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Transgene flow and persistence may be monitored by using *in vivo* markers such as GFP

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ABSTRACT

There is growing concern among scientists, regulatory officials, and environmentalists about the potential escape of transgenes and the resulting naturalization of transgenic plants. The current thought is that if and when genes are introgressed into wild relatives, the resultant plants could become more weedy and/or alter natural communities and ecosystems by their increased competitiveness. For many plant/transgene combinations that are commercialized or in the process of commercialization in the U.S. and in other countries, this scenario is very plausible. It is plausible because of the nature of the host plants' breeding systems, the ecological importance of nearby wild relatives, and properties of transgenes that may confer an increment of fitness to the host plants. Therefore, the availability of an easy and cost-efficient system to track genetically engineered plants containing potentially ecologically important transgenes would be beneficial for use in basic and applied research and for monitoring commercial releases. I introduce such a system, which consists of linking one or more ecologically important transgenes, such as those conferring herbicide-, disease-, or insect-resistance, to a transgene coding an *in vivo* marker, such as a green fluorescent protein (GFP) gene. In situations where, say, transgenic insect resistant canola plants are mixed with non-transgenic canola and/or wild mustard, the fate of transgenes may be traced by visual observation of canola or canola-

mustard hybrids under an ultraviolet or blue light. Such a system would be very useful in evaluating ecological hazards inherent in many crop/transgene combinations.

INTRODUCTION

The potential release and widespread use of transgenic plants have precipitated both regulatory concern and scientific research. A primary issue is the possible escape of transgenes into the natural environment, along with the ramifications of escape. One ramification is the possibility of increased invasiveness and competitiveness of transgenic genotypes, compared to conspecifics and congeners and the surrounding vegetation (Hoffman, 1990; Ellstrand and Hoffman, 1990; Raybould and Gray, 1993; Linder and Schmitt, 1994; Rogers and Parkes, 1995). A major segment of plant biotechnology risk assessment research has focused on the potential escape of transgenes and the fate of plant populations containing those transgenes (Raybould and Gray, 1993). Thus, the major risk of releasing many crop/transgene combinations into the environment may be equated with the probability of the extra-agronomic survival of transgenic crop plants and/or the probability of the introgression of transgenes into related species, coupled with persistence and selection of those populations containing the transgenes. Researchers have approached the problem of risk assessment in many ways. However, research has focused mainly on gene flow since this is the first tier of risk assessment for each species (Linder and Schmitt, 1994). Most methods for estimating gene flow are labor-intensive, which limits the amount of research that may be performed. Researchers typically use transgenes that are not neutral (e.g., herbicide resistance genes), or markers already resident in plants (i.e., phenotypic or molecular markers). These latter markers, though, have the disadvantage of not being universally identical among species. Furthermore, once transgenes have "escaped" there is no easy way to monitor their progression through ecosystems. This review will briefly discuss the potential problems of escaped transgenes into the environment and will introduce methodology to assay gene flow and monitor the persistence of transgenes in the environment that may be applied to any engineerable crop plant.

Will transgenes be transferred to wild relatives?

There is growing evidence that, for certain crop plants in certain areas of the world, there will be rapid transfer of transgenes to weedy relatives. In North America, some notable examples include rice in Louisiana (Langevin *et al*. 1990), sorghum (Paterson *et al*. 1995) and sunflower in the midwestern U.S. (Arias and Rieseberg, 1994), and canola in Canada and the southeastern and northwestern U.S. (Raymer *et al*., 1990; Mikkelsen, *et al*. 1996). Canola (predominantly *Brassica napus*) is especially problematic since herbicide resistant canola has already been commercialized in Canada and Europe and canola has complex taxonomic and breeding relationships with multiple weedy wild relatives.

The mustard (Brassicaceae or Cruciferae) family, to which the genus *Brassica* belongs, contain many important weeds and crop plants. The origin and description of the *Brassica* oilseed species has been reviewed by Downey (1983). *Brassica campestris (*syn. *rapa)* is a diploid species and *B. napus* is an amphiploid species which arose from naturally occurring interspecific hybridization between *B. oleracea* and *B. campestris*. *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 (Downey and Robbelen, 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 (Radford, 1974; Crawley *et al*., 1993). In addition, in many areas certain biotypes of *B. campestris* (wild mustard) are considered noxious weeds. Hybridization can occur between *B. napus* and *B. campestris*, *B. juncea*, *B. kaber*, *B. oleracea*, *B. adpressa Sinapsis alba*, *S. arvensis*, *Raphanus raphanistrum* and to a lesser degree, other members of the Brassicaceae (Bing, 1991; Kerlan *et al*., 1992; Adler *et al*., 1993; Raybould and Gray, 1993; J rgensen and Anderson, 1994; Scheffler and Dale, 1994; Mikkelsen *et al*., 1996). In addition, once a transgene has been introgressed into one of the above relatives, it can then be spread to other members of the Brassicaceae. Clearly, any transgene in canola will get shuffled around the Brassicaceae, and will have a strong likelihood of becoming fixed outside of cultivation.

Will plants with introgressed transgenes persist in the environment?

Although transgene movement among species may exacerbate transgene persistence, there is much less data on persistence compared with introgression. It is foreseeable that any fitness-related transgene such as those conferring herbicide resistance, disease resistance, and insect resistance could be rapidly spread from canola into a myriad of weedy relatives. Many authors cite the low frequency of hybridization and low fertility of hybrid progeny to support an argument of low ecological risk of engineered plants such as canola. However, if the transgene in question confers an increment of fitness to its host plant, then there will be a high likelihood the transgene will become fixed in the population of escaped plants. This is because it requires only a few fertile spontaneous hybrids to enable the transgene to spread and persist in nature if it confers increased competitive ability to its host. In an ongoing field experiment in Georgia, preliminary data show that canola harboring an insecticidal gene (a synthetic *Bacillus thuringiensis* crystal endotoxin gene (*Bt CryIAc*) (Stewart *et al*., 1996b) had better overwinter survivorship than non-transgenic canola when exposed to autumn insect feeding pressure (Stewart, *et al*., unpublished data). As the research continues we will better assess the persistence in the environment of the *Bt* canola. At this time it is uncertain whether *Bt* canola would be a weed in an agricultural sense, but the prospect of a gene conferring strong insect resistance in weedy wild mustard is not appealing. Because the spread of transgenes is highly likely to occur (Mikkelsen, *et al*., 1996), it is important to have some means of monitoring transgenic plants and/or transgene flow in order to be able to assess the consequences. Thus, although data are still needed to assess the risk of various crop-transgene combinations, a transgene monitoring

system is imminently required.

Monitoring transgenes

Current methods to monitor transgenes are identical to the methods that are used to estimate gene flow in biotechnology risk assessment experiments. None are quick and universally applicable. The aim of such experiments is typically to estimate the amount of gene flow from a pollen-donor population of transgenic plants or otherwise marked plants to non-transgenic plants grown at specified distances from the transgenics (Fig. 1).

> **Figure 1.**A typical experimental design used to assess gene flow from pollen-donor transgenic or 'marked' plants in the center 'bullseye' to concentric rings of non-transgenic pollen-recipient plants. Seeds are collected from pollen-recipient plants and progeny analyzed for transgene frequency.

The offspring of the "ring" (pollen recipient) plants are collected and analyzed. Hybridization frequency (F), the proportion of crossed or hybrid progeny and hybridization rate, defined as the total expected pollen flow at each distance if target populations are situated as concentric rings around the pollen donors (transgenics) are determined (Klinger *et al*., 1992; Arias and Riesenberg, 1994). Hybridization rate is defined as R = F pi x d, where d is the distance of the source to the target population (Klinger *et al*.,1992; Arias and Riesenberg, 1994). For either parameter, F must be determined using genotypic or phenotypic means. The means utilized is highly dependent on available technology.

In gene flow experiments researchers have used both transgenic and non-transgenic plants as pollen sources. In the cases of non-transgenics, researchers have utilized phenotypic (Manasse, 1992; Luby and McNichol, 1995), biochemical (Klinger *et al*., 1992; Arias and Rieseberg, 1994) or molecular markers (J rgensen and Anderson, 1994) already resident in the plants of interest. In assessing gene flow of transgenic plants, either specific PCR of the transgene or linked dominant marker gene products coupled with selection regimes have been used (Klinger *et al*., 1992; Morris *et al*., 1994; Paul *et al*., 1995; Paul *et al*., 1995). The disadvantages of using specific PCR markers are obvious. DNA must be isolated from plants and the transgene fragment must be amplified using PCR, which is a daunting and laborious task. Therefore, most researchers have used dominant markers such as a transgene conferring herbicide- or antibiotic-resistance. In this system, the transgenic plants survive an otherwise lethal dose of herbicide or antibiotic, while non-transgenic plants die. Although this approach is less labor-intensive and less expensive than PCR, intrinsic drawbacks exist. First, the seedlings lacking the transgene must be killed during the assay, so no persistence-in-the-environment questions may be directly answered using these plants. Thus, this approach is not suitable for the monitoring of putatively escaped transgenes in ecological settings. Second, herbicide and antibiotic resistance genes are not neutral markers under some conditions. For transgenic plant persistence experiments

neutral markers are desirable so there will be no interactions with the transgene of interest. Paul *et al*. (1995) side-stepped the first drawback by using plants engineered with beta glucuronidase gene (GUS) (Jefferson, 1989). Plants with this gene, upon application of the substrate (X-Gluc), turn blue. Plant snippets, therefore, were sampled and tested for GUS to determine the degree of persistence of the transgenic plants. There are problems with using GUS in this respect, though, because the price of the necessary substrate, X-Gluc, makes the assay very expensive. This assay also requires tissue sampling so it is therefore not an *in vivo* assay. It is clearly apparent that none of the above methods is universally suitable for monitoring the ecological fate of transgenes on a commercial scale.

In vivo monitoring of transgenic plants

A non-destructive *in vivo* assay using a transgene that could be inserted into any plant species is desirable. Such a transgene is the gene coding for green fluorescent protein (GFP), recently isolated and cloned from the jellyfish *Aequorea victoria* (Prasher *et al*., 1992). GFP is a 27 kDa monomer that has the unique characteristic of emitting green light when exposed to ultra-violet (395 nm) or blue light (490 nm). It has been genetically engineered into bacteria, nematodes, *Drosophila*, mice, and plants (Chalfie *et al*., 1994; Prasher, 1995; Haseloff and Amos, 1995). The distinguishing characteristic of GFP, compared with other reporter genes is its ability to glow with no added substrate, enzyme, or co-factor (Prasher, 1995). Thus, it is a unique *in vivo* transgenic marker. A transgenic marker having such characteristics is "universal", because it is species-independent. Any transgenic plant can be potentially visually tracked with the aid of this powerful *in vivo* reporter gene and using a generalized experimental plan. So, GFP could be fused translationally or transcriptionally, or simply linked on the same plasmid to another transgene of commercial or agronomic importance, and the resulting plants may be easily monitored (Fig. 2).

> **Figure 2.**GFP-transgenic (green) and non- transgenic (pink) tobacco (*Nicotiana tabacum* cv.'Xanthi) exposed to a hand-held ultraviolet light (UVP model B 100 AP, Upland, CA: 365 nm). Photos (except A) were taken with a yellow filter. The *mGFP4*construct (courtesy of Jim Haseloff), under the control of the *CaMV 35S*promoter was engineered into tobacco using *Agrobacterium*-mediated transformation. (A) Seedlings from tissue culture. (B) Whole mature plants of transgenic and non-transgenic plants. (C) A closer view of mature fluorescent and non-fluorescent inflorescences. Note that panicle and calyces express GFP.

When *GFP* is under the control of a constitutive promoter and when viewed under low ambient light, the leaves of *GFP*-transgenic plants fluoresce green when exposed to ultraviolet or blue light. The GFP gene must be highly expressed for its green color to mask the pink autofluorescence of chlorophyll; so that the plants appear green rather than pink under a UV light (Fig. 2).

Only recently have GFP genes been modified for expression high enough to be useful for ecological applications (i.e., whole plant fluorescence). In preliminary studies, transgenic plants engineered with the native GFP gene do not yield high expression (Haseloff and Amos, 1995) because of cryptic splice sites (Haseloff & Amos 1995) and poor codon usage (Sheen *et al*, 1995). Because of this poor processing in plants resulting in low expression, the gene has been modified by Jim Haseloff and coworkers (Haseloff and Amos, 1995) for higher expression in plants. This modified gene (mGFP4) has altered codons at the sites of prior mis-splicing, but has an unchanged amino acid sequence. The modified gene provides stable and improved expression in transgenic plants (Haseloff and Amos, 1995). More recently, versions of the gene with increased expression of wildtype have been produced (Sheen *et al*., 1995; Crameri *et al*., 1996). Sheen's version is a synthetic gene that has been codon optimized for high expression in plants (mentioned in Sheen *et al*., 1995). Crameri and colleagues (Crameri *et al*., 1996) took a different approach to modify the gene in order to increase expression. They used an approach called "DNA shuffling" to randomly mutagenize the coding sequence coupled with the direct selection of highly fluorescent transformed bacterial colonies. It is unclear, however, whether such a GFP gene which has been selected for higher expression in bacteria would also have higher expression in plants. From work in my lab, it seems that either mutagenized or synthetic versions of *GFP* should yield expression high enough for ecological monitoring (Stewart, 1996).

The process of creating and monitoring transgenic plants may be patterned after the following format. First, plasmid constructs are made by linking GFP and the commercially interesting gene (or creating transcriptional or translational fusions if appropriate), along with a selectable marker (Fig. 3).

> **Figure 3.**An example of a binary expression vector useful for in-vivo monitoring of transgenic plants. The HPH (hygromycin phosphotransferase) gene allows selection of transgenic cells or shoots. As an example of an economically- and ecologically important gene, Bt (synthetic *Bacillus thuringiensis cryIAc*, courtesy of Mycogen Genetics), is linked to the *sGFP*(a synthetic GFP, courtesy of Jen Sheen). Until such time as recombination occurs, one may assay for the presence of Bt by observing plants under an ultraviolet or blue light. B= *Bam*HI, C=*Cla*I H= *Hin*DIII, K=*Kpn*I N= *Nco*I p= *Pst*I, S= *Sal*I, X= *Xba*I.

However, in some applications the selectable marker may be omitted and visual selection of high-expressing transformants may be performed instead. The plasmid is then engineered into the plant of interest using *Agrobacterium*-, gene gun-, or other process-mediated transformation. Transgenic plants are selected, characterized and made homozygous. After the seeds are deployed in the field, gene flow experiments may be performed by visually observing progeny plants under an ultraviolet or blue lamp at night. Monitoring commercial plots or large fields may consist of aerial blue light deployment

coupled with aerial observation or remote sensing. The green-fluorescing plants should be apparent in the background of pink-to-red non-transgenic plants.

The described method should provide researchers a simplified method to perform gene flow and transgene persistence experiments. Instead of testing plant species one-at-a-time, many plant species may be tested in tandem and in the same field. Since the plants may be allowed to re-seed *in situ*, the composition of the population (transgenic vs. non-transgenic) may be assessed in real time, sidestepping the need for complex molecular or biochemical analyses. Thus, using such a system also opens the door for performing ecological experiments where single genes may be manipulated and the ecological significance of a gene or an allele may be rapidly assessed. Finally, and perhaps most importantly, biotechnology companies may use such a system such as this to "tag" the genetically engineered plants they produce. Thus, in the event that a gene is found to cause or be associated with an ecological risk, the plants may be monitored closely for escapees. Since transgenic plants would "stand out in a crowd" they could be easily identified and destroyed if needed. Phase shifted GFPs that emit red and blue light have been recently identified (Delagrave *et al*., 1995; Ehrig *et al*., 1995; Heim and Tsein, 1996). As a result, it may be advantageous to tag certain transgenes with different spectra. In time, other, more useful (perhaps needing no specialized lighting schemes) *in vivo* markers may be identified and characterized, providing researchers and companies additional tags with which to monitor transgenic plant releases.

In order for this scenario to be possible, agricultural biotechnology companies must have a long view of the commercialization of transgenic crops. That is, to assure the long-term success of individual products and, in turn, their respective company, a mitigating-plausible-ecological-effects view rather than passing-federal-regulations view must be in place (Kareiva *et al*., 1994). Perhaps more controversial paradigm shift is from a national perspective to an international perspective. For example, the commercialization of soybean containing a strongly insecticidal *Bacillus thuringiensis* (*Bt*) transgene (Stewart *et al*., 1996a) would have little ecological effect in North America with regards to gene transfer and persistence in weedy wild relatives, since there are none. However, if the same *Bt* soybean is grown in China, the center of genetic diversity of *Glycine max* and *Glycine soja*, then transgene transfer could have significant effects on decreasing genetic diversity; a difficulty for breeders (Angle, 1994). This scenario of illicit immigration of transgenic crops is quite plausible. More than one visiting Chinese scientist has asked me for *Bt* soybean seed to "help the farmers in his/her country." It would be helpful, from a long-term research point-of-view, to have the tools in place in which to monitor, and therefore evaluate transgenic plant releases.

Research still needs to be performed to assess the safety of GFP (food safety and ecological side-effects). In addition, it is important to determine the cost of highly expressing a GFP gene from a plant fitness viewpoint. It is possible there will be significant metabolic, reproductive, or photosynthetic costs to synthesizing GFP. However,

preliminary data form my lab show GFP expressing plants are apparently no different than non-transgenic plants (Stewart, 1996). Clearly all these items must be resolved before a GFP-tagging system could be used on a commercial scale.

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