizontal movement from the inoculated plots was detected, consistent with bulk soil movement during cultivation; also some vertical movement was apparent, probably

associated with migration on root surfaces.

A second *R. leguminosarum* biovar *viciae* release was performed on the same Rothamsted site in 1994. Peas were inoculated with CT0370, a strain cured of its pSym (thus unable to nodulate) and marked on the chromosome by insertion of *gus*A (Selbitschka *et al.,* 1995). Nodules were screened for rhizobia containing the *gus*A marker, as CT0370 could nodulate only if it received a pSym from RSM2004 or indigenous *R. leguminosarum* biovar *viciae*. More than 20,000 root nodules were screened for GUS$^+$ rhizobia but no indication of pSym transfer to CT0370 was found. After release, CT0370 numbers declined 100-fold but then remained fairly stable in the bulk field soil, around 10-fold higher than the population of RSM2004, despite their inability to nodulate. However, when plants growing on the site were investigated five years later, numbers of CT0370 in the rhizosphere of non-host plants were low and almost undetectable on pea roots, compared to RSM2004 (Clarke *et al.,* 2002). Subsequent experiments, where the pSym from RSM2004 was transferred to CT0370, confirmed that the pSym conferred an advantage to survival in non-host rhizospheres, a great advantage in the pea rhizosphere, but a disadvantage in bulk soil.

The number of indigenous *R. leguminosarum* biovar *viciae* in soil was monitored by a most probable number method based on nodulation of host plants using soil dilutions (Vincent, 1970). This method is time-consuming and cannot discriminate between individual strains within the populations: counting the CFU of the GM strains on selective agar was much easier. Together these methods allowed a comparison of the long-term fluctuations in the indigenous *R. leguminosarum* biovar *viciae* population and the survival of introduced inoculants (Figure 1).

Conclusions

The field releases of GM rhizobia have enabled the assessment of potential improvements to strains, illustrating that traits that appear advantageous under controlled conditions may not show a very clear benefit under the more variable conditions in the field. This applied to improvements in symbiotic efficiency (in the USA), competitiveness for nodulation (in Spain) and biological containment (in Germany).

The ecological studies of rhizobia have generally shown a sharp drop in numbers after application to the field and, as anticipated, where there was an indigenous rhizobial population, the inoculants have to compete for nodule formation. Soil cultivation resulted in movement of strains from the site of inoculation. Where there were very low competing populations of indigenous rhizobia, spread of the inoculants from the site of application to host plants in adjacent plots was observed. Where long-term survival has been monitored, the introduced populations appeared to stabilise after the initial drop, in the case of RSM2004, for more than 15 years. The transgenes used in the GM strains facilitated monitoring of spread and survival. The experiments also showed some unexpected results, indicating that pSym may play a role in the colonisation of non-host as well as host plant roots, but does not confer an advantage for survival in bulk soil.

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Table 1   Environmental Factors affecting survival of RSM2004

Site	Soil	Clay	Soil pH	Survival
Dijon* **France**	clayey eutric cambisol	40%	6.8	< 2 weeks
Bayreuth **Germany**	**loamy eutric cambisol**	**20%**	**5.2**	**30 weeks**
Rothamsted **UK**	**fine loamy chromic luvisol**	**32%**	**7.4**	**>15 years**

*Heavy rain following immediately after application may have washed away inoculant: subsequent trials survived >2 years

Figure 1   Survival of GM rhizobia in field release site
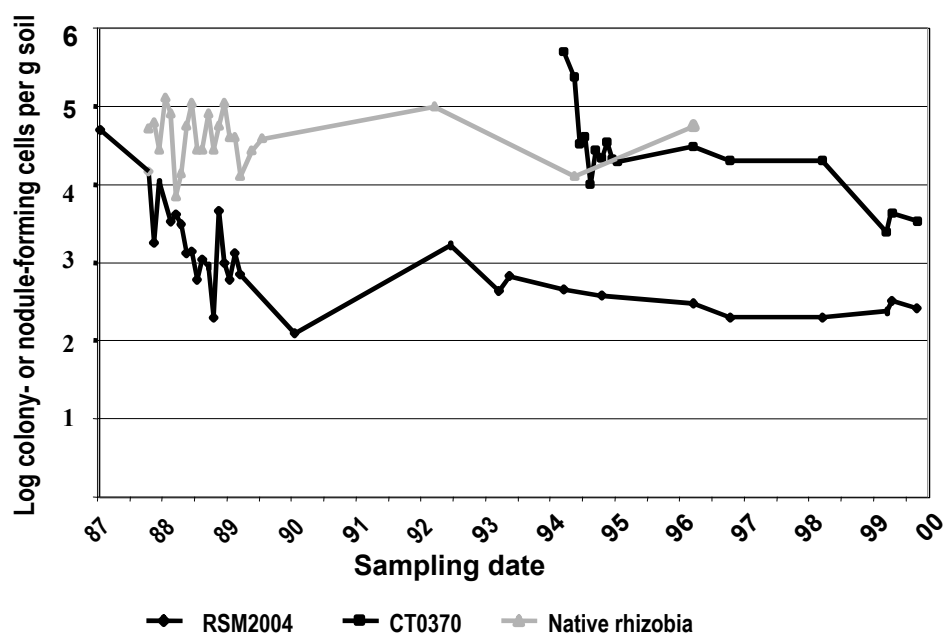
Monitoring the fate and ecosystem effects of genetically modified *Pseudomonas putida* producing phloroglucinol and phenazine in wheat rhizosphere.

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Introduction

There is increasing interest in applying microorganisms to control soil-borne plant pathogens. Performance and activity of such microorganisms was frequently inadequate. Combining several modes of action against plant pathogens in one single organism by genetic modification might improve their efficacy. Despite long-term experience with introducing non-modified microorganisms, concern about the ecological impact of large-scale release of genetically modified microorganisms (GMMs) has been raised. The impact of these genetically improved biocontrol strains may affect the soil microbial ecosystem. To date, field studies with genetically modified bacteria have focused mainly on microorganisms with markers, which are not expected to affect the indigenous soil microflora. Effects of GMMs have been studied mainly in microcosm experiments, however, these microcosms lack the full biotic and abiotic components of a field environment.

Pseudomonas putida WCS358r was modified to produce the antifungal compound phenazine-1-carboxylic acid (PCA) (Thomashow et al. 1990), or the antifungal and antibacterial compound 2,4-diacetylphloroglucinol (DAPG) (Bangera and Thomashow, 1999). These strains were applied as seed coating on wheat seeds. Field experiments were performed in the years 1997, 1998, 1999 and 2000. Our objective was to determine activity, survival and ecosystem effects of genetically improved biocontrol bacteria on the fungal and bacterial rhizosphere microflora, using cultivation-independent 18S and 16S rDNA analysis

Material and Methods

Strains

Pseudomonas putida WCS358r is a plant growth-promoting rhizobacterial strain with disease-suppressive properties, based on the production of its fluorescent siderophore (Bakker et al. 1986). A rifampin resistant derivative of WCS358, WCS358r, was used as the parental strain. The *phzABCDEFG* genes from *P. fluorescens* 2-79 (Mavrodi et al. 1998), under control of the P_{tac} promoter were introduced on a mini-Tn*5* LacZ1 transposon into WCS358r, resulting in two derivatives, GMM 2 and GMM 8, with different levels of PCA production (Glandorf et al. 2001). Likewise, WCS358r was modified to produce DAPG by inserting, on a kanamycin-resistant mini-Tn*5* lacZ1 transposon, the *phl*ABCDEF genes (Bangera and Thomashow 1999). The gene cluster contained its own promoter, however, the *phl*F gene, encoding a repressor of DAPG synthesis, was disrupted to promote constitutive production of DAPG. The DAPG producing derivative of WCS358r was labeled as GMM P. Bacteria were grown on Kings medium B (KB) containing the appropriate antibiotics.

Field experiment

In 1997 and 1998, the experiments were conducted on a site located at De Uithof, Utrecht, The Netherlands. A randomized block design was used with four treatments, with six replicates each, resulting in a total of 24 plots, each of 1 m^2. The four treatments were seeds treated with WCS358r, GMM 2, GMM 8, and non-bacterized seeds (control). In 1999 repeated introductions of GMMs were started. Again a randomized block design was used, with six replicates for each treatment. The treatments were seeds treated with WCS358r, GMM 8, GMM P, a mixture of GMM 8 and GMM P, and non-treated seeds. Each year following 1999 the same treatments were applied to the same plots. Per plot about 1750 wheat seeds were sown manually in 11 rows of 1 m length at a depth of 2-3 cm. Plots were separated from each other by 60 cm bare strips. The experimental field was fenced to block rabbits from entering the site and bird entry was prevented using nets.

Ecosystem effects

Total DNA was extracted from soil samples. Ribosomal DNA was amplified using primers specific for bacterial 16S rDNA or fungal 18S rDNA. Fungal PCR products were digested with *TaqI* and bacterial PCR products with *HinfI*. The fragments were separated on polyacrylamide gels. Dendrograms representing percentage similarity of banding patterns were constructed by UPGMA cluster analysis using the algorithm of Nei and Li or Dice.

The 1997 samples were further studied by specifically zooming in on the *Fusarium* population by sequencing *Fusarium*-like clones selected by ARDRA from a clone-library (Leeflang et al., 2002).

Results

Survival

In all years populations of WCS358r and the GMMs decreased from about 10^7 CFU per gram of rhizosphere sample to 10^2 -10^4 CFU per gram at harvest, and to near the detection limit (10^2-10^3 CFU/ g rhizosphere sample) one month after harvesting (131 or 139 days after sowing). In general no indications were found that the fitness of the GMMs was affected by the genetic modification, as numbers of CFUs of the parental strain and the GMMs were comparable. Also no differences were observed between numbers of the GMMs on rifampicin-containing KB with or without kanamycin, suggesting that the phz and phl genes were stable in the bacterial chromosome throughout the growing season.

Activity

Detection of PCA in rhizosphere extracts was done using HPLC and mass spectrometry. Rhizosphere extracts obtained in the field trial of 1998 twelve days after sowing were fractionated using reversed phase HPLC. In extracts of control- and WCS358r-treated wheat rhizosphere no PCA was present. HPLC chromatograms of rhizosphere extracts of wheat plants treated with GMM 2 and GMM 8 had peaks with the same retention time as standard PCA and the presence of PCA was confirmed by mass spectrometric analysis of these peaks. Comparison of the heights of the PCA peaks suggests that PCA production in the rhizosphere by GMM 8 is higher than the production by GMM 2.

Ecosystem effects

Seed application of both WCS358r and the PCA-producing GMMs caused a shift in the

fungal population of wheat roots, as indicated by cluster analysis of replicate ARDRA-generated profiles of rhizosphere samples. Treatments are considered to be different, if both replicate ARDRA patterns of one treatment cluster together, apart from patterns of other treatments. In this case the replicate ARDRA patterns per treatment are more similar to each other than to other patterns. Effects on the fungal microflora as a result of bacterization with WCS358r or the GMMs seemed differential, since the ARDRA profiles from the GMM-treated samples clustered separately from the WCS358r-treated samples and from the control treatment. Effects of the GMMs could be observed up to 40 days (1997) and 89 days (1998) after sowing, whereas WCS358r-induced effects were detectable up to 12 and 40 days, respectively. In both years all treatments cluster together one month after harvest, indicating that the effects induced by the bacterial treatments were transient (Glandorf et al., 2001).

The 1997 samples were further studied by specifically zooming in on the *Fusarium* population using a molecular method (Leeflang et al., 2002). Seventy *Fusarium*-like clones, selected from a library consisting of 1000 clones, were selected and sequenced. Analysis showed that both the WCS358r and the GMM inhibited the development of *Fusarium* type I. This probably allowed other *Fusarium* types to develop and resulted in a higher diversity of different *Fusarium* types in the WCS358r and the GMM treatments.

In 1999 and 2000, next to effects on the fungal microflora, effects on the bacterial microflora were detected. The DAPG producing derivative of WCS358r caused a shift in the fungal microflora that lasted until the end of the growing season. For the bacterial microflora a transient shift up to 40 days was observed for the treatments with the DAPG producers. In 2000, however, no distinct clustering patterns could be observed, and similarity between replicate samples was low, suggesting that the natural heterogeneity of microbial populations exceeded possible effects of the GMMs.

Discussion

No differences in survival between the genetically modified strains and the wild type were observed, indicating that the extra metabolic load did not affect the ecological fitness of the GMMs.

In the field trials of 1997 and 1998, both introduction of the modified and wild type strains resulted in a transient effect on the composition of the rhizosphere fungal microflora, as determined by 18S rDNA analysis. This was most prominent at the beginning of these field trials, when the numbers of introduced bacteria were relatively high (Glandorf et al. 2001). The WCS358r-induced effect on the fungal microflora is probably caused by the production of pseudobactin 358, the fluorescent siderophore of WCS358 (Bakker et al. 1986). GMM-induced impact on the composition of the fungal microflora lasted longer than the WCS358r-induced impact. The GMM-induced shift in the fungal microflora was longer lasting and differed qualitatively from the shift caused by the parental strain. This indicates that the PCA produced by the GMMs also affected the composition of the fungal microflora. The detection of PCA in the rhizosphere of GMM-treated plants and not in rhizosphere samples of WCS358-treated plants and control plants supports the role of PCA in these shifts in the fungal microflora.

In 1999, introduction of the DAPG producing GMM, either as a single application or in the combination with the PCA producer, had a long lasting effect on the rhizosphere fungal microflora, as determined by 18S rDNA analysis. For the same treatments a transient effect was observed on the bacterial microflora, based on 16S rDNA analysis. It was expected that the intensity of the effects would increase with repeated introduction of the bacterial strains in the same plot. However, in 2000 no clear effects of bacterial treatments were observed on either the fungal or the bacterial microflora.

Thus in this year of the experiment effects of the introduced GMMs did not exceed those of

natural variation. In the 2000 experiment we also observed that seed treatment with bacteria resulted in increased plant growth. This plant growth promotion was independent of the ability of the bacteria to produce PCA or DAPG.

Conclusions

Our results show that introduction of PCA-producing GMMs can transiently affect the composition of the rhizosphere fungal microflora of field-grown wheat. When introduced for the first time, the DAPG-producing GMM had a longer lasting effect on both the bacterial and fungal microflora, This effect was no longer observed at the beginning of the following season, and, contrary to our expectation, no enhanced effects were observed by repeated introduction.

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Revised risk assessment for GM-foods: The need to analyse unintended effects including consequences from gene flow

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Complementing previous concepts of the risk assessment of foods derived from modern biotechnology recently, an Intergovernmental Task force established by the Codex Alimentarius Commission agreed on the need to assess long term, unintended or unexpected effects in guidelines for the safety assessment. The substantial equivalent is no safety assessment but can be used as a starting point to instruct the safety assessment. Modern methods of molecular characterization have revealed pleiotropic effects on the expression of health related constituents of crops following methods of conventional breeding, biotechnological methods or genetic modifications. Moreover, recent research specified epigenetic effects such as silencing of gene expression and environmental effects on the transcriptional or translational regulation of the expression of traits. Modern profiling methods may be used to monitor such effects characterising gene expression modified by a genetic intervention or by specific environmental signals. Also new evidence about gene flow or pollen dispersal from GM crops necessitate the analysis of potential consequences for food safety. The consumer supported demand to assess long term effects for crops which are produced and marketed world wide under different ecological and social conditions may result in the need for more complex assessments addressing interaction of health and environmental objectives, as well as monitoring and management measures in an integrative way. The World Health Organization (WHO) supported concept to develop environmental health indicators may be used for the assessment and comparison of effects of modern and traditional technologies of biotechnology for food and feed production.

Redefining Biosafety and Risk Assessment

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Addressing the biosafety of genetically modified organisms has become an integral part of the progression of biotechnology and its applications. The increasing use of GMOs in agriculture and other fields includes both beneficial and potentially hazardous consequences. Much of the research in biosafety in the last decade has attempted to quantify potential risks to the environment and to consumers, and has greatly enhanced our ability to rationally evaluate the effects of widespread use of GMOs. Such risk assessments cannot be limited, however, to scientific data, as they are dependent on how we choose to define "risk" and "benefit". Additionally, to make sense of a quantitative analysis, it is imperative to avoid merely political assignments of risks and benefits. The application of biotechnology will have an impact that extends beyond gene flow, loss of genetic diversity or the improvement of current practices or crops. It will force the redefinition of some of our most basic motivations in science and could lead to changes in how we function as a society. In concert with scientific data, discussions of biosafety should include a consideration of some of these broader issues.

Transgenes in maize landraces in Oaxaca: Official report on the extent and implications

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In November 2001 a report was published indicating that transgenes had been found in landraces of corn grown in the sierra of Oaxaca, Mexico (Quist, D. & Chapela, I.H., 2001 Nature 414:541-543). This report initiated a long debate on three main subjects: 1) The technical qualification of the report; 2) The validity of the results presented and 3) The possible consequences and implications that such an event could have.

As a consequence of such report the Mexican Government initiated a preliminary sampling and analysis through the National Institute of Ecology which indicated the presence of transgenes in corn in two states. Based on these findings, the Secretary of Agriculture, requested an investigation into the subject. An "ad hoc" committee was formed which included experts from different areas of expertise. The first step was to devise an approach to obtain representative samples from the State of Oaxaca and the neighboring State of Puebla. Once the sampling strategy had been planned it had to be implemented ensuring the "chain of custody" and that all relevant information was obtained for each sample at each location. The samples where then processed and distributed to the institutions that were going to carry out the testing. Tests performed on the samples included PCR for general transgenic traits such as the 35S promoter, NOS terminator or cry genes; protein analysis using "strip tests" and ELISAs for specific proteins such as PAT, CP4, Cry1A and Cry9C; sequence and Southern blot analysis to confirm the findings and identity of some of the genes found.

Up to this moment, the results presented by the Mexican Government have shown that transgenes such as cry1A can be found extensively in land races throughout the State of Oaxaca. The presence of cry9C has not been detected in any of the samples tested. As for any apparent consequences to the landraces themselves, this so far has not been the case. The small growers have not reported any phenotypic changes in their crops that could suggest that a major modification could take place. The changes observed are those expected when the farmers use a hybrid to "enhance" or improve their landraces, a practice that is very common among small growers in this area.

Monitoring microbial inocula, activity and impact on ecosystem function and soil microbial diversity

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Pseudomonas fluorescens SBW25 (WT) and its genetically modified, phenazine-1-carboxylic acid (PCA) producing variant 23.10, are PGPR (plant growth promoting rhizobacteria) which protect a number of crop plant species from damping-off caused by *Pythium* species. The GMM, 23.10, carries on its chromosome a single copy of the *phzABCDEFG* genes under the control of the P*tac* constitutive promoter. 23.10 has improved biological control activity when compared to wild type SBW25, and effectively suppresses infestations of *Pythium sp.* at 100x normal back ground densities in soils. It also has an improved spectrum of activity over several plant phytopathogens including species of *Pythium*, *Fusarium* and *Rhizoctonia* spp. The inocula survive and persist in the rhizosphere of several crop plants and establish higher population densities in the rhizosphere of pea and wheat when compared to sugar beet, thus showing plant specific tropism. However, the total microbial densities in these rhizospheres were not effected by the addition of either WT or GMM inocula and any impact on microbial diversity (bacteria, fungi and mycorrhizae) was negligible to unobserved when assess plate count (CFU/g) and culture independent molecular assays (of 16S and 18S rRNA based PCR-DGGE). Greatest variation in the profiles of the different treatments were demonstrated by community metabolic profiling (CLPP), but variation directly correlated to plant type and plant age not the presence of bacterial inocula in disease free plants. Inocula suppressed infection and promoted plant biomass. Rhizosphere community profiles in infected plants in the presence of inocula were highly similar to disease free systems. Histological assessment of the impact of inocula on established mycorrhizae associations were also conducted on rhizosphere and soil cores collected from field margin grassland pasture.

In all instances the plant species and presence of a phytopathogen had a greater recordable impact on microbial diversity and function than the presence of inocula. The relevance of these data in respect to the impact of functional GMMs in agricultural systems will be discussed

Crop-to-Crop Gene Flow: Dispersal of Transgenes in Maize, During Field Tests and Commercialization

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Gene flow in maize (Zea mays L. ssp mays), among genotypes with varying level of hybridization and stages of evolution, including the wild relative teosinte (Zea mays ssp. mexicana (Schader) Iltis), is not new. Considerable research exists that evaluates the impact of improved, conventional maize cultivars on traditional landraces and teosinte in Mexico. Considerable research also exists concerning the ability of plant breeders and seedsmen to prevent the undesired transfer of genetics from unimproved varieties or wild relatives to elite genetics via the utilization of stringent pollen control techniques. Recently, these investigations have received renewed interest due to the possibility that transgenes may somehow affect the landraces and wild relatives. The purpose of this presentation is to provide a review of the literature on gene flow and pollen control in maize. Included will be a discussion of our research on maize pollen biology, flowering dynamics, and evaluation of several practical techniques for controlling pollen and therefore gene flow on a research scale. Results to date are consistent with observations that maize pollen is desiccation intolerant and loses water and viability due to desiccation rapidly after dehiscence as is found in Gramineae generally. Teosinte pollen generally desiccated more rapidly that maize pollen although the duration of shedding was typically longer due to the existence of multiple staminate inflorescences per plant. Stigma or 'silk' elongation in landraces and improved maize varieties was rapid and growth continued for approximately 10 days after initial emergence. Crossing occurred among improved cultivars and among improved cultivars and landraces equally in either direction. However, crossing of maize with teosinte typically involved teosinte plants fertilizing maize plants. The evaluation of two research scale methods of pollen control indicated successful pollen control could be obtained. The methods of pollen control that were evaluated included early detasseling of plants before pollen shed commenced and distance isolation. Prior literature regarding maize floral biology and the use of spatial isolation to control maize hybridization was consistent with our results. Our research demonstrates and documents that effective tools for managing research scale pollen flow exist and that these results are consistent with the floral biology of the crop. The only way to determine impact of transgenic gene flow is to be able to conduct further research. The extent to which precautions need to be applied to the pollen flow depends ultimately on the implications of the flow of novel genes. If the consequences of novel gene flow are biologically significant, more precaution will need to be exercised than if experiments demonstrate no significant biological impact of the novel genes beyond that of traditional breeding activities.

Farmer management of maize landrace diversity

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To assess the impact of farmer management on maize landrace diversity in the Central Valleys of Oaxaca, Mexico, where landraces comprise most of the maize grown, we interviewed farmers in six villages and took samples of seed. Among other things, we found that:

- The level of deleterious and lethal mutation is high in the landraces.

- Frequent seed and pollen exchanges result in extensive migration and gene flow among landraces, which are organized as a metapopulation.

- These same landraces show a strong differentiation for traits under selection by farmers (mainly ear traits).

We also report on prior studies in Cuzalapa village, southwest Mexico, and in Burkina Faso, both of which also point up the the impact of migration and geneflow in open genetic systems. Due to high levels of recombination, most genes introduced from exotic varieties will behave independantly and their diffusion is favored by seed and pollen exchanges. At the same time, these exchanges are part of mechanisms that maintain and enhance the genetic diversity and viability of these landraces. Strategies that would restrict them will erode geneflow and result in genetic erosion.

Biodiversity and biosafety e-learning at the Slovak Agricultural University of Nitra/Slovakia

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In the frame of the Slovak Agricultural University (SAU) of Nitra education program conditions were created allowing a lifelong education under a slogan, Education for each and all "This from of study was organized for different target groups" (agricultural and food-processing companies managers, state administration and self-governments representatives and others) by a method of distance learning composed by specialized courses. For participants of this study the relevant literature was prepared. Moreover, they have access to information systems on education and advisory services using sophisticated information and communication technologies (ICT). Up to now 87 items of study literature were edited.

In the lifelong education are aligned specialized courses on biodiversity conservation as follows: K1: Plant genetic resources utilization K2: Agricultural crops seed production, K3: Agroenvironmental aspects of biodiversity conservation and sustainable development, K4: Biodiversity conservation in agroenvironmental programs, K5: Agrobiodiversity and traditional agroecosystems conservation, K6: Biodiversity conservation in rural development programs. Up to now graduated 750 participants of such courses.

On the base of Ministry of Encironment of the Slovak Republic request we have organized the first course GMO- risk assessment and risk management "financed by the government of the Netherlands" (MATRA project). There were 25 attendants. Many of them are presently acting as members of the Expert commission of the Ministry of Environment for GMO risk evaluation issues.

From ecological model to regulation: The respective roles of science and values in the assessment of the environmental risks of transgenic organism release

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Ecological risk assessment will always be a highly controversial field. In the case of assessing the risks of transgenic organism release into the environment, judgments with potentially far-reaching implications must be made in advance of complete knowledge of the ecological impact. Some argue that quantitative methods are available, and these are foundational to a rational decision making process. Others deny this view, asserting that ecological risk assessment is a weakly inductive science unable to make solid predictions at the best of times, let alone in cases of uncertainty. This debate has become particularly acrimonious as polarized views have emerged in response to the widespread use of the precautionary principle's in developing regulations for transgenic organism release.

Evaluating and responding to ecological risk is not as simple as either polarized views suggests. The case of transgenic salmon shows that the spectrum from ecological model to conservation biology to regulation is a complex dynamic of science and values. Judgments are formulated on the basis of antecedent views about what constitutes scientific rationality, what makes good science, and the role the science should play in setting the science and technology agenda for society. No one is immune from making these judgments; what is relevant to science policy and environmental regulation is the effects of the wide range of interaction between science and values in competing responses to risk assessment.

Possible ways of using transgenic mosquitoes for malaria or dengue control and risk assessment[1]

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The *Anopheles* vectors of malaria can be controlled by insecticidal house spraying or treated bednets and the *Aedes* vectors of dengue and some *Anopheles* populations can be controlled by attacking larvae. However, sustaining such search and destroy operations with sufficient intensity to control or eradicate disease is problematic. It has long seemed an attractive alternative to release male mosquitoes (which do not bite) as the searching agents and to equip them with genetic factors, that cause sterility or inability to transmit disease, for the males to pass on with their sperm.

Transgenic *Aedes* and *Anopheles* have been produced and it is speculated that they could improve existing methods for:

1. Ensuring that no biting females are released and that the released males produce no female progeny: this might be done with a construct that causes selective female lethality whose expression is conditional on the absence of a dietary factor. This factor would be available to the maintenance colony but not to mosquitoes being reared for release or in the field. This technique might be targeted to eradicate urban vector populations which are absent from the surrounding countryside.

2. Producing mosquitoes not susceptible to malaria parasites or dengue virus: these would only be useful if the genes concerned could be linked to a genetic system for driving them through wild populations. However, use on an operational scale, with unbreakable linkage of the driving system to the gene to be driven, would be extremely difficult to achieve.

Before any releases of transgenic mosquitoes are made they should be tested for the unlikely possibility that the genetic modification has had the side-effect of enhancing susceptibility to transmission of any human pathogen.

Presumably there might be a risk of horizontal transfer of the artificial construct with method 2, but scarcely with method 1 because the construct would only be carried in the field by non-biting males.

Low establishment of hybrids between oilseed rape and wild radish

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The risk of gene flow between oilseed rape (*Brassica napus*) and wild radish (*Raphanus raphanistrum*), a wild relative of the crop that is widespread as a troublesome weed in arable fields, is investigated. Creating super weeds could result from the transfer of beneficial traits such as herbicide resistance. Preventing and managing this risk is necessary to make the best use of genetically modified crops. In previous works, spontaneous interspecific hybridization was shown to be very rare when the wild radish was the female, but very frequent when the female was a male sterile oilseed rape (which becomes popular in composite cultivars). The potential of hybrids and hybrid progeny to establish in controlled conditions and in the field is studied here.

The earliest life-history stages of F_1 hybrids are much reduced compared to both parents. The effect of the growth conditions on the difference between hybrids and parents is stressed. The reproduction of the hybrid is very difficult. Cytogenetics anomaly, as observed by genomic *in situ* hybridization, prevails when backcrossed seeds are produced. Therefore, the developmental cost of the interspecific hybridization is very high and is not counterbalanced by the advantage due to herbicide resistance. However, at the sixth generation of backcrossing to wild radish, there is no more apparent difference between hybrid progeny and wild parent. An important cytoplasm effect (rape versus radish) is observed on the fitness of the descendants. Although the location of the transgene on the genome can mitigate fitness, the combination of the known biological parameters predicts that herbicide resistant hybrid progeny can develop and infest fields.

Gene flow in autogamous cereals: facts and recommendations

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Autogamous cereals, such wheat, are the major crops on the world scale. They should benefit from genetic engineering, and, therefore, risk assessment of the release of transgenic cereals must be undertaken. Our group initiated a project on that topic under the framework of a joint European Union contract. This project investigated the possibility of transgene movement from biotechnologically derived herbicide-resistant wheat towards wild relatives through interspecific hybridization, and also gene movement from mutant *Setaria italica* to wild *Setaria* spp. as a model system.

Evidence from historical, experimental and present field observations of interspecific hybridization and possibilities of translocations has been obtained, at least for some weedy and ruderal species related to wheat. There is no evidence that such hybrids have stabilized in any population, but genes such as herbicide resistance can have a stronger selective advantage than other genes, and thus there is the possibility of stabilization under continuous selection pressure. Pollen dispersal causing hybrids was a few meters under natural conditions, but was greater where there was no naturally-competitive pollen. The role of polyploidy in the fertility of interspecific hybrids is stressed.

Three different approaches of testing and evaluating genetic tools to mitigate gene flow were studied in various ecological and experimental conditions: 1) The study of the effect of the genome location of the transgene (A, B or D genome); 2) The comparison of gene flow when the transgene inheritance is recessive or maternal compared to a dominant trait; 3) A tandem construct of a gene of choice and a gene deleterious to weeds.

Intraspecific gene flow during the large scale cultivation of transgenic oilseed rape varieties with different herbicide tolerances

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Gene flow between plant populations is influenced by crop characteristics like the kind of pollination or the pollen size as well as by environmental conditions, the size of the populations and the distance between them. Although oilseed rape (Brassica napus) is mostly self-pollinating, substantial outcrossing rates have been reported. Worldwide there is an increasing cultivation of transgenic oilseed rape, most of it with herbicide tolerances. In the EU several transgenic oilseed rape lines have a part C approval (90/220/EC), although to date no varieties are on the market. Still little information is available on outcrossing frequencies during agricultural cultivation of transgenic oilseed rape lines and on the consequences of outcrossing. Transfer of a herbicide tolerance gene to other oilseed rape varieties, for instance, causes seed contamination and can give rise to volunteer plants with an unexpected herbicide tolerance pattern.

In a large scale field release experiment with Glufosinate tolerant (LibertyLink[®], LL) and with Glyphosate tolerant (RoundupReady[®], RR) oilseed rape, outcrossing frequencies of the herbicide tolerance genes to neighbouring fields and the emergence and control of double tolerant oilseed rape volunteers were investigated. The size of each of the 4 transgenic plots was 0,5 ha, surrounded by 8 ha non-transgenic oilseed rape. The transgenic plots were either in direct contact with each other and with the non-transgenic field, or they were separated by 10 m fallow. Seed samples collected in the transgenic plots and in the surrounding non-transgenic field at different distances were screened for tolerant seedlings using herbicide germination tests and PCR.

It was found that a 10 m isolation distance reduces average outcrossing rates at the inner borders of neighbouring fields from about 1 % to about 0,5 %. Outcrossing then decreases exponentially within the field, and at 50 m it is clear below 0,1 %. Outcrossing frequencies could not be correlated with the direction of the wind during flowering time. In order to detect volunteers with a herbicide tolerance gene originating from outcrossing, oilseed rape plants emerging after harvest were treated with one of the complementary herbicides. Double tolerant volunteers could be detected in each of the transgenic plots, but were mostly limited to the inner field borders. After eradication of double tolerant volunteers by subsequent tillage and the emergence of the following crop (winter wheat) very few oilseed rape volunteers were observed.

The expression of the stacked transgenes in double tolerant oilseed rape plants was investigated at normal (22°C) and at elevated (37°C) temperatures. No gene inactivation has been found so far.

Airborne Pollen Dispersal Modeling: An Effective Tool For Regulating Gene-flow

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As part of the environmental risk assessment for plants with novel traits (Derived through recombinant DNA technologies or other methods) pollen flow from these plants to the surrounding environment may need to be considered. Where information is required on gene-flow frequencies at various distances, for totally- or partially wind-pollinated crops, regulators have sometimes relied on isolation zones used for seed production and confined field research trials. These zones were determined from information based upon breeder experience and a very limited number of field trials, and may not be applicable to locations, environmental conditions or field seasons other than those experienced during the study's execution. Since gene-flow and pollen dispersal are highly variable phenomena, instances of the breakdown of isolation zones have been noted. Segregation from sexually compatible plants is an important consideration, and is used to preserve seed purity, prevent the entry of unapproved events into the food and feed supplies and is also of interest to the organic farming community. Due to the zero tolerance policy for admixtures of novel traits in organic crops, adequate segregation may present a real challenge for organic growers. This has led to a controversy in north America with the Saskatchewan Organic Directorate attempting to launch a class-action law suite against two major biotech companies based upon gene-flow from GM canola.

We describe a novel application which quantifies the variability and frequency of gene-flow (and pollen-flow) for wind-pollinated plants based on well established principles from the air pollution field. These modeling principles are based upon the atmospheric physics of pollen particle flow. This quantification of variability indicates pollen movement under varying environmental conditions and at varying localities and thus determines probability distributions for pollen-flow. The use of this tool will allow regulators, or those with responsibility for crop segregation, to more accurately define gene-flow under differing levels of containment corresponding to differing levels of segregation or product "purity" and at different localities and conditions.

We describe some of the physical and biological mechanisms underlying pollen- and gene-flow in wind pollinated plants, discuss in general terms the variability found in previous measurements of pollen- and gene-flow and then describe how mechanistic modeling of the physical and biological mechanisms can greatly enhance risk assessments and the effectiveness of gene-flow management. We then describe on-going work on developing models for the forestry sector in Ontario, Canada, and then discuss the initial stages of work with the Canadian Food Inspection Agency in applying these methods to agricultural crops. Finally, we describe the applications of such a modeling system for regulatory authorities.

Detecting and Quantifying Genetically Modified Organisms (GMOs) in Foods and Agricultural Products — Analytical Tools to Support Regulators and Industry

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GMO testing is becoming an essential tool for farmers, agricultural traders, food manufacturers, food retailers, and government regulatory authorities. This is because, in virtually every area of the world, including Europe, Asia, the Middle East, and the Americas, government regulations and consumer requirements make it increasingly necessary for the GMO content of food products to be verified before they are imported or introduced into the marketplace. International accords, such as the Biosafety Protocol of the Biodiversity Convention, have further increased the need for methods to verify the genetic status, not only of seeds and other propagation materials, but also of foods and agricultural produce. Gene modification (gene splicing or recombinant DNA technology) introduces novel genetic information into the organism, which reprograms the organism to produce new proteins and express new functions. Tests for both genes and proteins are useful in distinguishing between genetically modified and conventional foods and agricultural products. The polymerase chain reaction (PCR), which detects transgenic (recombinant) DNA sequences, is widely used to identify and quantify the full range of commercialized GMOs at all points in the food production and manufacturing chain, while immunological methods, which detect transgenic proteins, are used today to detect a more limited selection of GMOs at early points in the food chain. We will discuss how these methods are currently employed by governments and in the agricultural and food industries, and will consider from a technical perspective the applicability of each to specific purposes. We will conclude with discussion of future directions and of the next wave of GMO analytical technology. This discussion will also consider the potential for methods developed for GMO analysis to be applied to other questions important to agriculture and the food industry, such as pathogen detection, detection of adulteration, and species identification.

"Production of Antibodies and Vaccines in Plants and Their Use for Global Health"

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Antibodies and vaccines are critical tools in human and animal health care, enabling us to treat many life-threatening diseases. Until now, the engineering and large scale production of recombinant antibodies and vaccines has been time consuming and expensive, prohibiting the wide spread use of these proteins throughout medicine. Recent developments in protein engnineering and production technologies have contributed to overcome many of these problems. Using molecular farming, i.e. the production of recombinant proteins in transgenic organisms, we can use plants to synthesize antibodies and vaccines on an agricultural scale. This technology will help to bring recombinant antibody and protein therapeutics down in cost, without sacrificing their quality or safety, enabling us to broaden our concept of what they can be used for.

Different antibody variants and vaccines are all produced in an active form and they join a growing list of recombinant proteins that can be functionally expressed in plants. The highest production yields can be seen with recombinant proteins that are retained within the cell's secretory pathway, and the lowest yields are seen in the cytosol. Importantly, recombinant protein expression can be used to modify the inherent properties of plants, for example by using expressed anti-pathogen antibodies to increase disease resistance. Plant transformation is technically straightforward for model plant species and some cereals and the functional expression of recombinant proteins can be rapidly analysed using transient expression systems in intact or virally infected plants. Protein production can then be increased using plant suspension cell production in fermenters, or by the propagation of stably transformed plant lines in the field. Transgenic plants can be exploited to produce organs rich in a recombinant protein for its long-term storage.

This presentation will focus on discussing the challenges involved in engineering of antibodies and vaccines and their expression in plants, how these challenges can be overcome and efforts to produce a series of recombinant proteins in different plant species. Issue relating to safety of GMO and their impact on consumer and environemnt will be adressed- Our long term perspective is that recombinant protein production in crop plants may create an opportunity to distribute these diagnostic and therpeutic proteins beyond the developed and into the developing world.

Rainer Fischer and Neil Emans (2000). Molecular farming of pharmaceutical proteins. Transgenic Research 9: 279-299.

Possible effects of transgenes on genetic diversity

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In this presentation, I will assess a number of issues related to the potential effect of transgenes on genetic diversity. Firstly, I will consider what is genetic diversity and how do we characterize it? Secondly, I will discuss to what extent genetic diversity is a good per se, which is important to maintain. Thirdly, I will examine which type of diversity might be threatened, mainly the domesticated or wild gene pools. Fourthly, I will ask whether transgenes or transgenic cultivars play a special role, distinct from that of genes incorporated into cultivars by classical plant breeding, in threatening genetic diversity. Throughout my talk, I will discuss published data as well as data from my own research program on gene flow between wild and domesticated beans in Mexico, one of the centers of domestication of this crop.

Deployment pathways of Bt potato in developing countries

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The cry1Ab5 gene was introduced in the potato crop to confer resistance to potato tuber moth pests. A total of 614 transformation events have been produced for 10 varieties adapted to different agro-ecologies. High level of resistance to Phthorimaea operculella was obtained in foliage and in tubers. Small-scale field trials were developed to confirm resistance and assess true-to-typeness. Reductions in insecticide use are expected to range between 50 to 100% leading to significant health and environmental benefits. These PTM-protected varieties are available for commercial production to developing countries with adequate biosafety regulations. However, none are today in the hands of those who need them the most.

Several scientific, regulatory, and policy questions or issues remain to be properly addressed: (1) the equivalence of the Cry1Ab5 protein in potato with other crops engineered with cry1Ab5 gene; (2) the crop management appropriate to the production system and environment; (3) the avoidance of negative impacts of gene escape; (4) the varieties targeted for genetic engineering; (5) the segregation of GE variety; (6) the opportunity cost of not deploying Bt potatoes; (7) the co-existence with alternative production systems. With the exception of the Bt toxin safety for human consumption, these issues are country- or region-specific and need to be addressed not solely by scientists but also by different actors of the civil society. Unless this is achieved in a timely manner, it is unlikely these PTM-protected varieties will be deployed in developing countries.

Common and country specific drivers for biosafety regulatory frameworks.

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Much is said about criteria to meet in building an efficient biosafety regulatory framework (BRF) such as science based approach to risk assessment, openness and transparency, public involvement, etc., etc. Considerable efforts as well as financial resource are spent to create an internationally acceptable system that would work in different countries (mainly in the developing world and countries with economies in transition) basing on existing approaches working in developed countries having long experience in regulating modern biotechnology.

It is however difficult to expect that these approaches would efficiently apply outside the developed world. Indeed, in developed countries regulations emerge from the free market economy ("Regulation is a means by which governments seek to gain the benefits and ameliorate the potential negative consequences of a market economy" - EU-U.S. Biotechnology Consultative Forum, Final Report, 2000). It is therefore clear that regulatory system is driven by three key forces (or their combinations), i.e. state (government), independent industry, and independent consumer having a chance to make an informed choice. Interrelations between these three forces will define the national specifics. Thus in US (producer driven society) product is safe unless proven unsafe. In EU which is public driven society, product is unsafe unless proven safe (with variations from country to country). With these three drivers efficient regulation requires professional (full time) regulatory body and strict split between risk assessment and risk management (decision making, etc.) procedures.

In the vast majority of developing countries and countries with economies in transition (DC&CET) economic situation is far from being free market; industry is lacking or underdeveloped and strongly depends on the governments, and people have no chance to vote with their money because of lack of it and shortages in product supply.

Thus, outside developed world an emerging BRF completely depends upon outspoken priorities of the country which are not necessarily technological and are often political. These priorities as well as interrelations between the governments, industry and the public define the national "flavor" of biosafety. The principal, and most probably inevitable, "evolutionary" mistake made in the DC&CET is that people making a decision are the same individuals that are involved in risk assessment, and very often in marketing biotechnology. Lack (often absence) of public funds makes independent expertise impossible, thus making communicating to public difficult

It could be said finally that the only domain of BRF that could be developed and accepted internationally independently on country nature is risk assessment (only those hazards and risks are meant which are subject to natural science).

Mitigation of transgene flow from crops to related weeds; tobacco as a model

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Some transgenic crops can interbreed with related weeds, or the crops themselves become feral, increasing the potential of problematic offspring (i.e. "superweeds"). Transgenic mitigation (TM) (1), in which a primary gene is coupled in tandem with mitigator genes that are positive or neutral to a crop but deleterious to offspring, was used as a failsafe mechanism to mitigate the effects of such problems. A gene conferring resistance to acetolactate synthase inhibiting herbicides was cloned in a tandem construct with a transgenetic mitigator antiweediness gibberellic acid-insensitive dwarfing gene. The tandem construct was transformed into tobacco. The dwarf T_1(=BC_1) transgenics could not compete with the wild-type segregants when co-cultivated in soil at different spacing. Most dwarf plants died at 1 cm, and more than half of them died when planted at 2.5 cm, but the survivors formed no flowers or seeds. Even at 10 cm spacing where few TM plants died, only those growing at the periphery formed flowers after wild-type plants stopped growth. The results demonstrate what would happen to TM hybrids and/or crops as volunteer weeds in a season when herbicides are not used. Thus, the Transgenic Mitigation concept was validated with tobacco as a model and is being tested using the same construct in regenerated transgenic oilseed rape crossed with the wild type and crossed with *Brassica campestris=rapa* as crop/related weed models. Adding further genes to the TM constructs, such as anti-shattering genes and anti-secondary dormancy genes would further guarantee the lack of survival of offspring of transgenic crops as volunteer weeds or from their crosses with related weeds.

(1) Gressel, J. (1999) Tandem constructs; preventing the rise of superweeds. Trends in Biotechnology 17:361-366

A proposed system for 'Biobarcoding'^{TM} organisms

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These are a variety of needs for devising simpler recognition methods for organisms marketed in commerce or released in the environment; whether they are conventionally selected, mutant, or transgenic bacteria, fungi, plants or animals. The needs include:

The need for protection for patented or other IP lines, where IP takes on either designation: "Intellectual Property" or "Identity Preserved". It is often hard to prove that a line has been 'miss-appropriated' by a competitor.

Labeling Regulatory authorities and various consumer groups are demanding labeling of transgenic commodities and may wish to require the use of consider simple, common recognition sequences for detecting transgenic or other organisms in the market place.

The need to trace organisms in the environment such as mycoherbicides and live inoculants, whether indigenous or transgenic, especially in cases where it might be construed that they damaged a crop. Complicated DNA fingerprinting can ascertain causality, but cannot be used to probe what released organism might be present.

The simplest detection system for differentiating a large number of products is the "bar code" system. A simple genetic analogy encoded in DNA sequences – "biobarcodes" is proposed. A set of two universal 'nonsense' (non-coding) nucleotide sequences is designed. These can be detected by a set of universal PCR primers that can be used to recognize all biobarcodes. The universal primers are long enough that a few mutational changes in the initial universal sequence will still allow it to be recognized by a PCR primer. The universal recognition codes are followed by a designed and assigned nonsense sequence that is long enough to allow tens of millions of different such sequences to be generated, and again allow for some mutational changes. Neither the initial universal recognition sequence nor the particular individual strain sequence should even vaguely resemble nonsense sequences reported in any gene data base. The algorithms used to generate the sequence are designed to exclude sequences that could self anneal, preventing the taq polymerase from amplifying the DNA. Frame shift mutations should not render any part of the biobarcode sequence as an open reading frame coding for a peptide – stop codons are interspersed so as to prevent frameshift mutations to form long open reading frames. The biobarcodes should be assigned by a single assigner, and the assigned codes are to be publicly available. The biobarcode DNA can be co-transformed with the gene of choice. In other cases, an excisable selectable marker will be needed, so that just the bar code remains after transformation.

The PCR amplified barcodes can be automatically sequenced and compared to the barcode database to ascertain the source of the organism. Should there be a possibility of introgression of the barcode from the initial organism into another strain or species, R or AFLP can be used to further verify the source

Transgene expression and field level hybridization between GFP/Bt transgenic *Brassica napus* and its wild relative, *B. rapa*

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Gene flow between domesticated and wild species occurs in areas where sexually compatible species are present. Although this has always occurred in conventional agriculture, plant genetic engineering adds the complexity of novel transgene flow. The level of transgene expression in hybrids and the frequency of hybridization and backcrossing are important factors in assessing the potential ecological and agricultural risks associated with genetic engineering. The average zygosity of transgenic hybrid populations change with the progression of generations, and the GFP transgene is an ideal marker to quantify transgene expression in advancing populations. In order to develop a model to study transgene flow, canola (*Brassica napus* cv Westar) was transformed with two GFP constructs, *mGFP5er* (GFP only) and pSAM 12 (GFP linked to a synthetic *Bacillus thuringiensis* (Bt) *cry1Ac* endotoxin gene).

Homozygous T_1 canola exhibited significantly greater fluorescence at 508 nm when compared with hemizygous individuals, and these data suggest that the GFP gene demonstrated additive transgene expression and fluorescence could be used to determine the effects of zygosity. Several hybrid generations were produced by backcrossing GFP/Bt transgenic canola (*Brassica napus*, cv. Westar) and birdseed rape (*B. rapa*) hybrid populations onto *B. rapa*. Average fluorescence of each successive hybrid generation was analyzed, and homozygous canola lines and hybrid populations that contained homozygous individuals (BC_2F_2 Bulk) demonstrated significantly higher fluorescence than hemizygous hybrid generations (F_1, BC_1F_1, and BC_2F_1). These data demonstrate that the generation of homozygous individuals within hybrid populations augments the average level of the transgenic phenotype above what would be expected after hybridization occurs.

Field level hybridization experiments have been performed to determine hybridization and backcrossing frequencies under agricultural conditions. In North Carolina, USA, hybridization was detected between ten canola events and B. rapa, and hybridization frequency ranged from 1.1% to 22.0%. Backcrossing occured at much lower frequencies, and averaged 1 backcrossed individual per 1400 B. rapa seeds.

Establishing a safety system for the production of proteins in plants by means of viral full-length clones by combining transgenic plants with modified viruses

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Infectious full-length clones of plant viruses are an attractive tool for the production of recombinant proteins in plants. However, the use of genetically modified viruses which are able to replicate in conventional plants raises concerns regarding the safety aspect. Therefore precautionary measures should be taken to ensure a safe production of foreign proteins in plants by means of gm viral full-length clones.

A possible safety system is based on using modified viruses which are not able to multiply in wild type plants. Different approaches were tried, with a full-length clone of potato virus X carrying the GUS gene (PVX/GUS) as a model virus. As a first approach, the viral sequence was put under control of a minimal promoter which allows transcription of the follow-up sequence only in the presence of a transcription activator. We could show that multiplication of the modified PVX/GUS did not occur in wild type plants. However, in plants expressing the transcription activator the modified PVX/GUS was able to cause a normal infection.

As a second approach, we are working on the combination of movement protein (MP) transgenic plants with movement deficient PVX/GUS constructs. PVX/GUS lacking the MP was not able to infect wild type plants systemically. In transgenic plants expressing the MP the deficiency could be overcome, and a normal infection could be established.

The biosafety system will be improved by putting the MP transgene under control of a minimal promoter, allowing its expression only in the presence of a transcription activator, which will be encoded by the MP-deficient PVX/GUS.

Fate of GM rhizobial inoculants: lessons from Europe and elsewhere

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The nitrogen-fixing symbiosis between leguminous plants and bacteria of the Rhizobiaceae has been exploited in agriculture for millennia. Commercial inoculants, applied to legume seed where insufficient compatible rhizobia are present in soil, have been available for over a century. Their agronomic importance, familiarity and the relatively good knowledge of their biology and genetics, has made rhizobia a target for improvement by genetic manipulation (GM). For example, Sinorhizobium meliloti genetically modified to increase nitrogen fixation, field-tested in the USA in 1994, was given commercial approval by the US Environmental Protection Agency in 1997.

In a programme of research funded by the EU, survival in the field of Rhizobium leguminosarum biovar viciae inoculants marked by GM has been monitored since 1987. At Rothamsted, populations declined sharply in the months following application but then stabilised although at two other sites the same inoculant could not be detected the year following application. Dispersal from the inoculation site was consistent with root growth and soil movement due to ploughing and no genetic interactions between the inoculant and native strains was detected in the field. There was evidence that the symbiotic plasmid of R. leguminosarum biovar viciae (which carries genes for host plant nodulation and nitrogen fixation) confers a survival advantage in the rhizosphere of both host and non-host plants. These observations are compared to results from other releases of GM rhizobia in Europe and the USA.

Analysis of risks of transgenic insects for pest management:

Past and future guidelines

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Genetic modification using recombinant DNA methods can now be used, almost routinely, to transform pest and beneficial insects that could be used to improve pest management programs. Goals include modifying mosquitoes, and other insects that transmit human and animal diseases, so that they are unable to transmit the causal pathogens. Transgenic methods could improve genetic control programs by producing sterile males or producing only females. Other goals include producing honey bees and silk moths that are disease resistant or have other desirable traits. Natural enemies used in biological control programs could be modified to enhance their effectiveness in several ways.

Risk assessments must be conducted prior to releasing transgenic insects into the environment for either short term experiments or permanent establishment. Potential risk issues to be resolved prior to releases include whether: the inserted gene(s) (trait) is stable; the traits (especially pesticide or antibiotic resistance genes) can be horizontally transferred to other populations or species; released insects will perform as expected with regard to their geographic distribution, host or prey specificity and other biological attributes; released insects will have unintended environmental effects; and, in the case of short term releases, the released insects can be recovered from field sites. Risk assessments of fitness and host specificity are relatively easy to assess in the laboratory, but horizontal gene transfer and unintended effects on ecosystem function are more challenging.

Permission to release a transgenic insect will have to be obtained from (several ?) regulatory agencies. Initial releases initially are being made into small plots, perhaps in cages and, in the USA, are intended to be short term experiments. Current regulations of the U.S. Department of Agriculture require the researcher to retrieve all transgenic insects from the environment at the end of the experiment. If transgenic insect strain(s) perform well and risk assessments are completed satisfactorily, permanent releases into the environment may be allowed, but guidelines for such releases are lacking. Many pest management programs, especially those involving replacement of pest populations by a transgenic population, will require permanent establishment in the environment. Several drive mechanisms have been proposed to insert genes into populations but analyses of the potential risks of such drive mechanisms have not been carried out. International guidelines are needed for risk analyses of transgenic insects because insects are highly mobile and could move beyond individual countries' boundaries.

Transgenic crops for resource-poor farmers.

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The world population is predicted to rise from the current approximately 6 billion to about 9 billion by the year 2050. The great proportion of this increase will be in tropical developing countries and especially in Sub-Saharan Africa. Populations in some countries in this region are expected to triple in the next 50 years leading to an exacerbation of the already impending food insecurity, especially in countries where most of the food is produced by resource-poor farmers. The deployment of transgenic crops is just one of the potential solutions to this problem and is being seriously considered in combination with other approaches. Initially transgenes protecting against biotic losses will help the situation and subsequently transgenes overcoming abiotic factors such as drought and salinity and those conferring useful agronomic and nutritional properties will be required.

The deployment of transgenic crops into these countries raises several issues that have to be addressed as soon as possible. These range from the production of suitable transgenic varieties, through the various biosafety issues associated with their acceptance, release and stewardship to socio-economic structures needed to get these crops to the resource-poor farmers and the ultimate consumers. These issues will be discussed.

Towards engineering the small brown planthopper to be vector incompetent for the transmission of *Rice stripe virus.*

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Rice stripe virus (RSV) is an important pathogen of rice in China and Japan. The virus is transmitted in the persistent circulative manner by the small brown planthopper, Laodelphax striatellus. In this novel approach to controlling the virus, bacterial symbionts of the planthopper are being transformed to produce antibodies or proteins that inhibit the ability of the insect to transmit the virus. RSV is a tenuivirus with a genome comprising four segments; RNA 1 encodes a single protein and RNAs 2-4 each encode two proteins using an ambisense strategy. A Chinese strain of RSV has been characterised and the six open reading frames of RNAs 2-4 have been cloned and the gene products expressed in E. coli. Using antisera to these products, the expression of these genes in plants and insects has been studied and two (P20 and P24) have been selected as likely to be important in the insect vector. Single-chain antibodies are being raised to these two proteins and their effect, and those of oligopeptides, on virus transmission is being tested by injection into the vector. The symbiont that is to be used to express RSV antibodies in the planthopper is Wolbachia, which controls sexual compatibility of the insect. Techniques for transformation of Wolbachia are being developed together with suitable vectors. The role of Wolbachia in controlling sexual compatibility will help to drive the transgene through the insect population.

To obtain base-line information on the transmission of RSV by L. striatellus and background information for controlled releases of transgenic insects cage experiments have been conducted using non-transgenic insects. A double-cage design is being tested to determine if it will contain transgenic insects through the rice growing season.

Global and multidisciplinary approach to study the feasibility of introducing transgenic landraces of maize in Mexico aimed to help small rural communities

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One of the most widely claimed benefits of agricultural biotechnology has been its potential to substantially contribute to alleviate the problems of hunger and poverty in developing countries. In the case of Mexico, maize has always been recognized as a staple crop and its main role in the diet of the Mexican people is evident. However, maize is also extremely important in Mexican tradition and folklore. It has been suggested that the introduction of high-yielding maize, whether hybrids or transgenic, may displace the local varieties and landraces which are normally less productive, thus leading not only to loose valuable sources of germplasm, but also to the lose of tradition and cultural identity.

Mexico adopted a de facto moratorium to any release of transgenic maize with the idea of first obtaining reliable information on the possible effects of the release of these materials on biodiversity as a whole, on teosinte in particular, and at the social and economic level. Because open pollination is the common behavior in maize varieties and more than 80% of our farmers keep seed for planting year after year, the possibility exists for gene flow to wild relatives and landraces with a potential impact on the use and sustainable conservation of biodiversity in the specific area of the release. The potential problems in each region depend on the particular socioeconomic and environmental conditions that prevail, as well as the scale of use. Thus, two types of concerns can readily be identified, those directly involving teosinte and maize biodiversity, and those related to changing the habits or impoverishing even more the rural populations that traditionally grow the landraces.

The purpose of this project is precisely to obtain preliminary data without the use of transgenic materials, in order to be in a position to develop science-based environmental risk assessments and risk-benefit evaluations. The approach involves the simultaneous evaluation of Social, Biodiversity and Biotechnology Issues through the concerted efforts of social scientists, ecologists, environmental scientists and biotechnologists from different institutions working together with selected rural communities who will play a prominent role in the development of this project as well as in deciding whether or not transgenic maize may help them to improve their way of life.

Effects of Bacillus thuringiensis (Bt) corn on soil Folsomia fimetaria, Folsomia candida (Collembola), Hypoaspis aculeifer (Acarina) and Enchytraeus crypticus (Oligochaeta)

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The effects of the Cry1Ab toxin from Bacillus thuringiensis (corn variety Cascade Bt MON810 and DeKalb variety 618 Bt) were studied on survival and reproduction of the soil collembolan Folsomia fimetaria, Folsomia candida, the collembolan predator mite Hypoaspis aculeifer and enchytraeids Enchytraeus crypticus. The toxicity was observed in Petri dishes with plaster of Paris substrate and in a soil-litter microcosm toxicity test system on both single species and in a predator-prey interacting test system. In petri dishes, roots and leaves of young plants (three weeks) were fed to F. candida for 16 days, dried leaves of mature plants collected after harvest and artificial food (mixture of dried leaves with yeast) were fed to F. fimetaria for 21 days. In the single species soil test system, F. fimetaria and E. crypticus were exposed for 21 days to increasing concentrations of young Bt plant material, max. 40 g kg^{-1} soil, and increasing concentrations of dried mature Bt plant material, max. 10g kg^{-1} soil. H. aculeifer and its prey F. fimetaria were used in the predatory-prey two species test system. They were exposed to increasing concentrations of dried mature Bt plant material of max. 20 g kg^{-1} soil for 21 days. Cry1Ab toxin was analysed by ELISA (PSB 05500 Agdia). After 16 day exposure to young plant, the reproduction of F. candida fed Bt roots significantly reduced, while there was no statistic difference between the reproduction of F. candida fed Bt and non-Bt leaves. There was a weak significant reduction by 30% on the reproduction of F. fimetaria fed Bt corn in Petri dishes for 21 days. Likewise there was a weak significant reduction by 40% of the reproduction of H. aculeifer by Bt corn in amounts corresponding to 20 g plant material kg^{-1} soil in the two species soil-litter microcosm systems. There were no effects of Bt corn materials on the reproduction of F. fimetaria and E. crypticus in the single species soil-litter microcosms. No effects of Bt corn materials on mortality of all the 4 species were observed in all treatments. The tendency of effects of the Bt corn on the reproduction of F. fimetaria, F. candida and H. aculeifer is discussed.

Elimination of transgenes in plant cells catalysed by the transient expression of bacteriophage P1 recombinase

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The Cre-lox recombination system of bacteriophage P1 is a powerful tool for genetic manipulation both in vitro and in vivo. Cre recombinase catalyses the site-specific recombination between two lox sites and mediates intramolecular and intermolecular rearrangements. The functions of the loxP-Cre recombination system have been applied for the production of transgenic plants with antibiotic- or herbicide-resistance genes removed. Our strategy makes use of transient expression of the Cre recombinase by means of plant virus vectors.

Transgenic tobacco (*N. benthamiana*) plants containing two directly repeated lox sites flanking GFP reporter gene were constructed. The Cre recombinase gene was placed under the transcriptional control of a subgenomic promoter in a plant virus vector based on PVX (strain PVX 201). The first progeny of transformed tobacco plants was inoculated with DNA of PVX-Cre virus. Systemic symptoms appeared on the plants at 10-14 days post inoculation. After the appearance of virus symptoms, extracts of systematically infected leaves were tested for the presence of PVX coat protein and Cre recombinase protein. Immunoblot analysis showed that accumulation of the Cre protein coincided with accumulation of PVX. The efficiency of recombination was estimated from the exchange of GFP gene activity and confirmed by PCR. Primers designed to flank the lox sites of the lox-GFP construct were used in PCR analysis. All plants infected with PVX-Cre produced PCR products indicating that DNA rearrangement had occured consistent with site-specific excision of the GFP gene.

Two conclusions may be drawn from these experiments. First, the Cre-recombinase protein can be functionally expressed by a plant virus vector. Second, Cre-mediated excision of the GFP gene resulted in plant cells free of marker gene.

Molecular means to prevent transgene escape from GM plants

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In addition to well documented advantages, Genetically Modified Organisms also bring new challenges. As the technology is being more widely adopted, it is important to pay attention to mitigating the risks associated with GM plants. The most pronounced risk involves gene flow from transgenic plants to cultivated or wild relatives. To prevent this risk we must be able to control transgene escape at a highly reliable level. Several approaches have been attempted to prevent gene flow, which can be broadly classified into agronomic and molecular methods. After a short overview of the well-known techniques of male/female sterility, chloroplast transformation, 'terminator' technology, and 'mitigation' technology the authors will concentrate on following methods for prevention of transgene escape: 'Disrupter Gene' technology (Zenega), 'Repressible Lethal Gene' technology (DOW Agroscience Canada), Recoverable Block of Function (UniCrop) and Repressed Excision System (UniCrop). A comparative review of these technologies in view of reliability and convenience for use in agriculture will be presented.

Development of a recombinant mouse virus for
control of mice in Australia.

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Introduced house mice periodically increase to plague proportions over a large region of Eastern Australia, causing significant damage to grain crops. Current management of mouse populations is costly, inefficient and employs non-specific toxicants. To provide a safe, viable, cost-effective mouse control alternative in these regions, a mouse-specific virus commonly found in wild populations has been developed as a vector for the delivery of immune mediated sterility (immunocontraception). An Australian strain of murine cytomegalovirus (MCMV) containing the gene for mouse ZP3 (an outer-egg protein) has been engineered. It is a strong inducer of infertility, is mouse specific, and the immunocontraceptive effect is observed in mice already infected with non-recombinant MCMV strains. The development of such an agent for field release requires that a number of regulatory and environmental concerns are met. To address such concerns, both scientific and management strategies have been initiated to provide a path to Australian field release approval. While Australia has a national interest in the control of introduced pest species, the development of infertility-inducing viruses must also take into account international concerns including any potential non-target species effects and general issues relating to the use of GMOs. The continued development of MCMV-based immunocontraception provides a conceptual research model for the targeting of other species such as the rabbit and fox with/by similar fertility control methods. The planned strategic and innovative use of a GMO for the control of mouse plagues in Australia provides the grains industry with a viable alternate to existing control measures.

Gene Flow Analysis from rice into wild/weedy relatives in the Neo-Tropics: Morphological and phenological characterization of red rice

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Transgenic crops offer an alternative for introducing traits which can reduce the need for chemical pesticides and fertilizers, and improved agronomic traits, thus contributing to slow down the conversion of currently unused land, which bears areas of biological diversity, into agricultural lands. Hybridization between crops and their wild relatives sometimes brings genes into wild populations, occasionally resulting in the evolution of aggressive weeds and/ or endangerment of rare species. Transgenic crops may also result in similar outcomes. The likelihood of crop-to-wild hybridization depends on the out-cross rate, and on distance and direction between wild and crop populations. Cultivated rice, *O. sativa* L*.,* is an autogamous plant, with a low out crossing rate of 0-1%. Rice is an introduced species in the Neotropics from Africa and Asia but with wild/weedy relatives including wild native species in Central and South America. Hybridization can be expected within the genomic group that includes *O. sativa*, viz., the AA group. The wild relatives of AA genome, which are found in Central and South America and may hybridize with the rice crop, include *O. rufipogon* (AA, hybrid seed set 19% without and 73% with embryo rescue), and *O. glumaepatula* (AA, hybrid seed set 39% without embryo rescue). Red rice (*Oryza sativa* f. *spontanea*) is weedy rice highly competitive, which seeds shatters readily and possesses dormancy. In contrast to Asia where manual transplanting is still predominant, in tropical America direct seeding of red rice-contaminated seed source is common for a high proportion of rice farmers in Latin America, ensuring field re-infestations and making it one of the most serious weed problems in this region. There are indications that genes placed in cultivated varieties of rice may transfer quickly into red rice (1% to 52% hybridization rate). However, most of the hybridization rate estimates have been done under controlled experimental temperate conditions. This work is part of a project directed to analyze the gene flow from non-transgenic or transgenic rice into wild/weedy relatives in the Neotropics, and its effect(s) on the population genetic structure of the recipient species. As a first step for setting up the tools to assess gene flow from transgenic and non-transgenic rice into red rice under controlled confined field plots, and under local agricultural field conditions, a morphological and phenological diversity characterization was conducted from field specimens collected from farmer's fields in the main rice-cropping region of Colombia. Results were analyzed to identify the most likely potential candidate biotypes to serve as recipient of gene flow from the cultivated crop, identify indicators for the occurrence of previous gene flow from commercial varieties, and use this information to provide guidelines for a safe use of transgenic rice in the Neo-tropics.

Molecular characterization of rice and rice wild/ weedy relatives using microsatellites and their use to assess gene flow in the Neo-Tropics

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A careful assessment of potential impacts of gene flow from transgenic plants on population genetics of natural crop plant biodiversity is needed in other to design strategies for the safe and durable use of these crops in the Neo-tropics. This work is part of a project directed to analyze the gene flow from non-transgenic or transgenic rice into wild/weedy relatives in the Neotropics, and its effect(s) on the population genetic structure of the recipient species. The research will provide guidelines for evaluating the potential risks of using transgenic plants in the tropics, and describe potential areas of gene(s) flow. The information will contribute to improve the risk assessment procedures in the region, in particular for the partner countries Colombia and Costa Rica, which both rank among the countries with the highest biodiversity in the world. The current report summarizes the progress on setting up the use of microsatellites markers to assess gene flow from transgenic and non-transgenic rice into wild *Oryza* species and red rice under controlled confined field plots, and under local agricultural field conditions in Colombia.

A molecular genetic diversity analysis is being conducted in order to determine the genetic structure prior gene flow, and to select the best combinations of transgenic or non-transgenic rice, and wild/weedy populations to assess the gene flow. Crop/ wild/ weedy specific microsatellite markers were identified and selected, allowing the identification of hand-made hybrids from individual genotypes. This set of microsatellite is being used to characterize the genetic structure of the red rice population prior gene flow and to detect out-crossing rate in the field. Conditions are being optimized to detect 1% of out-crossing rate. The spatial distribution of alleles will be used to study local gene flow, including pollen dispersal distances. Microsatellite is used to trace crop-to wild/red rice gene flow and red rice/wild-to-crop hybridization rate under confined experimental settings as well as under natural conditions. Similar analyses could be used to assess transgenic-to non-transgenic variety gene flow.

Structure and quantity dynamic of arthropod communities in paddy fields of insect-resistant transgenic rice producing CpTI or CpTI+Bt

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Approved by the Safety Committee of Agricultural Transgenic Organisms, environmental release of insect-resistant transgenic rice producing CpTI or CpTI+Bt was conducted in Fujian Academy of Agricultural Sciences, Fuzhou, Fujian. Field surveys of arthropod communities in paddy fields were carried out from 2000 to 2001. The arthropod guilds were defined as phytophages, parasitoids, predators and neutral insects (aquatic mosquitoes and scavengers). According to systematic investigations, arthropod communities in the paddy fields consist of 107 species, including 36 species of phytophages, 4 species of neutral insects, 31 species of parasitoids, 9 species of predatory insects and 27 species of spiders. A total of arthropod species recorded was 101,102 and 104 for Minghui 86$^{CpTI+Bt}$, Minghui 86^{CpTI} and Minghui 86, respectively. The individuals in the same rice habitat were ranked : neutral insects > phytophages> spiders > parasitoids > predators before the grouting stage and phytophages> neutral insects > spiders > parasitoids > predators at later stages. There were more neutral insects in the transgenic rice fields. The ratios of individuals of neutral insects against natural enemies in the transgenic rice fields were different significantly from that in the original rice fields, and were 1.505, 1.345 and 0.950 on average in fields of Minghui 86$^{CpTI+Bt}$, Minghui 86^{CpTI} and Minghui 86, respectively. Those neutral insects might play an important role as predator's foods to maintain the predator's populations, especially at the early rice growth stage. There were different dominant pests in different rice habitats. Before the booting stage, the dominant pests were *Reiclia dorsalis* and *Nephotettix cincticeps* in the fields of transgenic rice lines, but *Cnaphalocrocis medinulsis*, *Scirpophaga incertalas*, *Reiclia dorsalis* and *Nephotettix cincticeps* in the fields of the corresponding non-transgenic line. After the heading stage, the dominant pests were *Nilaparavata Lugen*, *Reiclia dorsalis*, *Nephotettix cincticeps* for transgenic rice lines, but *Nilaparavata Lugen*, *Reiclia dorsalis*, *Scirpophaga incertalas*, *Nephotettix cincticeps* for the corresponding non-transgenic line. There were obvious difference in quantity dynamic of communities of pests and parasitoids between the transgenic and the corresponding non-transgenic rice fields.

The investigations of the compositions and the dynamics of the arthropod communities in the paddy field were not only one of the basic jobs in assessment of ecological impact of insect-resistant transgenic rice, but also the base for the integrated pests management in the transgenic rice fields. Based on the results mentioned above, we can conclude that Minghui 86$^{CpTI+Bt}$ and Minghui 86^{CpTI} can control effectively the populations of *Cnaphalocrocis medinulsis* and *Scirpophaga incertalas* in paddy fields, but other measures should be taken to control the populations of *Nilaparavata Lugen*, *Reiclia dorsalis*, *Nephotettix cincticeps*. Although the insect-resistant transgenic rice producing CpTI or CpTI+Bt had greatly changed the quantity dynamic of arthropod communities in paddy field, but no obvious differences in species composition were observed between the transgenic and non-transgenic rice lines.

Full-length and truncated ice-nucleating protein-based cell-surface display of green fluorescent protein in Escherichia coli

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The truncated ice nucleation protein InaK from Pseudomonas syringae KCTC1832 strain with only N-terminal domain (InaK-N), was newly achived as an anchoring motif to display the noninvasive reporter, green fluorescence protein (GFP), on Escherichia coli cell external surface. GFP expression levels and cell-surface localization efficiencies were compared to two other anchoring motifs with full-length InaK, or truncated InaK carried both N-terminal and C-terminal domains (InaK-NC). Whole-cell's fluorescence intensity of cells expressing InaK-N-GFP fusion was as much as 1.5-fold higher compared to cells with InaK-GFP fusion, and was approximately 40% compared to InaK-NC-GFP fusion. Measurements of cell external fractionations showed similarly the fluorescence intensity among three fusions, revealed the efficient cell-surface localization property of InaK-N-GFP fusion. We deduced the secretion signal for membrane translocation might exist in N-terminal primary sequence in InaK, and C-terminal might play an essential role to protect fusion proteins from proteolytic degradation when expressed in E. coli strains. The surface localization was further verified by pronase accessibility assay, immunolabeling analysis as well as immunofluorescence microscopic examination. Surface-displayed GFP system might be used in a variety of biological applications such as live vaccines development, constructing and screening protein libraries, whole cell bioconversion and biocatalyst, as well as environmental bioadsorbent development.

Key words: Ice-nucleating protein; cell-surface display; anchoring motif; green fluorescent protein

Evaluation of Food and Pollen Allergenicity of
Genetically Modifified Organism

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For the safety evaluation of genetically modified plants, the potential allergenicity of the newly introduced protein(s) has become a important issue. In 1996, the International Food Biotechnology Council and Allergy and Immunology Institute of the International Life Sciences Institute (IFBC/ILSI) presented a decision-tree approach to the evaluation of the potential allergenicity of the novel gene products. This allergy assessment strategy focuses on (1)the gene source, (2)the sequence similarity of newly introduced protein to known allergens, (3)the immunochemical binding of the newly introduced protein with IgE from the blood serum of individuals with known allergies to transferred genetic material and (4) the physicochemical and digestive properties of the newly introduced protein. The FAO/WHO decision tree in 2001 builds upon previous approaches to examining allergenicity, but also encompasses several additional strategies. On the basis of above mentioned and our work on recombinant allergen, a database for assessment of GMO allergenicity has been set up in our lab with the website of http://ambl.lsc.pku.edu.cn.

There is a total of 198 known major allergen sequences available in our allergen database. Generally speaking, obvious sequence similarity requires at least 8 consecutive identical amino acids, since the shortest peptide chain that can interact with T cells and activate allergic reactions needs at least eight or nine amino acids, meanwhile the epitopes to interact with IgE need longer peptide chains. Therefore, the method of searching eight consecutive identical amino acids is relatively reliable. With the development of genome and protein bioinformatics research, and the understanding of GMO food allergen attributes, the materials will accumulate quickly. It is therefore necessary to collect and organize such materials. Our database collected more than 20 food allergen epitope amino acid sequences, and newly-discovered food allergen amino acid sequences will be added timely.

It should be noted that when doing similarity comparisons, the default standard is that the eight-residue sequence should be identical. That is, if a protein has no eight residue identical sequence with any of the sequences in this database, then it should be considered that the protein being evaluated is not homologous with any of the sequences in the database. The users can also obtain the homology information of the protein being evaluated and the known allergen epitopes (usually 8 residues). For instance, if seven consecutive residues on the sequence being evaluated are identical with a known allergen epitope, then this protein is relatively similar to allergens. In some situations (say among a specific group of people), it can induce allergic reactions.

It is well known that cross-reactivity of allergen-specific antibodies was caused by similar amino acid sequence and/or similar structures of related proteins that share common epitopes. Some cross-reactive structures in pollen and food are known. So we extended our research to cross-reactive allergens of food and non-food sources, especially the airborne pollens. The recombinant allergens derived from peanut, walnut, ryegrass,even from house dust mite have been investigated in our laboratory. That would be of use in the standerization of evaluation of allergenicity by immunological assay.

Study of PCR-ELISA in the detection of genetically modified
organisms

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Screening of food using a polymerase chain reaction (PCR) for the presence of transgenic components, such as the widespread cauliflower mosaic virus 35S promoter and agrobacterium

tumefaciens nos terminator introduced into genetically modified organisms (GMOs), has become a routine method in modern food analysis. For the aim of developing a high throughput method suitable to automation we established a PCR-enzyme linked immunosorbent assay (PCR-ELISA). It is based on specific hybridization of a biotinylated PCR product with a digoxigenin-labelled probe, the label then serves in colorimetric immunodetection.

According to the characteristics of CaMV 35S promoter & T-nos terminator which have been used in genetically modified crops frequently, two pairs of primers and two pairs of corresponding digoxigenin-labelled probes were designed and synthesized. The CTAB method of extracting DNA from GMOs were established and optimized, then a PCR amplification of the target gene coupled with Liquid-phase hybridization or Solid-phase hybridization ELISA method suitable for screening of transgenic components 35S & nos was achieved. In Liquid-phase hybridization, 5'- biotinlabeled amplified PCR products were mixed uniformly with 5'- digoxinlabeled probe in liquid, then the hibridized products were captured on streptavidin-coated microplate; in Solid-phase hybridization, 5'- biotinlabeled amplified PCR products were captured with 5'-digoxinlabeled probe immobilized in streptavidin-coated microplate. The hibridized products were detected with alkaline phosphatase (AP) and p-nitrophenylphosphate (pNPP) in the same way, and no remarkable difference was noted between the rapid Liquid-phase hybridization and the steady Solid-phase hybridization.

With those fast and convenient PCR-ELISA methods we analyzed 4 samples, laborious blotting procedures and hazardous ethidium bromide in gel staining were avioded. The results showed that 2 soybean and 1 corn samples were positive, 1 corn samples were negative. The described methods enabled a simple, specific and accurate detection of genetically modified organisms and thus provide a useful tool for routine analysis of raw and processed food products.

Transgenic sweet potato with human lectoferrin gene mediated by *Agrobacterium tumefaciens*

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Sweet potato (Ipomoea batatas LAM.) is among the world's most important, versatile, and under exploited food crops, which produce the highest fresh yield of edible leaf and storage roots even in marginal environments among all food crops. Meanwhile we expect to find a productional plant bioreactor for producing foreign protein.

A cDNA fragment encoding human lactoferrin (hLF) was stably integrated into the genome of sweet potato CV 9403-4 by Agrobacterium tumefaciens-mediated transformation methods. We have engineered 4 constructs, containing the signal peptide from sweet potato sproramin or including the encoding region fused to human lactoferrin encoding cDNA, driven by sproramin promoter, the other two containing lactoferrin encoding cDNA driven by the cauliflower mosaic virus (CaMV) 35S tandem promoter or by sproramin promoter. All of them use neomycin phosphotransferase (NPT II) gene as select marker gene. *Agrobacterium* tumefaciens strain LBA4404 harboring the plasmids transferred sweet potato cv. 9403-4. Two *Agrobacterium tumefaciens* mediated gene transfer systems have been developed through the use of leaf or stem as explants. The first method is cutting leaves of plant *in vitro*, were inoculated and cultured on selective medium with 50mg/L kanamycin and 200mg/L cefotaxmine to induce resistant callus and regenerated plant via somatic embryogenesis, After 4months of selection, 78 kanamycin-resistant callus were obtained, from which some intact plants were formed. The later method, the difference is forming resistant buds directly from the cut site of stem when 1-naphthaleneacetic acid (NAA) is used in the selective medium. We have obtained 29 putative plants in total from the above two methods. The presence of human lactoferrin cDNA in the plant genome was detected by PCR and DNA hybridization.

Human lactoferrin (hLF) gene has been transferred to tobacco, tomato, and rice. The transgenic plants exhibited disease resistance and high quality. It is the first report to transfer the gene to sweet potato and appears to have a potential for generating transgenic sweet potatoes with useful agronomic traits, which are expected to express bioactive protein.

Criteria for Evaluating Biosafety Frameworks:
Objectives and Standards

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Globally the acres planted with biotechnology derived crops (agriculture biotechnology) have grown exponential over the last 7 years. Adoption of new technologies within the U.S. agricultural sector has resulted in sustained increases in agricultural productivity, contributed to economic growth, and ensured an abundance of food (Economic Research Service, USDA, AER 810 – May 2002). Rapid developments in plant genomics will enable even greater expansion. Critical to this greater expansion are appropriate national biosafety oversight systems. The challenge for these regulatory systems is to ensure that the agriculture biotechnology products meet appropriate safety standards but not unduly inhibit technology innovation.

To meet this challenge, national authorities are establishing regulatory oversight systems based upon the end product and use; novelty of the enhanced trait; and method by which the product was developed . These different approaches reflect the variety of national authorities used as a basis for the biosafety systems. Although the national approaches are different, they should all share common objectives and appropriate safety. The national approach should seek to be science-based regulatory systems that are comprehensive, commensurate, transparent, inclusive and predictable. This paper will explore some of the common objectives and standards and offer recommendations about "model" biosafety frameworks.

Cross pollination of GM corn in adjacent non-transgenic corn fields

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In farm scale field trials performed in 2000 and 2001 with herbicide resistant corn and non-transgenic corn the level of cross pollination was determined by a germination test of corn samples taken at various directions in order to estimate the impact of wind in different distances.

A transgenic corn plot of 1 ha was surrounded with 5 ha non-transgenic corn. In the non-transgenic corn field more than hundred samples were taken. Around the transgenic plot at least six rows in doubling distances were determined, vovering a range of 3 m to 49.5 m away from the transgenic plot in the first year, and to 100 m downwind in the second year. In each row, sixteen sample points were evenly distributed.

Cross pollination from glyphosate resistant corn was evaluated by a highly reproducible germination test. After corn kernels were grown in the greenhouse on filter paper, glyphosate was applied at second leaf stage. Surviving plants were addressed as transgenic. Per sample point at least 2,500 kernels were tested.

Outcrossing rates strongly decay within the first few metres of the non-transgenic field. The level of cross pollination was less than 1% after 10 m upwind and after 50 m downwind. Downwind outcrossing was detected on a very low level up to 100 m.

The results provide useful data for mathematically modelling the gene flow as well as for the ongoing threshold discussion.

Cooperation among governments and technology developers to reduce the potential presence of non-approved biotechnology food and feed products

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The potential for the release of biotechnology-derived food and feed products prior to attaining regulatory approval is an issue of concern among government officials, food importers and exporters and biotechnology product developers. The basis for these concerns includes the introduction of new and novel products, the recent adoption of regulatory frameworks in many countries, and increased public scrutiny of the safety and regulatory assessment processes. The potential reasons for an unapproved product being encountered include gene movement through pollen flow, inadequate segregation of grains and the lack of regulatory approvals for a new product. In order to minimize the potential for adventitious presence, cooperation is necessary among the companies developing these products and the government agencies responsible for regulatory oversight of the products. Institutions involved in product development have a responsibility to adhere to stringent stewardship standards to minimize adventitious presence and an obligation to provide timely, high quality applications for the necessary government approvals. Maintaining a science-based approach to safety assessments will facilitate the technology developer's ability to provide high quality data and studies that meet the necessary regulatory requirements. Cooperation among governments to harmonize criteria for safety assessments will help to facilitate the global approval of new products. Clearly, the common goal of industry, government and the public is to ensure that the safety of new biotechnology-derived food products has been adequately evaluated before their release to the market.

Exploitation of genetically modified *Pseudomonas* for industrial ecology applications

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Rhizosphere competent fluorescent pseudomonads are ideal candidates for utilization as biocontrol and bioremediation inoculants. Direct links between biocontrol efficacy and production of anti-microbial compounds have emerged *via* utilization of recombinant DNA technology. Production of antimicrobial 2,4-diacetylphloroglucinol (Phl) is the central mechanism utilized by *P. fluorescens* F113 in biocontrol. Environmental and microbial signals modulate regulatory processes governing production of Phl at the functional genomics level. Innovative design strategies based on reprogramming regulatory mechanisms *via* manipulation of these signals can be employed to improve biocontrol efficacy of *Pseudomonas* inoculants. Further more *P. fluorescens* F113 can be used as an ideal carrier strain for polychlorinated biphenyl (PCB) degradative genes facilitating development of novel rhizoremediation bioinoculants for controlled degradation in contaminated biosystems. However, public concerns as to the biosafety of genetically modified bacterial strains in the environment must be considered. Developments in molecular microbial ecology have facilitated assessment as to the impact of bacterial inoculants on soil-borne non-target microbial communities. Plant root exudates together with microbial signals can regulate the composition of indigenous microbial communities in the soil. *P. fluorescens* F113 wild type strain had no significant impact on key microbiota ranging from arbuscular mycorrhizal fungi to fluorescent pseudomonads. Genetically modified derivatives of *P. fluorescens* F113 also had no significant influence on indigenous microbiota. An understanding of signalling mechanisms occurring in plant-microbial interactions using a functional genomic approach has the potential to improve biosafety of genetically modified inoculants and facilitate registration processes for utilization of plant microbial protection products in industrial ecological applications such as biocontrol and bioremediation

Field Releases of Genetically Modified Organisms and Regulatory

Biosafety Framework in Brazil

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Brazil, member of the Convention on Biological Diversity, has adopted national policies toward the conservation of biodiversity and the sustainable use of genetic resources. Significant government investment has been made on biotechnology research and capacity building programs. Since 1995, a national legal Biosafety Framework has been in place, setting the standards for controlling the construction, cultivation, manipulation, transportation, marketing, consumption, release and disposal of Genetically Modified Organisms (GMO), with the objective of protecting the life and health of humans, animals, plants and the environment. All activities with GMO are subject to regulation and they are applied to institutions (165), authorized by a National Technical Biosafety Committee (CTNBio). The biosafety framework includes a harmonized approach with other legal instruments emanated by competent Ministries. CTNBio, part of the structure of the Ministry of Science and Technology, is composed by 36 members, with multidisciplinary representations from: the scientific community working on biotechnology, government (Science and Technology, Health, Environment, Education, Foreign Affairs and Agriculture), consumers and workers health agencies and biotechnology business sector. CTNBio is responsible to propose the Biosafety Policy, the Code of Ethics on genetic manipulation, determine GMO risk levels and environmental studies. Over 1000 petitions have been approved for field releases of genetically modified (GM) plants, after an extensive case-by-case risk assessment procedure. The field releases of GM plants were: corn (85% of total releases), soya (7%), cotton (5%), sugarcane (2%), beans, Eucalyptus, potato, rice, papaya and tobbaco (1%). The main traits inserted in GM plants were herbicide tolerance (HT) and insect resistance (IR), the combination (HT+IR) and virus resistance. To date, no official commercial GM crops have been cultivated in Brazil. The petition for commercialization of glyphosate tolerant soybean, approved by CTNBio (1998) with a requirement to perform a 5 years environmental monitoring, has been questioned in justice. Four petitions to commercialize GM corn (IR, HT and IR+HT) are under evaluation. GM crops have been under severe scrutiny in Brazil. Food products with 4% or more GM derived content are legally subject to labeling. In contrast, recombinant products applied to health and industry are well accepted by the consumers. Governmental concerted actions are in progress to harmonize the legislation for licensing GM products. The stage has been set for the future development of biotechnology in Brazil.

Recombinase-directed transgene placement

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The exact placement of foreign DNA into the plant genome produces transgenes with greater structural fidelity and faithful expression. Recombinase-mediated site-specific integration has been reported for several plant species, including rice and maize. The next challenge will be to develop an integrated strategy to stack and translocate DNA. Being able to append new DNA sequentially to a target site permits the continual use of a previously characterized chromosome location, which justifies the initial investment costs in identifying favorable chromosome targets. Stacking transgenic traits at a limited number of target sites is also preferable to scattering transgenes all over the genome, as the clustering of transgenes expedites the introgression of bundled traits to elite cultivars, through a process mediated by the translocation of transgenes from one chromosome to another.

A tiered approach to risk assessment of virus resistance traits based on studies in wild brassicas in southern England

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One of the most frequently raised concerns about the introduction of genetically modified crops is that transgenes may be transferred to wild relatives, resulting either in the disruption of natural patterns of genetic diversity by "pollution" of species gene pools or in the addition of traits which may cause wild plants to become more invasive. This contribution will examine a tiered approach towards the necessary risk assessment.

Our studies on virus distribution (six viruses) and fitness impacts on wild/ naturalised *Brassica* species (*B. oleracea* , *B. rapa* and *B. nigra*) at six sites in Southern England showed that even when congeneric, viruses can have very different impacts - even among populations of the same species. Such results raise important questions concerning the approaches to risk assessment before planned release of genetically modified crop plants with "obtained" virus tolerance.

Our glasshouse-based pathogenicity tests showed that absence of a virus in a 'snapshot' survey in the field cannot be taken as meaning that there is no risk of consequential ecological release from that natural constraint following introgression of a resistance trait active against that virus.

Generic risk assessment may not be possible but we will discuss the tiered approach (like that used for Agrochemicals) that we are developing for the assessments of impacts of "obtained" disease resistance traits (genes).

For example, can contained tests of virus pathogenicity be usefully extended to the determination of whether or not plants will become infected in the field and, if so, what will be the impact of that infection. Is this an appropriate stage to determine the cost of resistance and can such knowledge be a basis for predictions about population dynamics?

Detection of Genetically Modified Roudup-reay and hosphinothricin - tolerant Rapeseeds by PCR

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With the presence of GMOs on the international market, the safety issues have attracted more and more public attention all over the world. As a result, many countries have been trying to formulate methods to detect GMOs. Roundup-ready and Phosphinothricin-tolerant Rapeseeds are two main genetically modified rapeseeds in the world. Over the past few years, a good quantity of transgenic rapeseed has been imported in China. Hence, we have established the qualitive PCR methods for detecting the foreign CaMV 35S Promoter, FMV 35 Promoter, NOS Terminator, NPTII, Modified CP4-EPSPS, Modified GOX, BAR, PAT, BARNASE, BARSTAR genes in GM Roundup-ready and Phosphinothricin-tolerant rapeseed, We also established a quantitative PCR method for GM Roundup-ready rapeseed by detecting exogenous GOX and endogenous PE3-PEPCase genes, and these methods have been confirmed by several prominent Chinese academic institutes, and applied to detect transgenic rapeseed in China. Currently, we are refining the said methods, which will be adopted as the Chinese national standard in the near future.

Detection of genetic modified soybean in Miso (bean paste) and Chou-Tou-Fu (Fetid bean curd) by real-time PCR

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In this study, we used the real-time polymerase chain reaction for detection of the transgenic component of Roundup Ready soybean (RRS) derivative food, Miso (bean paste) and Chou-Tou-Fu (Fetid bean curd).

We extracted the genomic DNA from Miso during fermentation processe, and also from Chou-Tou-Fu that was heat-treated by three different ways by CTAB method. The real-time PCR detection of DNA sequences present in RRS was undertaken using the ABI 5700 detection systems in combination with detection chemical SYBR Green I fluorescent dye. The ratio of transgene to soybean endogenous gene indicates the samples' transgene content and the variation of trangene content in fermentation processed Miso.

Our results revealed that the transgenic components of Miso decreased gradually until the 120th day of fermentation process, from then on, the 35S promoter can't be detected steady. The PCR tests which used primers specific for *lec* gene revealed that genomic DNA of Miso was degraded too seriously after 120th day of fermentation process. The fermentation process of Chou-Tou-Fu was short so the DNA does not degrade seriously. The destruction of Chou-Tou-Fu DNA by three different heat-treatments degraded more seriously than fresh Chou-Tou-Fu, but still could be detected.

New Substances Notification Regulations - Animate Products of Biotechnology

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As of September 1, 1997, certain new biotechnology products, namely organisms, microorganisms and products of microorganisms (biochemicals and biopolymers), have been regulated under the New Substances Notification Regulations (NSNR) of the Canadian Environmental Protection Act (CEPA). Products for uses regulated under other federal Acts are exempted from notification under CEPA if they are listed in Schedule 4 of CEPA, 1999. Biotechnology products subject to the CEPA regulations must be notified to Environment Canada by the importer or manufacturer. The information provided is assessed by both Environment Canada and Health Canada to determine whether the product will or may have adverse effects on the environment or human health. This poster will describe what products are subject to the CEPA regulations, the notification process and the risk assessment process.

Field trials, the permitting process, comments and risks

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We detail experiences in the process of designing and preparing experiments, obtaining permits and the perceived and potential risks associated with trial releases of genetically modified/transformed arthropods. What was first proposed as a free release was modified to an environmental release in very large field cages. The insects to be released were transformed with a *piggyBac* transposable element encoding the fluorescent protein EGFP. Biological characteristics of the transformed strain are detailed. Descriptions and responses to the comments and criticisms received during the public commentary and comment period associated with obtaining the release permit are detailed and analyzed. Certain of the critiques provided during the permitting process are summarized and discussed, as are our responses to those critiques. Discussion is made of some perceived hazards and estimates of risk associated with such a release along with a discussion of statistical analysis.

Evaluation of Genetically Modified Bacillus thuringiensis for Safe Release in Environment: Green Fluorescent Protein as Facile reporter

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Green fluorescence protein (GFP) has become popularity in recent years as a versatile reporter in biological researches. As a noninvasive fluorescent marker in living cells, it allows for wide range of applications where it may function as a cell lineage tracer, reporter of gene expression, or as a measure of protein-protein interactions, therefore it could play an important role in evaluation on safe release of genetically engineered *Bacillus thuringiensis* in natural environment. In the present study, GFP's encoding sequence was first attempted to construct two kinds of chimeric proteins which expressed under the control of *cry3A* promoter or *btI-btII* promoter of *B. thuringiensis*. Recombinant plasmid pGFPExpA contains *cry3Apro-gfp* fusion gene, and pGFPExpB contains *BtI-BtIIpro-gfp* fusion gene, were transferred into *Escherichia coli* and plasmid-free *Bacillus thuringiensis* strain 4Q7, respectively. The *btI-btII* promoter was found to drive *gfp*'s expression strongly either in *B. thuringiensis* or in *E. coli* strain. However, *cry3A* promoter can not drive *gfp*'s expression in *E. coli*, and the expression in *B. thuringiensis* strain is also much weaker than that driven by *btI-btII* promoter. RT-PCR strategy was used to detect *gfp*'s expression at transcriptional level, as a result of it, *gfp*'s expression occurred apparently at transcriptional level and at early stage of sporulation when driven by *cry3A* promoter, compared to it occurred at early and late stage of sporulation when driven by *btI-btII* promoter. Surprisingly, the fluorescent microscopic examination showed that cells carrying *cry3Apro-gfp* fusion emitted green fluorescence before sporulation while the cells containing *btI-btIIpro-gfp* fusion gene did before early stage of sporulation. We deduce that *gfp*'s expression might undergo different regulation in *B. thuringiensis* cells.

Studies on GFP expression in genetically modified *B. thuringiensis* with GFP as a sensitive reporter was expected to release in field (i) to assess the ability to survive and disseminate in the environment; (ii) to monitor the potential transfer of modified pesticidal toxin genes to related indigenuous microbial, plant or animal population at various levels; and (iii) to determine the impact of deliberately released GMM on indigenuous microbial communities that inhabit the soil.

Keywords, Green fluorescent protein, *Bacillus thuringiensis*, safe release, expression, evaluation

Concerns about the effect of transgene introgression in maize landraces and teosinte

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Mesoamerica is a region where plant domestication occurred about 10,000 years ago. Most scientists currently agree that maize was domesticated in Mexico and descended from an annual species of teosinte (*Zea mays* ssp. *parviglumis*). Costs and benefits of transgenic crops for Mexican agriculture have been the subject in several forums for the past ten years. Today, an intense debate continues, and this issue has been raised questions about the extent of the knowledge available about the long-term effects of this technology on biodiversity in centers of origin of cultivated plants, already threatened by habitat alteration. This paper presents data related to the importance of *Zea* species in Mexico and information about ongoing research that may help to conduct a scientific risk assessment for transgenic maize technology adoption. Great advances have been made in knowledge of the natural distribution of teosinte in Mexico, more gene-flow studies between maize and teosinte have been completed and more knowledge about genetic diversity, genetic incompatibility systems, and about insects that affect teosinte and maize are available. The most important concerns that have influenced the debate about the eventual release of transgenic maize in Mexico have been questions about the potential of transgenic maize to modify genetic diversity of landraces and their quality as food. Another set of concerns is related to the risks associated to transgene escape and its dispersal into teosinte species and potentially enhancing their ability to survive or compete with another species. As a result of several national and international conferences, consensus exists indicating that current knowledge in Mexico is insufficient for assessing risks and benefits of transgenic maize. It is critical to develop a system for risk assessment within the context of current practices and threats to understand the impact, if any, from modern varieties (conventional and transgenic) on genetic diversity of landraces and teosinte.

Minimizing transgenic DNA while maximizing function: a new cluster project funded by the German Ministry for Education & Research

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One of the key topics in today's discussions about genetic engineering in agriculture is the next generation of transgenic plants which are suitable for the EU market. Their outstanding characteristics:

- They are resistant to pathogens, adapted to the growing site, and of high yield.
- Transgenic sequences are minimised to ensure function. Resistance to antibiotics and other sequences which are no longer of use have been eliminated.
- Expression of transgenic traits is limited to the tissues and to the conditions where they are needed. The transgene should be expressed according to what is a biological must.
- Production, testing, and selling of new transgenic plants is effected according to the consumers' demands, offering as much transparency as possible.

There are available strategies in the design of GM plants, which can be considered best practice to reduce the identified risks of GM plants and to avoid some unidentified risks.[3]

Three principle ways can achieve this:

- ♦ Avoid or minimise the inclusion of superfluous transgenes or sequences;
- ♦ Avoid or minimise superfluous expression of the transgene;
- ♦ Avoid or minimise the dispersal of transgenes in the environment.

Combined efforts made by scientists, industrial companies, and regulating authorities are necessary to realise the next generation of transgenic plants which are suitable for the EU market. One step towards this objective was the "Biosafety Research and Monitoring" announcement by the German Ministry for Education and Research (BMBF). In April 2001 they started funding scientific investigations which, among others, deal with the following problems:

- Establishment of new strategies to limit transferred gene sequences to what is necessary to assure functionability: Development of alternatives to the marker genes available for the selection of genetically modified plants; Design of new strategies for the elimination of gene sequences which became void after successful selection; Development of optimised binary vectors to generate transgenic plants free from unwanted sequences;
- Development of methods for sequence-specific integration of transgenes into the plant genome and for *in situ* modification of plant genes;
- Limitation of spreadability of transgenes.

It is the aim of the BMBF-funded cluster project "Targetted transfer of minimised transgene sequences with optimised function" to develop new strategies covering the necessary range of different approaches and/or to test them for their applicability. The wide range of approaches is required since one cannot expect that a general solution for minimising transgene sequences while maintaining optimum function will be found.

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[3] ACRE March 2001, Guidance on principles of best practice in the design of genetically modified plants (www.defra.gov.uk/environment/acre/bestprac/guidance/index.htm)

Direct and indirect effects of herbicide resistance on biodiversity depending on the cropping system.

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Diverse test results are analyzed along the question whether environmental benefits can be achieved by the herbicide resistance technique. Thereby the toxicological effects and amounts or frequencies of applications as well as the effects of changes in farming practice are described and diskussed.

Long-term and large scale field tests with different weed control methods and agricultural vegetation surveys are used in order to compare biodiversity effects of different weed control strategies. Research results from agricultural sciences, plant sociology, nature conservation and biosafety research normally discussed separately are covered.

A great variation of biodiversity (baselines) has been found between regions and locations in Europe. Biodiversity effects of weed control do not appear quickly but on the long run. The cropping history and the loss of beneficial plant species invading from the vicinity often compensate effects of a beneficial actual farming practice. Prospects for an appropriate management of species in agriculture are pointed out.

Furthermore particular economic and ecological concerns for multifunctional European agriculture in contrast to American agriculture are identified.

Safety Assessment of Genically Modified *Bacillus thuringiensis* WG-001 on Environment

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For evaluating the variability of survival and dissemination of genically modified *Bacillus thuringiensis* WG-001 that marked with insecticidal gene *cry1Ac*, the GMM WG-001 was applied into the environment as a foliar spray on cotton at Baoding city, Hebei province. Aerial dispersal through airstream from the spray site was monitored with 3.5-cm-diameter petri dishes that placed on the ground in a spokelike formation around the experimental filed at the distance of 5m, 10m, 20m, 25m, 30m, 35m, 40m. Concentration of WG-001 sprayed on cotton leaves was determined by plate counts of serially diluted bacterial suspensions. We found fifteen-meter is the maximum dispersal distance in a downwind direction and five-meter is the minimum distance in a upwind direction. The maximum bacterial concentration on cotton leaves was 2.17×10^6 cfu/g fresh weigh as soon as we had sprayed the WG-001 in the experiment field. After 34 days, we couldn't detect the viable WG-001 on cotton leaves except for only one sample site where the bacterial concentration is 3.46×10^6 cfu/g fresh weigh. At the same time, to evaluate the impact of field release GMM WG-001 on indigenous microbial community that inhabit in soil, soil samples were taken to a depth of 3 5cm in different sites from the experimental field. 10-fold series dilution soil suspension was prepared and aliquots of appropriate dilutions were plated on different media for quantitative analysis of the total population of fungi and bacteria.

Meanwhile, we have constructed recombinant plasmid which contains *cry1Ac-gfp* fusion gene so as to monitor horizontal transfer of modified pesticidal toxin genes to related indigenous microbial populations, plant or animal population at various levels. All of relative researches are in process.

Keywords, GMM, Bacillus thuringiensis, aerial dispersal, survival, gene's horizontal transfer

Transparency in research - an innovative approach for communication on biosafety research

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A large number of scientific projects have been carried out since 1987 as part of the biosafety research activities sponsored by the BMBF (German Federal Ministry of Education and Research). However, the projects themselves and their results have hardly any place in public awareness as whole, and for this reason they play only a subordinate role in the public debate about plant gene technology. To close the gap, communication management is being called in for the first time to support a BMBF-sponsored biosafety research programme. The central task of this sub-project will be to link the programme and its individual projects with the public controversy on *gene technology in agriculture* with the aim of achieving widespread dissemination of the relevant information at all levels of society. Information on the projects must be freely accessible and presented in such a way as to arouse people's curiosity. In this way, citizens will gradually come to see that safety research is a constructive process with open results. It is also intended that the themes should be placed in the overall context of practical agriculture and breeding and not merely presented in isolation.

Communication management on biosafety research in cooperation with joint research projects provides an improved information status within the public, a better dialogue between society and economy for linking actors as well as a platform for public interacting. Communication management does not aim at creating acceptance on plant gene technology - as it cannot replace public discussion processes.

To realise this, an internet portal has been set up under the address www.biosicherheit.de. Users here have free access to a database in which they can retrieve information on the objectives and methods of the 40 associated projects, together with the results of their activities. In future, the database will be extended successively to include not only ongoing activities but also completed BMBF projects and selected international research activities. On top of this, special portals have been set up for all interconnected themes, in each of which an introduction is given in readily understandable terms to the general subject matter of the particular line of research, supported by portraits of individual projects and further information on the full background context. The texts were prepared journalistically and are accompanied by diagram illustrations for better understanding of the contents. Special information and working materials, suitable for use in biology and general topics classes, have been designed for teachers and pupils.

This information and education offer is supplemented by up-to-date reports on the public debate, an email subscriber scheme, a telephone hotline, a comprehensive online lexicon, and a moderated interactive discussion forum. A protected web communication forum has been set up for project scientists.

In its aim of making complex scientific issues available for discussion and controversy on a wide social basis, this project is unique throughout Europe and can be seen as a communication model for dealing with other complex subject areas.

Influences of T4-lysozyme producing potato plants on the endophytic fungi of the roots studied by a classical and a molecular approach

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T4-lysozyme expressed in potato plants seems to be a promising strategy to enhance the plants` resistance against phytopathogenic bacteria like Erwinia carotovora. However, lysozyme has unspecific effects on other bacteria and on fungi. The monitoring of bacterial and fungal rhizosphere communities of transgenic and non-transgenic plants with a cultivation-independent molecular fingerprinting technique (PCR-DGGE) revealed in no cases a plant line dependent influence.

The lysozyme is secreted into the apoplastic space where its concentration should be higher than in the rhizosphere. Therefore, the influence on endophytes which live inside of the apoplast could be stronger than that on the microorganisms in the rhizosphere. Endophytic fungi were isolated from roots of the parental and a transgenic line after surface sterilisation. The isolates were cultivated, identified and characterised by molecular methods. Here significant differences between the parental and the transgenic line could be found: the roots of the parental line were colonised with Verticillium dahliae around three times higher than those of the transgenic line. Additionally the surface sterilised roots were examined by PCR-DGGE. Fingerprints of the endophytic fungi could be shown for the first time. To associate the bands to fungal isolates clones were generated and sequenced. The results of the two methods of investigation are compared and discussed.

Effects of T4-Lysozyme producing Potato Plants on the composition of bacterial and fungal rhizosphere communities studied by molecular fingerprints

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T4-lysozyme expressed in potato plants seems to be a promising strategy to enhance the plants' resistance against phytopatho-genic bacteria. However, due to the non-specific effects of T4-lysozyme on bacteria and fungi it is important to consider potential effects on the populations of plant-associated microorganisms.

With a cultivation-independent molecular fingerprinting technique (PCR-DGGE of the 16/18S rDNA fragments) the microbial communities of the rhizosphere of field-grown non-transgenic and transgenic plant lines were monitored over two consecutive years. The bacterial community patterns varied little between the rhizospheres of the five plant lines. Differences between the plant lines at the same sampling time were much smaller than seasonal fluctuations. Even the use of group-specific primers (α-, β-*Proteobacteria*, *Actinomycetales*, *Pseudomonas* species) to enhance the detection sensitivity did not reveal an effect of T4-lysozyme on these groups of bacteria. In the pattern of β-*Proteobacteria* of flowering potato plants in 1999 two additional bands were visible for all plant lines which could not be detected in the rhizosphere of flowering potato plants in 2000. This shows the need to perform such investigations during several consecutive years.

The patterns of the fungal communities were somewhat more variable, perhaps due to the fungal distribution in the habitat. Again, the seasonal differences were more pronounced than those between the plant lines.

Effects of transgenic potatoes with a modified starch composition on the structural and functional diversity of soil and rhizosphere microorganisms

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Effects that transgenic potatoes with a modified starch composition may have on the soil and rhizosphere microflora were investigated by traditional cultivation and cultivation-independent molecular fingerprinting methods.

Microbial communities of field-grown transgenic and parental lines, and in addition of another potato variety were compared. The dynamics of bacterial communities were followed by denaturing gradient gel electrophoresis analysis of 16S rDNA fragments amplified from total community DNA extracts (PCR-DGGE) at different time points over the growing season. While in bacterial DGGE patterns a clear difference of the bacterial community of soil and rhizosphere became visible, no significant differences between the transgenic potato and the parental line could be detected. On the other hand, an influence of the potato variety on the bacterial community was observed. Additionally, group-specific primers (alpha-, beta-proteobacteria, pseudomonas and actinomycetes) were used to increase the detection sensitivity. Only the *Pseudomonas*-patterns indicated differences between the transgenic and the parental lines. Direct cloning and sequencing revealed that dominant representatives of the rhizosphere bacteria belonged to two phylogenetic groups: the gamma-proteobacteria (*Enterobacter amnigenus*, *Pseudomonas* spec., *Xanthomonas albilineans*) and the beta-proteobacteria (*Herbaspirillum frisingensis*, *Variovorax paradoxus*, *Janthinobacterium lividum*).

In parallel, dominant bacteria were isolated from R2A and identified by FAME. Furthermore, the isolates were tested for their ability to degrade starch. The analysis showed a higher proportion of starch degrading bacteria in the rhizosphere and soil of the transgenic potato line in comparison to the wild-type line.

Ecological effects of pest resistance genes that disperse into weed populations.

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Gene flow from transgenic crops to cultivated or free-living plants has been the subject of much recent research, but little is known about the ecological consequences of this process. Here we focus on the question of whether transgenes that confer resistance to herbivores or diseases are likely to affect the fitness and population dynamics of free-living plants. When pest resistance genes occur at high frequencies in free-living populations, these traits could also affect non-target organisms and ecological communities (e.g., effects of *Bt* toxins). An important first step in this research is to determine whether specific transgenes confer a strong fitness benefit on wild or weedy relatives of crop plants. For many resistance traits and species, the fitness consequences of particular resistance genes may be negligible. However, our studies of wild sunflowers (*Helianthus annuus*) show that a *Bt* gene for lepidopteran resistance can be associated with reduced herbivory and enhanced fecundity under natural levels of insect pressure. We did not detect any fitness costs of this transgene. Once it is known that resistance genes can enhance the fitness of wild or weedy plants, further studies are needed to assess whether these populations could become more widespread or invasive. In general, little empirical information is available about the extent to which various herbivores and diseases limit populations of wild or weedy relatives of crop plants. Due to the difficult, long-term nature of research on plant population dynamics, we recommend fitness studies as a key element in assessing the ecological effects of pest resistance genes. From a regulatory standpoint, it is also useful to examine whether new transgenic constructs could have greater ecological effects than ongoing gene flow involving nontransgenic resistance traits.

Key words: crop-wild hybridization, gene flow, transgenic resistance, herbivory, disease, fitness effects, population-level effects, ecological consequences of gene flow, *Bacillus thuringiensis* (*Bt*)

Release and Risk assessment of GMOs in the Italian environment: an overview

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The aim of our work is to give an overview of the present situation in Italy regarding the release of Genetically Modified Organisms (GMOs) in the environment and the related biosafety studies.

In the European Community the deliberate release of GMOs in the environment is regulated by the Directive 90/220/EEC that will be repealed the 17th of October 2002 by the Directive 2001/18/EC. In Italy, the Ministry of Health authorizes and regulates the release of GMOs in the environment operating through the Interdepartmental Commission for the Risk Evaluation (CIV) composed by representatives of research centers and different ministries (Environment, Agricolture and others). The main role of CIV is to evaluate the notifications submitted and to authorize the related field trials to ensure the correct risk assessment. Since 1992 CIV has permitted 289 enviromental releases of 273 GM plants and of 16 other GM organisms. Among these 289 releases, 88 were presented by public institutions and 201 by private companies.

In our work we will illustrate the 9 field trials that have been authorized in 2002 and describe two national projects on Biosafety that have been approved recently. The first project (SAFE) coordinated by the Società Consortile Metapontum Agrobios, will verify the safety of food derived from GM Plants and will assess the impacts of these plants in the mediterranean environment. The second project, coordinated by Università di Milano, will evaluate all environmental risks correlated with the release of GM organisms including inferior and superior plants, microorganisms and animals, will produce Guide Lines for the complete risk evaluation.

In the future our objective is to create a database containing all information and scientific data available on risk assessment and, using genetic algorithms, to calculate the potential impacts of GM plants in the environment. This will be useful to establish post release monitoring plans and to obtain a correct and quick risk management, in compliance with the Directive 2001/18/EC.

Minimizing Transgenic Pollen Dispersal Now

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The cultivation of genetically modified (GM) crop varieties has raised public concern and political debate in many countries as the dispersal of GM pollen can contaminate non-GM crop plants on adjacent fields with grave consequences for the product acceptance or the seed purity and genes can be transferred by GM pollen to related weed species.

W could show recently that the problems linked to the release of GM pollen from maize and rape seed can be reduced or even eliminated by existing conventional breeding methodology. This can be achieved by growing male sterile GM plants in a mixture with male fertile non-GM plants, which act as pollen donors for the GM plants. That this is already feasible now is demonstrated by the fact that male sterile hybrids of rapeseed in Europe and oil maize in the USA are already being successfully cultivated in mixtures containing 20% or less male fertile pollinator varieties, the latter being well able to pollinate the whole field. Therefore it should be feasible to convert GM maize and rapeseed for such mixtures wherever and whenever GM pollen dispersal is a problem. We are confident that this is a way to assist coexistence of farming systems which rely on agronomic benefits of GM crops or which have to produce strictly GM free products.

Evaluation of control efficacy and biosafety of genetically modified *Helicoverpa armigera* nucleopolyhedrovirus

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Cotton is host to several species of heliothine moths, whose larvae can cause devastating crop losses and trigger therefore considerable investments in crop protection in the form of crop breeding (Bt-based molecular resistance), frequent pesticide sprays, and the application of biological agents. A host-specific nucleopolyhedroviruses, *Helicoverpa armigera* nucleopolyhedrovirus (HaSNPV), has been developed as a commercial biopesticide to control the pest in China. To improve its insecticidal properties, HaSNPV has been genetically modified by deletion of the ecdysteroid UDP-glucosyltransferase (*egt*) gene from its genome and insertion of an insect-selective scorpion toxin (AaIT) gene at the *egt* gene site. In laboratory bioassay, the virulence of these recombinants is similar to the wild-type HaSNPV. The acting speeds of the recombinants were significantly quicker than that at of wild-type HaSNPV. Field release experiments indicated that recombinants carrying a toxin gene provided a distinguishable better protection of cotton bolls from damage of bollworm than wild-type virus.

For the aim of development of this genetically modified viruses as commercial insecticides, following experiments related the biosafety are carrying out: Toxicity, allergic response and pathogenicity against experimental animals; impact on nontarget parasitoid and predator; spread and persistence in the environment; competitive ability with the wild-type virus, possibility of shift of exogenous gene to adjacent organism.

Slovak regulation and international cooperation in biosafety isues

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Slovak Republic has to create and adapt a number of acts and regulations to be compatible with European Union directives. One of the most important group of standards represents environment and health protection including safety in biotechnology. On the February the 19th Slovakia adopted a new Act on using of genetic technologies and genetically modified organisms (Act No. 151/2002). There is fully defined the regulation of all steps concerning the handing, utilization, transport of GMO, risk assessment and management issues and the relevant national authorities competence. The first experience will be analysed including the cooperation with scientific institutions and with different stakeholders.

Slovakia participates on many regional and international activities oriented on biosafety. In cooperation with all countries of the Central and Eastern Europe there are every year starting from 1995 regional conference organized helping to solve problems and support further progress in field of biosafety. We have participated on the Matra project "Implementation of national biosafety frameworks in preaccession countries" if CEE, What fairly helped us by the elaboration of the Gene Act. Presently is under processing the UNEP-GEF project on National Biosafety Frameworks.

Recombinant DNA technology is in our country used mainly for research purposes, the study objects being bacteria, yeasts, plants and mammalian cells. Biosafety of new biotechnologics was included into teaching program of the Slovak Agricultural University of Nitra.

Bt hybrid rice is safe to mammals: a conclusion drawn from completed toxicological assessments

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We previously demonstrated that the *Bt* hybrid rice we developed is highly resistant against rice leaf-folder and stem borers without reducing yield potential, indicating its great application value in rice production. However, whether the insecticidal protein-containing rice is safe as food to mammals including human is of a great concern to scientists, administrators and consumers alike. In order to address this concern, we carried out toxicological assessments on food safety of insecticidal protein-containing rice harvested from the homozygous line of *Bt* transgenic Minghui 63. The animals tested were Wistar rats and the entire assessment procedures consisted of four stages: acute, genetic, sub-chronic, and chronic toxicity tests, which took approximately three years to complete. The assayed quotas in the first two toxicity tests included acute toxic responses, reverse mutation rate of histidine-defective salmonella, percentage of micronucleus of bone marrow cells and percentage of abnormal sperms. The assayed quotas in the late two toxicity tests were body weight, blood and biochemical indices including haemoglobin content, red and white blood cell count, platelet count, blood-urea nitrate content, total cholesterol content, creatinine content, glucose content, albumin/globulin ratio, and organ coefficient. The results obtained from all the tests confirmed that the insecticidal protein-containing rice had no acute, progressing or irreversible toxic, tumour-inducing, or teratogenic effects on the tested Wistar rats including their embryo in any generation, thus indicating that it is safe to mammals.

Gene Flow: a hot topic in public debates concerning environmental biosafety of Genetically Modified Plants (GMP's)

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The Committee on Genetic Modification (COGEM) advises the Dutch Minister of Environment on potential risks of genetically modified organisms, and on ethical and social issues that may be involved.

Gene flow happens as common biological phenomenon in population genetics or population dynamics studies. Selection and gene flow provide both the existence and evolution of new species. Important in risk assessment studies on gene flow is: 1- is the modified gene a potential risk for the environment -2- If it is a risk, isolation distances are required and –3- always assume the 'worst case scenario': gene flow will happen.

There has been a lot of concern on biosafety of genetically modified plants in public debates. The background of this concern is often that people are afraid of genetic pollution, for instance in organic farming or in wild relatives. Therefore should gene flow be restricted as much as possible.

An important measure to restrict gene flow on the long term is to use isolation distances between fields with GM crops and other agricultural fields or wild relatives.

Extrapolation of isolation distances from experiments is difficult because experiments are usually specific for a certain place and time.

The Botanical Files (de Vries et al., 1992)* could be a guideline for obtaining the isolation distances. The development of a model based on important factors (reproduction strategy, pollen vitality etc.) calculating isolation distances might be a solution for the future.

The variational expression of Bt proteins within transgenic canola and its movement through trophic levels

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The levels of gene expression have been reported to vary between different species of transgenic plant, different parts and different life stages of the plant. In this paper, the variable expression level of CryIAc protein within transgenic oilseed rape (*Brassica napus* cv. Oscar) is quantified by ELISA immunoassay. Plants of Bt OSR are grown in the glasshouse, and are sampled at 2,4,5 and or 6 weeks to be quantified of Bt concentration in basal/previous top/current top leaf. The CryIAc protein concentration is expressed as percent of total soluble proteins (or ng per mg total protein) and as ng per gram fresh leaf weight. It is demonstrated that the Bt protein concentration increased significantly, as the leaf grew older, and that the Bt protein in basal leaf is significantly bigger than that in top leaf. The affect of biotic/artificial damage on the expression level of Bt proteins is also studied.

Studies of the effects of insect-resistant transgenic plants on beneficial insect among tritrophic interaction had mainly on the developmental characteristics. We presented a research on the movement of Bt proteins through trophic levels, which might help us reveal the mechanism of the 'cause and effect' of Bt toxins on nontarget insects. The diamondback moth larvae, its parasitic wasps and its predatory lacewings were used as a model system in this study. When the resistant Plutella larvae fed on Bt OSR before and after parasitized by Cotesia plutella wasps. A large amount of Bt proteins were taken up by the Plutella xylostellae larvae, a big proportion of Cry1Ac endotoxins were quantified in their faeces and only a few inside their bodies, Cry1Ac were failed to be detected in the newly emerged parasitoids larvae. In contrast to that, an amount of Cry1Ac toxins were quantified when the predatory Chrysoperla carnea fed on the resistant P. xylostella larvae reared on Bt OSR. However, no Bt toxins were detected when the lacewings were transferred to susceptible diamondback larvae reared on WT OSR and collected after 24hrs and 48hrs, respectively.

Effects of Transgenic Poplars to the Structure of Insect Community

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The study revealed that there was no much insect and spider community difference between transgenic and non-transgenic poplar trees of the small planted area while in large planted area obvious difference of that was observed. Through the comparison of insect and spider community structures between pure transgenic poplar stand and 1:1 mixed stand of transgenic and non-transgenic poplars, it is clear that Bt transgenic poplar can influence the insect community structure. In pure transgenic poplar stand, dominant species is poplar sawfly (Pristiphora benjingensis Zhou et Zhang) while in mixed poplar stand is poplar prominent (Clostera anachoreta (Fabricus)). It demonstrates that transgenic poplar can suppress the Lepidopteran species and at same time may favorable to other defoliators. To reduce the risky of being damaged by other non-lepidoptera defoliators resistant species to this kind of pest species should be used when B.t. gene is transferred. The damage rate of pure transgenic poplar stand and mixed transgenic poplar stand were 11.26% and18.48% respectively, the former was better but no significant difference. The diversity and uniformity of pure transgenic poplar stand were higher than that of Mixed transgenic poplars stand which leads to higher stability. However the density of lady beetles was 10 times higher in mixed transgenic poplar stand than pure transgenic poplar stand, averagely 0.21 per twig and 0.021per twig respectively while the density of spiders in pure transgenic poplar stand was higher than that in mixed transgenic poplar stand, averagely 0.125 per twig and 0.0625 per twig respectively. Further study is needed in the effect of transgenic poplars to the non-target insects and natural enemies.

Inheritance and Expression of a foreign gene (*Bt*)in the progenies of transgenic poplar palnts

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Plant engineering developed rapidly during recent 20 years.The safety of transgenic plant commercialized in a large scale received much concern since it has a close relationship with creatures and environment protection and human health.The goal of this paper is to provide a scientific framework to assess potential environment risks associated with transgenic woody biomass crops. Trees different from most agricultural crops in that they persist in the landscape for long periods of time.This different timeframe not only increase the probability that any one tree crop will be subjected to a much wider range of stress conditions,but also that any stress-induced side-effect of GM technology will be much harder to detect and address.Meanwhile,remote location and less intense management regimes mean limited opportunities for monitoring ,control and enforcement of regulations while making early detection of unanticipated problems highly unlikely.

The study is to detect the stability of foreign gene in Bt poplar,the transmission of the transgene to the tree progenies and develop a protocol of detection Bt gene in tree sample by means of PCR technique.PCR is adopted to analyze the expression of insectidal protein gene Bt in the transgenic poplar plants.The results show that the expression of the protein gene was stable at the seven year of the plantation.Several flowers were collected from a male transgenic tree and pollinated in controlled conditions.Progeny seeds were germinated and grown in vitro.Analyses revealed that segregation appreas to follow Mendal's rule as 67 out of 120 individual tested exhibit transgene expression in accordance with the awaited 1:1 segregation.A stable expression of the Bt gene was detected in both the parent and F1 progenies.The application of PCR detection in safety assessment is discussed.

Bacillus thuringiensis (Bt) toxin released from root exudates of Bt corn has no apparent effect on rhizobacteria community confirmed by DGGE of 16S rDNA –PCR

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The toxin from Bt corn is introduced into soil primarily in root exudates and by incorporation of plant residues after harvest of the crop, with probably some input from pollen during tasseling. The toxin was also present in the rhizosphere soil of grown Bt corn plants throughout their growth and several months after their death and subsequent frost. In vitro and in situ studies indicated that the toxin released in root exudates and from the biomass of Bt corn adsorbs and binds rapidly on surface-active particles in soil and remains larvicidal for at least 180 days. As the result of the binding of the toxins on surface-active particles, the toxins could accumulate in the environment to concentrations that may constitute a hazard to soil microbiota. Saxena and Stotzky's (2001) preliminary studies indicated that the toxin released in root exudates of Bt corn or from the degradation of biomass of Bt corn is not toxic to cultivable bacteria. However, cultivation dependent methods are mostly biased towards the selective enrichment of fast-growing bacteria adapted to high substrate concentrations, which often represent only a numerically minor fraction of the total microbial community. This preliminary work needs to be reevaluated by molecular techniques to investigate the non-culturable part of complex microbial communities.

To investigate the potential impact of Bt toxin on rhizosphere bacterial community, we choose 3 Bt corn cultivars and their parental varieties (Pioneer Company) to compare the rhizobacterial community using 16s DNA-PCR DGGE method in order to check the difference of whole rhizobacteria community between Bt and Non-Bt corn at different growth stages, compare the rhizobacteria community between different root types, explore the effect of hybrid corn varieties, come from crossbreed and genetic modified techniques, to rhizobacteria community.

The Bt toxin immunological assay carried out at V6 (50 d after germination) showed that all samples of radicle, seminal, and nodal roots' rhizosphere soil from 3 hybrids of Bt corn were positive for the presence of the toxin when assayed immunologically with the Quickstix test. No toxin was detected immunologically in any root type and soil of 3 non-Bt corn hybrids. The results are in agreement with two reports of Saxen and Stotkzy. The paired T test of the couple of Bt and Non-Bt corn rhizosphere soil DNA concentrations showed that there was no significant difference between B23 and B24, G26 and G30, P67 and P71 from V1, V2, V3 and V6 except B23 and B24 at V2, V3 stages. The ANOVA Tukey's Studentized range test comparison through V1 to V6 indicated that the rhizosphere soil DNA concentration of different varieties has subtle significant deferent from V1 to V3. It should be noted that the rhizosphere soil DNA concentration were no consistent statistically significant present between Bt corn and Non-Bt corn or 6 varieties throughout V1 to V6. Both of the UPGMA and PCA of DGGE fingerprints comparison of 6 varieties throughout V1 to V6 indicated that corn's rhizobacteria community was different between different hybrid varieties, different growth stages, and different root types; but the Bt toxin released from root exudates has no consistent and lasting effects on rhizobacteria community.

The rhizobacteria community is a very sensitive and complex system, any environmental factors change, even if the nutrient condition will alter them. We always change the rhizobacteria community through different fertilizers, and different plow ways, no matter Bt corn or non-Bt corn varieties were planted. The key question is what species had been changed by Bt corn or non-Bt corn varieties, or different management practices and what are the consequences of this change. More long-term studies are obviously necessary to carry out in the real Bt corn field and focus on the function of the soil process.

Risk Analysis Paradigms for Plant Biotechnology

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A key challenge in considerations of risks and benefit for products of modern plant biotechnology is to devise and implement risk analysis approaches that avoid "second order risks," that is, the risk of not recognizing a serious risk or the risk of overestimating a minor risk. This requires due consideration of the interrelated concepts of risk, benefit, and safety which underlie the process whereby risks are assessed, managed, and communicated within widely recognized risk analysis frameworks. Recognition of risk assessment as a formal, objective, science-based consideration used within risk analysis frameworks places risk-benefit issues within context by describing what is known as well as what is variable and uncertain with respect to biosafety questions. To properly inform risk management decisions and public communication of these decisions, the risk assessment process must be transparent, follow well-recognized national and international frameworks, and, wherever possible, it must utilize quantitative methods describing the likelihood of risk and the magnitude and nature of uncertainties. Quantitative risk assessment methods, in particular, are broadly applicable to the evaluation of human, animal, and ecological safety of products of modern plant biotechnology. Dietary consumption of protein, non-target organism effects, and gene flow are all examples of biosafety issues related to plant biotechnology that are readily amenable to quantitative risk assessment.

Detection of genetically modified organisms (GMOs) in soybean and processed products by PCR

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PCR methods for detection of genetically modified organisms (GMOs) were developed that can be used for screening purposes and for specific detection of glyphosate-tolerant soybean in foods. Primers were designed to amplify parts of the 35S promoter derived from *Cauliflower Mosaic Virus*, the NOS terminator derived from *Agrobacterium tumefaciens*. PCR protocols were established for the detection of soybean and proceeded products. Besides, confirmation of the results using restriction analysis was also done.

50 kinds of soy proceeded foods bough from the pitch in Beijing were analysed. Firstly, DNA were extracted by modified CTAB. Polymerase chain reaction (PCR) were done by the cited method. 7 samples were founded positive. That was to say 14 percent of the total samples were GM foods.

The described methods enabled a highly sensitive and specific detection of GMOs and thus provided a useful tool for routine analysis of soybean raw and processed food products.

Research on biosafety of transgenic Bt cotton

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Our research interests mainly focus on three aspects. The first one is biosafety assessment of transgenic Bt cotton plants; the second one is variance of Bt pesticidal protein content in different Bt cotton plant organs and variance of Bt content in cotton plants in different developmental stages; the third one is the difference of volatile chemical compositions between Bt transgenic cotton plants and regular cotton plants and the antennal responses of cotton bollworms to these substances.

We have detected the toxicity of Bt pesticidal protein to mammals, fish and invertebrates. Mice, zebra-fish and eelworms were chosen as experimental model animals respectively. To evaluate the biosafety of Bt protein expressed in Bt transgenic cotton plants, we did numbers of toxicity tests including acute tests, chronic tests, gene toxicity tests and so on. The results showed that there was no acute toxicity, chronic toxicity or gene toxicity to each kind of animals mentioned above.

By using ELISA method we found that transgenic Bt cottons had different Bt pesticidal protein content in different organs and developmental stages. Leaves and petals contained more Bt protein than bolls and bracteal leaves. In the late growing period, Bt protein content in all organs decreased significantly. It was also found that Bt pesticidal

protein could be detected at the early stage of sprout of seeds although seeds which had not sprouted contained so little Bt protein that it couldn't be examined.

In order to provide chemical and ecological evidence for the ecological risk assessment of transgenic Bt cottons, we analyzed volatile chemical compositions in Bt and regular cotton plants. We discovered that the ratios of á-pinene and â-pinene were much higher in the volatiles of transgenic Bt plants than in those of control plants, and two compounds found in the transgenic Bt cotton volatiles were absent in the control cotton plants. Nine compounds in volatiles of transgenic Bt cottons were responsible for EAD peaks in GC-EAD tests.

Transgenic: A necessity in chickpea crop

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Food legumes are important for sustainable crop production system. Worldwide, approximately 10 million ha. is under chickpea cultivation which yields more than 8 million tons of grain annually. Indian sub continent accounts for more than than 70% in both area and production of legumes. During last three decades, technolgical advances in biology have provided new tools that are impacting on plant breeding. Consequently, many transgenic varieties of different plants have been marketed. More than 40 million ha is under transgenic crops in different countries. However, transgenic plants of food legumes imortant to third world except soybean are yet to be constructed. Pod borer Helicoverpa causes almost 20% damages annually in chickpea crop alone in India. The resistance against pod borer in chickpea germplasm is not available. Therefore, it is important to construct transgenic chickpeas that are resistant to pod borer Helicoverpa. Chickpea is prominently a dryland crop. The tools of new biology may be used to construct Chockpea plants tolerant to biotic and abiotic stresses, and storage pest. Presently, ICAR has more than 4000 germplasm lines of Kabuli and desi chickpea. all lines were evaluated against pod borer infestation. The damage was recorded between 15-40 percent. Desi medium seeded types chickpea with tall erect and open canopy showed less damages in comparison to bold seeded and spreading types. The biotic and abiotic problems vary from region to region. Many sources of genes tolearant to abiotic stresses are available and can be used to expand the list of already available durable and effective chickpea cultivars. Improvement of the protein quality and many other characteristics of chickpea offers intriguing possibilties.

Preliminary research on ecologic effect in the rhizosphere of transgenic rice expressing modified antifungal genes

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Rice blast, sheath blight and other diseases caused by fungi are among the most important diseases which threaten rice production. Genetic biotechnology is one of the effective methods to breed resistant rice now. "Zhongda 2" is a genetically modified rice strain (GMR) produced by the introduction of "RC24" (a rice alkaline chitinase gene) into a indica rice cultivar, "Zhuxian B". "Qizhuan 39" is another GMR produced by the introduction of "RC24" and "â-1,3-Glu"(a alfalfa â-1,3-Glucanase gene) into a indica rice cutivar, "Qisiranzhan". The two GMRs have high levels of resistance to rice blast and sheath blight. The expression of the modified antifunal genes not only effects the rice resistance to the diseases, but also induce the changes of microbe community of rice and probably proceed to induce series changes of soil ecology. Preliminary analyzsis of the ecologic effect on soil after growing the GMRs showed that there were marked differences between GMRs and their non-transgenic rice control in the microbe communities of root and rhizosphere. The total numbers of endo-fungi in the roots of tow GMRs were markedly lower than the non-transgenic controls when the microbe were isolated and cultured from roots, or the mycelia in roots were observed under microscope after dye of roots. The total number of endo-bacteria in roots of "Zhongda 2" was not different from the controls, but that of "Qizhuan 39 " appeared much lower than the controls. Analysis of microbe community in the soil of rice rhizosphere showed that the total numbers of bacteria and fungi in the rhizosphere of "Qizhuan 39 " were markedly lower than those of the controls, but those of "Zhongda 2" were not markedly different from the controls. The further experiments of the determinations on some soil nutrients and the activities of some soil enzymes in the GMR rhizosphere were conducted. The results showed there were no marked differences between GMRs and the control rice in the concentrations of the dissolved organic matter, total and dissolved nitrogen, total and dissolved phosphorus, and in the activities of the polyphenol oxidase, hydrogen peroxidase, sucrase and urease of rhizosphere soil.

Biosafety study in field trials of releasing Bt transgenic Psedomonas fluorescens

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Anti-fungi P. fluorescens P303 strain (Nal^r, Rif^r) was transformed by plasmids (pZY604 and pJM6α) carrying different Bt cry genes. Transgenic bacteria BioP202 (Nal^r, Rif^r, Km^r) expressed two Bt cry genes (cry1Ac, and cry2Aa), and BioP8 (Nal^r, Rif^r, Km^r, Spe^r) contained an additional cry1Ab gene. Both BioP202 and BioP8 expressed appropriate anti-pests activities as well as maintained the anti-fungal property. In June 1999, BioP202 was released in a small-scale field trial in Beijing, and BioP8 was released in the second trial in Lianyungang City, Jiangsu Province in June 2000. Triplicates of five test areas (333 m^2 each) were performed in both trials: (1) P. fluorescens P303 spray, (2) Bt HD-1 commercial spray, (3) pesticide (fenvalerate) spray, (4) undiluted BioP202 (or BioP8) culture spray (100 mL/m^2), and (5) diluted (1: 10) BioP202 (or BioP8) spray. Sprayed areas were separated to each other by segregation zones (no treatment) of 10 m in width. In the BioP202 trial, soil samples were randomly collected from tested areas and segregation zones in the following spring (March 2000). In the BioP8 trial, random collections of soil samples were made twice, in autumn (October 2000) and in early spring (February 2001). P. fluorescens in soil (1 g) were extracted in sterile water (10 mL) and screened on antibiotic plates for resistant profiles. Tetrad resistance phenotype as BioP8 was not detected in any sample. Triple resistant phenotype as BioP202 was recovered once in a segregation zone sample of October 2000. Single and double resistances were more frequently detected. None of the recovered bacteria colonies was positive for Bt cry genes using PCR-RFLP identification system. We conclude that the Bt transgenic P. fluorescens can not persist in soil after a cultivation season. Because the plasmids carrying the Bt cry genes (also carrying the antibiotic genes) are reliable, lack of Bt cry genes in the recovered bacteria does not support that the antibiotic resistances in the recovered bacteria may be mediated by plasmids from the released inoculants. Our data confers to the notion that kanamycin resistance and spectinomycin resistance are widely existing in soil bacteria.

Performance of Transgenic Bt Cotton in Field and Its Effects on General Predatory Natural Enemies

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There are three main viewpoints about the interactions between transgenic plants and arthropod natural enemies, which is advantageous, dis-advantageous or neutral. To study the interactions of transgenic Bt cotton, pest insects and arthropod natural enemies, from the view of tritrophic system and integrated pest management (IPM), we investigate the population dynamics of beet armyworm (*Spodoptera exigua* Hübner) and cotton bollworm (*Helicoverpa armigera* Hübner), and of two predatory natural enemies—ladybird (*Propylaea japonica* Thunberg) and lacewing (*Chrysoperla phyllochroma* Wesmael) in a field test. We have four field plots of cotton in Shandong Province, each in 1/15 hm^2. Which included 'GK19' (transgenic Bt cotton) without pesticide application (treatment 1), 'GK19'with pesticide application (treatment 2) 'Simian 3' (corresponsive non-transgenic cotton) with pesticide application (treatment 3), and 'Simian 3' (corresponsive non-transgenic Bt cotton) without pesticide application (treatment 4).

We find out that: 1) Although there are similar eggs oviposition in all of the four treatments during the growth season, the number of cotton bollworm larvae and beet armyworm larvae on per hundred plants of transgenic fields is less than that of non-transgenic fields. 2) Although the number of ladybirds on per hundred cotton plants of treatment 1,2 and 3 is not different, there are much more ladybirds in the field of treatment 4. 3) The number of lacewings in all of the fields of treatment 1,2 and 3 is significantly less than that of treatment 4, while the number of lacewings in the field of treatment 2 is slightly higher than that of treatment 1 and 3. The results show that the transgenic cotton line 'GK19' performs resistance to both of cotton bollworm and beet armyworm, and that there are less predatory natural enemies in the field of transgenic cotton without pesticide application than that of non-transgenic cotton without pesticide application. The latter is quite opposite to other current references in this field on the biosafety assessment of transgenic cottons. It suggests that further detailed works should be taken on to make that clearer.

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Effect of the Bt toxin on microbial composition in the rhizosphere soil of transgenic Bt canola

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Because trace amounts of the Bt toxin released from the roots of transgenic Bt canola (Brassica napus) in hydroponic culture were detected by immunological assay, we investigated the effect of Bt canola on the microbial composition of rhizosphere soil in a field study. Bt canola and isogenic non-Bt canola were grown on a sandy loam soil and the rhizosphere soils were collected for microbial analysis. The results showed that there were no significant differences in the colony-forming units of culuturable bacteria, actinomycetes, and fungi between soils under Bt and non-Bt canola. Microbial biomass carbon in the rhizosphere appeared to be higher in the soils under Bt canola than in the non-Bt canola. The results of this study suggest that the composition of the microbial community of rhizosphere soil was influenced by the endotoxin released from the roots of Bt canola. The effects of Bt toxin on the composition and function of unculturable microbial community in rhizosphere soil need to be addressed in future research in the laboratory and field studies

Elimination of Marker Genes by Site-Specific DNA Recombination in Higher Plants

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We have developed a chemical-regulated, site-specific DNA excision system. In this system, a kanamycin-resistance marker gene was placed between two copies of *loxP* sequence, which can be specifically recognized and cleaved by a DNA recombinase Cre. Expression of Cre was tightly controlled by a chemical inducible prompter *LexA-46*. The latter can be specifically activated by a chimeric transcription factor XVE, whose activity, in turn, is controlled by the mammalian hormone estrogen, a chemical with no detectable non-physiological effects on plant growth and development. When this test DNA construct was introduced into the model plant *Arabidopsis thaliana* by a standard transformation protocol, application of -estradiol to the resulting transgenic plants led to the activation of XVE. The XVE transactivator then promoted a high level expression of Cre, which subsequently excised the *loxP*-sandwiched kanamycin-resistance marker gene and other "used" components of the system. Upon site-specific DNA excision and recombination, a promoter-less *GFP* (green fluorescent protein) reporter gene was brought directly downstream of a strong promoter, leading to *GFP* expression in marker-free transgenic plants. Genetic and molecular analyses indicated that the system is tightly controlled, showing high-efficiency inducible DNA excision in all tested transgenic events. An additional advantage of this system is that it is feasible to use any conventional marker genes, thus providing a convenient method to remove selectable markers from transgenic plants generated with different approaches (e.g., organogenesis or somatic embryogenesis).

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