SESSION 6

CONSEQUENCES OF GENE FLOW BETWEEN HIGHER PLANTS AND OTHER ORGANISMS

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Insecticidal transgenes into nature: gene flow, ecological effects, relevancy, and monitoring

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ABSTRACT

Highly effective genes conferring pest resistance have been and are being engineered into crop plants. There is a strong likelihood that these transgenes will be transferred from agronomic ecosystems into natural ecosystems. There will be ecological risks ranging from creating more invasive weeds to affecting beneficial insects. I argue for the need of relevancy in choosing appropriate experimental systems for assessing ecological risks of commercial transgenic insecticidal crops. Finally, I will describe a transgene monitoring system based upon green fluorescent protein (GFP), and how it may be used commercially.

THE CURRENT INSECTICIDAL CROP PIPELINE

During the past 10 years, transgenic crop development effort has increased exponentially. In the USA alone, there are now four different transgenic pest resistant crops that have been deregulated (commercialised): corn, cotton, potato, and tomato. These four, all containing *Bacillus thuringiensis* crystal toxin (Bt *cry*) transgenes, are the first of many pest-resistant crops to flow from the industrial R&D pipeline. To date, there have been eighteen plant species transformed with Bt cry genes and field tested in the USA: alfalfa, *Amelanchier*, apple, belladonna, cabbage, cranberry, corn, cotton, eggplant, grape, oilseed rape, *Populus*, potato, rice, soybean, tobacco, tomato, and walnut (USDA APHIS permits, Oct., 1998). This represents a 50% increase of plant species during the last three years. Most of these plants are slated for commercialisation with insecticidal genes. While the arsenal of insecticidal genes is growing, Bt will be the primary insect resistance transgene on the market for the next 10 years.

Bacillus thuringiensis crystal endotoxins

The first Bt transgenic plants were produced over 12 years ago (Vaeck *et al.*, 1987). These first plants contained native Bt genes that were not expressed very well. Now the genes have been codon-optimized for high expression in plants and have proven to be very effective to controlling specific insects (Perlak *et al.*, 1991; Adang *et al.*, 1993). Indeed, exclusively synthetic, codon-optimized Bt genes are now used for plant nuclear transformation. The class of compounds that are responsible for insecticidal activity are crystalline proteins also known as Cry proteins or delta-endotoxins. The mode of action of Bt endotoxins is the disruption of cellular membranes in the midgut. Endotoxins are proteolytically converted into small polypeptides in the midgut. These bind to glycoprotein receptors and disrupt osmotic processes (Adang, 1991). Bt Crys have high specificity of toxicity, a highly desirable trait.

As with any insecticide that has extensive use, insects can acquire resistance to Bt. Many have warned that Bt resistance genes will become fixed in insect populations rendering both Bt transgenics and Bt sprays ineffective (Whalon & McGaughey, 1993; Tabashnik, 1994; Whalon,

1994), although there are a minority of scientists who do not seem to be very concerned about Bt resistance management (Altman *et al.*, 1996). Both diamondback moth (*Plutella xylostella*) and tobacco budworm (*Heliothis virescens*) biotypes have been found to be resistant to several Bt toxins (Gould *et al.*, 1992; Heckel, 1994; Gould *et al.*, 1997; Tabashnik *et al.*, 1997b; Liu *et al.*, 1998). Indeed, the genetic loci responsible for resistance in both species have been mapped (Heckel *et al.*, 1997; Tabashnik *et al.*, 1997a). Thus, several strategies have been proposed, including refugia, high dose, mosaics, rotations, and transgene combinations (reviewed in Tabashnik, 1994). Currently, high dose (high Bt expression) and refugia are used to manage resistance in all Bt transgenic crops grown in the USA (Gould, 1998), but industry is moving toward transgene combinations (pyramiding) to manage Bt resistance. Pyramiding, in this case combining Bt genes with other transgenes has been proposed as a necessary strategy to prevent the development of Bt resistance (Wilson *et al.*, 1992; Boulter, 1993; McGaughey 1994). A notable advantage to pyramiding is that it would be transparent to the grower and not have the associated yield loss the refugia strategy demands.

New genes for insect resistance

There are three main reasons why several groups are racing to discover novel insecticidal genes. The first is to engineer plants insecticidal to control insects that are not susceptible to Bt Cry. The second is to discover candidate genes to pyramid with Bt. A third reason, related to the first, is to capture a unique market niche. The benchmark of any new gene candidate in transgenic plants that the gene product should have at least equal toxicity compared with Bt. However, one potential problem is that new toxins have less specificity than Bt. This means that insecticidal transgenic plants of the future will likely have an increase of non-target effects. I will briefly review the best candidate genes for commercialisation in transgenic plants. More extensive reviews have been published recently (Estruch *et al.*, 1997; Gatehouse & Gatehouse, 1998; Jouanin *et al.*, 1998; Schuler *et al.*, 1998).

Cholesterol oxidase

Cholesterol oxidase (CO) from *Streptomyces* culture filtrate has been found to be highly toxic to boll weevil (*Anthonomus grandis*) (Purcell *et al.*, 1993). Monsanto is presumably developing CO for the control of this economic insect on cotton (Purcell *et al.*, 1993; Greenplate *et al.*, 1995). However, it also has activity against southern corn rootworm (*Diabrotica undecimpunctata*), tobacco budworm (*Heliothis virescens*), and yellow mealworm (*Tenebrio molitor*) (Shen *et al.*, 1997). The mode of action is the lysis of midgut epithelial cells (Purcell *et al.*, 1993).

Vegetative Insecticidal Proteins

In contrast to Bt endotoxins, which are accumulated to high amounts when Bt sporulates, Bt also produces vegetative insecticidal proteins (Vips) when it is not sporulating (Estruch *et al.*, 1996; Yu *et al.*, 1997). While Vips come from Bt, they are unrelated to Bt endotoxins (Estruch *et al.*, 1996). Similar to cholesterol oxidase, the mode of action is midgut cell lysis (Yu *et al.*, 1997). The Vip3A insecticidal protein has been shown toxic to black cutworm, (*Agrotis ipsilon*), fall armyworm (*Spodoptera frugiperda*), beet armyworm, (*Spodoptera exigua*), tobacco budworm (*H. virescens*), and corn earworm, (*Helicoverpa zea*), a broad range of hosts (Estruch *et al.*, 1996). Novartis is apparently developing Vips for controlling corn insects.

Photorhabdus luminescens toxins

Newly discovered toxins from the bacterium *Photorhabdus luminescens*, which make their living in gut of entomophagous nematodes have been shown to be toxic to several orders of insects (Bowen *et al.,* 1998). Similar to cholesterol oxidase and Vips, midgut epithelial cells are damaged in insects that have consumed the toxins. DowAgroSciences has acquired an exclusive license for the use of *P. luminescens* toxins, and will presumably move towards the goal of commercialisation of transgenic plants expressing toxin-encoding genes.

Other-bioactive proteins for insect control

There are several insect-control proteins that have been proposed to be pyramided with Bt. None of these have the toxicity of Bt Cry proteins, cholesterol oxidase, Bt Vips, or *P. luminescens* toxins. The most studied of such proteins are proteinase inhibitors. The other insect resistance proteins that have been received attention are lectins and chitinases.

Proteinase inhibitors (PIs) from plants have been studied as candidates for insect control for over 20 years (Ryan, 1990) The mode of action is the overstimulation of the production of trypsin, chymotrypsin and other proteases in the insect gut (Broadway & Duffey,1986). Most PIs inhibit insect growth but are not antibiotic. Recent data supports the hypothesis that insect digestive physiology has memory of sorts. That is, certain insects can alter their arsenal of digestive enzymes (e.g., trypsins) to overcome specific PIs. However, if an insect does not feed on a certain plant, it may not be able to readily overcome the PI and as a result, its growth and development will be inhibited (Jongsma *et al.,* 1995; Broadway, 1995; Broadway & Villani, 1995). Therefore, certain insects may be preadapted for resistance to PIs. This fact and their relatively low toxicity to insects hamper their commercial feasibility.

Lectins, carbohydrate-binding proteins, from various plant species such as the snowdrop (*Galanthus nivalis*) also decrease insect growth (Powell *et al.,* 1993). Lectins bind brush border membrane proteins of various insects, but there is no apparent relationship between the ability to bind and toxicity to the host (Harper *et al.,* 1995). Lectins are generally more effective in transgenic plants than PIs. Lectins in transgenic crop plants might also be of special biosafety concern because of reports they can act as mitogens to human T-cells (Peumans *et al.,* 1997).

Chitinases have also received attention recently as possible insect control agents that could be used in transgenic plants (Kramer & Muthukrishnan, 1997). Chitinases from insects digest chitin, an important constituent of insect exoskeletons and gut linings. Similar to PIs and lectins, chitinases affect a broad range of insects. They have apparent toxicity between PIs and Bt Cry for insects they affect. Unlike PIs, chitinases seem to be effectively synergistic with Bt Cry toxins (Kramer & Muthukrishnan, 1997; Santos *et al.,* 1997).

TRANSGENE FLOW FROM CROPS TO WEEDS

There are several crops in the US and Europe that have the potential to hybridise with wild relatives. The crops that have sexually-compatible wild relatives growing in proximity to them are at risk for receiving fitness-enhancing transgenes such as insecticidal genes, which could alter ecological parameters. Some examples in the USA and Europe are rice (Langevin *et al.,*

1990), sorghum (Paterson *et al.*, 1995), sugar beet (Bartsch *et al.*, 1996; Bartsch & Pohlorf, 1996; Bartsch & Schmidt, 1997), and sunflower (Whitton *et al.*, 1997). There are several incidental crops, vegetables and fruits that also have neighbouring wild relatives (reviewed by Raybould & Gray,1993). Of course, the largest concern in the USA and Europe has been over oilseed rape (OR) (*Brassica napus*), a crop with numerous wild relatives and increasing world-wide cultivation. OR has been the subject of extensive research, is relatively easy to transform, and has been the model of choice for biotechnology risk research. I will briefly review its breeding and biology and some of the research that has been recently accomplished in OR transgene flow and insecticidal transgenic OR.

The case of *Brassica napus* (oilseed rape)

The mustard (Brassicaceae or Cruciferae) family, to which the genus *Brassica* belongs, contains many important crop plants and weeds. *Brassica napus*, is an amphiploid species (AACC 2n=38) which putatively arose from naturally occurring interspecific hybridization between *Brassica oleracea* (AA) and *Brassica rapa* (CC). Both winter and spring forms exist within *B. napus*. Subsequent growth patterns differ depending on the climate of the production region and the form grown. Winter forms are fall-seeded and spring-harvested, while spring forms may be either grown as a spring-seeded annual crop in temperate regions or as a fall-seeded crop in milder climates, such as the southeastern USA. *Brassica napus* is a self-pollinating species that outcrosses readily with the assistance of wind and insect pollinators. Outcrossing frequencies as high as 30% for directly adjacent plants have been reported (Robbelin & Downey, 1989). *Brassica napus* can be a volunteer in other crops and along roadsides but it is not considered to be a frequent invader of non-disturbed ecosystems (Rich ,1991; Crawley *et al.*, 1993).

OR is known to be interfertile with wild *B. napus, B. rapa, B. oleracea, Brassica nigra, Brassica kaber* (*Sinapsis arvensis*) *Brassica juncea, Brassica adpressa*, and *Raphanus raphanistrum* (Bing, 1991; Kerlan *et al.*, 1992, 1993; Scheffler & Dale, 1994; Eber *et al.*, 1994; Darmency *et al.*, 1995; Mikkelsen *et al.*, 1996; Chevre *et al.*, 1997, Metz *et al.*, 1997). One conclusion of these studies is that there is significant maternal effect in the efficacy of the crosses, with highest hybridisation potential being when *B. napus* is used as the pollen recipient. Agronomically-important transgene gene flow from OR will likely initially occur with OR as the pollen donor. Even though hybridisation frequencies were low, all the authors warned of significant introgression possibilities, as several of the resultant hybrids were fertile, especially under open pollinating conditions. The complicated taxonomy and tractable biotechnology of OR and its relatives have made this system a popular one for researching the risks of agricultural biotechnology.

Transgenic insecticidal OR, wild relatives and herbivory

There are at least three foreseeable effects of the gene flow of insecticidal genes from crop to wild relative. First, the persistence of transgenes may skew transgenic crop:refuia areas, which could slightly affect resistance management (Wearing & Hokkanen, 1995). Second, insecticidal transgenes could decrease beneficial and/or non-target insect populations. The published studies of Bt side-effects to beneficial insects are sparse. However, where they have been studied in the field, significant effects to beneficial insects have not been found (Flint *et al.*, 1995; Sims, 1995; Arpaia, 1996; Orr & Landis, 1997). One example contrary to this is a recent report (Hilbeck,

et al., 1998) that involved laboratory experiments using Bt corn, the pest/prey insect *Ostrinia nubilalis* (European corn borer, ECB), and the lepidopterous predator *Chrysoperla carnea*. While no direct effect of Bt was found on *C. carnea*, the predator grew more slowly and had greater mortality when fed Bt-exposed prey. The authors hypothesised that the Bt-stunted ECB were sick, leading to indirect toxicity to the non-target insect. While it is doubtful that direct effects of Bt on non-target pests will be obtained, the toxins entering the pipeline may have increased side-effects. Since they have a broader host range, there is a higher probability of increased risks on non-target insects; risks that will need to be explored on a case-by-case basis. The third and potentially the largest effect of gene flow of insecticidal transgenes into wild relatives will be the alteration of fitness or increased invasiveness of the plant host. As above, the OR/wild relative system is our best model.

There are very limited data on insect herbivory on the fitness of OR relatives. One study in small field plots in England showed that foliar insecticide did not increase fitness of wild radish (Rees & Brown, 1992). However, ambient insect levels were low and little damage (<5% defoliation) occurred on no-insecticide-treatment plants. However, because of higher ambient slug populations, higher fitness was observed on molluscicide-treatment plants (Rees & Brown, 1992). These results show that insecticidal (Bt) *R. raphanistrum* and *B. rapa* could be new risks that should be assessed. Certainly others have warned that insect resistance in weeds would not be a desirable modification (Kareiva *et al.*, 1994; Stewart *et al.*, 1997). My lab is in the process of testing the ecological performance of Bt-transgenic *B. rapa* in the field; the litmus test of gene flow/transgene effect in OR.

Relevancy of biotech risk research

Three field trials have been performed by companies (AgrEvo and Calgene) in 1998 using insecticidal OR in the USA (USDA APHIS permits, Oct., 1998). These companies tested lepidopteran-resistant transgenic OR with unspecified genes (confidential business information) in California. We can assume that these field trials are a precursor to eventual commercial releases. It is evident that any commercial transgenic OR will donate genes to wild relatives grown in proximity. It is also evident that insecticidal genes with broader pest toxicity will be used more often in the future. Insecticidal proteins in transgenic plants that affect a greater number of pests will lead to greater uncertainty in two risk factors. The first is the effect to non-target or beneficial insects. The second is herbivore-mediated selection effects that favour insecticidal transgenic plants over non-transgenic plants. These effects could likely interact with each other. Added to these will be the effects of scale (Dale,1997). Commercialisation area is much larger than field trial area. Simply, the ability for the transgenic plant to affect negatively more organisms will lead to greater complexity of risks.

The interaction of gene flow and pest resistance, therefore, needs to be studied using relevant insecticidal genes, promoters, and hosts. I believe that the scientific community has responded appropriately for the most part. However, the public availability of genes coding for cholesterol oxidase, Vips, and *P. luminescens* toxins will likely be limited because of intellectual property concerns and other business reasons. Therefore, I predict that increased co-operation between industry and public researchers will be necessary to thoroughly test new transgenic insecticidal plants for ecological risks. The days of using PI genes with the *CaMV* 35S promoter are over. These experiments are no longer highly relevant to tomorrow's agbiotech products. Can we envision the day when multiple transgenes of broad effects will put be into crop plants such as

OR? Will we be able to even thoroughly test for and predict complex ecological interactions that are set-off from such a GMO introduction? The answer is both yes and no. To manage for unintended effects of gene flow, it seems to be desirable to use monitoring tools to track transgenes. Transgene monitoring may not be able to mitigate for increased side-effects to non-target pests, but it would enable the management of potentially problematic genes flowing out of the host crop. One group has suggested an alternative to monitoring (Daniell *et al.,* 1998) consisting of transferring transgenes into chloroplasts, which are assumed to be maternally inherited. This approach is a controversial solution to transgene containment that is based upon faulty assumptions (Cummins, 1998; Stewart & Prakash, 1998). However, the most pragmatic pitfall to this possible solution is that tobacco is the only plant amenable to chloroplast transformation today. Since the ability to transform any crop using chloroplast transformation is at least a decade away, it should not be considered a commercially viable alternative.

TRANSGENE MONITORING

In the past, studies of gene flow and transgene persistence in the environment have been monitored largely using phenotypic (Manasse, 1992; Luby & McNicol, 1995), biochemical (Klinger *et al.,* 1992; Arias & Reiseberg, 1994), or molecular markers (Jorgensen & Andersen, 1994; Rogers *et al.*, 1996). These often require expensive substrates (in the case of GUS (Jefferson, 1989)), are not suitable for real-time and *in vivo* detection, and are not universal. However, with the recent availability of green fluorescent protein (GFP) encoding genes, a tractable monitoring system is feasible and has been recently introduced (Stewart, 1996). In this method GFP in transgenic plants can be visualised on a macroscopic scale non-destructively using ultraviolet light. GFP from jellyfish is a 27 kD monomer that has the unique characteristic of fluorescing green when excited with ultra-violet (360-400 nm) or blue (440-480 nm) light. This fluorescence depends only upon elemental oxygen, necessary for double-bond formation between the a and b bond of Tyr 66 (Ormo *et al.,* 1996; Yang *et al.,* 1996), and UV or blue light. Therefore, the protein does not need any substrate, enzyme, or co-factor for fluorescence, making it a genuine, *in vivo* marker (Niedz *et al.,* 1995).

Transgenic plants expressing GFP can be detected using only a hand-held UV light *in vivo* and in real time. Transgenic plants appear green under ultra-violet light amidst non-transgenic plants that appear red due to the red autofluorescence of chlorophyll when excited with UV light. Green fluorescent protein is stably inherited in progeny and is useful as a tag to mark transgenic plants *in vivo*. My lab (Stewart, 1996; Leffel *et al.,* 1997) and others (Pang *et al.,* 1996; Haseloff *et al.,* 1997) have shown that whole plant fluorescence with GFP is a powerful tool, and that GFP is stably inherited (Leffel *et al.,* 1997). We are now employing an improved chimeric gene with a endoplasmic reticulum retention signal (Haseloff *et al.,* 1997), *mgfp5-er,* which has proven to be much improved over *mgfp4* as a whole plant marker. (Mabon *et al.*, submitted). This procedure could be of great benefit in a larger transgenic crop management system (Marshall, 1998).

For this method to be successful a GFP gene is linked or fused to a gene of interest, e.g., an insecticidal transgene on the same plasmid, prior to introduction into plants. The gene of interest is then monitored by visual observation of GFP in the plant. My lab has shown that this procedure functions as expected (Mabon *et al.*, submitted). In addition, there is no evidence that GFP is toxic or has any costs to the host plants in the field (Harper *et al.*, submitted). These two

facts greatly enhance the usability of a GFP monitoring system. One can envision releasing plants with blue, cyan, green and yellow fluorescent proteins linked to genes of different agronomic traits. One can also envision remote sensing technology and precision agricultural applications of the technology.

CONCLUSION

In conclusion, it is clear that the pipeline of pest-resistant transgenic crops is full. These crops have the potential to greatly decrease conventional pesticide inputs into the environment, and to improve agronomic efficiency. There are, however, real risks that arise from the novel gene introductions and gene flow from crop to wild relatives that will persist in the environment. It is clear these risks need to be assessed, and science-based decisions made with regards to real biosafety issues. It also seems prudent to develop monitoring methods to track transgenes in the environment. Monitoring can serve as a long-term ecological research tool, and as a means to control for unintended problems that might arise from commercial releases.

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