Scientific Methods Workshop:

Ecological and Agronomic Consequences of Gene Flow from Transgenic Crops to Wild Relatives

Meeting Proceedings

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SUMMARY

Gene flow from transgenic plants to wild relatives is one of the major research areas targeted by USDA's Biotechnology Risk Assessment Research Grants Program (BRARGP). We received funds for a two-day workshop that brought together researchers who study the prevalence and consequences of gene flow from transgenic crops to weeds and other wild relatives. On the first day, speakers discussed the general context for gene flow research, the information needs of USDA-APHIS, EPA, and the biotechnology industry, and case studies of specific crop-wild complexes, including cucurbits, brassicas, sunflower, sorghum, rice, wheat, maize, strawberry, poplar, and turfgrasses. Written summaries of these talks are included below. On the second day, breakout groups discussed the advantages and disadvantages of various approaches for studying the occurrence of gene flow and various effects of gene flow (fitness effects of transgenes in wild relatives, effects on population dynamics, indirect community effects, and effects on the genetic diversity of wild relatives). The crops, wild relatives, and regulatory issues we discussed focused on the USA, but much of the workshop was also relevant to similar situations in other countries. Proceedings and abstracts from the workshop are available for download from the workshop website (www.biosci.ohio-state.edu/~lspencer/gene_flow.htm). Bridging the fields weed science and plant ecology, we discussed the most appropriate and rigorous empirical methods available for studying questions related to gene flow from transgenic crops to weedy and wild relatives.

BACKGROUND AND GOALS

Gene flow between crops and free-living, noncultivated plants is often considered to be an undesirable consequence of adopting transgenic crops (e.g., NRC 1989, NRC 2000). This process occurs when pollen moves from a crop to its wild or feral relative – or *vice versa* – and genes from their offspring spread further *via* the dispersal of pollen and seeds. In addition, some crops, such as oats, radish, and oilseed rape, can proliferate as feral weeds. Although crops and weeds have exchanged genes for centuries, transgenes can confer novel, fitness-related traits that were not available previously, and the same transgenes can be introduced into many different crops, increasing the potential for their escape (e.g., resistance to the herbicide glyphosate). A fundamental question, then, is what impacts could single or multiple transgenes have on the abundance and distribution of wild relatives? From a regulatory perspective, it is useful to compare the effects of transgenes to effects of nontransgenic crop genes that spread to wild and/or weedy populations, keeping in mind that certain traits developed through the introduction of transgenes (e.g. herbicide tolerance, herbivore and pathogen resistance, and resistance to harsh environmental conditions) have been produced through traditional breeding as well.

As a starting point, we need to determine which crops hybridize spontaneously with wild and/or weedy relatives in a given country or region. In cases such as sunflower, squash, and radish, the crop and the weed represent different forms of the same species, and crop-to-wild plant gene flow occurs whenever these forms grow near each other. In sunflower and radish, crop genes are known to persist for many generations in wild populations, even when first-generation wild-crop hybrids produce fewer seeds per plant than wild plants (e.g., Whitton et al. 1997, Snow et al. 2001). Gene flow can also occur when crops and weeds are more

distantly related, for example between wheat (*Triticum aestivum*) and jointed goatgrass (*Aegilops cylindrica*), sorghum (*Sorghum bicolor*) and johnsongrass (*Sorghum halepense*), or oilseed rape (*Brassica napus*) and field mustard (*Brassica rapa*) (Zemetra et al., 1998; Arriola and Ellstrand, 1996; Jeorgenson and Anderson, 1994). On the other hand, gene flow from maize, cotton, soybean, potato, and many other species is not a problem in the USA because wild or weedy relatives of these crops do not occur nearby. Thus, the extent of gene flow between crops and weeds is expected to vary among crops and geographic regions.

Currently, it is not possible to prevent gene flow between sexually compatible species that occur sympatrically. Pollen and seeds disperse too easily and too far to make containment practical. Therefore, it is important to determine which types of transgenic crops have novel traits that might enhance the vigor or invasiveness of wild or weedy relatives or have other detrimental effects. In the short term, the spread of transgenic herbicide resistance may create logistical and/or economic problems for farmers. For example, transgenes that confer resistance to glyphosate (Roundup) or glufosinate (Basta, Liberty) are expected to spread to weedy crop relatives that could otherwise be controlled by these commonly used herbicides, thereby requiring applications of alternative herbicides. Herbicide resistance could also spread to other plantings of the crop and to volunteer or feral crop plants (e.g., Hall et al., 2000). Delaying increases in populations of herbicide-resistant weeds is a basic goal of sustainable agricultural practices.

Over the longer term, certain weeds could benefit from transgenes that confer resistance to herbivores, diseases, or harsh growing conditions. Initially, the effects of one or a few transgenes may be difficult to detect unless weed populations are released from strongly limiting factors (e.g., drought stress, salinity). For most weeds, we know little about the extent to which various ecological factors limit the weed's abundance, competitive ability, or geographic range. This makes it difficult to predict whether transgenic weeds could become more difficult to manage than those that lack novel transgenes. Nonetheless, ecological research can provide helpful information for risk assessment.

For each type of transgenic crop, the following questions should be addressed:

1) Will the transgene(s) spread to free-living populations of plants and persist?
2) Are the transgenes likely to enhance the survival or seed production of weedy relatives?Could the proliferation of such transgenic weeds lead to serious environmental or agronomic problems?
3) Could transgene introgression affect the genetic diversity of wild relatives?
4) Are risks outweighed by the expected benefits of adopting particular types of transgenic crops, or should the release of these crops be prevented? How do these risks compare to those posed by conventional crop genes?

To date, most research has focused on the occurrence of gene flow and very little is known about its consequences (e.g, Wolfenbarger and Phifer, 2000). Even questions about gene flow can be complex, however. For example, empirical studies of spontaneous hybridization between a crop and its wild relatives sometimes reach very different conclusions about the frequency at which gene flow occurs (Jeorgenson and Anderson, 1994 *vs.* Timmons et al., 1996; Belanger et al. 2001 *vs.* Wipff and Rose-Fricker, 2001). Nonetheless, the state of knowledge about crop-wild hybridization is improving as more studies are carried out on economically important crops. We also know more about the *persistence* of crop genes in wild

and feral populations. Genes that have neutral or beneficial effects on fitness and are not linked to deleterious crop traits have the potential to persist indefinitely. The biggest gap in gene flow research is determining the ecological and agronomic consequences of this process. This important area of research is still in its infancy, and it is premature to draw conclusions based on existing scientific evidence. Much of the workshop was devoted to discussing possible ecological, agronomic, and evolutionary effects of crop-to-wild gene flow.

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Molecular Genetic Assessment of the Risk of Gene Escape in Strawberry, a Model Perennial Study Crop

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Dedication:

This work and presentation are dedicated to Dr. Anne Westman who passed away in November of 2001. We will always remember Anne as a true friend and excellent scientific colleague.

ABSTRACT

Concerns about genetically engineered crop plants are focused on three potential risks: (1) that transgenic crop plants will become aggressive weeds of agricultural fields or invasive in natural habitats; (2) that their engineered genes will be transferred by pollen to wild relatives whose hybrid offspring will then become more aggressive or invasive; or (3) that the transgenic crops will be a direct hazard to humans or domestic animals. The focus of our work is on the second of these risks, that of gene flow from a genetically engineered crop, strawberry, to a related wild species. Utilizing the molecular marker technology of Amplified Fragment Length Polymorphism (AFLP) and direct studies of the fitness of laboratory produced hybrids, we have documented that hybrids between cultivated and wild *F. virginiana* occur commonly and that they are highly fit, with the potential to persist and reproduce. In our molecular marker analyses, we have confirmed that *F. virginiana* populations near strawberry farms in our region contain substantial numbers of hybrid plants displaying DNA markers from cultivars currently grown cultivars no longer grown in our region. Additionally, studies of chloroplast DNAs polymorphism have allowed us to evaluate the contribution of seed dispersal versus pollen dispersal mechanisms for gene escape. Strawberries, unlike many crops, are perennial and clonal, and both these traits increase the risks of hybridization, introgression, and persistence. Our research is measuring the additional effects of these two life-history traits, and should provide guidance on how best to mitigate the risks of transgene escape from strawberries and perhaps from other outcrossing perennials as well.

INTRODUCTION

The opportunity for transgene escape via hybridization depends first on the presence of wild relatives capable of crossing with the crop under natural conditions. In fact, almost every crop is capable of hybridizing with at least one wild species; moreover, sexually compatible crops and related wild species may co-occur in agroecosystems (Ellstrand, 1988; National Research Council, 1989). However, in only a few instances have actual hybridization rates been quantified (e.g., Langevin et al., 1990; Klinger et al., 1992; Till-Bottraud et al., 1992), or subsequent introgression documented (reviewed in Doebley, 1989, 1992, Ellstrand, Prentice and Hancock, 1999). Similarly, while fertile hybrids between many crop plants and their wild congeners have been produced experimentally, the fitness of these hybrids relative to wild plants has been measured almost exclusively in annual species (Langevin et al., 1990; Klinger and Ellstrand, 1994; Snow et al., 1999; Madsen et al., 1998).

Nevertheless, hybridization and introgression between crops and related wild species are thought to have played a major role in the evolution of crop-weed complexes (Barrett, 1983; Harlan, 1983; Small, 1984) and therefore must occur at least occasionally.

Many of the traits incorporated into crop plants by traditional breeding methods (e.g., lack of seed dormancy, dwarfing, dependency on nutrient-rich soils) are likely to be disadvantageous in wild plants (Ellstrand and Hoffman, 1990). Thus, hybrids between crops and their wild relatives may be poorly adapted to non-cultivated environments which in turn may slow down or even prevent the transfer of crop genes into natural populations (Doebley, 1992; National Research Council, 1989). In contrast, traits that are targeted for gene transfer by biotechnologists such as increased resistance to herbicides, pests, and diseases may confer a fitness advantage to wild plants (Gasser and Fraley, 1989). If such traits are introduced into crop plants via genetic engineering, then hybrids produced by crop-weed matings have the potential to become serious pests (Ellstrand and Hoffman, 1990; Rissler and Mellon, 1993). Ironically, the same technology that has led to the need for studies such as ours on gene flow from transgenic crops has also provided more sensitive assays of this process than the morphological measures used in the past (e.g., Nason et al., 1992).

The most likely avenue of gene escape from crops to related wild species is gene flow by pollen (Kareiva et al., 1991; Ellstrand, 1992). The rate of gene flow by pollen may be influenced by a number of ecological and genetic factors. For example, floral traits such as self-incompatibility, high outcrossing rates, and biotic pollination will increase the probability of gene escape whereas self-compatibility, high selfing rates, and limited pollen dispersal will substantially reduce the potential for gene flow. Many crop plants are outcrossing and insect pollinated (Fryxell, 1957); for example, 8 of the 10 most important vegetable crops in California are either obligately or predominantly outcrossing (Frankel and Galun, 1977). This suggests that many crops are capable of substantial gene export if wild compatible relatives occur within mating distance. Other factors that may influence gene flow by pollen include pollinator type and behavior (Schmitt, 1980; Peakall, 1989), the spatial structure and density of plant populations (Levin and Kerster, 1974; Handel, 1983), the degree of overlap in flower phenology, and the compatibility level between the cultivar and its wild relative (Hancock, Grumet and Hokanson, 1996).

In addition to the gene flow risks posed by pollination, many crop plants (e.g. strawberry) produce attractive edible fruits, which can be dispersed by animals, often over long distances. This significantly increases the risk of gene movement to wild populations. In comparison to pollen gene flow, seed dispersal mechanisms may pose a more significant risk for gene escape at substantial distances from the cultivated fields (Ellstrand and Hoffman, 1990).

The persistence of engineered genes in wild populations will depend, in part, on the fitness of hybrid progeny. Fruit and seed abortion, seed germination and seedling establishment, and plant growth and fecundity may differ between hybrids and nonhybrids, especially in the F1 generation. The progeny from interspecific hybridizations may be markedly less fit than non-hybrids (Levin, 1978; Grant, 1981). However, if some portion of the hybrid progeny are as vigorous as their wild progenitors, or if certain traits make them more successful, genes from hybrid individuals may persist in natural populations (reviewed in Rieseberg and Wendel, 1993). While most crop-wild F1 hybrids are weak, at least a few instances have been reported where an initial F1 barrier to gene flow has been absent (Klinger and Ellstrand, 1994; Arriola and Ellstrand, 1996,1997, Snow et al., 1998), and heterosis has even been observed in a few crop-wild F1 hybrids (Langevin et al., 1990; Klinger and Ellstrand, 1994). Also, because hybrids may backcross with other individuals, engineered genes may enter the wild population through the process of introgression (Ellstrand and Hoffman, 1990, Ellstrand, Prentice and Hancock, 1999). Depending on the effects of their newly acquired traits, hybrid or introgressed plants have the potential to become aggressive weeds in both agricultural and nonagricultural ecosystems.

The cultivated strawberry (*Fragaria x ananassa*) and its wild relative *F. virginiana* represent a particularly suitable system for investigating the potential for transgene escape and its possible

environmental consequences. The two species are sexually cross-compatible such that fertile hybrid offspring are produced. Moreover, they often occur in close proximity, their flowering times overlap, they have floral characteristics that promote outcrossing, and they share the same major pollinators. Consequently, the potential for interspecific pollen flow between these two closely related species is high. While several authors have described natural populations of possible hybrid origin (Luby et al., 1991; Stahler, 1990), prior to our initial work, the incidence, mechanism, rate and consequences of gene flow between the cultivated strawberry and its wild relative had not been directly documented. The relative fitness of hybrid individuals was also unknown. This information is of critical importance, as the first transgenic strawberries are nearing deployment (Morgan, 2001; Hancock, 1999).

In this communication, we present our studies on: the measurement of genome flow between cultivated and wild strawberries in the Southeast; the relative fitness studies of laboratory produced hybrids; and an evaluation of the extent of genome flow resulting from seed dispersal vs. pollen dispersal mechanisms.

MATERIALS AND METHODS

Plant material: Fifty-two single-plant entries were evaluated (Table 1). The 13 *F.* x *ananassa* entries comprised all major commercial cultivars historically grown in the region (Caldwell 1989; Galletta 1997; Poling 1998), including cultivars developed in the eastern US, primarily grown until the late 1980s, and cultivars developed in California and Florida, primarily grown since the late 1980s. *Fragaria virginiana* was sampled from 18 populations that were selected (by the authors and regional strawberry expert J. Ballington, North Carolina State University) as remote from past and present cultivation (one to five plants sampled/site). For comparison, representative genotypes of *F. chiloensis* and *F. vesca* (a diploid putative ancestor of the octoploid species; Darrow 1966) were also evaluated.

DNA isolation: For each entry, leaf tissue (100 mg) was immersed in liquid N_2 and finely ground, then genomic DNA was extracted and treated with ribonuclease A (Promega). Samples were extracted in DIECA (diethyldithiocarbamic acid, sodium salt) buffer, using the protocol in Lamboy and Alpha (1998). Isolated DNA was suspended in TE buffer and DNA concentration was measured on a fluorometer (Hoefer TKO 100). Sample quality was assessed by digesting 100 ng DNA with *Eco*RI (Promega), then electrophoresing digested and undigested DNA in an agarose gel.

AFLP analysis: Protocols were modified from Vos et al. (1995), using oligonucleotide primers and PCR amplification cycles described therein. For each sample, 165 ng of genomic DNA were digested with 3.3 U *Eco* RI (*E*) and 1.65 U *Mse* I (*M)* (New England Biosystems) in a 16.5 µl reaction [10 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate, 4.4 mM NaCl, 0.09 mM DTT (dithiothreitol), 8.8 µM EDTA, 8.8 ng/µl BSA] for 3 h at 37° C. Four microliters of the digestion product were evaluated in an agarose gel. The remaining 12.5 µl were combined with 12.5 µl ligation mixture [2.5 pmol of *E* adapter, 25 pmol of *M* adapter, 1 U T4 DNA ligase (Promega), 65 mM Tris-HCl (pH 7.8), 25 mM magnesium acetate, 20 mM DTT, 1 mM ATP] and incubated at 25° C for 12 h, then diluted 1:10 with TE buffer. Diluted ligation products (2.5 µl) were 'preamplified' using two primers based on adapter sequences, each with a single-nucleotide 3' extension (*E*-A, *M*-C), in a 25.1 µl reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 15 ng of each primer, and 0.5 U Ampli*Taq*® DNA polymerase (PE Biosystems). Preamplification products were diluted 1:50 or 1:30 in TE buffer. The dilutions were used as templates for selective amplification, with primers based on preamplification primers with a 3' extension of two nucleotides. Four pairs were used: *E*-ACC/*M*-CAG, *E*-ACT/*M*-CAA, *E*-ACT/*M*-CAC, and *E*-AGG/*M*-CAT. In each pair, the *E* primer was

5' end-labeled for fragment detection: 2.5 ng of primer were incubated with 0.5 µCi [γ^{33}P ATP] and 0.1 U T4 polynucleotide kinase (Promega) in 0.25 µl volume (1X manufacturer's buffer) at 37° C for 1 h, then 70° C for 10 min. Each PCR reaction (10 µl) contained 2.5 µl of diluted preamplification product, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 ng of labeled *E* primer, 15 ng of unlabeled *M* primer, and 0.15 U Ampli*Taq*® DNA polymerase.

Reaction products were mixed with equal volume of tracking dye (98% formamide, 10 mM EDTA, 0.25% each of bromphenol blue and xylene cyanol), denatured at 90° C, and evaluated by vertical electrophoresis on 6.0% denaturing gels (20:1 acrylamide-bisacrylamide, 7.5 M urea) in 1X TBE buffer (50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 8.0) at constant power (70 W, 40-50 V/cm). After electrophoresis, gels were transferred to filter paper and dried. Xray film (Kodak) was exposed to dried gels. Each primer pair x entry was evaluated on at least two gels.

Data analysis: For each primer pair, presence/absence of each fragment was recorded for each entry. Fragments of the same size in different entries were considered homologous. Pairwise distances between entries were computed using distance metric D_D, the complement of Dice's (1945) similarity coefficient, defined as $1-[2n_{xy}/(n_x + n_y)]$ (n_{xy}=number of fragments common to plants x and y; n_x and n_y= total number of fragments in *x* and *y*). Distances were calculated for each primer pair separately and for combined data of all pairs. Correlations between distance matrices for the separate pairs were evaluated with Mantel (1967) matrix correspondence tests, using The R Package (Casgrain et al. 1999). Subsequent analyses were conducted with the combined data. The distance matrix was used to conduct principal coordinate (PC) analysis, with Splus5 (MathSoft), and neighbor joining (NJ) analysis, with PHYLIP version 3.5c (Felsenstein 1995). Consensus analysis was conducted from ten replicate NJ analyses, with data entered in a different order each time. Geographic distances between the *F. virginiana* entries' collection sites were computed from latitude and longitude, using RAPDistance version 1.04 (Armstrong et al. 1996). Correlation between geographic and marker distances was evaluated with the Mantel test. For nine cultivars, the relative contributions of 29 parental clones (Sjulin and Dale 1987) were used to calculate pairwise distances, with $D_{ij}=\sum_k |(x_{ik} - x_{jk})|/100$ (x_{ik} and x_{jk}=contribution of parental clone *k* to cultivars *i* and *j*) (Graham et al. 1996). Correlation between pedigree and marker distances was evaluated with the Mantel test.

Fragments were selected as 'cultivar markers' for detecting crop-wild gene flow, based on stringent criteria (fragments present in at least one *F.* x *ananassa* cultivar and absent from all 31 *F. virginiana* entries) or on frequency (present in at least three cultivars and fewer than four *F. virginiana* entries). Fragments were selected as wild *F. virginiana* markers based on stringent criteria (present in at least one *F. virginiana* entry and absent from *F.* x *ananassa* and *F. chiloensis*). We used the program Resampling Stats (Resampling Stats, Inc.) to examine the relationship between the number of *F. virginiana* entries evaluated and number of cultivar markers selected. From the set of 31 *F. virginiana* entries, subsets of *n* entries were randomly sampled without replacement (*n*=1, 3, …, 31). For each subset, the number of fragments selected as cultivar markers under the stringent criteria was determined. For each value of *n*, sampling was repeated 1000 times.

RESULTS AND DISCUSSION

Determine the extent of gene flow from cultivated strawberry to the wild strawberry in an extensive sampling in the Piedmont region.

To develop cultivar-specific markers, we assessed AFLP marker variation in a diverse germplasm array (Table 1, Fig. 1): strawberry cultivars in our region (1930 to the present); *F. virginiana* from sites isolated from cultivation; and *F. chiloensis*. (cultivated strawberry is a hybrid of *F. virginiana* and *F. chiloensis*.) Four AFLP primer pairs were evaluated. All fragments were scored, and marker distances between entries were calculated (proportion of fragments not shared between entries). Using 4 primer pairs, 254 fragments were generated. Marker variation was extensive, and 89% of the fragments were polymorphic. The diploid putative ancestor *F. vesca* was evaluated, and 92% of its fragments were shared with the octoploid species. Fragment number and polymorphism were similar among primer pairs. In cluster analyses (Fig. 2), *F. vesca* was distinct from the other species, and *F. virginiana* and *F.* x *ananassa* were closely grouped. AFLP distances between cultivars were correlated with distances based on pedigrees (Sjulin and Dale 1987) (Mantel r = 0.571, $P \leq 0.003$).

Twenty-six of the fragments were selected as markers characteristic of the cultivars, appropriate for measuring gene flow. Each marker was present in ≥1 cultivar and absent/rare in wild *F. virginiana* (Table 3). Two thirds of the markers were shared with the *F. chiloensis* parent (and thus may derive from it). Nine markers were specific to (SE) cultivars grown in the Southeast, primarily until the late 1980s, and two were specific to California and Florida (CA/FL) cultivars, almost exclusively grown since the late 1980s.

To assess gene flow from cultivated to wild strawberry, we are using 24 of these markers to evaluate plants from wild *F. virginiana* populations located at distances of 15 m to 7 km from 10 strawberry farms (Fig. 1). To date, we have evaluated 107 plants from 10 populations (Tables 4,5). In each population, 83% to 100% of the plants had at least one cultivar marker. Twenty-one of the cultivar markers were present, including markers for modern cultivars and markers for cultivars no longer widely grown. Marker frequencies were variable but not random, suggesting variable introgression and persistence. Within populations, plants had different marker combinations. Seven populations had plants with markers derived from >1 cvar, which thus appeared to be second- or later-generation hybrids. The frequency of markers from modern cultivars was highest in populations ≥100 m from strawberry crop fields. Overall, however, cultivar marker frequency was not well correlated with a population's distance from the crop fields, suggesting that introgression of crop genes was not limited to the vicinity of cultivated fields.

Assess the relative contribution of gene flow by pollen vs. seed in wild populations containing hybrid plants.

To develop cultivar-specific markers for maternally-inherited organelle genomes, we used PCR-based markers to assess polymorphism in *Fragaria* entries (3 *F. chiloensis*, 6 cultivars, 6 wild *F. virginiana*). We evaluated 12 PCR-RFLP markers for intergenic and intron regions in the chloroplast and mitochondrial genomes (Taberlet et al. 1991, Demesure et al. 1992, Dumolin-Lapegue et al. 1997). The markers were amplified using primers based on conserved gene sequences. PCR products (500-3800 bp in length) were digested separately with 8 restriction enzymes and 3 enzyme combinations, and digestion products were electrophoresed. Polymorphism was detected for one marker in the chloroplast intergenic region $trnS_{UGA}$-trnfM (Sfm), and it was used to evaluate additional entries (Table 6). Plants had either one or two copies of a 32 bp sequence (haplotype A or B respectively). Primers were designed to amplify a 300-350 bp product including the polymorphic region, and this STS marker was used to evaluate wild *F. virginiana* plants from sites near strawberry farms. In the species entries (Table 6): Haplotype A was present in *F. vesca*, all *F. chiloensis*, 5 *F.* x *ananassa* cultivars and 18 *F. virginiana* entries. Haplotype B was present in 8 cultivars (including the major CA/FL cultivars) and 11 wild *F. virginiana* entries. Most of the cultivar haplotypes were consistent with published pedigrees. Results for *F. virginiana* population samples: All plants in Pops 1 and 2 (Fig. 1) were haplotype B. At Ferguson farm, most plants were haplotype A. At Robertson farm there were four populations sampled. All

plants in 3 populations were haplotype B. The fourth population was comprised of two subpopulations separated by 30 m each. One subpopulation displayed only the A haplotype, the other only the B haplotype. Thus wild strawberry chloroplast genomes (derived from seed parent) were polymorphic, within and between populations and farms.

Simple sequence repeat (SSR) markers: Bryan et al. (1999) recently developed 36 PCR markers based on SSRs in intergenic and intron regions of the *Nicotiana tabacum* chloroplast genome, and 26 were polymorphic in Solanaceous spp. Three of these markers have been evaluated for polymorphism in *Fragaria*, using primers and protocols modified from Bryan et al. (1999). Two of the primer pairs (NTCP3 and NTCP30) amplified polymorphic products (Table 6). Combining Sfm and SSR markers, 10 haplotypes were present in the 52 *Fragaria* species entries. Four haplotypes were present in cultivars and absent from *F. virginiana*, and 2 were only in *F. virginiana* entries.. If hybrid *F. virginiana* plants display any of these haplotypes,we can determine if they result from a past seed dispersal event or from pollination from the field. For those hybrids displaying haplotypes present in both cultivars and wild *F. virginiana* , we will not be able to determine the mode of genetic dispersal.

Work is complete for evaluation of these markers in wild *F. virginiana* plants from sites near strawberry farms . Final stages of the work, currently underway, include verification of maternal inheritance of the markers in interspecific crosses, and use of the markers characteristic of cultivars to evaluate maternal parentage of wild *F. virginiana* plants from sites near farms.

Assess the relative fitness of F1 hybrids and nonhybrids in the natural populations.

During each of three years, flowers of *F. virginia*na plants were pollinated with one of four different pollen donor types: other *F. virginiana* plants (wild-wild crosses) and three cultivars (Apollo, Cardinal and Chandler; wild-cultivar crosses. For each parameter measured (fruit set, mean seed mass per fruit, seed germination and seedling survival), seed derived from wild-wild crosses performed better than wild-cultivar crosses in some years, but not in others (Table 7). For several parameters, wild-Chandler crosses performed better than the other cultivar-wild crosses, and were competitive with wild-wild crosses. In a field experiment in which hybrid and non-hybrid plants were intermixed, there was no significant difference between wild and hybrid plants in stolon number, leaf number and in above ground biomass after three years. Fruit production was higher in wild-Chandler crosses than in wild-wild crosses. Overall, the results of this study indicate that hybrid plants are viable and vigorous and in many respects comparable to that of wild plants

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Table 1. *Fragaria* species array.

ID	#plts	Location	County	Comments	Collected	Source	cptype[2]
F. vesca	1			cultivar Mignonette	1998	NC	A
F. chiloensis							
Fchil38	1	coastal N. CA		Del Norte; aphid resistance	1981	USDA	A
Fchil358	1	coastal N. CA		Cape Mendocino	1984	USDA	A
Fchil1377	1	Los Lagos, Chile		Valcano Michimahuida	1992/95	USDA	A
Fchil372	1	Cuzco, Peru		selection from cultivated type	1965	USDA	A
Fchil31	1	coastal CA		Dry Lagoon	1981	USDA	A
Fchil1330	1	coastal OR	Lane	OR Dunes Natl Rec Area	1994	USDA	A
Fchil33	1	coastal OR	Lincoln	Yachats State Park	1977	USDA	A
F. virginiana							
959	1	central west SC	Greenwood	Sumter Natl Forest, ditchbank	1995	USDA	B
9511	1	central west SC	Abbeville	Sumter Natl Forest	1995	USDA	A
Ocon (1-3)	3	northwest SC	Oconee	Oconee Station, forest edge	1998	Clemson	BBB
FHD (1-5)	5	northwest SC	Oconee	Sumter Natl Forest, forest edge	1999	Clemson	AAAAA
CHD3	1	northwest SC	Greenville	roadside near Caesar's Head	1999	Clemson	A
9613	1	northeast NC	Bertie	woods near stream	1996	USDA	A
9617	1	northeast NC	Pasquotank	roadside near woods	1996	USDA	B
1627	1	northeast NC	Jones	woodland edge	1996	USDA	B
Math (1-4)	4	northwest NC	Jackson	woodland edge	1997	Clemson	BBBB
BSO (1-4)	4	northwest NC	Transylvania	Blue Ridge Pkwy, forest edge	1998	Clemson	ABAA
DC4	1	northwest NC	Jackson	Blue Ridge Pkwy, forest edge	1998	Clemson	A
RTF1	1	northwest NC	Haywood	Blue Ridge Pkwy, forest edge	1998	Clemson	A
Fv21,22	2	northwest NC	Transylvania	Blue Ridge Pkwy, forest edge	1997	Clemson	AA
9619	1	southeast AL	Lee	open woodland + roadside	1996	USDA	A
9625	1	southeast AL	Tallapoosa	roadside	1996	USDA	A
9627	1	southeast AL	Tallapoosa	roadside	1996	USDA	A
9633	1	south central AL	Dallas	roadside+limestone cedar glade	1996	USDA	A
9510	1	GA			1995	USDA	B
F. x ananassa cultivars							
ID		cultivar name	cultivated at present?	original maternal parent	date intro-duced	breeding program	
SwtCh[1]	1	Sweet Charlie	moderate	FL 80-456 (breeding line)	1994	FL	B
Cam[1]	2	Camarosa	moderate	Nich Ohmer	1992	CA	BB
Chand[1]	3	Chandler	widespread	Nich Ohmer	1983	CA	B
Card	1	Cardinal	seldom	The Native Iowa (*F. virginiana*)	1975	AK	A
Eglow	1	Earliglow	seldom	Aberdeen	1975	MD	B
Titan	1	Titan	seldom	Chesapeake	1971	NC	B
Apollo	3	Apollo	seldom	Chesapeake	1970	NC	BB
Atlas	1	Atlas	seldom	Chesapeake	1970	NC	B
Seq[1]	1	Sequoia	none	Hudson Bay (*F. virginiana*)	1968	CA	A
Ebelle	1	Earlibelle	none	The Native Iowa (*F. virginiana*)	1964	NC	A
Sun	1	Sunrise	none	Missionary	1964	MD	B
Alb	1	Albritton	none	The Native Iowa (*F. virginiana*)	1951	NC	A
Blake	1	Blakemore	none	Missionary	1929	MD	A

[1]Indicates cultivars developed in CA or FL, grown since the late 1980s (CA/FL cultivars).
[2]Indicates the chloroplast phenotype for the Sfm marker (see previous specific aim 2).

Table 2. Summary of the weedy *F. virginiana* populations sampled near strawberry farms.

County	Farm	yrs cultivated	present cultivars	#sampled pops	distance from *Fxa* field	#plants/ population	
						total	sampled
Anderson	Acker	>10	Card, Apollo	1	≤50 m	±10	1
	Cedar Ridge	±4	Chand	1	50-200 m	>200	8
	Hardy	20	Card, Eglow	1	200 m	300	8
	Stoneybrook	6	Chand, Cam, SwtCh	1	≤50 m	10-20	3
Pickens	Hunter	2	Chand	1	≤50 m	25-50	5
	Trotter	7	Chand, Cam, SwtCh	3	≤50 m	20	3-5
Greenville	Beechwood	19	Chand, Cam	1	≤1 km	>100	10
	Sandy Flats	15	Chand	2	300-400 m	50-100	5
	Ferguson	14	Chand	4	50-135 m	200-300	14-33
					3-6 km		21
Greenville/ Pickens	Robertson	20	Chand	4	100-400 m	50-500	6-24
					3-6 km		19

Table 3. Markers present in cultivar(s) and absent/rare in wild *F. virginiana* (*Fv*). Number of plants containing the marker, for each species/group.

Marker[1]	STS[2]	*Fchil*	SE[3]	CA/FL[3]	Type[4]	*Fv*	Pop 1	Pop 2	Ferguson[5]	Robertson[5]
ACC21	•	1	5	**2**	A	0	2	7	+	+
ACC43		6	0	**3**	C	0	0	0	--	+
ACC80	o	2	5	1	A	0	0	0	--	+
ACT23		1	3	0	B	1	0	0	--	--
ACT45	•	2	0	**4**	C	0	2	0	+	--
ACT52		1	8	**4**	A	3	8	4	+	+
ACT63	•	3	7	**4**	A	1	4	1	--	--
ACT68		7	6	0	B	2	12	3	--	+
ACT83	o	1	1	0	B	0	0	0	--	--
ACT91	•	2	8	**4**	A	1	2	0	+	--
ACT108		1	1	0	B	0	0	0	--	--
AGG16		0	4	0	B	0	4	0	--	--
AGG30		4	4	0	B	1	0	0	--	--
AGG39		7	5	**3**	A	2	0	1	--	--
AGG63		1	7	0	B	2	5	5	--	--
AGG66		0	7	**4**	A	3	3	0	--	+
AGG70	•	1	3	**2**	A	0	11	1	--	+
AGG89		0	2	**4**	A	0	3	0	--	+
AGG104		0	4	0	B	0	4	0	--	--
CAC7		0	3	**3**	A	0	0	0	--	--
CAC18	o	0	5	0	B	0	4	0	--	+
CAC36	o	1	2	**4**	A	2	0	0	--	+
CAC40	o	0	8	**4**	A	2	14	3	+	+
CAC67	o	7	6	1	A	1	0	0	--	+
CAC73		0	3	**1**	A	0	11	1	--	--
CAC110	•	7	6	**3**	A	1	5	1	--	+

[1]Markers from primer pairs: ACC=*Eco*RI-ACC/*Mse*I-CAG; ACT=*Eco*RI-ACT/*Mse*I-CAA; AGG=*Eco*RI-AGG/*Mse*I-CAT; CAC=*Eco*RI-ACT/*Mse*I-CAC. The first three pairs were used in cluster analyses.

[2]Markers that are cloned and sequenced (•) or in progress (o).

[3]The markers present in Chandler are indicated in bold. SE = cultivars traditionally grown in the Southeast, CA/FL = cultivars from CA or FL, grown since the late 1980s.

[4]Type A = present in both SE and CA/FL cultivars (≥1 cultivar from each group); type B = present only in SE cultivar(s); type C = present only in CA/FL cultivars.

[5]+ indicates marker present in (+) or absent from (--) weedy populations near the farm.

Table 4. Cultivar markers in weedy *F. virginiana* populations (M=Pop1, S=Pop2). Marker ACT45 is in modern (CA/FL) cultivars only.

	Markers present in SE and CA/FL cultivars											Markers in SE cultivars only						# Markers
	ACC21	ACT52	ACT63	ACT91	AGG39	AGG66	AGG70	AGG89	CAC40	CAC73	CAC110	ACT68	AGG16	AGG63	AGG104	CAC18	ACT45	
Albr	■	■	■	■	■	■			■	■	■					■		10
Eglow		■	■	■				■	■		■		■	■	■	■		10
SwCh		■	■	■		■		■	■		■						■	8
Apollo	■		■	■	■	■			■		■	■	■	■	■	■		13
Atlas		■	■			■	■		■		■	■	■	■	■			11
Earl	■	■	■	■	■	■		■	■			■		■				10
Card	■	■	■	■	■	■			■			■						8
Sun	■	■		■					■			■		■		■		7
Blake		■		■		■					■			■				5
Titan		■	■	■	■	■	■		■	■	■	■	■	■	■	■		14
Seq	■	■	■	■	■	■	■	■	■								■	10
Cam		■	■	■	■	■		■	■		■						■	9
Chand	■	■	■	■	■	■	■	■	■	■	■						■	12
M1									■			■						2
M4									■									1
M5			■			■	■		■	■		■	■		■			8
M6												■		■				2
M7		■					■	■	■	■	■	■						7
M8		■					■	■	■	■	■			■				7
M9						■	■		■	■		■	■		■			7
M10							■			■	■			■				4
M11			■			■	■		■	■		■	■		■			8
M12		■					■	■	■	■	■	■						7
M14		■							■			■		■		■	■	6
M15							■		■	■						■		4
M16							■		■	■		■						4
M63	■	■	■							■		■	■		■	■		8
M64		■	■				■		■								■	5
M66	■	■		■					■			■				■		6
M68		■		■					■			■						4
M75							■			■	■			■				4
Mtotal	2	8	4	2	0	3	11	3	14	11	5	12	4	5	4	4	2	15
S8	■				■							■						3
S9	■	■																2
S20							■			■	■			■				4
S23	■	■																2
S32	■								■			■		■				4
S44																		0
S47																		0
S48	■	■																2
S50	■													■				2
S52									■			■		■				3
S55	■	■																2
S75			■						■					■				3
Stotal	7	4	1	0	1	0	1	0	3	1	1	3	0	5	0	0	0	10
total	9	12	5	2	1	3	12	3	17	12	6	15	4	10	4	4	2	17

Plants M7, M8, M12, M14, and M63 could not be first-generation hybrid progeny of a cross between wild *F. virginiana* (containing no cultivar markers) and one of the listed cultivars.

Table 5. Chloroplast PCR-RFLP and SSR marker polymorphism in *Fragaria* entries.

			Chloroplast SSR markers				multilocus
Species	Entry	Sfm	NTCP3-I	NTCP3-2	NTCP8	NTCP30	phenotype
Fchiloensis	33	A	a	a	a	ab	1
Fchiloensis	38	A	a	a	a	a	2
Fchiloensis	372	A	a	a	a	ab	1
cultivar	Chand	B	null	a	*no data*	a	3?
cultivar	Card	A	a	a	a	a	2
cultivar	Cam	B	null	a	a	a	3
cultivar	Sun	B	b	null	a	*no data*	4
cultivar	Ebelle	A	a	a	a	a	2
cultivar	Atlas	B	a	a	a	a	5
Fvirginiana	9627	A	b	a	ab	a	6
Fvirginiana	9619	A	b	a	a	a	7
Fvirginiana	BSO2	B	b	ab	ab	a	8
Fvirginiana	BSO3	A	b	ab	a	*no data*	9
Fvirginiana	Math3	B	null	ab	a	a	10
Fvirginiana	959	B	b	a	a	*no data*	11

Table 6. Relative fitness of wild *F. virginiana* and hybrid plants: fruit set, seed mass/ fruit, seed germination and progeny seedling survival at 6 weeks, for wild-wild and wild-hybrid crosses.

	Fruit set (%)		Mean seed mass per fruit (mg)			Seed germination (%)		Seedling survival (%)		
year	1995	1996	1995	1996	1998	1995	1996	1995	1996	1998
pollen donor:										
Apollo	59.0	29.2	26.7	12.5	26.5	59.0	11.8	14.5	42.0	0.0
Cardinal	53.6	23.6	31.2	22.7	27.8	53.6	40.8	15.6	64.6	73.3
Chandler	53.8	52.8	48.2	21.3	51.0	54.8	48.7	9.3	53.8	71.7
wild	83.3	58.3	50.8	19.2	36.5	83.3	42.1	10.6	82.8	100
significant difference:[1]										
among all donors	yes	yes	yes	no	no	yes	no	no	yes	yes
among cultivars	no	yes	yes	no	yes	no	yes	no	yes	yes

[1]Analysis of variance, differences among all pollen donors and among cultivars: not significant (no) or significant (yes) at $P<0.05$.

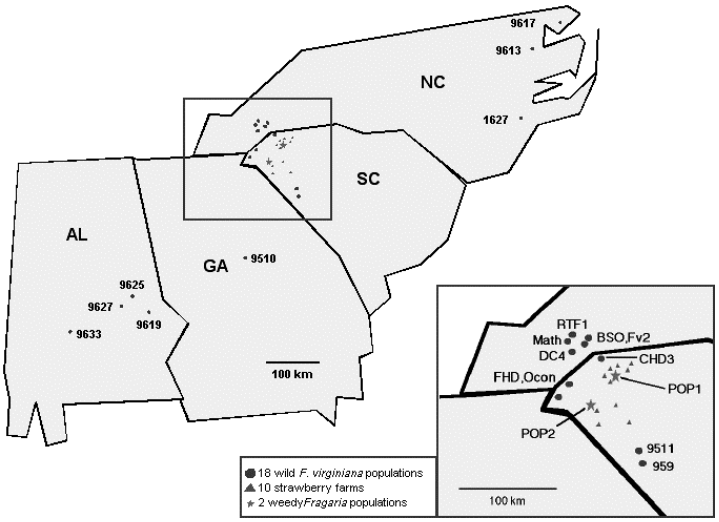

Figure 1. *Fragaria virginiana* collection sites, weedy populations and strawberry farms.

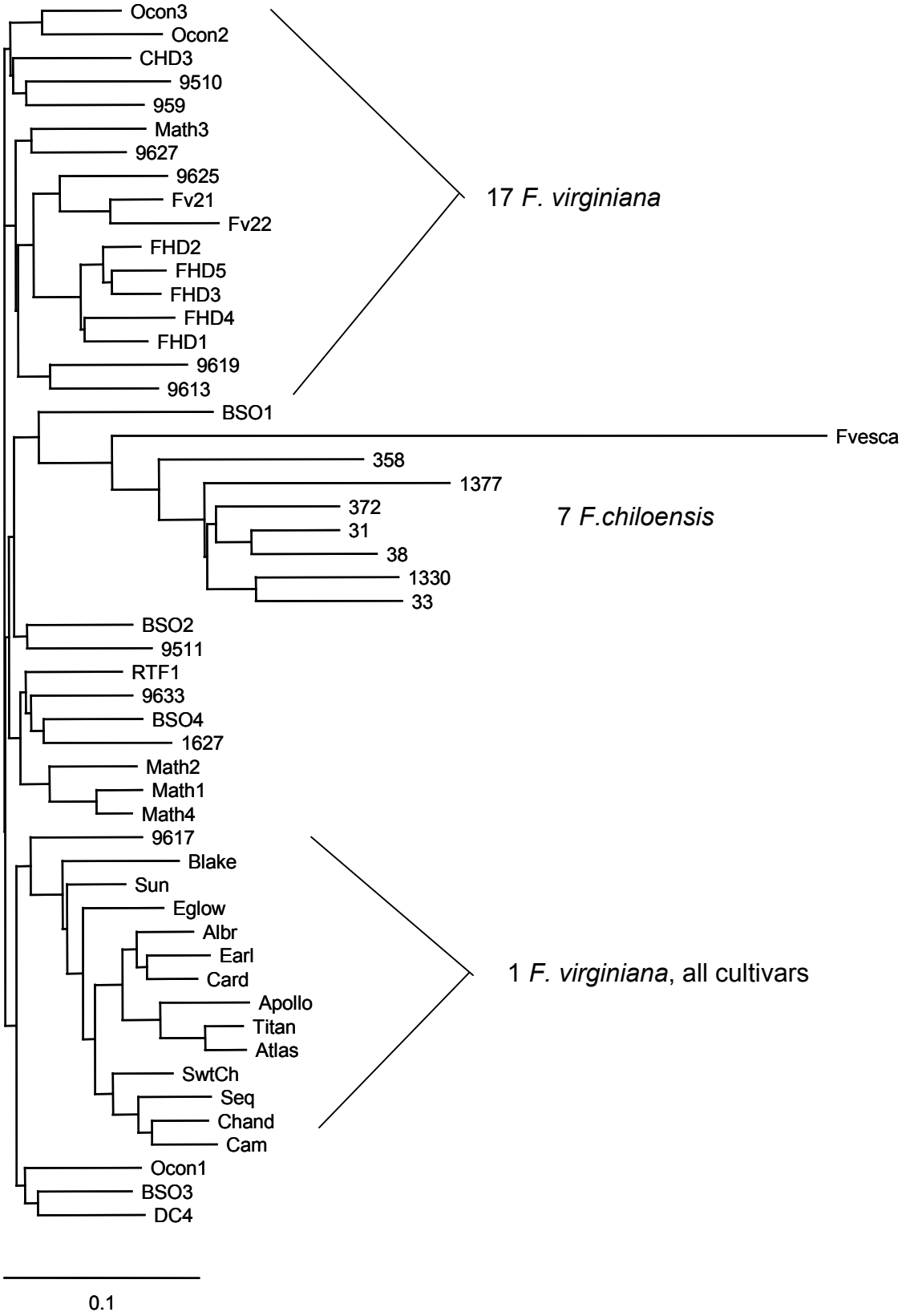

Figure 2. Neighbor joining cluster analysis of (Nei's) pairwise distances between *Fragaria* entries, based on 201 AFLP marker fragments, 3 primer pairs.

Gene flow and hybrid fitness in the *Sorghum bicolor* – *Sorghum halepense* complex.

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ABSTRACT

The experimental evidence that cultivated plants and their wild relatives regularly exchange genes through pollen transfer under natural conditions is well documented in the literature. However, there has been no consensus regarding long-term ecological or evolutionary consequences of gene flow from cultivated plants into populations of their wild relatives. We review what is known regarding gene flow from crop sorghum, *Sorghum bicolor*, into populations of its feral congeners, particularly johnsongrass, *S. halepense*. Descriptions are given of studies conducted on crossability, spontaneous hybridization and fitness of hybrids. The data presented support the hypothesis that crop sorghum commonly contributes alleles to wild/weedy sorghums under field conditions. We focus on the possible consequences of continued gene flow from the crop to wild populations and point out future research needs in this crop/wild complex.

Sorghum bicolor is one of the world's most important crops with some 42 million hectares dedicated to its cultivation last year. 2001 World production leaders include India with 10.3 million hectares, followed by Nigeria - 6.9 million Ha, Sudan - 4.2 million Ha, and the United States - 3.6 million Ha (FAOSTAT, 2001). Sorghums are wind pollinated, predominantly selfing plants with an n number of 10 ($2n = 2x = 20$); however, exceptions do occur as in the case of the tetraploid *Sorghum halepense.* In virtually all cases crop sorghum is sympatric with sexually compatible, cultivated and feral relatives. For example, in the United States, one can identify three congeners commonly growing near fields of sorghum, *Sorghum sudanense, S. almum,* and, *S. halepense*. The potential for gene flow among this group of plants is generally recognized to be high.

The history of the sorghums may provide some insight into this high potential crossability in the genus. *Sorghum bicolor* appears to have been first domesticated in sub-Saharan Africa perhaps as recently as the turn of the Common Era (Kimber 2000). It was later traded overland to India and China where it was likely used to develop and improve local species. This migration may have provided the opportunity for further natural

interspecific hybridization between the African and the Asian sorghums (Rooney and Smith, 2000). There is some genetic evidence to support this hypothesis, for example, *Sorghum halepense* is reported to be an allopolyploid result of interspecific hybridization between African *S. bicolor* and Asian *S. propinquum* (Paterson et al. 1995). This perhaps was an inauspicious mating given the fact that johnsongrass is now recognized as one of the world's worst weed pests (Holm *et al.*, 1977). The sorghums a represent fairly unique crop-wild complex with common ancestry that may be much more recent than that of many crops with similar acreage dedicated to their cultivation. Doggett (1988) confirms that the members of the genus are quite interfertile when given the opportunity to mate in the wild, thus gene flow events will likely be common.

Natural hybridization between the wild races and species of sorghum has been implicated as a potential source of new genetic variation by a number of authors (e.g. Baker, 1972; Harlan 1992). Several studies have focused on crossability between crop sorghum and its weedy relatives primarily because wild sorghums are often viewed as potential sources of new traits that might be exploited for breeding programs. For example, reports of forced crosses between johnsongrass and crop sorghums have yielded the production of viable hybrids (Hadley 1953; Sangduen and Hannah 1984). However, rogue hybridization events in either direction may be problematical. Unintended hybridization in the wild-to-crop direction can be the source of contamination of breeding plots. While pollen flow from the crop-to-weed direction may provide the means of introduction of crop alleles in to weedy populations which may enhance weediness. The present discussion centers on consideration of the later.

Doggett (1988) claims that North American johnsongrass often contains pools of stable crop-weed introgressants. Further, Harlan (1992) has suggested that continued crop-to-wild hybridization has likely been the key to the continued success and aggressiveness of johnsongrass in the United States. To evaluate the potential for spontaneous pollen transfer between crop sorghum and johnsongrass, Arriola and Ellstrand (1996) used an experimental system to measure the incidence and rate of hybrid formation in the field. The authors used an allozyme marker to directly measure the movement of pollen from a field of crop sorghum to a series of johnsongrass plants placed at intervals surrounding the field at distances ranging from 0.5 - 100 meters. Crop and johnsongrass plants were fixed for alternate alleles at an adenylate kinase (ADK) locus. Electrophoretic analysis of the johnsongrass progeny allowed identification of putative hybrids. Hybrid seeds were detected on panicles of johnsongrass plants at distances up to and including 100 meters from the crop. Rates of hybrid formation were idiosyncratic varying with field location, year, and distance of target from the crop, but occurred at a range of 0 - 12 % overall. These rates are consistent with reported sorghum natural outcrossing rates which vary from 0.1 - 30% (Ellstrand and Foster 1983; Doggett, 1988; Pederson *et al.*, 1998).

Gene flow requires not only a hybridization event, but also incorporation of the migrant alleles into the gene pool of the recipient population (Slatkin, 1987). Hence, the hybrids produced must persist in the wild if there is to be the opportunity for continued gene exchange in subsequent generations. To estimate the likelihood of persistence Arriola and Ellstrand (1997) conducted a fitness study of sorghum crop-weed hybrids grown under field conditions. Randomized rows of crop sorghum, johnsongrass and crop-weed hybrids were grown to flowering and seed set. Several measurements of fitness correlates were made

including biomass, seed production, tillering, and pollen stainability. No significant differences were reported among any of the plants measured (Table 1). Crop-weed hybrids were determined to be as fit as either parent plant. It was concluded that hybrid progeny of sorghum and johnsongrass would persist in the field and that crop-to-weed gene flow will likely occur at measurable rates.

Given the common ancestry, and likely high level of pollen exchange between crop sorghum and the johnsongrass, it is difficult to accurately estimate the consequences of continued gene flow events. Morden *et al.* (1990) have suggested that johnsongrass is likely of recent origin and thus is not well differentiated from the crop. There are data that support this conclusion at the molecular level as well (Hoang-Tang and Liang, 1988). To complicate matters, population genetic analyses of the sorghums demonstrate relatively low levels of allozyme diversity across species particularly in johnsongrass (Warwick 1990, Morden *et al*., 1989,1990; Aldrich *et al.* 1992; Arriola, unpublished data). Comparisons of johnsongrass allozyme diversity with plants possessing similar life history characteristics illustrate this point (Table 2). Of the possible explanations for these low levels of variation it is useful to consider gene flow. Continued pollen flow from the largely monomorphic crop may have a swamping effect on the smaller wild populations. The sharing of crop alleles may be causing, and maintaining a certain level of homogenization of the two gene pools. Perhaps johnsongrass and crop sorghum need more time to become more genetically differentiated than they are at present.

The promiscuity of sorghum with its congeners leads to a final consideration, the potential for transgene escape from a genetically modified *Sorghum bicolor*. The global economic importance of sorghum makes it a prime candidate for genetic transformation. However, genetically modified sorghum has not been commercially produced to date. Casas *et al.,* (1993) have transformed a variety with a herbicide (Ignite/Basta) resistance marker gene, but there has been no mention of this plant line in the literature since that time. The reasons for a lack of a modified sorghum appear to be centered on technique. Subudhi and Nguyen (2000) report that there has been little success in developing stable gene transfer methods for sorghum in spite of the report mentioned above. Perhaps, however, it is fortunate that no GM sorghums have appeared in the marketplace, as one can be confident that whichever gene is inserted into the sorghum genome, it will likely appear in the johnsongrass gene pool as well.

The *Sorghum* crop/wild complex poses many interesting problems. One can generally find all of the proper conditions for gene transfer and persistence, but it is difficult to predict when such events will occur. The similarities between the crop and feral relatives pose problems in elucidating the frequency of gene flow events in the past. One can be fairly certain, however, that sorghum has been exchanging genes with its wild relatives for some time, and there is no reason to suggest that this will stop in the future. Possible consequences of continued gene flow are varied with Harlan (1992) suggesting that johnsongrass will continue to be enhanced by gene flow from the crop and subsequent selection in its agricultural habitat. More work is need in this crop/wild complex including studies re-examining the rate of hybrid formation using molecular markers. Also, a more detailed exploration of the johnsongrass genome might enable one to determine the degree of introgression between these species and historical levels of gene flow.

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Table 1[1]. Comparisons of measured sexual and vegetative reproductive characters between hybrid and non-hybrid progeny of forced crosses between crop sorghum and johnsongrass. Date to flowering (DTF); panicle number per plant (PAN); number of seed per panicle (SEED); pollen stainability (POL); number of tillers per plant (TLR); total above ground biomass (AGB); total below ground biomass (BGB).

Character	Test	Constant[b]	N	P
DTF	M-W[a]	3319.5	55	0.11
PAN	t-test	-0.930	55	0.35
SEED	M-W	3258.0	55	0.22
POL	M-W	3372.5	55	0.056
TLR	t-test	0.229	55	0.82
AGB	t-test	-0.453	55	0.65
BGB	t-test	0.0162	55	0.99

[a]M-W = Mann-Whitney rank sum test.
[b] Constant = U for Mann-Whitney; t for t-test.
[1] Arriola, P. E., and N. C. Ellstrand. 1997. Fitness of interspecific hybrids in the genus *Sorghum*: persistence of crop genes in wild populations. Ecological Applications **7**(2): 512-518.

Table 2. Comparisons of species level measures of genetic diversity with values reported for plants with life history character similar to johnsongrass (Hamrick and Godt, 1990). Abbreviations: P, percent polymorhic loci; Ae, effective number of alleles; He, genetic diversity.

Class	P_S	Ae_S	He_S
Johnsongrass	0.181	1.17	0.119
Monocots	0.592	1.27	0.181
Widespread range	0.589	1.31	0.202
Mixed-wind breeding system	0.735	1.28	0.194
Sexual and asexual reproduction	0.438	1.20	0.138

Patterns of genetic diversity in sympatric and allopatric populations of maize and its wild relative teosinte in Mexico: evidence for hybridization

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Genetic diversity in maize and its wild relative teosinte (*Zea mays* ssp. *mexicana*) in Mexico is important for the sustainability and improvement of the third most important crop in the world. This study examines the genetic diversity of sympatric and allopatric populations of maize and teosinte and evidence for hybridization in Mexico using isozyme electrophoresis. We take a population level approach to the analysis of genetic diversity in standing Mexican populations. Our data provides evidence that hybridization contributes to the genetic similarity between sympatric pairs of maize and teosinte populations compared with allopatric populations of either subspecies. Introgressed populations of teosinte show an increase in diversity relative to isolated populations. To date, introgression from teosinte does not appear to play an important role in the genetic diversity of sympatric maize. Patterns of differentiation among sympatric and allopatric populations of maize and teosinte indicate that introgression occurs more often than previously thought. Additionally, recent introgression between maize and teosinte has resulted in morphological hybrids that often have genetically transgressed allele frequencies compared to adjacent maize and teosinte populations.

INTRODUCTION

In the Central Plateau and Valley of Mexico, maize (*Zea mays* L. ssp. *mays*) grows in sympatry with *Zea mays* ssp. *mexicana* (Schader) Iltis) one of the several teosinte taxa providing the opportunity for hybridization (Wilkes 1967). There is some evidence to suggest that *Z. mays* ssp. *mays* hybridizes more frequently with *Z. mays* ssp. *mexicana* than with other taxa of teosinte (Wilkes 1967; Doebley 1990). This gene flow between *Z. mays* ssp. *mays* and *Z. mays* ssp. *mexicana* could result in introgression, the incorporation of new genes from one taxon into the population of the other (Jarvis and Hodgkin 1999). Therefore hybridization between maize and teosinte is expected to alter the genetic diversity of sympatric populations. The impact of ongoing gene flow between these taxa on patterns of genetic diversity is of applied importance since collectively Mexican maize landraces and teosinte are potentially the most genetically diverse resource of *Zea* germplasm in the world (Senadhira 1976; Hancock 1992).

Evidence for hybridization and introgression in populations of maize and *Z. mays* ssp. *mexicana* in Mexico however remains inconclusive. There is no direct molecular evidence of hybridization at the population level by previous isozyme studies of *Z. mays* ssp. *mexicana* and maize. The purpose of this research was to seek genetic evidence for hybridization at the population level. We evaluate the current population genetic structure of maize (*Zea mays* ssp. *mays*) and its wild relative teosinte (*Z. mays* ssp. *mexicana*) from allopatric and sympatric populations in Mexico. We compared the population genetic structure of sympatric and allopatric populations of maize and *Z. mays* ssp. *mexicana* using isozyme electrophoresis. We considered evidence of hybridization and its effect in altering patterns of genetic variation in sympatric populations.

MATERIALS AND METHODS

Plant material, Allozyme electrophoresis, and Data analysis

Samples were collected from Mexican populations of teosinte (*Zea mays* ssp. *mexicana*) and cultivated maize (*Z. mays* ssp. *mays*) in the autumn of 1998 and 1999. Locations represent the geographical range of *Z. mays* ssp. *mexicana* in the Valley of Mexico and the Central Plateau and include moderate to large population sizes for both taxa. Allopatric populations of *Z. mays* ssp. *mexicana* are rare and presently appear limited to the state of Morelos. Each collected individual was assayed directly from fresh mature leaf material for twelve enzyme systems revealing eighteen loci. A full description of the plant material, electrophoresis protocol, and methods of analysis are published in Blancas (2001).

SUMMARY OF RESULTS

Genetic frequencies for each population are summarized for all polymorphic loci in Table 1 (Blancas 2001; Blancas et al. in review). Genetic diversity statistics are summarized in Table 2 (Blancas 2001; Blancas et al. in review). A hierarchical cluster dendogram of Nei's genetic sitances for the six major *Zea mays* L. groups is shown in Figure 1 (Blancas 2001, Blancas et al. in review).

DISCUSSION

Our data substantially support the long held, but controversial, supposition of hybridization between maize and teosinte. Our results show that allopatric and sympatric teosinte differ slightly in overall levels of diversity, measured in terms of polymorphic loci and estimated heterozygosity. Maize in populations sympatric with teosinte did not have higher levels of diversity than allopatric maize not currently associated with teosinte. Allozyme diversity, in terms of A and A_p, in morphological hybrids assayed from sympatric populations is equal to or greater than maize and teosinte from sympatric populations.

Typically, most population genetic studies comparing crops with their wild relatives contrast patterns of genetic diversity measures without addressing issues of introgression. Allozyme diversity within populations of maize is higher than within populations of teosinte when observed heterozygosity of all loci is examined. Allopatric teosinte populations, however, are small and isolated by comparison, and subject to loss of variation due to increased inbreeding and genetic drift (Ellstrand and Elam 1993). In this study, no two maize populations shared similar patterns of gene frequencies. In sympatric populations, the distribution of genetic variation in maize is different from teosinte. Allele frequencies at polymorphic loci can substantially vary between maize and teosinte from an associated sympatric population.

In 1984, Doebley et al. reported H_e of 0.234 and 0.249 and H_o of 0.231 and 0.215 obtained from bulk seed collections for Chalco and Central Plateau *Z. mays* ssp. *mexicana*, respectively. We obtained H_{ep} of 0.16 and 0.10 and H_{op} of 0.09 and 0.10, from our samples of sympatric and allopatric teosinte, respectively. The most polymorphic group was allopatric maize, with $H_{ep} = 0.22$ and $H_{op} = 0.05$. In general, genetic variation does not appear to be greater than the average outcrossing species as Doebley et al. (1987) suggested or according to the allozyme literature review by Hamrick and Godt (1989). Due to the nature of the local populations sown from an original seed source for consecutive years for many of the maize landraces, samples from a single population have a close genetic ancestry (i.e., correlated haplotypes) which contributes to a high F_p and an incongruity between H_{op} and H_{ep}.

A more detailed discussion of these findings can be found in Blancas (2001) and Blancas et al. (in review).

EVIDENCE FOR AND PATTERNS OF INTROGRESSION

Our data provide evidence for introgression of maize and teosinte in that sympatric teosinte populations are more genetically similar to maize than to allopatric teosinte. This observation seems to contradict evidence (Doebley et al. 1984) that sympatric teosinte maintains allozymic distinctness from maize (Doebley 1990). It seems instead that there is genetic exchange between maize and teosinte growing in close proximity or sympatrically. On the other hand, transgressed allozyme frequencies in hybrids indicate that hybrids are more distantly introgressed and maintain genetic differentiation thus behaving as a separate lineage. Additional evidence for introgression is observed in moderate to high genetic differentiation between hybrids of sympatric populations compared to allopatric populations of maize or allopatric teosinte. These data support previously obtained allozyme data (Doebley et al. 1984, 1987) that hybridization and introgression occurs between sympatrically located populations of maize and teosinte.

While our data confirm similar findings, our population level sampling suggests that hybridization events and introgression occur more often than previously estimated (Doebley et al. 1984, 1987). Extensive sampling of sympatric pairs and incorporation of morphologically intermediate hybrids with their segregation in their analysis shows a more complete picture of the genetic relationship between sympatric maize and teosinte. Doebley et al. (1984) hypothesized that continual introgression should result in a short genetic distance between sympatric pairs than non-sympatric pairs. However, the genetic distance between sympatric pairs can appear more distant if introgressed alleles of low or moderate frequency are not sampled. Small scale sampling of accessions and careful exclusion of hybrids from sympatric populations is likely to underestimate introgressed alleles, which explains why the results of Doebley et al. (1984) differ from ours.

In the absence of an appropriate diagnostic marker, accurate estimates of hybridization are difficult, especially in regions of Mexico where the maize seed is "recycled". However, we observed allele frequencies in the hybrids that transgress (frequencies that are not intermediate to the parents) those in the local parents at a number of loci. Gene flow (N_m) estimates based on F_{ST} would indicate that there are a higher number of migrants per generation among sympatric maize populations than among any other group. Continual migration among genetically similar (i.e., short genetic distance) populations reduces genetic divergence among populations (Slatkin 1985). Interestingly, allopatric teosinte populations have the second highest estimated gene flow rate of the groups we analyzed. This would suggest that the allopatric teosinte populations sampled here, although entirely allopatric with maize, are not isolated from one another.

Senadhira (1976) concluded that genetic variation of teosinte and its contribution to maize are small but represents a sizeable genetic resource based on her results. Fewer populations of teosinte both sympatric and allopatric to maize exist today (Wilkes 1967; Sanchez Gonzalez 1997; and pers. obs.). Although we can not determine the exact extent of hybridization, introgression in sympatric populations is evident, and hybridization is potentially more than the rare occurrence suggested by Doebley et al. (1987) contributing to the demise of teosinte populations. The role of introgression is significant in influencing the variation maintained in sympatric populations and in *Z. mays* ssp. *mexicana* as a whole. It is clear that there is a genetic difference between teosinte populations that are sympatric to those that have been historically isolated from maize. Although this first population genetic study of maize and teosinte in Mexico is based on populations that represent a small portion of the total number, our study shows that in addition to gene flow there are geographic genetic differences that contribute to differentiation at the population level.

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Table 1[1]. Allele frequencies observed for allopatric maize and teosinte, and maize (M), putative hybrids (H), and teosinte (T) from sympatric populations (**AA**, **Pi**, **Pñ**, **SF**, and **TM**). *N* is the number of individuals per population group analyzed.

			AA			Pi			Pñ		SF			TM			
		Maize	M	H	T	M	H	T	H	T	M	H	T	M	H	T	Teosinte
N		**304**	**21**	**21**	**21**	**19**	**22**	**29**		**20**	**4**	**16**	**24**	**21**	**20**	**27**	**21**	**222**
GOT1	1	0.26	0.19	0.14	0.17	0.03		-			0.17	-	-	0.17			
	2	0.69	0.81	0.83	0.83	0.92	1.00		1.00	1.00	0.83			0.50	0.96	0.75	0.98
	3	0.05		0.03		0.05								0.33	0.04	0.25	0.02
GOT2	1	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	2	0.01															
GOT3	1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.95	1.00	1.00	1.00	1.00
	2												0.05				
LAP1	1	0.99	1.00	-	1.00	1.00	1.00	-	1.00	1.00	1.00	1.00	1.00	0.25	1.00	-	1.00
	2	0.01												0.75			
MDH1	1	0.58	1.00	1.00	0.02	0.58		-	0.17		0.38			0.21	0.31	0.78	0.95
	2	0.41			0.98	0.42	1.00		0.83	1.00	0.62	1.00	1.00	0.79	0.69	0.22	0.05
	3	0.01															
MDH2	1	0.79	1.00	1.00	1.00	1.00	1.00	-	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.93	0.97
	2	0.21														0.07	0.03
PGD1	1	1.00	1.00	1.00	1.00	1.00	0.98	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	2						0.02										
PGM1	1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.84
	2																0.16
UDP1	1	0.10	0.07	0.02		0.48		0.47	0.32	-	0.47	0.81	0.57	1.00	1.00	1.00	0.87
	2	0.01			0.02			0.53	0.54								0.10
	3	0.71	0.88	0.55	0.84	0.21	0.91				0.47	0.19	0.43				0.03
	4	0.04		0.05	0.05	0.05			0.14		0.06						
	5	0.14	0.05	0.33	0.09	0.26	0.09										
	6			0.05													
UDP2	1	0.68	1.00	0.98	0.86	0.74	0.11	1.00	0.71	-	0.91	0.88	0.71	1.00	0.92	1.00	0.82
	2	0.32		0.02	0.14	0.26	0.89		0.29		0.09	0.12	0.29		0.08		0.18

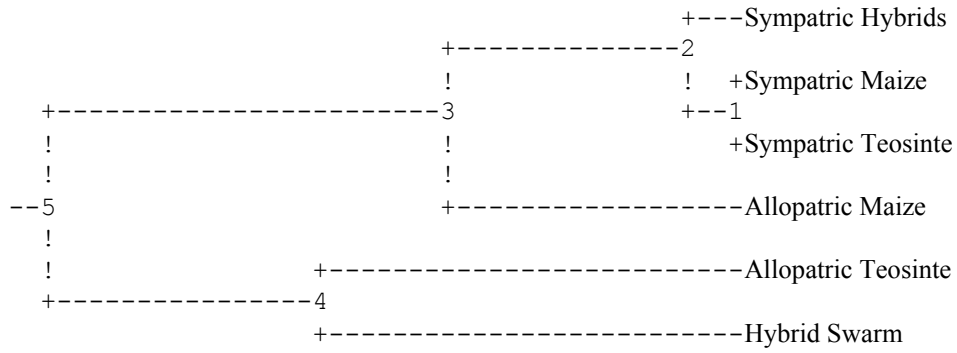
= allele frequency not reported.

Table 2[1]. Nei's (1987) genetic diversity statistics derived from allele frequency analysis at 18 allozyme loci for *Zea mays* L ssp. *mays* and *Z. mays*. ssp. *mexicana* and putative hybrid populations in Mexico.

Zea mays	**N**	$\mathbf{N_a}$	**A**	$\mathbf{A_p}$	**P**	**H**	$\mathbf{F_{ST}}$	$\mathbf{N_m}$
Z. mays ssp. *mays* (allopatric)	6	50.8	1.22	2.63	0.44	0.12	0.505	0.245
Z. mays ssp. *mays* (sympatric)	4	17.75	1.17	2.60	0.28	0.07	0.290	0.611
Z. mays ssp. *mexicana* x *Z. mays* ssp. *mays* (sympatric)	5	22.8	1.16	3.00	0.28	0.04	0.497	0.253
Z. mays ssp. *mexicana* (sympatric)	5	19.6	1.17	2.67	0.33	0.04	0.751	0.083
Z. mays ssp. *mexicana* (allopatric)	4	55.5	1.07	2.20	0.28	0.05	0.382	0.403
Z. mays ssp. *mexicana* x *Z. mays* ssp. *mays* (swarm)	1	20	1.22	2.00	0.22	0.05	*	-

N = Number of populations sampled; N_a = Average number of plants sampled per population; A = Average number of alleles per locus;
A_p = Mean number of alleles per polymorphic loci (total number of alleles from polymorphic loci divided by the total number of polymorphic loci);
P = Proportion of polymorphic loci; H = Nei's estimated heterozygosity; F_{ST} = Nei's (1987) summary F statistics for population differentiation between populations within *Z. mays* ssp. *mays* and *Z. mays* ssp. *mexicana*; * F_{IT} = measure of individual diversity relative to the total population.
Observed $F_{IT} = 0.202$; $\mathbf{N_m}$ = estimated genetic migration where $\mathbf{N_m} = [(1/ F_{ST}) - 1]/4$ (Slatkin and Barton, 1989).

Figure 1[1]. Hierarchical cluster dendrogram based on Nei's genetic distances for six major *Zea mays* L. groups sampled.



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Gene Flow from Transgenic Crops to Wild Relatives: What Have We Learned, What Do We Know, What Do We Need to Know?

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ABSTRACT

I present a brief history of the science of crop to wild gene flow and a glimpse into its future. Scientists have long recognized that crops can spontaneously mate with their wild relatives. However, for reasons probably associated with the cultures of basic vs. applied science, study of that process was largely neglected. The realization that transgenes could end up unintended in wild plants generated a closer examination of the process of crop-to-wild gene flow and its consequences. I expect that this field will continue to grow, with increased emphasis on using transgenic crops themselves as experimental organisms. However, a sister phenomenon, crop-to-crop transgene flow, and its consequences, may prove to be a more urgent emerging issue to be addressed by specialists in transgene flow.

INTRODUCTION

You would think that spontaneous gene flow from crops into wild populations would be an ideal research topic for basic and applied plant evolutionists, ecologists, and population geneticists. Crops are among the best studied plant species; their genetics and ecology have been studied for centuries. Since many wild relatives of crops are important weeds and/or important sources of germplasm, they are also better studied than your average plant species. The various research questions associated with the process are obvious: What are the factors that influence hybridization between evolutionarily distinct lineages? What is relative hybrid fitness in the field? Do different immigrant alleles introgress at different rates? What are ecological impacts of immigrant alleles at the level of the population, community, or ecosystem? Do any of these immigrant alleles contribute to weediness, invasiveness, niche shifts, or increased extinction risks? Have wild populations collected "heirloom" alleles from past varieties?

Then why did it take the advent of transgenic crops to put the spotlight on a field that had attracted the attention of no more than a handful of plant evolutionists? The key is the phrase "basic and applied". There has been a chasm between "basic plant biology" whose tradition comes from the "ivory tower" field of botany and "applied plant biology" whose tradition comes from the "practical" agricultural fields of agronomy and horticulture. The cultures of the two fields marked their territories; splitting plants into (1) those that have a direct impact on human affairs, the useful and noxious ones, and (2) those with no practical significance, such as *Arabidopsis*. The cultures also generated a bit of disdain for scientists working in the other field. Since spontaneous hybridization involves plants from each of the two groups, it doesn't fit well into either

category. While it is surprising that any work at all was done on crop to wild gene flow given these cultural barriers, it might not be surprising that the handful of scientists who published in this area prior to the advent of transgenics are among the brightest lights of their fields: examples include Edgar Anderson, Herbert Baker, Spencer Barrett, J. M. J. De Wet, Jack Harlan, and Charlie Heiser.

Let's take a brief look at the development of the field, the principles that have emerged, and the most important research questions that remain unanswered.

PAST

The fact that cultivated plants naturally mate with their wild relatives has been recognized for a long time (De Candolle 1886). In fact, decades ago Edgar Anderson (1949) described what he called "superweeds", especially noxious plants resulting from the hybridization between domesticated plants and their wild relatives. For most of the 20th century, what research was done on the topic concerned itself largely with two areas, (1) the introgression of domesticated alleles into weed populations resulting in increased crop mimicry (e.g., Barrett 1983) and (2) the spontaneous introgression of wild alleles into crop landraces that might serve as a substrate for crop improvement (e.g., Jarvis and Hodgkin 1993). A few scientists took a broader view, writing papers suggesting that domesticated plants, their associated weeds, and wild relatives form actively evolving complexes of plants, joined through both shared ancestry and occasional hybridization (e.g.,De Wet and Harlan 1975, Small 1984), similar to the what "basic" plant evolutionists call a "syngameon" (Grant 1981).

Despite these contributions, the prevailing view of both basic and applied plant scientists at the time of the creation of the first transgenic plants was that spontaneous hybridization between domesticated plants and their wild relatives was rare and idiosyncratic. Most likely, this attitude among basic plant scientists was part of a broader backlash to decades of enthusiasm for identifying natural hybrids and describing their evolutionary impact, only to have genetic analysis topple some of the "classical" examples, and the attitude among applied plant scientists probably resulted from the frustrations some breeders encountered in attempts to make wide crosses. This now-outdated view has persisted in some quarters. For example, Martina McGloughlin (2000), Director of Biotechnology at the University of California at Davis, recently wrote in a guest editorial for the *Washington Post*, "Breeders have found that, with rare exceptions, the crops do not successfully cross-breed with other plants in the environment, especially plants in crop-growing regions."

PRESENT

Given that common view, it is surprising that much thought was given to what might happen if transgenes found their way into natural populations. Interestingly, among the first to address the issue were two Calgene scientists (Goodman and Newell 1985) who wrote, "The sexual transfer of genes to weedy species to create a more persistent weed is probably the greatest environmental risk of planting a new variety of crop species". For reasons that are still somewhat unclear to me, of all the environmental concerns voiced about crop biotechnology, those associated with transgene flow into the

populations of wild relatives have received the most attention. Indeed, almost every general treatment of the environmental impacts of plant biotechnology gives some consideration to the topic (e.g., Colwell et al. 1985, Hails 2000, Keeler and Turner 1990, Marvier 2001, McHughen 2000, National Academy of Sciences 1989, 2000, 2002, Rissler and Mellon 1996, Scientists' Working Group on Biosafety 1998, Snow and Moran-Palma 1997, Tiedje et al. 1987, Traynor and Westwood 1999, Van Aken 1999, Wolfenbarger and Phifer 2000).

The renewed focus on gene flow has had important consequences. Gene flow is a primary concern for regulatory oversight of transgenic plants in the United States and other countries. It is a consideration for regulatory decisions made about transgenic plants grown under notification and permit as well as those being considered for deregulation (USDA-APHIS 1997). In addition, renewed research effort has been focused on the topic.

Much of the effort has been to address the question of whether domesticated plants are capable of spontaneously mating with wild relatives under field conditions. Indeed, there is now substantial evidence that at least 44 cultivated plants mate with one or more wild relatives somewhere in the world (Table 1). The picture that is emerging is that most of the world's domesticated species probably mate with one or more wild relatives somewhere in the world (reviewed by Ellstrand et al. 1999). Hybridization rates measured by experimental studies vary with the study system, ranging from exceedingly low to quite high. It is not unusual for hybridization to be detected at distances of one hundred meters or more. An increasing number of hybrid fitness studies have been conducted under field conditions. The hybrids are rarely fully sterile; in certain instances they are actually as fit or more fit than their genetically pure wild siblings (reviewed by Ellstrand et al. 1999). Additionally, plenty of evidence has been uncovered demonstrating hybridization between crops and their wild relatives has served as a stimulus for the evolution of increased weediness and invasiveness (Ellstrand and Schierenbeck 2000). Another problem associated with crop to wild gene flow has been uncovered, the increased risk of extinction due to hybridization; many examples of this problem have been noted (reviewed by Ellstrand et al. 1999).

In summary, crop-to-wild gene flow is not uncommon, and on occasion, it has caused problems. Would we expect transgenic plants to behave any differently? The answer is "no". And indeed, experimental work on transgenics is beginning to trickle onto the scene. Transgenics spontaneously hybridize with wild relatives, their hybrids are somewhat or fully fertile, and those hybrids generally pose about the same kinds of risks as those posed by conventional crops (reviewed by Ellstrand 2003). But specific varieties may present unique risks. In many cases, transgenes will present unique phenotypes in the wild. After all, if the trait could have been obtained from a cross-compatible wild relative, it is unlikely that it would have been obtained at great expense and hassle through transformation. Also, there is a growing body of data showing unintended biochemical, physiological, anatomical, and morphological phenotypes in transgenic plants (examples in Kuiper et al.2001). It is not clear whether the rate of such pleiotropy is higher in transgenics than nontransgenics, and it is not clear how such unintended traits will be expressed in crop-wild hybrids. What is clear is that the lessons

gleaned from conventional crops can only go so far in predicting the impacts of transgenes that end up in unintended genomes.

FUTURE

What does the future hold for research on gene flow from transgenic crops to wild relatives? There is no doubt that researchers in this area will continue on the research themes established over the last decade regarding hybridization rates and hybrid fitnesses. But I do not doubt that the research will increasingly utilize transgenic crops themselves instead of nontransgenic surrogates. Since it is almost a certainty that transgenes have entered natural populations, I suspect that the first report will come in the next five years. Additional key areas that are vastly underexplored are the genetic basis of traits that contribute to the evolution of weediness and invasiveness (see discussion in Ellstrand and Schierenbeck 2000) and gene flow by seed.

But the most important contribution in this area is apt to be via the evolution of a sister field on the unintended gene flow from transgenic crops to other crops (transgenic or not). This kind of gene flow has received almost no attention from those who address the risks of transgenic organisms, but crops of the same species growing in proximity are much more likely to mate with one another than with a wild relative. A few recent events have demonstrated that intercrop gene flow has already delivered transgenes into plants for which they were not intended and that gene flow may have environment, economic, agronomic, or social impacts:

- Spontaneous sequential cross-pollination between three varieties (two transgenic) of canola resistant to different herbicides resulted in the evolution of multiple herbicide resistant volunteers (Hall et al. 2000). While other herbicides are available for controlling these new weeds, the range of options has now been reduced, especially for farmers who want to remove volunteer canola from other crops resistant to the three herbicides in question.
- Apparent spontaneous intercrop gene flow of an herbicide resistant transgene into a nontransgenic variety of canola has already resulted in at least one lawsuit involving the unapproved use of intellectual property (Clark 2001).
- Interestingly, the same case drew a complaint from the defendant of genetic pollution by the transgenic variety.
- Reports of the occurrence of transgenes in remote Mexican maize landraces (Dalton 2001, Quist and Chapela 2001) after years of that country's moratorium on transgenic maize have raised discussion about whether post-commercialization transgene containment is likely or even possible (Hodgson 2002).

In addition to the foregoing events, the unintentional spread of transgenes through intercrop gene flow may have other important consequences:

- The pollination of seed crops intended for human or animal consumption by plants transformed to create industrial biochemicals may pose human or animal food safety issues (Ellstrand 2001).

- The pollination of crops intentionally grown for organic produce may have their certification jeopardized if that produce contains seeds or seed products resulting from seeds that were sired by transgenic plants (National Academy of Sciences 2002).
- Seed from plants that have been unintentionally pollinated by transgenic plants may serve as "genetic bridges" that transfer transgenes to other varieties or wild relatives (National Academy of Sciences 2002).

While there is certainly no reason to abandon research on crop-to-wild gene flow, the data and skills accumulated in this endeavor, may prove helpful in addressing the simpler, but perhaps more urgent, issues of transgene flow among crops.

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Table 1 There is more than circumstantial evidence for natural hybridization between the following domesticated plants and one or more wild relatives (Ellstrand 2003)

Cultigen	Scientific name
Alfalfa	*Medicago sativa*
Apple	*Malus x domestica*
Avocado	*Persea americana*
Banana	*Musa acuminata*
Bean, common	*Phaseolus vulgaris*
Beet, sugar	*Beta vulgaris*
Bentgrass, creeping	*Agrostis stolonifera*
Cacao	*Theobroma cacao*
Cane, sugar	*Saccharum officinarum*
Cassava	*Manihot esculenta*
Cocona	*Solanum sessiliflorum*
Coffee, arabica	*Coffea arabica*[b]
Cotton	*Gossypium barbadense*
Cotton	*Gossypium hirsutum*
Elm, Siberian	*Ulmus pumila*
Fescue, tall	*Festuca pratensis*
Gourd	*Cucurbia pepo*
Grapes	*Vitis vinifera*[b]
Juniper	*Juniperus chinensis*
Lettuce	*Lactuca sativa*
Maize	*Zea mays* ssp. *mays*
Millet, foxtail	*Setaria italica*
Millet, pearl	*Pennisetum glaucum*

Mushroom, button	*Agaricus bisporus*
Oats	*Avena sativa*
Potato	*Solanum stenotomum*
Potato	*Solanum tuberosum*
Quinoa	*Chenopodium quinoa*
Radish	*Raphanus sativus*
Rape, swede	*Brassica napus*
Rape, turnip	*Brassica campestris*
Raspberry	*Rubus idaeus*
Rhododendron, catawba	*Rhododendron catawbiense*
Rice	*Oryza glaberrima*
Rice	*Oryza sativa*
Rye	*Secale cereale*
Ryegrass	*Lolium perenne*
Salsify	*Tragopogon porrifolius*
Sorghum	*Sorghum bicolor bicolor*
Soybean	*Glycine max*
Squash	*Cucurbia pepo*
Strawberry	*Fragaria x ananassa*
Sunflower	*Helianthus annuus*
Walnut	*Juglans regia*
Watermelon	*Citrullus lanatus*
Wheat, bread	*Triticum aestivum*
Wheat, durum	*Triticum turgidum durum*

Plenary Address:

Prevalence and Management of Herbicide-Resistant Weeds

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ABSTRACT

Transgenic and non-transgenic herbicide resistant crops are increasingly being planted commercially in North America. The widespread use of herbicide resistant crops has the potential to increase or alter the use pattern of the herbicide to which a crop is resistant, which has implications for the further evolution of resistant weed species. Gene flow from the resistant crop to related weeds is another potential impact of adoption of this technology, although the relative importance of this phenomenon to increases in weed resistance is unknown. Weed management in agricultural systems is achieved through a combination of methods that include prevention, eradication, and control (reduction of weed densities to economically acceptable levels). Methods of weed control include biological, chemical, cultural, and mechanical techniques, as well as combinations of these approaches. In many parts of the world, however, chemical control with herbicides is the predominant method of weed management. Currently in the US and worldwide, herbicides outrank insecticides and fungicides in both volume used and total sales. As use of herbicides has increased, increased cases of selection for resistance in weeds have been documented. Since the first reported case of weed resistance in 1970, 258 weed species have evolved resistance to one or more of 18 herbicide classes. One of the most recent cases documented is weed resistance to glyphosate, the active ingredient in Roundup©, which has been found in the US and four other countries. To preserve the utility of herbicides in agriculture, active resistance management is essential, using methods such as herbicide rotation, mixing herbicides with different mechanisms of action, and employing combinations of chemical and nonchemical control techniques.

OVERVIEW OF WEED MANAGEMENT

Weed management can be defined as a farming system that uses all available knowledge and tools to produce crops free of unwanted vegetation (Zimdahl 1993). Integrated weed management or integrated pest management are often used synonymously with weed management (Anderson 1996). In practice, weed management consists of several categories of activities. Prevention involves practices that avoid, inhibit, or delay weed infestations (Radosevich *et al.* 1997). Practices such as using clean field equipment, planting weed-free crop seeds, enforcing weed quarantines, and abiding by weed and seed laws are all forms of weed prevention. Eradication is total elimination of a weed species from an area and is rarely feasible or even attempted except in small areas or in high-value crops because of the difficulty and high cost of such an extreme measure. Weed control is the reduction of weeds to economically acceptable levels without necessarily eliminating all weeds from an area. Methods

of weed control include biological, chemical, cultural, and mechanical or physical (Anderson 1996, Radosevich *et al.* 1997, Zimdahl 1993).

Although the use of herbicides is only one method among many available methods for controlling weeds, it is generally the most prevalent method used in developed countries and many developing countries. Among all pesticides (fungicides, herbicides, and insecticides), herbicides rank number one in user expenditures as well as volume of active ingredient (Table 1) (US EPA 2002). In 1997, herbicides constituted 58% of the US market and 46% of the world market for pesticides in user expenditures, and 46% of the US market and 40% of the world market for pesticides in volume of active ingredient used. Additionally, when considering all pesticides, the top 12 most used conventional pesticides in US agricultural crop production are herbicides (Table 2) (US EPA 2002). Thus, in spite of the range of weed control methods available in an integrated weed management program, US agricultural production is heavily dependent on herbicides.

HERBICIDE RESISTANCE IN WEEDS

Herbicides are active at one or more target sites within a plant, such as enzymes, proteins, membranes, or other sites where herbicides bind and thereby disrupt normal plant functions. Resistance to a particular herbicide may occur in plants as the result of a random and infrequent mutation or through genetic manipulation; there has been no evidence to date that demonstrates herbicide-induced mutation (Prather *et al.* 2000). Over the past 30 or more years, continual use of herbicides has imposed selection pressure for increased resistance within weed species that were formerly susceptible. Herbicide resistance has become well known since the discovery of triazine resistance, which was first reported in 1970 (Holt 1992, LeBaron and Gressel 1982, Powles and Holtum 1994). In general, resistance is documented using standard, published procedures for determining that a particular plant survives herbicide treatment (Heap 1994, Moss 1995).

Over time, several organizations have published definitions pertaining to herbicide resistance in an attempt to define and standardize terminology in this area. The term tolerance is used loosely throughout the weed resistance literature and is generally used synonymously with resistance or not at all. Herbicide resistance may be defined as the naturally occurring inheritable ability of some weed biotypes to survive a herbicide treatment that should effectively control that weed population (i.e., the normal field dosage, or the recommended rate on the herbicide label) (HRAC 2002). Cross resistance is resistance to two or more herbicides resulting from the presence of a single resistance mechanism, that is, the herbicides are in the same chemical class. Most cases of herbicide resistance in weeds involve a single mutation or modification in some function so that the weed is resistant or cross resistant. Less commonly, a single plant expresses resistance to several herbicides that affect different target sites. Multiple resistance is resistance to several herbicides resulting from two or more distinct resistance mechanisms in the same plant (Holt *et al.* 1993, HRAC 2002). The mechanism of multiple resistance in many of the cases that have been studied is detoxification of herbicides by cytochrome P_{450} mixed-function oxidases (MFOs), similar to those found in many insects resistant to insecticides.

Several characteristics of herbicides and their use are thought to contribute to a high risk for selection of resistance in weeds. These include: (1) single target site and specific

mechanism of action, (2) very active and effective in killing a wide range of weed species, (3) long soil residual activity and season-long control of germinating weeds, and (4) applied frequently and over several growing seasons without rotating, alternating, or combining with other types of herbicides (Holt and LeBaron 1990). All of these characteristics would likely cause intense selection pressure for the evolution of resistance. Herbicides that meet some or all of these criteria would be more likely to select for resistance than would those that meet few or none of the criteria. Such herbicides as glyphosate and the triazine, sulfonylurea, and imidazolinone chemical classes meet most of these criteria; these are also some of the herbicides or herbicide classes to which resistant weeds have evolved (Heap 2002).

Currently, 258 unique weed biotypes have been reported with resistance to one or more of 18 herbicide classes (Table 3) (Heap 2002). Taxonomically, these biotypes comprise 156 species and include 94 dicots and 62 monocots. The appearance or documentation of herbicide resistance in weeds is increasing at a linear rate (Figure 1), which mirrors the trends previously seen with insecticide and fungicide resistance (Holt and LeBaron 1990). Several common weed genera include species in which resistant biotypes have been documented numerous times in many different locations (Table 4). Most of these genera with the greatest number of documented cases of resistance are also among the world's worst weeds (Holm *et al*. 1977), regardless of the presence of resistance. Among the most important herbicide resistant weed species around the world, all but two (*Chenopodium album* and *Amaranthus hybridus*) have been documented with multiple resistance (Table 5) (Heap 2002). Other data regarding herbicide resistance by weed species, herbicide mechanism of action, country, or US state can be obtained from the International Survey of Herbicide Resistant Weeds, available online at: http://www.weedscience.org/in.asp (Heap 2002).

Herbicide resistant weeds can have significant impacts on crop production (Orson 1999, Powles *et al.* 1997). These include the loss of utility of specific herbicides to which weeds have become resistant, an increase in difficult-to-control weeds, the need to develop alternative control methods, and potentially greater costs for weed management. Many strategies have been proposed for prevention and management of herbicide resistant weeds (Boerboom 1999, Friesen *et al*. 2000, Nalewaja 1999, Prather *et al.* 2000, Retzinger and Mallory-Smith 1997, Shaner *et al*. 1992). These include: (1) rotation of herbicides, (2) use of herbicide mixtures, (3) use of herbicides with short-term soil residual activity, (4) use of crop rotation, (5) use of non-chemical weed control methods, (6) use of certified, weed-free crop seed, (7) use of clean field equipment free of weed seeds, and (8) use of integrated weed management strategies. All of these methods will theoretically lower the selection pressure for resistance, thereby maintaining refuges of susceptible alleles in a weed population (Christoffers 1999).

HERBICIDE RESISTANT CROPS

A number of herbicide resistant crops with resistance to one or more herbicides are now commercially available in North America. These include canola (*Brassica napus*), cotton (*Gossypium* spp.), maize (*Zea mays*), rice (*Oryza sativa*), and soybean (*Glycine max*) (Duke 1999). Among these crops, only canola has a congener among the weeds that have been documented with herbicide resistance (Heap 2002). The herbicides that have been used in the development of resistant crops include bromoxynil, glufosinate, and glyphosate, as well as herbicides in the cyclohexanedione, imidazolinone, sulfonylurea, and triazine chemical classes. All of these except glufosinate have also selected resistant weed biotypes in field situations

(Table 3). The imidazolinone and sulfonylurea herbicides are in the ALS inhibitor group (inhibition of acetolactate synthase or acetohydroxyacid synthase, AHAS), which has selected the largest number of resistant weed biotypes, while the triazines are in the photosystem II (PS) inhibitor group, which has selected the second largest number of resistant weed biotypes (Table 3). The cyclohexanedione herbicides are in the ACCASE inhibitor group (inhibition of acetyl CoA carboxylase), which has selected the third largest number of resistant weeds. Biotypes of four weeds species have been selected for resistance to glyphosate while one weed species has been selected for resistance to bromoxynil (Table 3).

There are a number of potential benefits to crop production and management that might be realized by using herbicide resistant crops. The use of post-emergence, non-selective herbicides such as glufosinate and glyphosate on resistant crops could provide a broader spectrum of weeds controlled; allow greater use of reduced- or no-tillage cultural practices resulting in reduced soil erosion; reduce the use of older, less environmentally safe herbicides; allow greater timing flexibility for growers; and allow reduced herbicide usage overall (Duke 1999, Powles *et al*. 1997, Shaner 2000). The use of herbicide resistant crops also carries potential risks, however, including over-reliance on fewer weed management strategies, selection of weed resistance or weed population shifts to naturally resistant weed species; pleiotropic effects of transgenes; and development of resistant crops as volunteer weed problems (Duke 1999, Liebman and Brummer 2000, Powles *et al*. 1997). Most reports indicate that the greatest potential risk of the use of this technology is over-reliance on a few herbicide chemistries and the threat of selecting new resistant weed problems.

The potential also exists for gene flow from transgenic crops to wild or weedy relatives, resulting in the development of new resistant weed problems (Duke 1999, Dyer 1993). While few crop-weed congeneric or conspecific pairs exist for currently available herbicide resistant crops, other crop species under consideration for engineering or commercializing resistance have weedy relatives with which they might hybridize. These include the amaranths, cucurbits, millet, oat, potato, rapeseed and vegetable crucifers, rice, sorghum, sunflower, tomato, and wheat (Liebman and Brummer 2000). Although herbicide resistance should confer no fitness advantage in the absence of the herbicide, the impacts of gene flow could be significant in agricultural settings if new weed problems were created. Theoretically, gene flow for resistance could substitute for and accelerate the natural mutation rate for resistance, which is thought to be extremely low (K. Mercer, personal communication). Regardless of the cropping system or management practices used, therefore, resistance management should always be practiced (Powles *et al*. 1997, Preston and Rieger 2000, Shaner 2000). The specific benefits and risks of use of any herbicide resistant crop also must be determined on a case-by-case basis.

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Table 1. U.S. and world pesticide sales at user level, 1997 estimates.

Pesticide Class[1]	U.S. Market		World Market		U.S. % of World Market
	Million	(%)	Million	(%)	
User Expenditures *(millions of dollars)*					
Herbicides	$6,846	58%	$16,886	46%	41%
Insecticides	$3,553	30%	$11,592	31%	31%
Fungicides	$802	7%	$6,037	16%	13%
Other	$696	6%	$2,533	7%	27%
Total	***$11,897***	***100%***	***$37,048***	***100%***	***32%***
Volume of Active Ingredient *(millions of pounds)*					
Herbicides	568	46%	2,254	40%	25%
Insecticides	129	10%	1,470	26%	9%
Fungicides	81	7%	539	9%	15%
Other	453	37%	1,421	25%	32%
Total	***1,231***	***100%***	***5,684***	***100%***	***22%***

NOTE: Totals may not add due to rounding

SOURCE: EPA estimates of world market based on Wood Mackenzie staff input, SRI Consulting staff input, American Crop Protection Association (ACPA) annual surveys. Estimates of U.S. market are from Tables 2 and 3.

FOOTNOTES: 1 - See definitions of pesticide classes below Tables 2.

2 - Includes sulfur and petroleum/other chemicals but does not cover industrial wood preservatives, specialty biocides and chlorine/hypochlorites.

(US Environmental Protection Agency. 2002. Pesticide Industry Sales And Usage: 1996 and 1997 Market Estimates. Office of Pesticide Programs. Available online: http://www.epa.gov/oppbead1/pestsales/)

Table 2. Quantities of most commonly used conventional pesticides in U.S. agricultural crop production **(approximate quantities in 1997, 1995, 1993, and 1987).**

	1997	Earlier Years					
		1995		1993		1987	
	Mil. lbs. AI	Rank	Mil. lbs. AI	Rank	Mil. lbs. AI	Rank	Mil. lbs. AI
1. Atrazine	**75 - 82**	1	68 - 73	1	70 - 75	1	71 - 76
2. Metolachlor	**63 - 69**	2	59 - 64	2	60 - 65	3	45 - 50
3. Metam Sodium	**53 - 58**	3	49 - 54	8	25 - 30	15	5 - 8
4. Methyl Bromide	**38 - 45**	4	39 - 46	3	49 - 57	---	NA
5. Glyphosate	**34 - 38**	7	25 - 30	11	15 - 20	17	6 - 8
6. Dichloropropene	**32 - 37**	5	38 - 43	6	30 - 35	4	30 - 35
7. Acetochlor	**31 - 36**	11	22 - 27	---	0	---	0
8. 2, 4-D	**29 - 33**	6	31 - 36	7	25 - 30	5	29 - 33
9. Pendimethalin	**24 - 28**	9	23 - 28	10	20 - 25	10	10 - 13
10. Trifluralin	**21 - 25**	10	23 - 28	9	20 - 25	6	25 - 30
11. Cyanazine	**18 - 22**	8	24 - 29	5	30 - 35	7	21 - 25
12. Alachlor	**13 - 16**	12	19 - 24	4	45 - 50	2	55 - 60
13. Copper Hydroxide	**10 - 13**	16	7 - 11	20	4 - 7	40	1 - 2
14. Chlorpyrifos	**9 - 13**	14	9 - 13	13	10 - 15	14	6 - 9
15. Chlorothalonil	**7 - 10**	13	8 - 12	14	10 - 15	19	5 - 7
16. Dicamba	**7 - 10**	18	6 - 10	16	6 - 10	23	4 - 6
17. Mancozeb	**7 - 10**	13	6 - 9	19	4 - 7	21	4 - 6
18. EPTC	**9 - 13**	16	7-11	12	10 - 15	8	17 - 21
19. Terbufos	**6 - 9**	19	6 - 9	17	5 - 8	11	8 - 10
20. Dimethenamid	**6 - 9**	40	2 - 4	---	NA	---	NA
21. Bentazone	**6 - 8**	23	4 - 8	18	4 - 7	15	6 - 9
22. Propanil	**6 - 8**	17	6 - 10	15	7 - 12	13	7 - 10
23. Simazine	**5 - 7**	29	3 - 5	23	3 - 8	28	3 - 4
24. MCPA	**5 - 6**	28	4 - 5	22	4 - 5	25	4 - 5
25. Chloropicrin	**5 - 6**	33	3 - 4	39	2 - 4	---	NA

(US Environmental Protection Agency. 2002. Pesticide Industry Sales And Usage: 1996 and 1997 Market Estimates. Office of Pesticide Programs. Available online: http://www.epa.gov/oppbead1/pestsales/)

Table 3. Summary of herbicide resistant weeds by mechanism of action (Heap 2002).

HERBICIDE RESISTANT WEEDS SUMMARY TABLE Monday, April 15, 2002				
Herbicide Group Click for details	**Mode of Action**	**HRAC Group**	**Example Herbicide**	**Total**
ALS inhibitors	Inhibition of acetolactate synthase ALS (acetohydroxyacid synthase AHAS)	B	Chlorsulfuron	72
Photosystem II inhibitors	Inhibition of photosynthesis at photosystem II	C1	Atrazine	63
ACCase inhibitors	Inhibition of acetyl CoA carboxylase (ACCase)	A	Diclofop-methyl	28
Bipyridiliums	Photosystem-I-electron diversion	D	Paraquat	21
Synthetic Auxins	Synthetic auxins (action like indoleacetic acid)	O	2,4-D	21
Ureas and amides	Inhibition of photosynthesis at photosystem II	C2	Chlorotoluron	20
Dinitroanilines and others	Microtubule assembly inhibition	K1	Trifluralin	10
Thiocarbamates and others	Inhibition of lipid synthesis - not ACCase inhibition	N	Triallate	6
Triazoles, ureas, isoxazolidiones	Bleaching: Inhibition of carotenoid biosynthesis (unknown target)	F3	Amitrole	4
Glycines	Inhibition of EPSP synthase	G	Glyphosate	4
Chloroacetamides and others	Inhibition of cell division (Inhibition of very long chain fatty acids)	K3	Butachlor	2
Nitriles and others	Inhibition of photosynthesis at photosystem II	C3	Bromoxynil	1
PPO inhibitors	Inhibition of protoporphyrinogen oxidase (PPO)	E	Oxyfluorfen	1
Carotenoid biosynthesis inhibitors	Bleaching: Inhibition of carotenoid biosynthesis at the phytoene desaturase step (PDS)	F1	Flurtamone	1
Mitosis inhibitors	Inhibition of mitosis / microtubule polymerization inhibitor	K2	Propham	1
Organoarsenicals	Unknown	Z	MSMA	1
Arylaminopropionic acids	Unknown	Z	Flamprop-methyl	1
Pyrazoliums	Unknown	Z	Difenzoquat	1
Total Number of Unique Herbicide Resistant Biotypes				**258**

Table 4. Weed genera with the greatest number of occurrences of resistant biotypes worldwide and in the US. Some of the occurrences for a genus may include the same biotype but documented in different fields (compiled from Heap 2002).

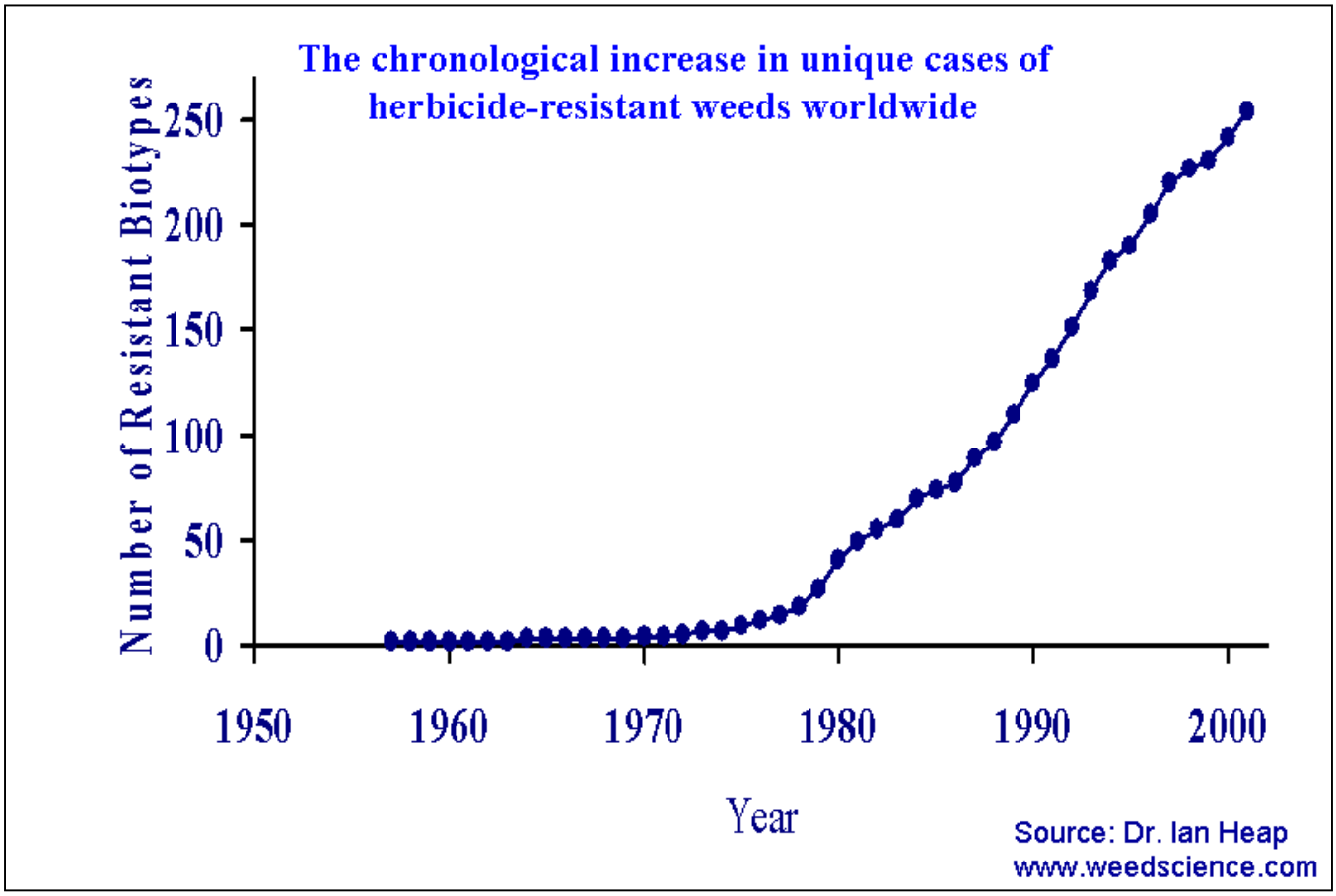
Genus	Common name	Number of Occurrences	
		Worldwide	US
Amaranthus	Pigweed	86	50
Chenopodium	Lambsquarters	44	20
Lolium	Ryegrass	43	14
Avena	Wild oat	41	13
Kochia	Kochia	32	28
Setaria	Foxtail	28	11
Echinochloa	Barnyardgrass, watergrass	27	9
Conyza	Fleabane or horseweed	24	2
Alopecurus	Blackgrass	17	0
Eleusine	Goosegrass	12	8

Table 5. Most important herbicide resistant weed species worldwide (Heap 2002).

Most Important Herbicide-Resistant Species

1.	Rigid Ryegrass	*Lolium rigidum*
2.	Wild Oat	*Avena fatua*
3.	Redroot Pigweed	*Amaranthus retroflexus*
4.	Common Lambsquarters	*Chenopodium album*
5.	Green Foxtail	*Setaria viridis*
6.	Barnyardgrass	*Echinochloa crus-galli*
7.	Goosegrass	*Eleusine indica*
8.	Kochia	*Kochia scoparia*
9.	Horseweed	*Conyza canadensis*
10.	Smooth Pigweed	*Amaranthus hybridus*

Figure 1. The chronological increase in unique cases of herbicide resistant weeds worldwide (Heap 2002).



Fitness and population effects of gene flow from transgenic sunflower to wild *Helianthus annuus*

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ABSTRACT

A widely acknowledged risk associated with transgenic crops is the possibility that hybridization with wild relatives will cause fitness-related transgenes to persist in wild populations. If wild plants acquire transgenes coding for resistance to herbivory, disease, environmental stress, and/or commonly used herbicides, they could become more abundant in their natural habitats or invade previously unsuitable habitat. In addition, wild populations containing transgenes that provide resistance to herbivores or disease may have additional effects on natural insect and pathogen populations. However, little is known about whether these concerns are justified. For example, many weedy species are affected by herbivores, yet the impact of insect damage on population densities and invasiveness has rarely been examined. In order to determine if a transgene poses a risk to wild populations, or to the species with which the wild plant interacts, three questions must be addressed. These are: 1) Are there genetic or geographic barriers to the escape of the transgene from the crop into wild populations? 2) Is the transgene expected to increase in frequency in wild populations? And 3) What are the ecological consequences of escape? We are investigating these questions in the cultivated/wild sunflower system. In answer to the first two questions we have found: few barriers to gene flow between the crop and wild population, and decreased lepidopteran herbivory and increased seed production in wild plants containing a Bt-toxin gene specific to lepidopterans. In answer to the third question, preliminary analyses suggest that increased seed production in individual plants (caused by, for example, a Bt gene) will lead to an increase in the size of wild populations. We are currently doing experimental and modeling work to determine if larger local populations will lead to larger or more persistent metapopulations.

INTRODUCTION

Evaluating the ecological risks of transgenic crops has become increasingly important as more varieties are released, and as more acres are planted in these varieties. A widely acknowledged risk associated with transgenic crops is the possibility that hybridization with weedy relatives will cause fitness-related transgenes to persist in wild populations. Despite this understanding there exist almost no data appropriate for evaluating the ecological effects of the escape of transgenes from crops into wild populations. We argue here that assessment of the risks associated with the escape of transgenes must sequentially address three questions (Snow et al. 1999).

The first of these questions is: Is the transgenic variety of the cultivated plant sexually compatible with wild relatives? Clearly, if cultivated and wild populations are not sexually compatible, there exists no risk of escape of a transgene, and risk assessment need go no further. However, many crop species, including rice, sorghum, canola (oilseed rape), sugarbeet, oats, squash, carrot, radish, strawberry, clover, sunflower, and others are known to hybridize with their wild relatives (Snow and Moran-Palma 1997; Zemetra et al. 1998). The fecundity of crop-wild hybrids is often found to be lower than the fecundity of purely wild types (e.g. Snow et al. 1998). However, fecundity in F_1s must be zero to prevent the transfer of beneficial alleles into wild populations.

If the crop can successfully hybridize with its wild relative, it is necessary to answer the second question: Does the transgene confer a fitness benefit on the wild plant? If the transgene is of no benefit (or is always costly) to the wild plant, genetic drift (or purifying selection) will determine its fate in the wild population. For example, it may be that transgenes controlling traits important to harvesting the crop and shelf-life of the product, such as those associated with fruit-ripening, will be neutral or costly in wild populations. Consequently, these traits are not expected to increase in frequency, and may pose little ecological risk. By contrast, transgenes for characters like insect or pathogen resistance and drought tolerance may be beneficial to wild populations, and for this reason they may increase in frequency in wild populations by natural selection. Transgenes for herbicide tolerance are unlikely to increase fitness in purely wild populations, but weedy populations containing these genes may be more difficult to control with herbicides. Very little is known about the fitness effects of transgenes in wild populations; studies of the fitness effects of transgenes in wild relatives have been performed for no commercially released transgenes.

Finally, if a transgene confers a fitness benefit on a wild relative it is necessary to answer a third question: What are the ecological consequences of the escape of the transgene into a wild population? Specifically, it is necessary to determine if the transgene alters interactions between the wild plant and its biotic and abiotic environment. A transgene that increases in frequency in wild plants does so, by definition, because it increases survival or fecundity, and one risk that has been discussed in the literature is the effect of this increased individual fitness on the population size, dynamics, and habitat use in the wild plant. In addition, transgenes that confer resistance to herbivores and pathogens will have effects on native species using the wild plant as a host. Clearly, these questions must be the crux of any ecological risk assessment. However, virtually no work has been done in these areas.

We have been addressing these three questions in using *Helianthus annuus*, which is the wild progenitor of cultivated sunflower, and a Bt transgene that confers resistance to lepidopteran herbivores. Here we summarize the work we have done towards answering each of these questions.

SUNFLOWER AND ITS INSECT HERBIVORES AS A MODEL SYSTEM

Wild sunflower represents an excellent model system with which to address these questions. Wild *Helianthus annuus* is a native, self-incompatible, annual plant that is widespread throughout much of the USA, reaching its greatest abundance in midwestern states (Heiser 1954), where most cultivated sunflower is grown (Schneiter 1997). Wild sunflower is a disturbance specialist, and populations are typically patchy and ephemeral, relying on the soil seed bank and long-distance dispersal for opportunities to become established in new areas. In the absence of tilling or other types of disturbance, population size declines. In agricultural areas, however, repeated tilling allows wild sunflower populations to persist for many years, especially along field margins.

Wild sunflower is host to many insect herbivores, and many of these species are also pests in the crop. Some herbivores have negative effects on fitness in wild populations (Pilson 2000), and they can also substantially reduce yield in some years and locations (Charlet 1997). 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). In wild *H. annuus*, insect resistance is typically polygenic, and 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 (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 (e.g., Stewart 1999) may also be developed in the future.

ARE WILD AND CULTIVATED SUNFLOWER SEXUALLY COMPATIBLE?

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. 1997, Morán Palma 1997, 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. Moreover, cultivated sunflower, which is primarily planted in North and South Dakota, Nebraska, Kansas, and eastern Colorado, is nearly always sympatric with wild *H annuus* (Schneiter 1997; Heiser 1954). Clearly, both genetic and geographic barriers to gene flow from crop to wild sunflower are minimial. Thus, the answer to the first question is yes, and further studies of pre-commercial transgenes in the wild genetic background are necessary.

WHAT IS THE FITNESS EFFECT OF THE BT-TRANSGENE IN THE WILD BACKGROUND?

Because it is clear that any transgene deployed in cultivated sunflower planted in North America will escape into wild populations, it is necessary to determine if the transgene(s) increases the fitness of wild plants. In the case of a Bt-transgene we need to know if plants carrying the gene are resistant to herbivory (by the species specifically targeted by the transgene), and further, whether this reduction in damage leads to an increase in fitness. We have addressed these questions for a Bt-transgene that is specific to Lepidoptera (Snow et al. submitted; Pilson, et al. in preparation). Our study involved the Bt protein Cry1Ac, which is toxic to many lepidopteran species but is not expected to affect other insect taxa (Estruch et al. 1997). Ingesting a very small amount of Bt toxin (e.g., parts per billion) typically causes susceptible insects to stop feeding and die within a few days (Estruch et al 1997), or move to a nontoxic host plant (Davis and Onstad 2000).

Determining the ecological effects of pre-commercial transgenes is inherently difficult due to biosafety and regulatory concerns. Uncaged plants must be exposed to natural levels of insect damage and cross-pollination, yet dispersal and persistence of the transgene(s) must be prevented. Our solution to this difficult problem was to use male sterile plants for the field experiments so that the possibility of transgene escape through pollen could eliminated.

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 and BC_3 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 near Burlington, in eastern Colorado, and BC_3 progeny were planted in Nebraska in 2000. The effect of the transgene was examined by comparing insect damage and fecundity between Bt+/male-sterile and Bt-/male-sterile plants,. We also compared Bt-/male-sterile and Bt-/male-fertile plants to determine the effects of male-sterility on herbivory and seed production (some seed predators feed on pollen [Korman and Oseto 1989; Delisle et al 1989] and might avoid male-sterile plants).

The Bt transgene led to reduced lepidopteran damage at both field sites and in both years (Figure 1 for data from the Nebraska 1999 experiment). However, the Bt toxin had no effect on amounts of damage caused by four non-lepidopteran species (Figure 2 for Nebraska 1999; Colorado 1999 and Nebraska 2000 show similar patterns). Although some of the non-lepidopteran species are negatively affected by competition with lepidopterans (M. Paulsen and D. Pilson, in prep.), they did not cause more damage on Bt plants than on controls. As expected, damage by some herbivores was reduced on male-sterile plants relative to male-fertile plants (Figures 1 and 2). Bt+ plants produced an average of 55% and 23% more seeds per plant than Bt- plants in Nebraska in 1999 and 2000, and 14% more seeds per plant in Colorado in 1999 (Figure 3). The reduction in herbivory on male-sterile plants suggests that using male-sterile plants to test for Bt effects may underestimate the fecundity advantage associated with the transgene. Had we been able to use pollen-producing Bt plants in the field, we might have documented more dramatic fecundity benefits of 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 (Snow et al. in prep.; Figure 4). 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.

This study shows that selection favoring an increase in the frequency of a Bt transgene has the potential to be quite strong. If herbivores cause more damage to F_1 (Cummings et al. 1999) and BC_1 sunflowers than to wild genotypes, it is possible that the fecundity advantage associated with Bt would diminish with subsequent generations of backcrossing. On the other hand, because male-sterile plants had less damage from lepidopterans than those with pollen, we may have underestimated the fecundity advantage of Bt in this study. In addition, we have observed higher levels of lepidopteran damage on wild plants in other years Pilson 2000; D. Pilson and M. Paulsen, in prep.). 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. If so, the transgene is expected to spread quickly and kill susceptible, native lepidopterans that feed on wild sunflower.

WHAT ARE THE ECOLOGICAL CONSEQUENCES OF THE ESCAPE OF A BT GENE INTO WILD POPULATIONS?

For transgenes to have important ecological effects in wild populations, not only must they increase the fitness of wild plants, they must also alter interactions between the wild plant and its biotic and abiotic environment. Arguably, it is the potential ecological effects that should be the focus of attention in risk assessments. Although the escape of a transgene into a wild population, and its subsequent increase frequency by natural selection, are necessary, they are not sufficient to predict the environmental consequences of escape. Specifically, these processes are only important to the extent that they lead to the alteration of existing ecological interactions among species.

In ongoing work we are examining two types of ecological consequences of the escape of a Bt transgene into wild populations. These are 1) the effect on the population dynamics of wild sunflower, and 2) the effect on the community of native herbivores that feeds on wild sunflower, and we present preliminary results from these studies here.

THE EFFECT OF A BT-TRANSGENE ON THE POPULATION DYNAMICS OF WILD SUNFLOWER

We have shown that a Bt gene increases seed production in wild plants, and is therefore likely to increase in frequency in wild populations. However, increased seed production by individual plants will only lead to larger populations, or more populations, if wild sunflower is currently seed limited. Currently, little is known about processes controlling the population

dynamics of wild sunflower. Thus, it is currently unclear what effect, if any, that a Bt transgene will have on the dynamics of wild populations.

Surprisingly, there are few data with which to evaluate the importance of seed limitation on the population dynamics of any plant species. Silvertown and Franco (1993) and Silvertown et al. (1993) compared the sensitivities of demographic transitions in herbs and woody plants and found that population growth rates were more affected by changes in fecundity in semelparous species, suggesting that annual species, such as sunflower, are more likely than other plants to be seed limited. Louda and Potvin (1995) found that elimination of herbivores by application of insecticide increased not only individual fitness but also local population size in a native thistle. Crawley and Brown (1995) found that weedy roadway populations of oilseed rape were seed limited at the landscape scale. In contrast, Bergelson (1994) found that population size was not seed limited in experimental plantings of a diminutive cress, *Arabidopsis thaliana*, a result that is likely due to insufficient open space and competition from surrounding vegetation.

To evaluate the effect of increased seed production on the population dynamics of wild sunflower we are using the following approach. First, in western Nebraska (in 2000 and 2001) and eastern Kansas (in 2000) we established experimental populations with varying amounts of seed production, and we are examining the effect of seed production in one year on population size and seed production in the following years. Second, we are using these experimental populations to derive parameters for spatially explicit metapopulation models. We will use these models to make predictions about the effect of seed production on metapopulation dynamics at our two study sites. And third, we will test our metapopulation models by comparison with empirical observation of metapopulation dynamics in both western Nebraska and eastern Kansas. Our two study sites are in very different environments (historically mixed short-grass prairie in western Nebraska, and tall grass prairie in Kansas), and we expect that the dynamics will be different in these habitats as well. This multi-year research program is in its early stages, so here we only present data documenting the effect of seed production in one year on population size the following year.

In 2000 we established 48 experimental populations at each of our study sites (at the Cedar Point Biological Station, near Ogallala, in western Nebraska, and at the Kansas Ecological Reserves, near Lawrence, in eastern Kansas); here we will only discuss preliminary data from the Nebraska populations. Each experimental population consisted of either 16 (n=32) or 21 (n=16) experimental plants. In each population the experimental plants were surrounded by either 45 or 40 plants that were not allowed to disperse seed, and which served to maintain a similar competitive environment for all experimental plants. All of these plants were sown in a central (rototilled) 2m x 2m square (experimental plants in the central 1m x 1m) located in the center of a larger 10m x 10m square. Half of the experimental populations with 16 plants were sprayed with a broad-spectrum insecticide to reduce herbivory (and increase seed production). Because sunflower is a disturbance specialist, and requires a recent disturbance for germination, we rototilled a 2m x 4m strip in one cardinal direction from each of the 48 plots (i.e. 12 tilled north, 12 tilled east, 12 tilled south, and 12 tilled west). The 45 or 40 competitor plants were removed before dispersing any seeds, and experimental plants were allowed to disperse their seeds naturally. Due to a severe drought during the 2000 growing season many plants performed very poorly, and the treatments (number of experimental plants and spraying) had no effect on seed production. Nonetheless, seed production (estimated by the number of heads dispersing seeds and an estimate of mean head area) varied by approximately two orders of magnitude (from ~200-20,000 seeds).

In 2001 we counted the number of seedlings emerging in each experimental population (in both the original square and in the tilled strip). (Presumably, seeds were dispersed in all directions. However, with few exceptions, seeds only germinated in the tilled squares and strips.) We also counted the number of seedlings that survived to reproductive age and the number of inflorescences produced by each plant that survived to reproductive age. We measured the inflorescence diameter of 10 randomly chosen inflorescences in each experimental population, and estimated seed production in 2001 by estimating the total inflorescence area produced by each population.

We used regression analysis (SAS 1989) to evaluate the effect of seed production in 2000 on the number of seedlings, number of reproductive plants, number of mature inflorescences, and the estimated number of seeds produced in 2001. Because the direction in which the strips were tilled had a significant effect on all of these response variables (probably due to a consistent prevailing wind direction during seed dispersal in 2000), we performed these analyses on residual values after the effect of direction had been removed in an ANOVA (PROC GLM in SAS). While it may seem redundant to evaluate the effect of seed production in 2000 on all of these population responses in 2001, it is only redundant to the extent that there are no density dependant processes affecting seed production. For example, it might be the case that many seeds germinate, but that intraspecific competition in high-density populations reduces the number of plants surviving to reproductive age to the same seen in an originally lower density population. Similarly, many plants might survive, but plants might produce fewer inflorescences in higher density populations.

Preliminary analyses suggest that increasing seed production in one year increases both population size and the number of seeds dispersed the following year (Figure 5). Moreover, the relationships between seed production in 2000 and number of seedlings, number of reproductive plants, number of inflorescences, and estimated head area in 2001 appear to be very similar, suggesting that density-dependant factors are not important in these populations. From these data we tentatively conclude that sunflower populations are seed-limited, and that increased seed production may lead to increased local population size. Additional analyses of these data, and of the Kansas and second set of Nebraska populations, are necessary before we can draw firm conclusions.

We are also using these experimental populations (and additional manipulations of these populations in later years) to derive parameters for our metapopulation models. Specifically, we are evaluating the effect of seed production on patterns of seed dispersal, the establishment and decay of the seed bank, and the longevity of our experimental populations (both with and without additional disturbance). These empirically derived parameters, measured on the scale of our 2m x 2m populations, will be used in a spatially-explicit metapopulation model. Metapopulation models were originally developed by Levins (1969), and in recent years have been a major focus of ecological inquiry (Hanski and Gilpin 1991, 1997; Tilman and Kareiva 1997). These models are often used in studies of organisms with very distinct habitat patches (e.g. frogs in ponds, Sjogren Gulve 1994), but Antonovics et al. (1994) have illustrated how empirical metapopulation studies can be conducted with organisms that are patchily distributed but have broad habitat requirements. Our model will be written at the 2m scale, because that is the scale at which we are deriving our parameters, but it can be scaled up from 2m to 80m and beyond, enabling us to make predictions at larger spatial scales.

If the escape of a Bt gene into wild sunflower populations leads to an increase in the size or number of wild populations, there will likely be additional ecological consequences. For example, to the extent that sunflower increases in abundance, plant species that commonly co-occur with sunflower will probably be affected, and may decrease in relative frequency. These

community level effects of the escape of a transgene are currently beyond the scope of our work.

THE EFFECT OF A BT TRANSGENE ON HERBIVORE COMMUNITY STRUCTURE

Clearly, if a Bt transgene escapes into wild sunflower populations it will have a negative impact on the suite of susceptible native herbivores. Our data indicates that native herbivores will be killed by the Bt-protein (Figure 1), and as the frequency of a Bt gene increases in wild sunflower, the negative impact on these native herbivores will increase as well. In addition to the taxa shown to be affected in the present study, we have wild sunflower at our study sites is also attacked by two other native lepidopterans (*Homoeosoma electellum*, *Eucosma womonana*), and these species are also likely to be negatively affected by a Bt gene. Moreover, these species are specialists, feeding on only *H. annuus*, or on only *H. annuus* and other species in the genus *Helianthus*, and for this reason they cannot easily find food on other host plants. Although these species may evolve resistance to Bt, at least initially the suite of native lepidopterans will be severely impacted by the escape of a lepidopteran-specific transgene into wild populations.

The effect of a lepidopteran-specific Bt gene on the non-lepidopteran members of the native herbivore community depends on existing interactions between these suites of herbivores. If the lepidopterans have negative competitive effects on any non-lepidopteran species, the release from competition provided by a Bt gene might allow these non-lepidopterans to increase in abundance. Experimental manipulations of damage by three of the lepidopterans (*Plagiomimicus*, *Homoeosoma*, and *Cochylis*) indicate that only *Plagiomimicus* competes with other herbivores. Damage by *Neolasioptera* (sunflower seed midge) and *Smicronyx fulvus* (red sunflower seed weevil) is lower in inflorescences with *Plagiomimicus* than in inflorescences without this species (M. Paulsen and D. Pilson, in prep.). However, because *Plagiomimicus* abundance is typically low, these interactions are probably only important every 5-10 years when *Plagiomimicus* reaches high abundance during population outbreaks (M. Paulsen, et al. in prep.).

SUMMARY

We have argued that assessing the ecological risks of the escape of transgenes into wild populations must sequentially address three questions. With respect to the question of cross-compatibility, we have found few barriers to gene flow between crop and wild sunflower, We also provided an unambiguous affirmative answer to the question of whether the transgene is positively selected in the wild. Wild plants containing a Bt-toxin gene specific to lepidopterans exhibited decreased lepidopteran herbivory and increased seed production. Thus, we expect that Bt-transgenes released in commercial sunflower will escape into and increase in frequency in wild populations (at least in locations and in years in which susceptible herbivores are abundant). Next it is necessary to evaluate the ecological consequences of transgenes in wild populations; however, virtually no work has been done in this area. Preliminary analyses suggest that increased seed production in individual plants (caused by, for example, a Bt gene) will lead to an increase in the size of wild populations. We are currently doing experimental and modeling work to determine if larger local populations will lead to larger or more persistent metapopulations.

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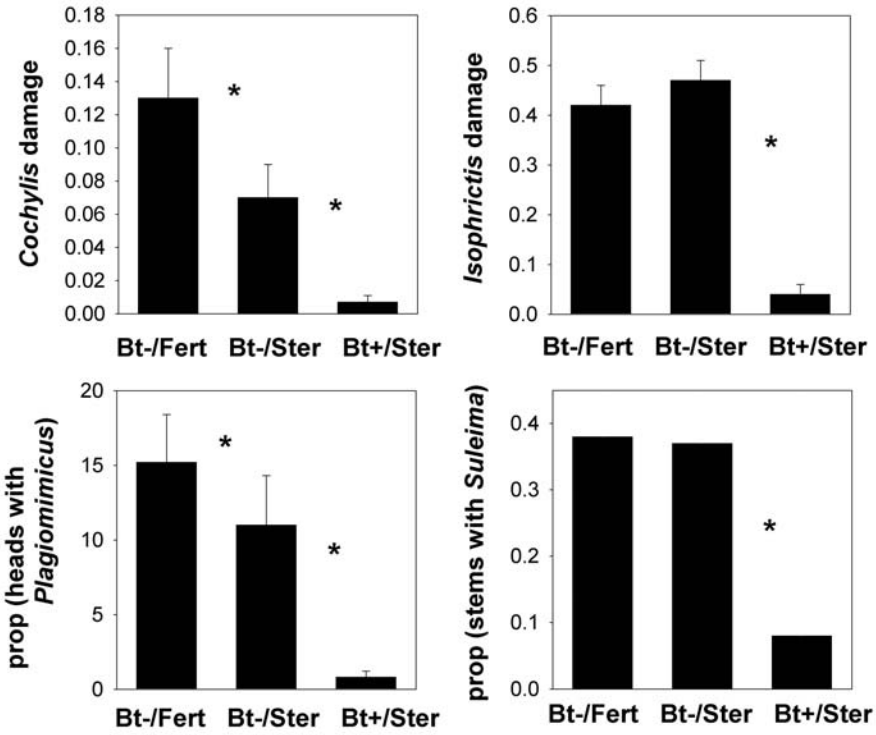

Figure 1 – Lepidopteran damage in Nebraska in 1999. *Cochylis hospes* (Chchylidae) and *Isophrictis similiella* (Gelechiidae) damage were categorized as 0, 1, or 2 (0, 1-30, or >30 seeds eaten) for each inflorescence, and the mean value over all inflorescences on each plant was analyzed by ANOVA. The proportion of heads on each plant attacked by *Plagiomimicus spumosum* (Noctuidae) was analyzed by ANOVA. The proportion of plants with stem damage by *Suleima helianthana* (Tortricidae) was analyzed by categorical ANOVA. In all analyses we made planned contrasts between Bt-/male sterile and Bt-/male fertile plants, and between Bt-/male sterile and Bt+/male sterile plants, and significant contrasts (at $p<0.05$) are indicated by asterisks.

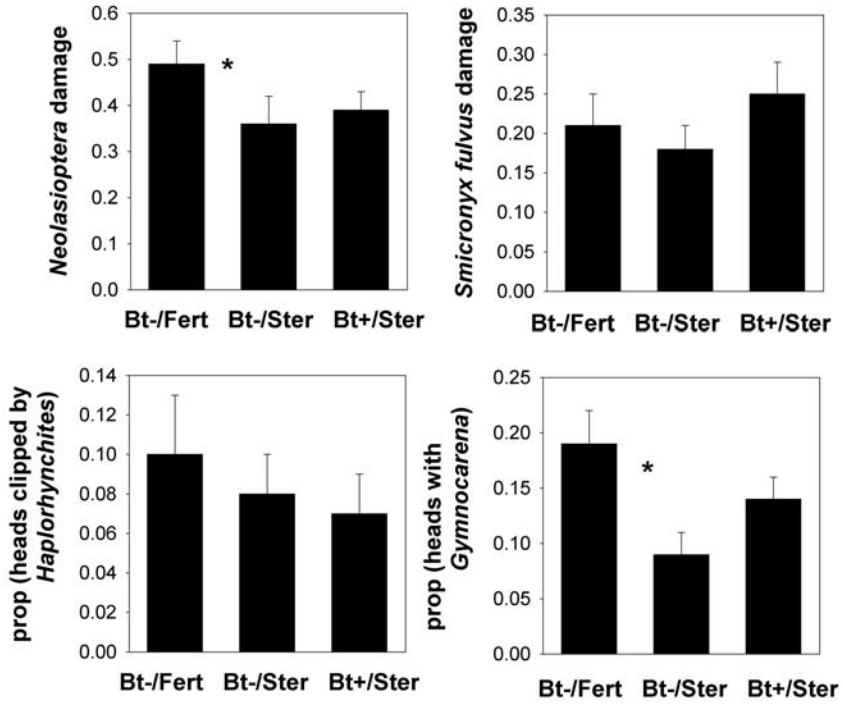

Figure 2 – Damage by non-lepidopteran herbivores in Nebraska in 1999. Damage by *Neolasioptera helianthi* (Cecidomyidae) and *Smicronyx fulvus* (Curculionidae) was quantified categorically (as described in Figure 1). These data, as well as data for *Haplorhynchites aeneus* (Curculionidae) and *Gymnocarena diffusa* (Tephritidae), were analyzed by ANOVA with planned contrasts (as described in Figure 1).

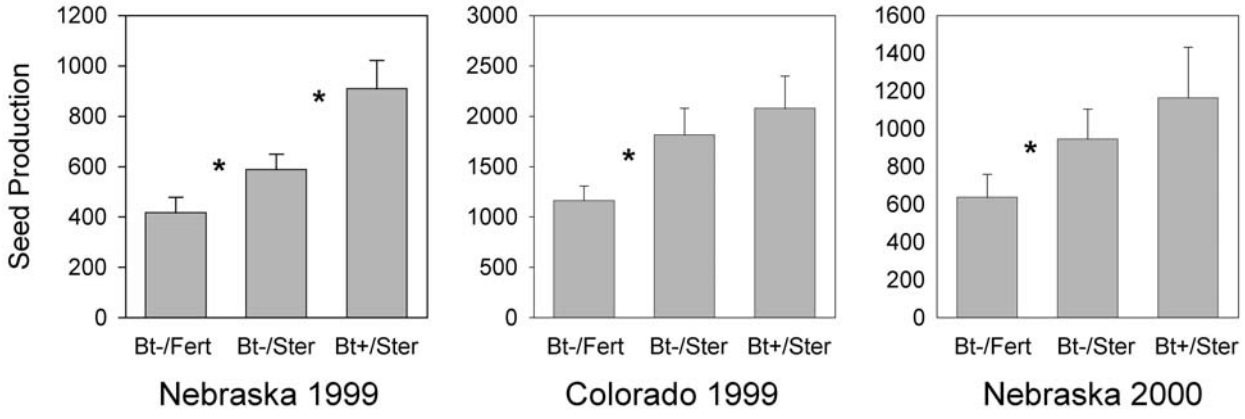

Figure 3 -- Effects of the Bt transgene and male sterility on seed production per plant in Nebraska and Colorado in 1999 and Nebraska in 2000. Untransformed means and 1 SE are shown; N = 58-60 in Nebraska in 1999 and 47-49 in Colorado in 1999 and Nebraska in 2000. Data were analyzed by ANOVA followed by planned contrasts as described in Figure 1. When both years and sites were combined in a single ANOVA the planned contrast between Bt+/male sterile and Bt-/male sterile plants was significant at $p<0.0206$. The selection coefficients favoring the Bt gene were 0.35 in Nebraska in 1999, 0.13 in Colorado in 1999, and 0.19 in Nebraska in 2000.

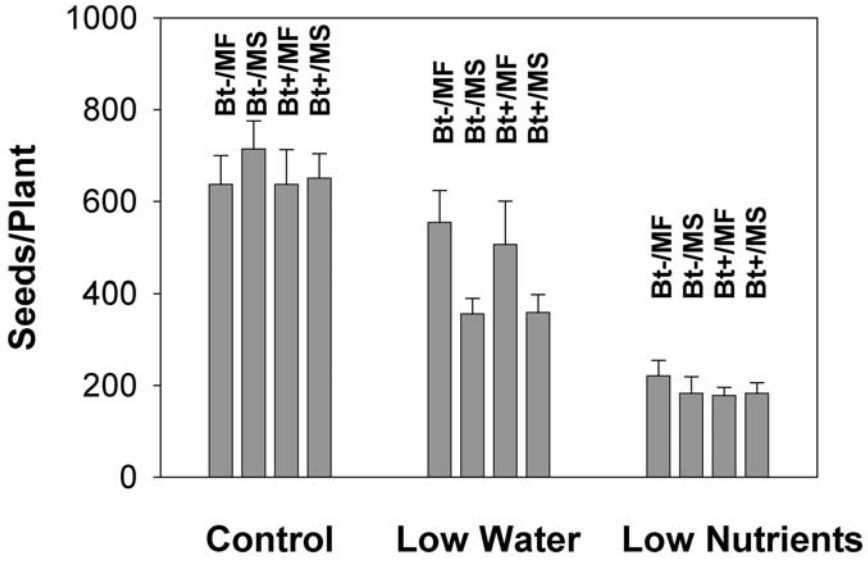

Figure 4 – Effects of the Bt transgene and male sterility on seed production of plants grown under three conditions (Control, Low Water, Low Nutrients) in a greenhouse. Means and 1 SE are shown. The only significant effects in a three-way ANOVA were growing condition ($P<0.0001$) and the interaction between growing condition and male-fertility ($P<0.0042$) Sample sizes from left to right were Control: 19, 24, 16, and 27 plants per treatment; Low Water: 17, 27, 12, and 24; Low Nutrients: 14, 11, 14, and 12.

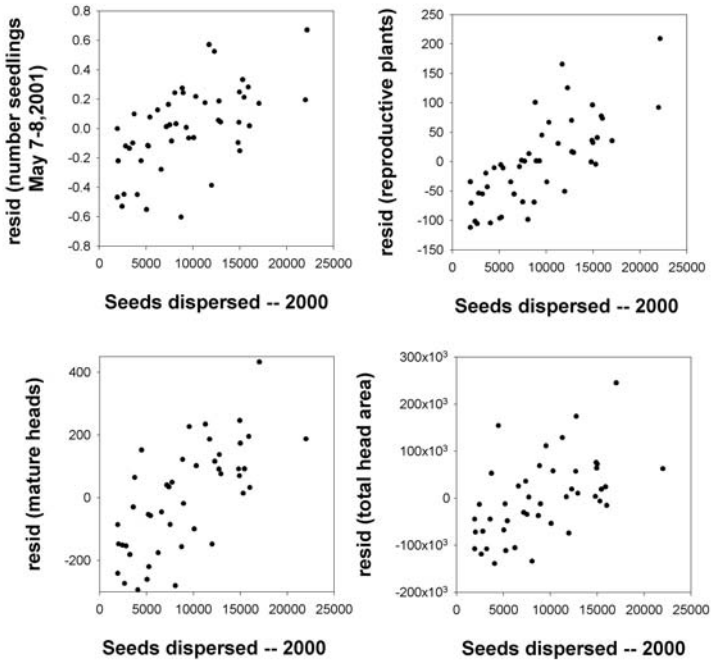

Figure 5 – The effect of the number of seeds dispersed in each of the 48 experimental populations in Nebraska in 2000 on seedlings, plants surviving to reproductive age, number of inflorescences with seeds, and total head area in 2001. Because tilling direction (see text) had a significant effect on these responses, data plotted are residual values after tilling direction was removed by ANOVA. Correlations coefficients (from upper left to lower right) are: 0.59, 0.75, 0.69, 0.54; all are significant at $p<0.0001$.

Case Study: Gene flow from commercial transgenic *Cucurbita pepo* to "free-living" *C. pepo* populations

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ABSTRACT

Field observations of natural populations of sexually compatible wild and weedy relatives of crop plants can provide useful data for assessing the risk of gene flow to these relatives, and the consequences of that gene flow. Surveys conducted and samples collected during the summer and fall of 2000 and 2001 allowed an assessment of the opportunity for gene flow, as well as its potential consequences. The data from these field observations were supplemented by plot experiments to assess the relative impact of virus infection on free-living, nontransgenic and transgenic genotypes of *C. pepo*.

GEOGRAPHIC DISTRIBUTION OF *CUCURBITA PEPO* POPULATIONS

The geographic distribution of the free-living *Cucurbita pepo* populations studied extended from the Mississippi, Illinois, and Ohio River drainages in southeastern Missouri, southwestern Illinois, and northwestern Kentucky, south through Arkansas, western Mississippi, Louisiana, and Texas (Figure 1). Thirty-three populations were studied, and samples were taken for virus ELISA assays. Sixteen populations were surveyed twice (2000 and 2001). Samples were also taken for DNA marker studies to assess genetic relationships among these populations. Free-living *Cucurbita pepo* plants occur as small (the majority in the range of 1-10) populations, with occasional large populations growing in disturbed unmanaged sites (Figure 2).

RATES OF VIRUS INFECTION IN NATURAL POPULATIONS

One factor that could influence the fate of transgenes introgressed into these free-living populations is the extent to which virus infection imposes a selective pressure upon these populations. In this study, the viruses of primary interest were cucumber mosaic virus (CMV), watermelon mosaic virus (WMV2), and zucchini yellow mosaic virus (ZYMV), since squash with transgenes conferring resistance to these viruses are presently in commercial production.

ELISA assays to date of samples from free-living *C. pepo* collected in 2000 and 2001 revealed that 32% of individuals assayed were infected with at least one of the three viruses of interest. However, infection was not uniform throughout all populations. In many populations, no virus infection was detected by ELISA (Figure 3). Furthermore, infection levels differed from year to year (Table I). In contrast to the ELISA results, visual assessment of the same populations for viral symptoms resulted in only 2% symptomatic plants. These data show that much of the virus infection in free-living *C. pepo* plants is from viral strains that produce very little phenotypic effects.

EFFECT OF VIRUS INFECTION IN EXPERIMENTAL POPULATIONS

To assess the impact of virus infection on free-living *C. pepo* genotypes, individuals from populations previously collected throughout this species' geographic range were planted in a common field. Two separate plots were planted in the field. One was inoculated with ZYMV, while the other was uninoculated. Various parameters that could be correlated with fitness were measured. One of those parameters, analysis of variance, including pairwise comparisons between inoculated and uninoculated plants showed that fruit production did not differ (Figure 4), but the amount of seed produced by inoculated plants was significantly less for most populations (Figure 5).

Data from *in situ* experiments provide an interesting contrast. These experiments were designed to study the long-term fate of transgenic plants under normal conditions, either in wild (unmanaged) populations or in agricultural (weedy) habitats. Three of these experiments were attempted in 2000. One experiment was planted in a newly cleared field in Mayflower AR, where a farmer was growing ornamental gourds. Only transgenic plants were planted at this location, providing the opportunity to assess the competitive ability of these plants in the presence of other weeds. The field was relatively unmanaged (i.e. no weed control), and the farmer removed a few of the ornamental gourds (easily distinguishable from fruit of the experimental plants) at the end of the season. The remainder was left to overwinter. A census was taken of the surviving transgenic plants; data collection will begin in the spring of 2002. Another experiment was planted at the Louisiana State University research station in Alexandria, LA, but plants failed to survive. A third experiment, planted at the University of Arkansas research station in Hope, AR, was established in a field of soybean—a crop in which *C. pepo* is known to be a weed. In this case, transgenic (introgressed) free-living *C. pepo* plants were planted along with the corresponding nontransgenic genotype. Virus symptom data were taken at the end of the season, as well as fruit and seed production. In contrast to the fruit data collected under conditions of high (100%) infection, the data collected in this field, indicate that transgenic plants might be at a disadvantage in the absence of virus infection. This population, as well as others to be established in 2002, will provide further data to either confirm or contradict this early result.

EFFECT OF VIRUS INFECTION IN NATURAL POPULATIONS

Data gathered from the surveys provided information on the consequences of virus infection in natural populations. At the end of the season in 2001, sixteen populations that had been surveyed in 2000 were re-visited. A census was taken again, as were samples for to assay for virus infection. Examination of population size and the frequency of virus infection in 2000 versus populations size and frequency of infection in 2001 was unable to reveal any relationship

between these two factors from one year to another (Table I). For the twelve populations that had no CMV, WMV2 or ZYMV infection in 2001, the populations declined or no plants were found at all. Of particular interest is the extremely large population found in 2000 that appears to have disappeared in 2001. In that case, no CMV, WMV2, or ZYMV infection was found in the samples taken from that population in 2000. Therefore, the decline in these populations cannot be attributed to infection by these viruses. A two-way test of independence (Sokal and Rohlf, 1995) was non-significant, supporting the hypothesis that change in population size was independent of infection by the viruses studied. This result does not rule out the possibility that other viruses might be infecting these populations and causing this decline, but if that is indeed the case, they are not the viruses to which the transgenes would provide resistance. Follow-up surveys of these populations in 2002 would be of interest to assess the contribution of the seed bank to the persistence of these populations. However, the relatively small size of most of these populations raises the possibility that genetic drift may have a significant effect on frequencies of any gene in these populations (Wilson and Bossert, 1971).

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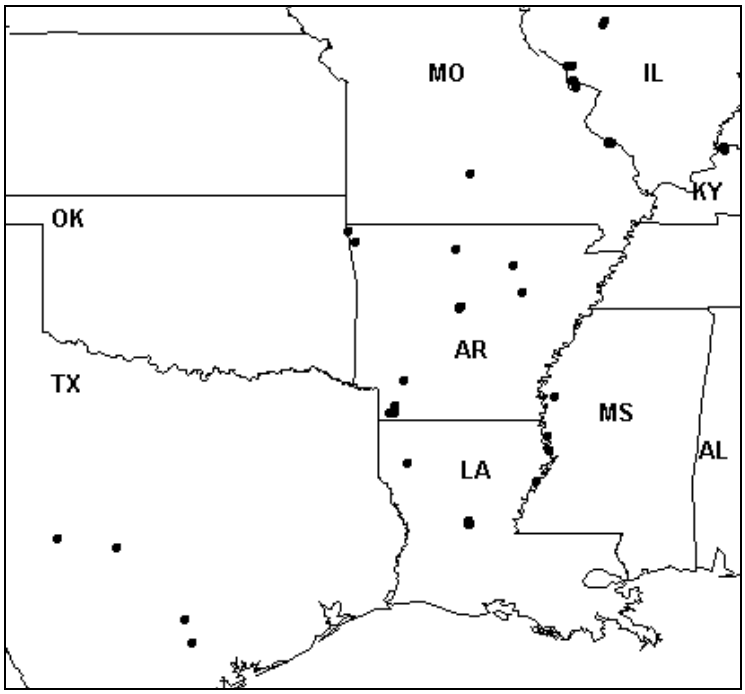

Figure 1. Location of *Cucurbita pepo* sites surveyed in 2000 and 2001.

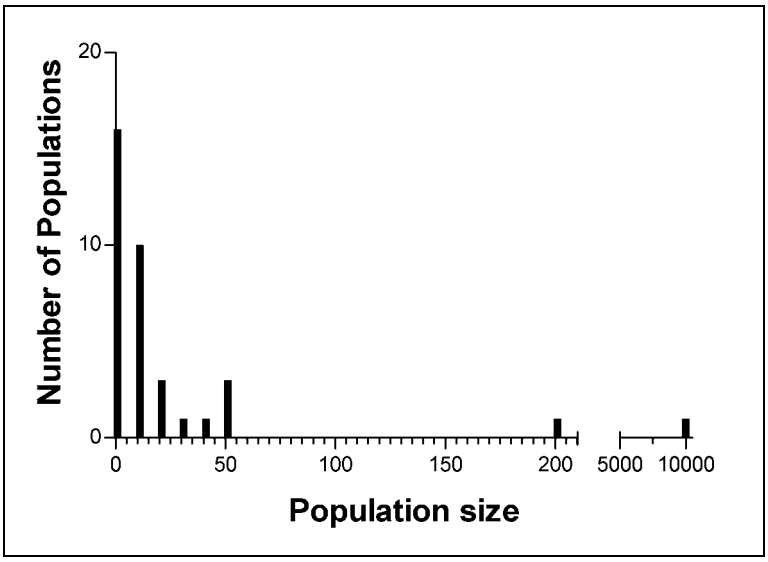

Figure 2. Distribution of population sizes free-living of *C. pepo*.

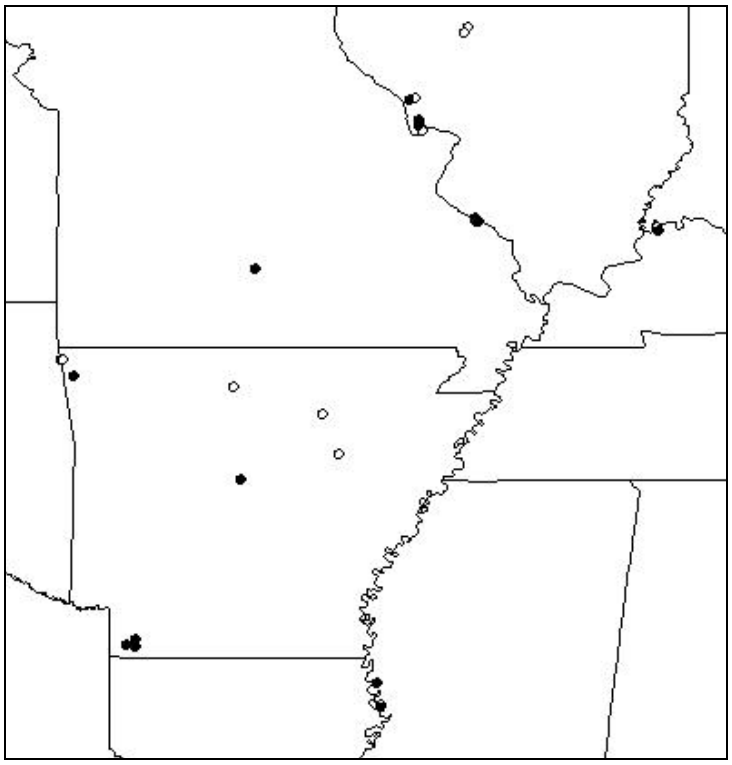

Figure 3. Populations assayed for CMV, WMV2, and ZYMV infection. ○ = no virus infection, ● = at least one virus-infected plant.

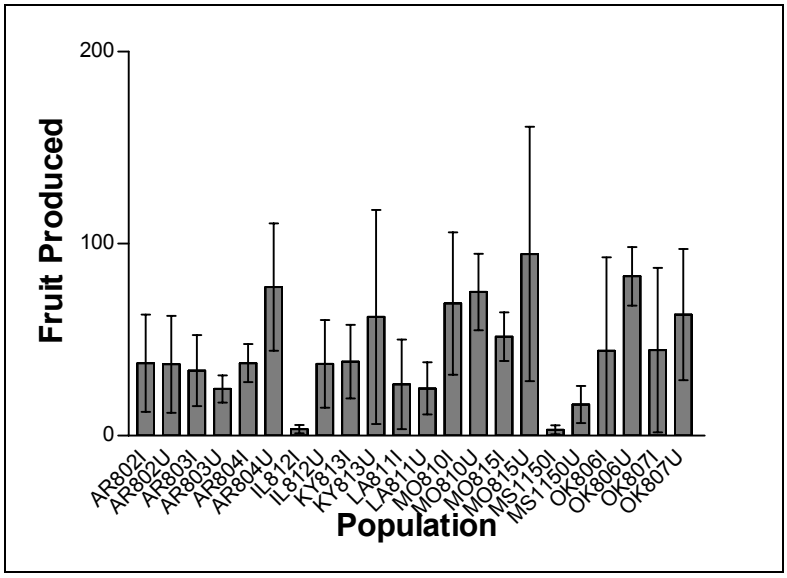

Figure 4. Fruit production of different populations of virus-inoculated (I) and uninoculated (U) free-living *C. pepo*. Plants were inoculated with ZYMV, and fruit counts were taken at maturity. None of the pairwise comparisons between inoculated and uninoculated plants of the same genotype were significantly different at P=0.05. Data presented are means ± 1 standard deviation.

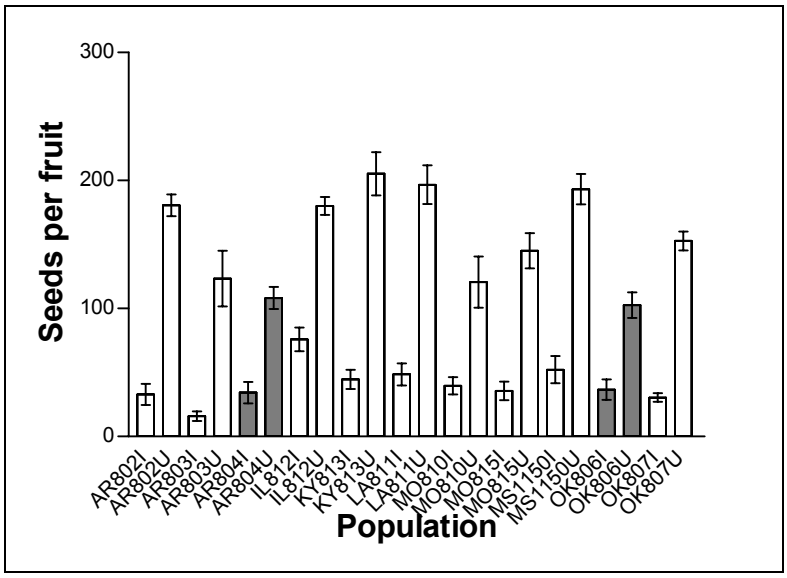

Figure 5 Seeds per fruit produced by different populations of virus-inoculated (I) and uninoculated (U) free-living *C. pepo*. Plants were inoculated with ZYMV, and seeds from up to ten fruit (all fruit if less than 10) from each population were counted. Pairwise comparisons between inoculated and uninoculated plants of the same genotype were significantly different at P=0.05, with the exception of the populations represented by the shaded bars. Data presented are means ± 1 standard deviation.

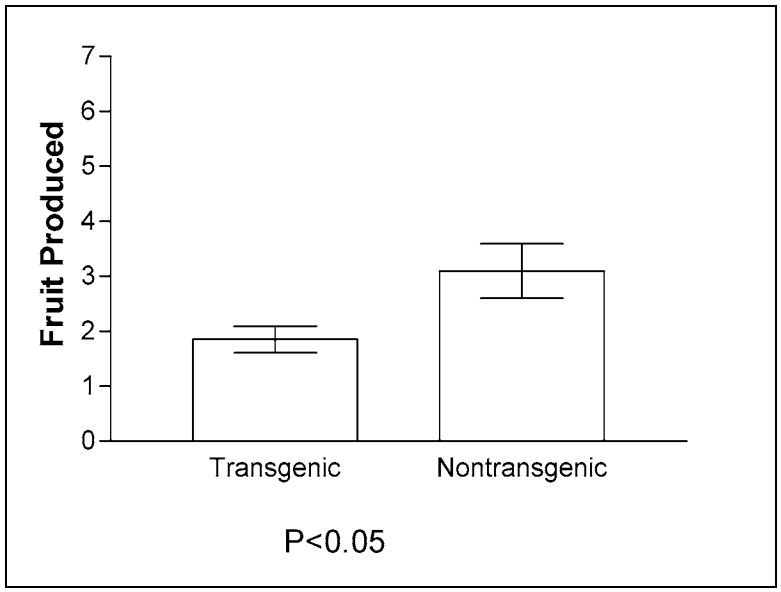

Figure 6. Fruit Production of transgenic (introgressed) free-living *C. pepo* and the corresponding nontransgenic genotype under conditions of no virus infection. Nontransgenic plants produced more fruit that transgenic plants (P<0.05). Data depicted are means ± 1 SEM.

	2,000		2001	
Population	Population Size	Virus frequency	Population Size	Virus Frequency
27A	6	0	3	0.67
27B	2	0	0	
27C	2	0	4	0.25
27D	8	0	0	
27E	3	0	4	0.75
28	42	0	6	0.00
29D	2	0	0	
30	5	0	6	0.50
31	12	0	7	0.00
32	35	0	7	0.14
33	50	0.05	0	
34	10,000	0	0	
35	23	0.13	0	
36A	3	0	0	
36B	11	0.2	0	
36C	2	0.5	0	

Table I. The size of populations and the frequency of CMV, WMV2, and ZYMV infection, as determined by ELISA assay, in 2000 and 2001.

Observed			
	Increase	Decrease	Total
Infected	0	4	4
Uninfected	3	9	12
Total	3	13	
Expected			
	Increase	Decrease	Total
Infected	0.75	3.25	4
Uninfected	2.25	9.75	12
Total	3	13	

Table II. Two-way table to test independence of population change and virus infection. The null hypothesis (H_0: population increase/decrease is independent of virus infection) cannot be rejected at P=0.05 ($G_{adjusted}$=1.556; $Chi^2_{0.5,1}$=3.841).

Monitoring the Environmental Consequences of Gene Flow from Transgenic Sugar Beet

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ABSTRACT

Gene flow via seed or pollen is a basic biological principle of plant evolution. The genetic and ecological consequences of gene flow depend on the amount and direction of gene flow as well as on the fitness of hybrids. In Europe, wild relatives of cultivated beet are important plant genetic resources; the conservation of wild beet diversity has become an important task in biosafety research. We have recently shown that a century of gene flow from *Beta vulgaris* ssp. *vulgaris* has not altered the genetic diversity of wild *Beta vulgaris* ssp. *maritima* in the Italian sugar beet seed production area. The assessment of potential risks of transgenic plants also has to take into account that conventional crops cross with wild plants. Unintended products of these crosses are weed beets that bolt and flower during their first year of planting. Weed beets cause harvest delays and yield losses. Gene flow is hard to control in wind-pollinated plants. At the same time, wild and weed beet populations undergo evolutionary changes and may expand their geographical distribution areas. The precautionary approach to risk management necessitates monitoring the local wild and weed populations that might be affected by transgene escape. Here, we present the methodology used for monitoring the geographical distribution and diversity of *Beta* populations in California and Italy. Future research should focus on the evolution of wild beet populations in comparison to baseline data. Two monitoring models are presented showing how endpoints can be measured: 1) "Prior-After" crop commercialization against today's baseline and 2) "Parallel" to crop commercialization against GMO-free reference areas/populations. Model 2 has the advantage of taking dynamic changes into account. Model 1 is superior if gene flow is so strong that unaffected areas/populations will not be found. Any assessment should be carried out realistically in comparison to natural variation of plant population parameters.

1. FOCUS OF BIOSAFETY RESEARCH AND MONITORING

Gene flow is by definition the active or passive dispersal of genes via seed, pollen or clonal parts of a plant within the environment. Since risk is a product of both exposure and hazard, it is clear that biosafety research on environmental effects should not only target the probability of gene flow, but must also focus on the consequences (and potential hazards) of successful transgene flow to relatives of transgenic crops (Fig. 1). Gene flow via seed or pollen is a basic biological principle in plant evolution. The ecological and genetic consequences of gene flow depend on the amount and direction of gene flow as well as on the fitness of hybrids (Bartsch et al. 2001). The assessment of potential risks has to be performed taking into account that

also conventional crops cross with wild plants (Saeglitz and Bartsch, 2002). This means that biosafety research should address the phenotype (especially the fitness phenotype) of the transgenic hybrid versus that of non-transgenic controls.

Biosafety research cannot solve every open or basic question of general ecology (Kareiva et al. 1997). Even after the best pragmatic use of a case–by–case and step–by–step approach, a well-designed monitoring program is necessary after commercialization. This monitoring must prove, now on a larger scale, the prognostic assumptions made by former biosafety research and assessment (Marvier et al. 1999). We know for sure that containment strategies do not work properly and provide no justification for avoiding monitoring (Sukopp and Sukopp 1993; Saeglitz et al. 2000). Monitoring must be flexible enough to recognize unforeseeable phenomena such as pleiotropic effects, although currently, we have no evidence that transgenic plants systematically express more pleiotropic effects than plants from classical breeding programs (Bartsch and Schuphan 2002).

2. BIOSAFETY EVALUATED CASE-BY-CASE AND STEP-BY-STEP: THE SUGAR BEET EXAMPLE

Beets have been cultivated for more than 2000 years in the eastern Mediterranean region. *Beta vulgaris* comprises an extraordinary variable group, in which it is often difficult to distinguish between cultivated and wild forms (Bartsch and Ellstrand 1999). This is mainly due to the extensive use of sea beet (*B. vulgaris* ssp. *maritima* ARCANG.) gene resources in conventional breeding programs. Sea beet is largely a coastal taxon, with a wide distribution from the Canary and Cape Verde Islands in the west, northward along Europe's Atlantic coast to the North and Baltic Seas. It also extends eastward through the Mediterranean region into Asia where it occurs in Asia Minor, in the central and outer Asiatic steppes, and desert areas as far as western India. Sea beet varies from self-compatible annuals to self-incompatible, iteroparous perennials with a life span of between one and more than eight years (Desplanque et al. 1999). Cultivated *B. vulgaris*, including Swiss chard, red garden beet and sugar beet, are biennial. The latter is partially self-incompatible due to the extensive use of male sterility genes in sugar beet breeding. All cultivated and wild subspecies of *B. vulgaris* are mostly wind-pollinated, although some insect pollination has been noted.

Conventional sugar beet (*B. vulgaris* ssp. *vulgaris*) has now been cultivated for 200 years. This cultivar has not shown unwanted ecological effects despite the introduction and spread of this European species to the New World (Bartsch and Ellstrand 1999). The only realistic way of assessing the environmental effect of transgenic beets is a comparison with classically bred cultivars. Transgenic attributes are genetically dominant in heterozygotes and inherited like conventional genes by wild beet populations. In our biosafety studies, the transgenic beets expressed tolerance to rhizomania, a disease caused by the Beet Necrotic Yellow Vein Virus (BNYVV), which has spread through the sugar beet fields of Europe, California, Japan, and China. Rhizomania is transmitted via the soil fungus *Polymyxa betae* KESKIN (Cooper and Asher 1988). The disease leads to decreased sugar beet yields and a loss of up to 30% sugar content (Guinchedi et al. 1987). Once infestation has occurred the disease persists for up to several decades despite eradication of all susceptible host plants. The advantages of tolerance against BNYVV may influence ecological performance parameters at different life stages of beet, including first year's vegetative growth, hibernation, and second year's bolting performance and

seed formation. Tolerant genotypes are infected to a lesser degree, but still act as a potential host for replicating the virus. Based on more than nine years of experience in biosafety field-testing of transgenic sugar beet, we wanted to know whether transgenically-mediated virus tolerance has a superior ecological effect on naturally virus-tolerant beet genotypes, especially wild beet hybrids. In particular, we compared the ecological performance of rhizomania resistant genotypes under various environmental conditions, focusing on parameters such as competitiveness, winter hardiness and seed production. We also studied natural gene flow between beet populations in Italy and California.

2.1 Germination of beet seedlings

Since young beets are confronted first with the soil borne rhizomania disease, the germination and vitality of young seedlings were measured under infestation and non-infestation conditions. We found no difference in seedling performance even under virus infestation (Bartsch et al. 1996).

2.2 Competitiveness

We tested the competitive performance of beet against *Chenopodium album*, a common weed in sugar beet fields and young fallow. Field experiments carried out between 1993 and 1999 demonstrated that transgenic beets grew better than virus-susceptible beets only when the virus was present. The difference between susceptible and resistant beets declined as more competing weeds were placed nearby. No differences were observed if the virus was absent (Bartsch and Brand 1998; Pohl-Orf et al. 2000).

2.3 Winter hardiness and seed production

The biennial sugar beet needs to survive cold winter temperatures in order to produce offspring. Winter hardiness is an important ecological factor for the geographical distribution of cultivated and wild beet in Europe. The natural distribution range is limited to mild areas at the seacoast in the Northern hemisphere. Some of our experiments focused on over-wintering of transgenic and non-transgenic sugar beet at different locations in Europe representing mild to cold winters in the years 1994-1999. We found no survival differences even under virus infestation conditions (Pohl-Orf et al. 1999, and Fig. 2 A). At a given virus infestation level, no significant differences among the three plant genotypes were found in hibernation rate, in biomass production of bolters resulting from the surviving beets, or in seed production, with the exception of better bolter biomass performance of the beet cultivar under virus infestation (Fig. 2B and 2C). There was a clear effect of the field location on hibernation rate. The plant survival rate was significantly lower at the virus-free site in comparison to the infestation site, which was most likely due to the colder winter at the virus-free site (winter cold sum: – virus / + virus: -27 °C / -17 °C). The sugar beet cultivar showed a significantly weaker hibernation rate at the virus free site but had a better bolter biomass production under virus infestation; otherwise there was no difference in comparison to the two wild beet hybrids. No effect was found on seed biomass production (Fig. 2).

Due to the potential for hybridization between cultivated and wild beets, it is important to know whether transgenic virus tolerance could also increase the fitness of wild beet populations. The fact that we found no difference between transgenic and non-transgenic hybrids was most likely due to the genetic wild beet background of the hybrids. Natural virus tolerance was inherited from wild beet by all three genotypes tested: the transgenic and isogenic control genotype by our hybridization with wild beet, and the virus-tolerant cultivar by recent hybridization with wild

beet and subsequent backcross-breeding to a high-performance cultivar. BNYVV tolerance has been widely found in wild beets (Whitney 1989), although no selection pressure from this disease is observable. An ecological examination of potential virus distribution in sea beet habitats had shown the absence of virus due to soil conditions unfavorable for virus infection (Bartsch and Brand 1998). The general lower hibernation rate at the virus free site was most probably due to lower winter temperatures. Survival rates fit well with the known correlation between survival and winter cold sum (Pohl-Orf et al. 1999). In any case, we found no costs associated with resistance – a result similar to that observed by Snow et al. (1999).

2.4 Development of weediness due to early bolting

Weeds are simply plants in the wrong place, either in agricultural or nature conservation areas. Interestingly, the same species can be protected as a plant genetic resource in one country and eradicated as a weed in another. Seed bolters pose problems for mechanical harvest machinery as well as reducing yields, and are therefore regarded as weeds in sugar beet fields. Early bolting and seed production in the first vegetation period is an important attribute for the ecological distribution of beet, since freezing temperatures can be better tolerated as a seed in the Northern hemisphere. In addition, the development of an annual habit is also important for the weediness of beet in disturbed habitats such as agricultural fields (Fig. 1).

The unwanted annual habit can evolve in two ways: random introgression of genetically dominant genes from wild beet (see next Section) or selective re-evolution towards wild characteristics (genetic draw-back). The later phenomenon was targeted by one of our field experiments. We found that the transgenic genotype had a much "safer" performance due to its higher resistance to early pre-bolting than the isogenic control (Bartsch et al. 2001). Since the physiological background is still unknown, this pleiotropic effect should be carefully considered and cannot be related to transformation events *per se*.

2.5 Sexual reproduction

Transgenic attributes are subject to natural reproduction and gene flow to all sexually compatible relatives. One prerequisite is sympatric growth of cultivars and their hybridization partners. There are only a few plants that can cross with sugar beet: Swiss chard, Fodder beet, Table beet and wild *Beta* species belonging to the section *Vulgaris*. No difference was found in the hybridization ability of transgenic, in comparison to non-transgenic controls (Bartsch and Pohl-Orf 1996; Dietz-Pfeilstetter and Kirchner 1998). The resulting hybrids between sugar beet and relatives were also the subject of experiments investigating biosafety in terms of germination, competitiveness, winter hardiness, seed production (Pohl-Orf et al. 2000, see Fig. 2). We found no special transgenic effect.

2.6 Consequences of gene flow and pathogens in Italian wild beet habitats

There is no evidence that the rhizomania disease studied here has any ecological role in non-agricultural areas, since the virus cannot be found in sea beet populations (Bartsch et al. 1996). We conclude that the ecological implications of the introduction and spread of virus resistant transgenic hybrids will be minimal in this special case. However, long term monitoring by collecting basic data on geographic distribution and genetic diversity of wild plant populations is absolutely necessary for the detection of any effect (Table 1).

Wild sea beet habitats are naturally and artificially disturbed areas. Many populations rarely comprise more than 100 individuals. Since year-to-year monitoring data demonstrate no clear

picture concerning population establishment and spread, plant genetic data best reveal the evolutionary history. For this reason we studied the ecological impact of a century of gene flow from traditionally bred cultivated beets into the wild sea beet populations of north-eastern Italy (Bartsch et al. 1999b).

We demonstrated that gene flow from a crop to a wild relative did not necessarily result in a decrease in the genetic diversity of the wild plant, although the cultivated beets were less diverse and outnumbered the wild relatives by a factor 10,000 to 1. These data support the view that gene flow alone should not be regarded as an adverse environmental effect of transgenic plants. In addition, we found no relationship between genetic diversity (Fig. 3, in terms of heterozygosity) and population size. An explanation for this phenomenon could be a high amount of gene flow. Founder effects or genetic bottlenecks seem to play a minor role in the Italian area, in contrast to sea beet in Germany where the wild species actually expand their geographical distribution eastwards without any sign of transgene related effects (Driessen et al. 2001).

2.7 Gene flow from cultivars to weed beet in California

Californian wild beets belong to two different taxa, and have at least three different origins. We found wild beet evolved from (1) escaped Swiss chard or Red beet, (2) *B. macrocarpa*, presumably introduced from Spain, and (3) hybridization of *B. vulgaris* with introduced *B. macrocarpa*. Although wild sea beet probably played some role in the origin of Californian wild beets, our genetic information is insufficient to determine the extent to which hybridization of cultivated beet with sea beet and/or direct introduction of sea beet from Europe contributed to contemporary *B. vulgaris*-type wild beets in California. A hot spot for gene flow is the Imperial Valley in Southern California. Here, the *B. macrocarpa* species grows as a weed in sugar beet fields. In the Imperial Valley sugar beet is grown in winter culture, and vernalization of the biennial plants is a common phenomenon (bolting) due to moderately cold winter temperature. In 1998 examination based on 15 sugar beet fields (representing an area of approximately 2 million m^2) showed a sugar beet bolting rate of 0.6 plants/m^2. This rate seems to be higher than typical for this area, probably due to an extraordinary cool winter of 1997/98 with periods of low freezing temperatures in some parts of the area. The density of the annual weed *B. macrocarpa* is in the range of 2.7 plants/m^2 (representing an area of approximately 1 million m^2 of sugar beet plantation examined). Although the annual *B. macrocarpa* usually flowers earlier than sugar beet bolters, a flowering time overlap could be detected in May 1998. Based on 9 specific isozymes, introgression in this area was detected at a rate of 2% of wild beet individuals (13 out of 594 Californian plants examined), which were morphologically similar to *B. macrocarpa*, but had isozyme alleles specific to *B. vulgaris* (Fig. 4). This past gene flow has led to a *de facto* increase of genetic diversity in the *B. macrocarpa* weed.

Engineered cultivars grown in California, especially the Imperial Valley, would have an increased probability of gene escape to wild relatives if their bolting properties were similar or higher than the tendencies of the current (non-engineered) cultivars planted in this area. Because of this increased tendency for beets to bolt in this area, the impact of an engineered trait escaping into wild populations would have to be assessed. Isozyme alleles specific to *B. vulgaris* were found in 2% of Californian *B. macrocarpa* individuals, leading to a higher genetic diversity of these accessions in comparison to European accessions (Table 2).
A question remains: can Californian wild beet be regarded as a plant genetic resource? If wild beet in this area is commonly designated as a weed (according to Figure 1) population genetic monitoring endpoints are redundant (Fig. 5). However, in Europe this genetic monitoring will

play an important role even in agricultural areas, since nature conservation includes rare plants in agricultural systems.

3. MONITORING SCOPE AND THE ROLE OF BASELINES

Monitoring is used for any post-commercialization measure that provides data on the fate or effects of transgenes in the environment. Monitoring needs baseline data on the evolution of a given (eco-) system structure and system process (Fig. 5). Indirect and direct methods are both helpful for detecting the possible impact of transgenes or their products. Environmental monitoring of agricultural crops and crop production practices is generally needed, not because of any specific, identified risk, but to enhance our ability to develop more sustainable food production practices.

Monitoring of transgenes is conducted to accomplish four specific objectives: 1) confirm compliance with regulatory requirements; 2) collect information necessary for controlling and managing potentially adverse environmental situations or systems; 3) assess environmental quality, and 4) detect "unexpected" and potentially damaging effects (Suter, 1993). Monitoring may be recommended to reduce uncertainties remaining after risk assessment, confirm conclusions with additional data, or provide informational feedback on system status or condition. Monitoring is not a substitute for biosafety research or risk assessment. Rather, it is integrated with research and risk assessment to ensure that ecological systems and processes are being protected. A decision that monitoring is required is ideally based on the scientific information provided in the risk assessment or some other scientific rationale suggesting a risk is possible. Where a conclusion based on scientific data confirms minimal risk, no monitoring should be *required,* thus allowing concentration of limited resources on more significant areas.

Nickson and Head (1999) have formulated two basic approaches to monitoring GMPs: general or specific. General Monitoring, which is also referred to as surveillance in the new EU directive 18-2001, is not necessarily based on any specific hypothesis of risk. It could be carried out using expertise and infrastructure already present in agricultural systems and within conservation efforts. By gaining familiarity and experience with GMPs through general monitoring, one can conduct "range-finding" and possibly better define the nature of a perceived risk and benefit. Specific monitoring must be based on a scientific hypothesis. It is science-based monitoring where a protocol with specific interpretable endpoints is used. Eventually, information from general monitoring could be refined through the development of specific monitoring protocols designed to determine what, if any, correlations exist between practices, technologies, activities, etc. used in agriculture and the overall condition of the system (Nickson and Head, 1999).

In the case of beet, any monitoring should focus on the evolution of transgenic wild beet populations in comparison to baseline data. Two monitoring models can be discussed in terms of how endpoints could be measured: 1) “Prior-After” crop commercialization against today’s baseline and 2) “Parallel” to crop commercialization against transgene-free reference areas/populations. Model 2 has the advantage of taking dynamic changes into account, e.g. point III or IV as baseline (Fig. 5). Model 1 is superior if gene flow is so strong that unaffected areas/populations will not be found and natural variation has to be assessed at the point I and II baseline (Fig. 5). In every case a baseline is needed in order to detect any deviation from natural variation of certain endpoint parameters (Table 2).

Causal analytic approaches need to compare transgenic gene flow with conventional gene flow effects. Finally, the additional effect of any transgene - if detected – needs broader, not only scientific, assessment as to whether this effect is unwanted or acceptable. The monitoring will be – in the case of beet – a combination of experimental biosafety research, ecological field observation and population genetic analysis.

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Table 1: Population size of representative sea beet populations in north-western Italy. This area is of special concern since gene flow from sugar beet seed production affects wild beet populations (Bartsch and Schmidt 1997). Individual numbers >90 are in most cases estimates, missing data are marked as "?"

	Year	1994	1995	1996	1997	1998	1999	2000	2001	Sum over
	Location									years
1	Cervia Saline	0	?	?	200	400	30	8	6	644
2	Bocassette	70	87	51	20	21	5	14	100	368
3	Scanarello	5	4	0	6	3	4	6	25	53
4	Porto Levante (sea dike)	5	70	1	1	0	0	30	35	142
5	Porto Levante (harbor)	3	3	6	13	32	23	25	21	126
6	Albarella (harbor)	2	27	6	6	1	6	8	2	58
7	Albarella (near P.Levante)	18	?	2	2	12	?	4	12	50
8	Albarella (Yachting club)	5	5	29	49	53	?	150	100	391
9	Albarella (inland dike)	100	100	28	61	83	5	55	107	541
10	Chioggia	100	30	100	17	200	?	?	46	495
11	Pellestrina	23	30	12	12	0	?	0	88	165
12	Porto di Malamocco	70	?	8	18	20	?	20	54	190
13	Fusina	60	89	10	124	203	?	?	123	609
14	Cimetero San Michele	5	?	601	46	200	?	90	170	1112
15	San Erasmo (sea dike)	?	?	20	38	12	?	?	200	270
16	San Erasmo (inland field)	?	?	?	3000	400	?	?	3000	6402
17	Torcello	?	?	71	600	1450	?	101	3000	5222
18	Punta Sabbione	10	1	1	0	0	0	?	0	12
19	Bilione (Valpelina)	?	?	1200	500	60	?	?	27	1789
20	Ausa Corno	18	4	20	12	0	2	?	66	122
21	Grado (sea dike)	50	200	100	51	65	?	5	49	522
	Approx. sum over locations	546	652	2269	4778	3215	?	?	7232	

Table. 2: Genetic diversity data of Californian wild beet **in comparison to other *Beta* groups. Number of populations examined (n), proportion of polymorphic loci (*AP*), the mean number of alleles among all loci (*A*) and among polymorphic loci (A_P), estimated heterozygosity (*H*), and total number of alleles found within a population group (*U*). Highest values within *B. vulgaris* are underlined.**

	A	*AP*	*P*	*H*	*U*
***B. vulgaris* USA/Europe (n_{all} = 47)**	**2.92**	**3.08**	**0.923**	**0.330**	**36**
Sugar beet USA/Europe (n = 16)	**2.23**	**2.42**	**0.923**	**0.343**	**29**
Swiss chard USA (n = 4)	**1.85**	**2.30**	**0.769**	**0.248**	**24**
Red beet USA (n = 5)	**2.15**	**2.60**	**0.769**	**0.250**	**27**
Sea beet Europe(n = 13)	**2.69**	**2.83**	**0.923**	**0.304**	**35**
Sea beet California (n = 9)	2.38	2.64	0.846	0.284	30
B. macrocarpa California (n = 9)	2.31	2.89	0.682	0.125	28
***B. macrocarpa* Europe (n = 4)**	**1.62**	**2.13**	**0.615**	**0.145**	**13**

Table 3: Suggestions for endpoint parameters at any one time-scale. Shaded cells represent areas where data are available for the Genus *Beta* (see Section 2 of this paper). Abbreviations: T = population(s) with transgene introgression and C = population(s) without transgene introgression as control.

Time/stage Endpoint Parameter	I before transgene release	II first transgene relase	III after transgene release	IV after transgene release	Remarks and special tasks
a. Number of individuals	C	C	C T	C T	population monitoring
b. Geographic dispersal	C	C	C T	C T	estimation of spread
c. Genetic diversity	C	C	C T	C T	population monitoring
d. Level of gene flow	C	C T	C T	C T	cause–effect analyses
e. Fitness		C T		C T	mostly experimental

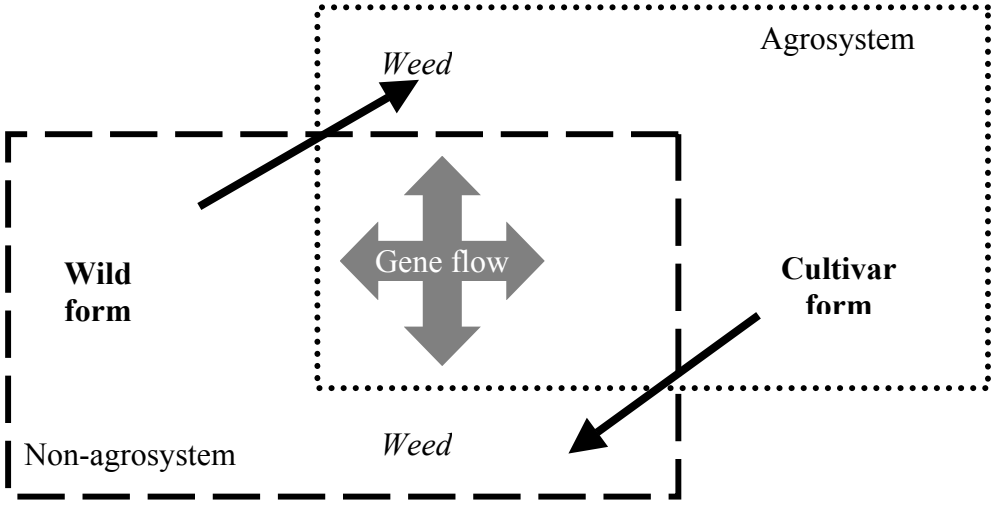

Figure 1: Intra-specific gene flow in a wild-weed-cultivar system. Weeds are plants in the wrong place, here, either as cultivars outside agricultural areas or wild plants in cultivated ground. Weeds may also evolve due to gene flow (Desplanque et al. 1999, Mücher et al. 2000). Wild and cultivar forms of a single species can be protected as a plant genetic resource in one place and be eradicated as weed in another (figure taken from Bartsch and Schmitz 2002).

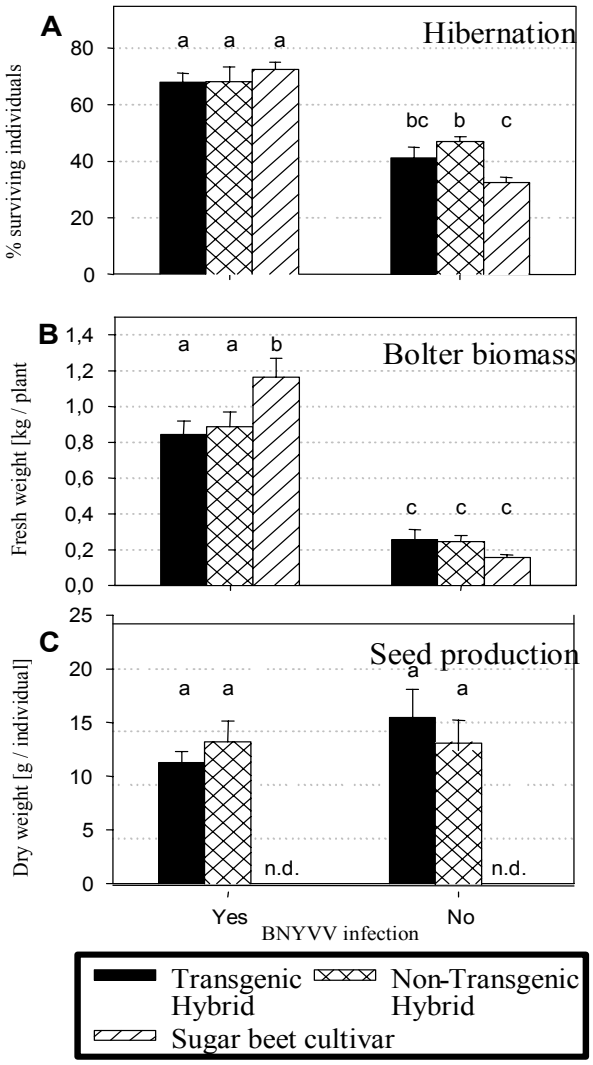

Figure 2: Ecological parameters (hibernation, bolter biomass and seed production) of two wild beet hybrids and a sugar beet cultivar grown with or without virus infection. Mean levels of characters with the same indicator letter are not significantly different by 2-way ANOVA / Tukey-test (SEM = Standard Error of the Mean, n.d. = not detected)

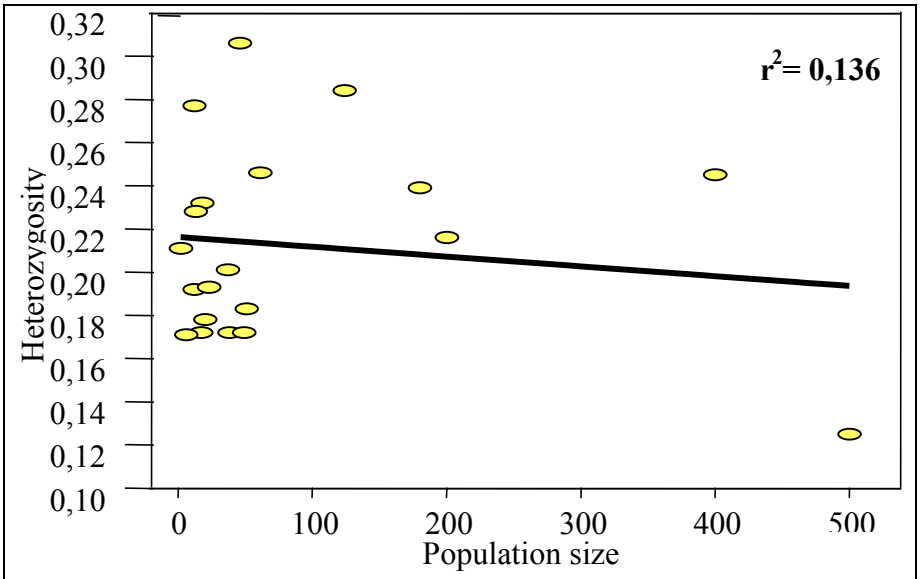

Figure 3: Relationship between heterozygosity calculated from isozyme data (Bartsch et al. 1999a) and population size [number of individuals] of sea beet in NE Italy in 1998. There is evidence that high rates of gene flow surpass loss of genetic diversity from genetic drift and founder effects.

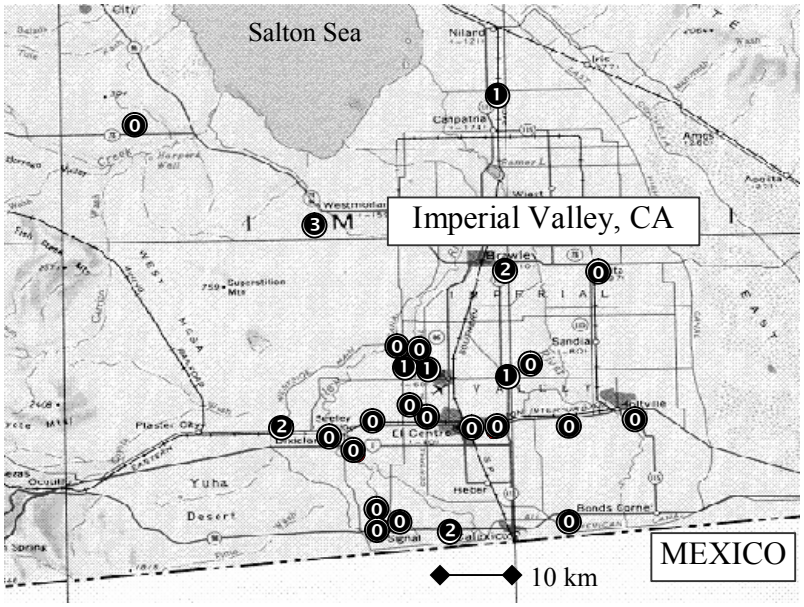

Figure 4: Gene flow from cultivar to weed in California's genus *Beta*: Circles indicate number of individuals of a *B. macrocarpa* population with gene introgression from *B. vulgaris*. Total number of populations: 20; Sample size 20 individuals per population.

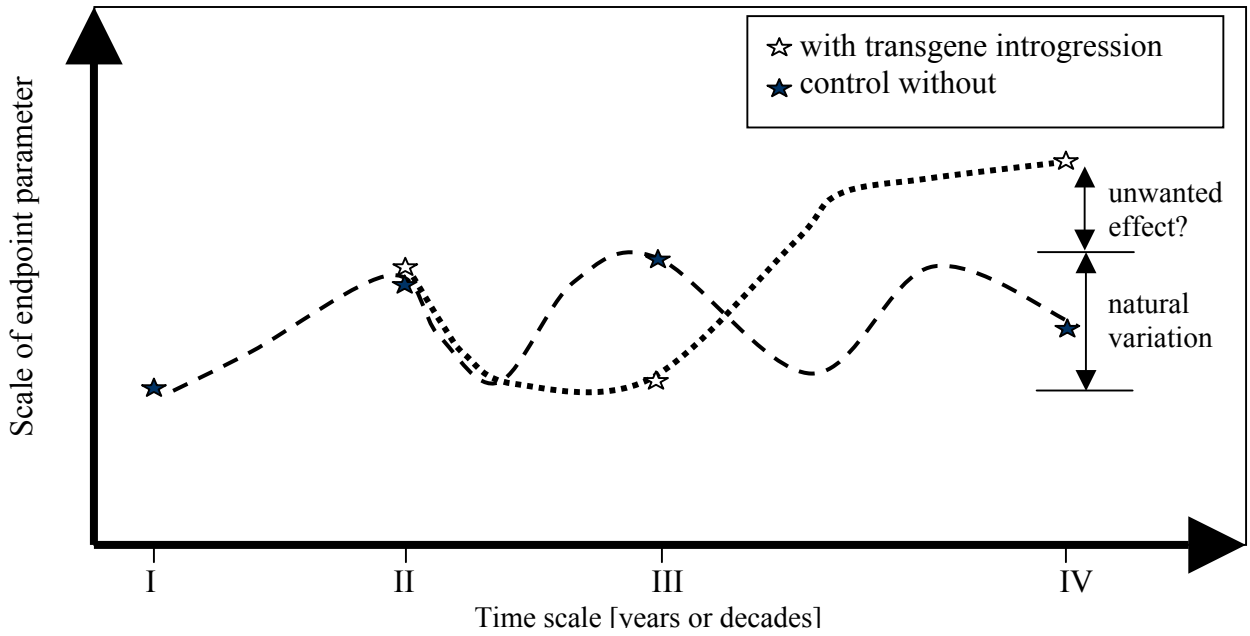

Figure 5: Hypothetical monitoring model. Critical points for measuring the potential impact of transgene escape into a wild plant population: I. before transgene release, II. at first commercial transgene release, III. and IV. at representative times after transgene release. Suggestions for endpoint parameters are listed in Table 3.

Ecological Risk Assessment for the Release of Transgenic Rice in Southeastern Arkansas

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ABSTRACT

Swarms of hybrids between cultivated rice and its weeds have been known in Taiwan, Thailand, China and India since the 1920's and appear now to be resident in the state of Arkansas. The USDA is currently funding work to evaluate the rate of pollen movement out of cultivars and into weeds, largely because of its interest in commercializing genetically modified rice. Greenhouse and limited field trials will determine, to some extent, the movement of cultivated genes into naturalized populations, but will provide only a snapshot of their history. Here we propose an approach to investigate the competitive dynamics and degrees of introgression between cultivated and weedy rice to assess the movement of transgenes in cultivation. Competition experiments provide a measure of population growth parameters for rice and red rice, as well as an indication of the commercial impact of transgenic-weed hybrids on rice production. Further, sampling genetic variation in hybrid swarms will provide an integrated, historical assessment of the movement of cultivated genes into weedy rice populations. The results of this work will assist in making decisions regarding the locations, and likely safe cultivars to consider for release as genetically modified organisms.

"Is crabgrass (*Digitaria sanguinalis*) so excellent a weed because it was once cultivated for food, or was it once cultivated as a millet because it is so excellent a weed?"

Protère 1955

OVERVIEW

Most domesticated plants hybridize naturally with wild relatives in some part of their range (Langevin et al. 1990, Hauser et al. 1998, Ellstrand et al. 1999). As a result, alleles from domesticated crop species are often found in natural populations (Oka and Chang 1961, Morishima et al. 1963, Chu and Oka 1970, Sanders 1987, Jorgensen and Andersen 1994, Landbo et al. 1996, Xu et al. 1997, Luby and McNichol 1995). The flow of genes from crop to weed has not been considered a risk in the past, primarily because organisms modified by traditional breeding were thought to be under the same selective controls as domesticated rice (Brill 1985, National Research Council 1989). Transgenics are different, however, in that plants created by modern technologies could not have been produced by traditional means. Crop-to-weed flow of transgenes carries the threat of creating more aggressive weeds, and accelerating the extinction of native species (Ellstrand and Hoffman 1990, Raybould and Gray 1993, 1994, Snow and Palma 1997, Ellstrand et al. 1999).

Red rice (*Oryza sativa* L.) is a leading weed pest in the cultivated rice fields of the Southeastern U.S. making it a legitimate system with which to evaluate the ecological risk of release of a genetically modified organism. Red rice and cultivated rice are now considered a single species (Grist 1986), although this has not always been the case (Dodson 1898, Quereau 1920, Copeland 1924). Control of red rice in cultivated rice fields is exacerbated by the similar biologies of the two varieties. Further, hybrid plants frequently demonstrate heterosis (Langevin et al. 1990) and their seeds may remain in the seed bank for up to 12 years (1984a). The release of transformed rice cultivars into areas where red rice has been naturalized may provide an avenue for introgression of novel genes into naturalized populations and presents a clear risk of building populations of aggressive weeds that are resistant to traditional means of control.

We are working to evaluate outcrossing rates of transformed rice lineages to assess the likelihood of release of transgenes into naturalized red rice populations. We will evaluate the ecological risks of releasing genetically transformed rice with a rice cultivar transformed with a biologically neutral genetic marker, GUS protein. Further, we posit that progressive approaches to the analysis of molecular data can provide a more accurate estimate of the movement of a neutral marker into weed populations. At present, there is no widely available report of the ecological risks of the release of transformed rice. The results of this project will assist in making decisions about the safety of introducing into the environment genetically modified organisms.

PROBLEM STATEMENT

Beneficial alleles increase in frequency under selection. Transgenes are likely to confer a selective advantage to hybrids in crop fields and in marginal habitats, altering competitive relationships and promoting the persistence of hybrid populations. The model in Fig. 1 illustrates the expected change in frequency of a beneficial allele under selection. An allele increases in frequency in this model as a function of the initial allele frequency, the mode of inheritance and the intensity of selection. In this example, initial allele frequency (p_0) for a dominant allele is set at either 1% or 50%, values similar to those reported by Langevin et al. (1990) for populations of red rice in Louisiana. The selective advantage (s), an estimate of competitive superiority, is set here at either 0.1 (a 10% advantage) or 0.5 (a 50% advantage). A clear outcome from this simulation is that beneficial alleles, once introduced, will go to fixation regardless of initial frequency or selective advantage (Oka and Chang 1961, Wright 1969, Diggle and Neve 2001). With substantial gene flow (p_0 = 0.5) and strong selection (s = 0.5), a beneficial transgene could be fixed within 10 years, all else equal.

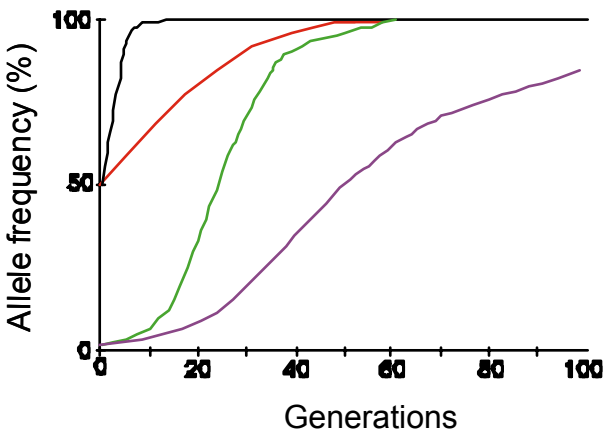

Fig. 1. Expected change in frequency of a favorable allele under selection. Curves from right to left reflect greater selective advantage and higher initial allele frequency. Model conditions are:
— p_o = 0.5, s = 0.5 — p_o = 0.5, s = 0.1
— p_o = 0.01, s = 0.5 — p_o = 0.01, s = 0.1
where p_o = initial allele frequency, s = selective advantage. (Simulation generated by "Populus," courtesy of Don Alstad, U. of Minnesota.)

Hybridization creates an avenue for the introduction of transgenes into natural populations making it likely that alleles from cultivated plants are resident in naturalized populations. A recent review from the United Kingdom reports that approximately one third of the domesticated species spontaneously hybridize with one or more species of the local flora (Raybould and Gray 1993). For the Netherlands, that fraction is about one quarter of the crop species reviewed (de Vries et al. 1992). (Similar reviews are unavailable for the U.S.) Hybridization rates of this magnitude argue strongly that outcrossing and introgression between crop plants and natives is common. Introgression, gene flow between populations whose individuals hybridize, is achieved when hybrids backcross to one or both parental populations (Rhymer and Simberloff 1996). Previous introgression makes the production of successful hybrids even more likely, since hybrids, which are more similar genetically to crops than are weeds, are less likely than weed races to encounter reproductive barriers with cultivars. By this mechanism, genes from cultivars are increasingly more likely to be added to the gene pool of the weed populations as introgression proceeds.

Transgenic weeds may persist in marginal habitats. Marginal habitats represent safe sites (*sensu* Harper 1977) for the successful completion of the life cycle of agricultural weeds (Aldrich and Kremer 1997). The persistence of weed populations in such locations may have several unwanted ecological consequences, however. Ditches and canals can serve as dispersal routes

for the spread of weeds out of cultivation or, alternatively, may serve as reservoirs for weed populations that may invade native vegetation. Escape of aggressive weeds constitutes a threat to native biological diversity and may negatively impact native ecosystem processes (Raybould and Gray 1994). Additionally, weeds from such waste places may represent a seed source for the re-colonization of agricultural fields--either into the same field from which transgenes were obtained, or into other fields of the same crop, or into fields of an entirely different crop plant.

In the U.S., the degree of introgression between rice and red rice is unknown. Introgression between rice and rice weeds has been well characterized in the Old World. Populations of hybrids resident in marginal habitats have been characterized from India (Roy 1921, Bhlerao 1928, Mitra and Ganguli 1932, Hector 1935, Sampath and Govindaswam 1958, as cited in Oka and Chang, 1961), Thailand and Formosa (Oka 1956, Harlan 1965, 1969), Taiwan (Oka 1956) and West Africa (Chu and Oka 1970), and now appear to be established in the State of Arkansas. A large number (87, D. Gealy, personal communication) of recognizable morphotypes of red rice suggests that rice and red rice weeds have a complex history of introgression in Arkansas. This seems likely given that outrcrossing rate from domesticated rice to red rice may reach 54% (Langevin et al. 1990, Oard et al. 2000), and given that introgression, once begun, proceeds at an accelerating rate. Further, herbarium records provide evidence that weeds and putative rice-red rice hybrids escape from cultivation. *Oryza sativa* has been collected from marginal habitats in six Arkansas counties largely, but not exclusively, in rice producing regions of the State (University of Arkansas Herbarium, unpublished data).

OPERATIONAL HYPOTHESIS

A conceptual model illustrates the primary issues in assessing the ecological risks of releasing transgenic rice in a system where red rice Is common (Fig. 2). Unidirectional arrows indicate movement of transgenes, hybridization (introgression) by circular arrows. The size of each box represents the relative size of the source of transgenic-weed hybrids. Transgenes are introduced to the system through agriculture, and may move out by pollen movement or dispersal of seeds. Populations in marginal habitats may serve as a reservoir of transgenes, which have four possible fates: go extinct, move back into culture, persist in marginal sites or, conceivably, escape into more naturalized landscapes. Factors influencing the rate of movement from one section to the next include the size of red rice populations, competition and gene flow. The size of the population in each section will largely influence the persistence time of the hybrid swarm. The only known component of this diagram at present is the introduction of transgenes through agriculture; transgenic rice plants have not yet been released in the state of Arkansas. Because there are as yet no fields of transgenic rice, our experiments will focus on experimental plots to assess competitive advantage, and sampling natural populations to assess the degree of introgression between cultivars and weeds. Our assessment will address the competitive advantage of transgenic rice hybrids in culture and in marginal habitats, and in determining the degree of hybridization in red rice populations.

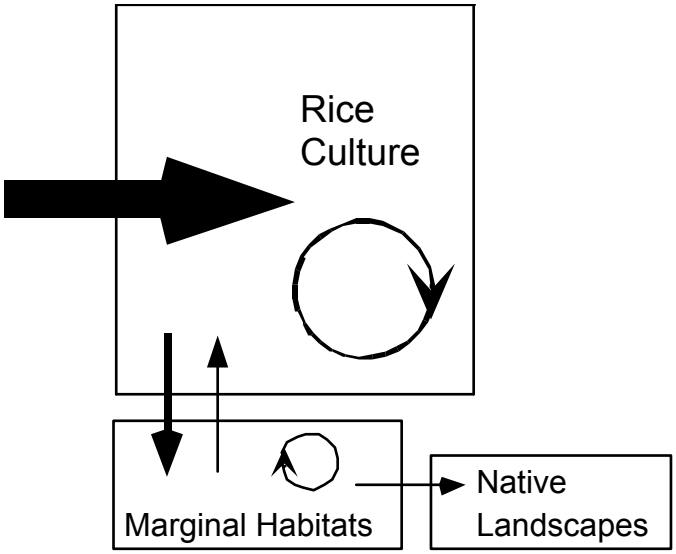

Fig. 2. Identifying the risks of releasing transgenes into culture. Boxes represent habitats. Arrows indicate movement of transgenes between habitats by pollen or seed dispersal (unidirectional arrows) or within habitats through hybridization and introgression (circular arrows). Size of arrow suggests relative contribution to gene flow. Reducing the ecological risks of releasing transgenes involves reducing movements and population sizes of hybrid plants.

STUDY SYSTEM

Rice is a staple food for most of mankind, second only to wheat in global production (FAOSTAT website 1998). Rice is grown on more than one million ha in the US with average yields of 6048 kg/ha. Arkansas is the leading rice producing state in the U.S. contributing approximately 45% of the total yield, or more than 27 million metric tons per year. As such, rice is an important agricultural product of the state and contributes substantially to the economy of the region.

Rice cultivation was introduced to the U.S. from India through South Carolina in 1686, and was firmly established there by about 1690. Rice culture became established in southwestern Louisiana and Texas by 1888, and in Arkansas by about 1904. Introduction to the remaining rice-producing states occurred later: California in 1912, and Mississippi in 1948. Red rice was a weed of rice fields in the U.S. as early as 1846 (Constantin 1960), brought from abroad by means of red-rice contaminated seed. Red rice was observed in new rice fields in the east central region of Arkansas shortly after the introduction of cultivated rice, probably through rice seed trade among farmers (Saldain 1997). Given this history, red rice has persisted in naturalized populations in the Delta region of Arkansas for no more than 150 years.

Like cultivated rice, red rice is an annual plant that reproduces by seeds (Smith 1981). Although once thought to be different species, red rice and cultivated rice are now treated as conspecific (*Oryza sativa* L.) (Grist 1986). Red rice is characterized by the red color of its pericarp when the grain is hulled (Dodson 1898 as cited in Saldain 1997). The red rice plant has longer, narrower, and rougher leaves than rice, and the foliage is very light green (Diarra et al. 1985a). Red rice has a spreading habit and its culms are prone to lodge near maturity. A reduction in plant height of red rice plants over a 20 year period provides some evidence that red rice is under breeders' selection for the smaller stature of domesticated lines (Yoshida 1981). As with other weedy forms of crop plants, the seeds of red rice are shed easily and a dormancy mechanism is present (de Wet 1975, Ladizinsky 1985, Langevin et al. 1990). Red rice is a proposed progenitor of cultivated rice and, therefore, has few reproductive barriers with it. Two forms or biotypes of red rice are common in the southeastern U.S.: strawhull and blackhull, although more than 80 types can be recognized (D. Gealy, personal communication). The two common forms are morphologically distinct; strawhull is usually awnless and blackhull has long awns (Smith 1981, Smith et al. 1977). Of the two, strawhull is more common (Diarra et al. 1985a) and will be used in the experimental trials described below.

Red rice is a serious weed pest throughout much of the southeastern U.S., and any place where rice is grown. The percent infestation rates are, by state, Arkansas - 30-40%, Mississippi - 50%, Texas - 40-50%, and Louisiana - 100%. The presence of red rice in cultivated fields limits rice production and quality, reduces yields and overall market value of the crop (Diarra et al. 1985b, Kwon et al. 1991, Pantone and Baker, 1991, Estorninos 2000). For example, Diarra et al. (1985a) found that five rice plants/m^2 reduced the rice grains per panicle yields by 8 to 18%. Likewise, Kwon et al. (1992) reported that grain production of the 'Lemont' cultivar was reduced by 90% at the highest red rice densities studied (35 plants/m^2), while 'Newbonnet' was reduced by 67% with 40 red rice plants/m^2. Similar reductions in the parameters used to measure vegetative growth, such as plant height, leaf area index, tiller density and plant weight, are also reported as a result of red rice interference with cultivated rice (Diarra et al. 1985b, Kwon et al. 1991, Pantone and Baker, 1991, Estorninos 2000).

The rate of crop-to-weed gene flow is influenced by three criteria (Ellstrand et al. 1999):

- interfertility of breeding populations
- degree of overlap in flowering phenologies
- densities of interbreeding populations

By these criteria, cultivated rice is likely to have a high rate of genetic exchange with its red rice counterparts. Hybridization rates between rice and red rice, reported as the mean proportion of the harvested seeds, indicate that 1-52% of red rice seeds may receive pollen from domesticated rice donors (Langevin et al. 1990). Outcrossing rate varies among rice varieties because late season varieties have greater phenological overlap with red rice (Langevin et al. 1990). In addition to the high potential for outcrossing, two additional characteristics make red rice difficult to control. First, seeds may remain dormant in the seed bank for up to 12 years (Jennings and Vergara 1968, Diarra et al. 1985b). Dormancy precludes complete eradication in a single season, and suggests that even a rare hybrid may remain undetected for a long period. Second, hybrid offspring of cultivated and wild rice demonstrate heterosis (Langevin et al. 1990). Hybrids are larger and produce more seeds than their wild progenitors, making them good competitors with cultivars, and increasing the probability of crop-to-weed gene flow. Because of measurable outcrossing rates, long-term storage in the seed bank, and vigorous hybrids, there are few barriers to gene flow from crop to naturalized weed populations creating an exceptional opportunity for the release of transgenes into weedy populations (Langevin et al. 1990, Ellstrand et al. 1999).

Recent successful transformations of cultivated rice include the insertion of genes for resistance to virus (Upadhyayaet al. 1998), blight (Datta et al. 1999), phloem feeders (Rao et al. 1998, Lee et al. 1999), stem borers (Xu et al. 1996, Alam et al. 1998, Datta et al. 1998), nematodes (Vain et al. 1998) and herbicides (Kim et al. 1999). Release of these traits into naturalized populations of a hybrid weed may seriously compromise the benefits of the transformation effort by creating new weeds, and radically altering the biologies of naturalized populations. Further, rice-red rice hybrids are fertile, vegetatively robust and appear to demonstrate heterosis (Langevin et al. 1990), suggesting a competitive advantage of red rice over cultivated rice can only become larger. The impending release of transgenic cultivated rice is a clear and

obvious system in which to evaluate the ecological risk of the release of a genetically modified organism.

WORK IN PROGRESS

We have begun to address concerns regarding the release of genetically modified rice by developing a biologically neutral marker to track the rate of gene flow through pollen movement into naturalized, weedy rice populations. To date, we have followed three selfing generations of two lineages of Nipponbare cultivar and established that they are stable and homozygous, and seven other lineages are likely to be stable and homozygous. A second greenhouse effort is underway to investigate causes of instability in lineages that fail to breed true. Establishment of stable, transformed lineages is an essential first step in tracking gene flow and in understanding the behaviors of transformed varieties under field conditions.

In addition, we believe this neutral marker system is a tool that may be used to follow transgene transfer and migration out of cultivated materials. Tissues with GUS protein stain brilliant blue in the presence of an enzyme, X-Gluc. Staining procedures are straightforward and inexpensive, and, because the marker is biologically neutral (i.e., no pest or herbicide resistance), it poses little ecological risk should escape occur during field trials. Clegg et al. (1993) recommended that neutral markers be developed for ecological risk assessment, and this GUS marker system is an exceptional candidate;

A further effort in the greenhouse is directed toward understanding the reproductive biology of transgene X red rice hybrids. Pollen viability, panicle production, allocation to reproductive vs. vegetative biomass, and seed germination success will be compared among rice, transformed rice, red rice and hybrids of rice X red rice, and transformed rice X red rice. At present, we have begun creating hybrid lines for the heterosis studies.

Field studies to estimate the rate and dynamics of pollen movement will begin in 2003. GUS - transformed plants will be surrounded by red rice target plants following Manasse (1992).

PROPOSED EXPERIMENTS

In the future we propose a somewhat different approach to understanding the ecological risks associated with the release of genetically modified rice. We will evaluate competition between transgenic crops, red rice and their hybrids, and investigate "naturalized" populations of red rice to evaluate the gene transfer rates from cultivated rice to red rice weeds.

Our objective, broadly, is to develop a clear understanding of the dynamics of the rice-red rice system in an effort to better characterize the risks associated with releasing genetically modified rice. An emerging issue, then, is whether transgenic rice is capable of competing with transgenic X red rice hybrid. Therefore, we will conduct a series of competition experiments between rice transformed for herbicide resistance, red rice and hybrids between them. Experiments will take place under field conditions, including the application of herbicide in cultivated fields for which both plants have been made resistant. The results of these experiments will allow us to assess productivity of transformed rice in cultivated fields when

transformed weeds are present. These experiments are feasible now that the release of "clearfield" rice, a glufosinate resistant variety, has been approved.

Further, establishing large-scale plots of transgenic plants to monitor gene flow is not feasible at present. Instead, we will take advantage of information contained in the genomes of red rice weed populations to reconstruct the history of inbreeding between cultivars and weeds. We propose to evaluate genetic variability for both nuclear and chloroplast markers in cultivated and red rice populations. Such an approach promises to reveal important information regarding the movement of genes from cultivar to weed. First, microsatellite analyses will allow us to characterize the degree of introgression and to evaluate the genetic structure of red rice populations (Rieseberg and Brunsfeld 1992). This project is facilitated by the fact that more than 600 microsatellite primer sequences have been identified and are commercially available in rice (http://www.gramene.org/microsat/microsats.txt). Results from this portion of the project will provide an estimate of the relative frequency of cultivar genes resident in weed populations. Second, microsatellite analyses will result in an understanding of the phylogenetic relationships among red rice populations (Meyer et al. 1995, Zhu et al. 2000), as well the histories of establishment and colonization by those populations (Petit et al. 1997). Further, and in combination with locality data, these analyses may also reveal information regarding the history of gene flow into red rice (Templeton 1998). Such an understanding will enable us to identify where and through which cultivar the highest rates of gene flow have occurred. Finally, gene flow in rice fields arises as a consequence of the movements of two life stages, seeds and pollen. To evaluate the relative contributions of seed and pollen movement to total gene flow, genetic structure will be estimated from nuclear markers, which can move in either seeds or pollen, or chloroplast DNA, which is typically maternally inherited can move only in seeds (McCauley 1997, Oddou-Muratorio et al. 2001). Differences between estimates of genetic structure between nuclear and chloroplast marker is largely attributable to pollen movement. Each of these analyses will provide insight into the evolution of red rice weeds, but will also provide clear management recommendations in reducing the risks of genetic escape.

JUSTIFICATION AND SUMMARY

The ecological risks of genetically modified crops are of greatest concern when there are no inherent barriers to the spread of transgenes through sexual reproduction (Bergelson et al. 1998). The proposed research tests whether the release of genetically transformed lineages posses a risk of genetic escape into natural populations of *Oryza sativa*. There are three significant aspects of this work. First, the potential for release of transgenes through a conspecific will have tremendous implications for weed control in the future. Secondly, gauging the outcrossing rates in one year may provide guidelines for release of other target genes in the future. Finally, although nearly all crop species hybridize with natives in some part of their range, few commercially viable crop species are sympatric with conspecifics. If the ecological risk of release of genetically modified organisms can be anticipated by taxonomic relationships, then rice is certain to be a candidate. In conclusion, the proposed research will be an essential first step in understanding the risk of release of modified rice varieties in the delta region of the southeastern U.S., with relevance to agricultural production and management. The results of this study will help address concerns about the effects of introducing genetically modified

organisms into the environment, and to help regulators develop policies concerning such introductions.

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Gene flow and its consequences: *Brassica napus* (canola, oilseed rape) to wild relatives.

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ABSTRACT

Gene flow from transgenic canola (*Brassica napus*) to weedy relatives is an extant risk in implementing biotechnology in agricultural systems. Wild relatives, such as *Brassica rapa* and *Raphanus raphanistrum*, have been shown to hybridize with transgenic canola, and future research must characterize the possible ramifications of transgenic phenotypes contained within introgressed weed populations. We have developed a system to track the movement of an insecticidal Bt transgene from genetically modified canola into relatives by a visual marker, green fluorescent protein (GFP). Whole plant fluorescence has been used to track gene flow into *B. rapa*, and future studies will use these transgenic hybrid lines to test the impact of an insecticidal transgene on agricultural crops through competition studies.

INTRODUCTION

Genetically modified plants have been a focus of controversy since many people believe them to be inherently risky. Data are needed to address concerns (Stewart et al. 2000). Canola or oilseed rape (*Brassica napus*) is an interesting crop for the study of transgene escape into wild relatives. Canola is an allotetraploid (AACC, 2n = 38) and has many wild weedy relatives such as birdseed rape or field mustard (*Brassica rapa*, AA, 2n = 20), wild radish (*Raphanus raphanistrum*, RrRr, 2n = 18), dog mustard (*Erucastrum gallicum*, 2n = 30), *Hirshfeldia incana* (2n = 18), and wild mustard (*Sinapis arvensis*, 2n = 18) persisting in or near areas of cultivation. Birdseed rape is a common weed in many places where canola is grown (Holm *et al*. 1997), and wild radish is a noxious cosmopolitan weed that can exist outside the agricultural setting. These species have been shown to hybridize with canola under both laboratory and field conditions (Chevre *et al*. 1997, Darmency *et al*. 1998, Eber *et al*. 1994, Jorgensen and Anderson 1994, Lefol *et al*. 1996, Metz *et al*. 1997, Mikkelson *et al.*1996, Reiger *et al.* 1999 & 2001). Also, canola transformation protocols have been developed and transgenic varieties have been widely studied under field conditions (Darmency *et al*. 1998 & 2000, Paul *et a*l. 1995, Ramachandran *et al*. 2000, Stewart *et al*. 1997). Canola has been transformed with fitness enhancing transgenes such as herbicide, disease, and insect resistance (Metz *et al*. 1997, Stewart *et al.* 1996, Harper *et al.* 1999).

It is generally accepted that transgenes will move from canola to *B. rapa*. Transgenic hybrids have been produced between transgenic canola modified with herbicide resistance genes and *B. rapa* (Metz *et al*. 1997, Mikkelsen *et al*. 1996). After one backcross generation, many of the progeny were morphologically and cytologically similar to the *B. rapa* parent (Metz

et al. 1997). After successive backcrosses into the weedy parent, it was found that 10% of the subsequent BC_3 and BC_4 hybrids had resistance to the herbicide (Metz *et al*. 1997). This illustrates that a transgene can be passed between species and are active in successive generations. To this point few studies have been performed to produce transgenic hybrids that would have a putative selective advantage outside agriculture.

With the evidence that transgenes will move into weedy *B. rapa* from canola, it is important to assess the enhanced weediness potential of insecticidal transgenic *B. rapa*. Bt transgenic hybrid lines have been developed with the assistance of a transgenic visual marker for selection, green fluorescent protein (GFP)(Halfhill *et al.* 2001a). Rather than relying on negative selection with antibiotics, these lines were developed by visual assays selecting for the GFP phenotype. In future field-level experiments, the weediness potential of these insecticidal hybrid populations will be determined in competition experiments with different crop species.

Transgene flow from canola to wild radish (*R. raphanistrum*) occurs at a much lower level. Depending on the wild radish genotype, the cross can occur in the field between 1 in 198,084 crosses (Chevre *et al.* 2000). In Australia, the number rose to 1 in 25 million (Chris Preston, pers. comm.). The fertility of the hybrids was also diminished. However, given the nature of the severity of wild radish as a weed it seems prudent to also assess the weediness potential of insecticidal transgenic wild radish.

While it is most important to assess the weediness potential of transgenic weeds, long-distance pollen dispersal and pollen viability are also important factors to determine. While numerous studies have investigated interplant pollen flow and dispersal, few studies have examined empirical spatial patterns of pollen dispersal and viability. Long distance dispersal rates range from 0.6% at 366 m (Stringham and Downey 1982) to 0.0038% at 400 m (Scheffler *et al.* 1995) and as far as 2.5 km (Timmons *et al.* 1995) and 4 km (Thompson *et al.* 1999).

GREEN FLUORESCENT PROTEIN TRANSGENE TAGGING

An essential component of our gene flow research has included tagging fitness-enhancing genes, such as a *Bacillus thuringiensis* (Bt) cry1Ac gene that encodes an endotoxin protein for caterpillar control, with a transgene encoding the green fluorescent protein (GFP). GFP can be visualized in mature plant tissues macroscopically in real time by illuminating the plants in the dark with a long wave UV or blue lamp. Therefore, for the first time, transgene segregation and expression can be simultaneously assessed in all plant tissues in real time (Stewart 1996, 2001). GFP could also be used to mark seeds, flowers or other organs. For example, pollen could be painted with GFP to study gene flow and pollination behavior, especially long distance gene flow (Hudson *et al.* 2001). There is no fitness cost with expressing GFP in plants either cytoplasmically or targeted to the endoplasmic reticulum. However, ER targeting yields better expression in the field (Harper *et al.* 1999). Furthermore, we can assess the zygosity status of transgenic plants, no matter whether they are the initial recipient species or hybrids with wild relatives by simple observation or using more sophisticated fluorescence spectrometry (Halfhill *et al.* 2001a).

SAFE SPOT HYPOTHESIS

Our research has focused on two particular elements of *B. napus* risk assessment. The first component tests for the equal probability of gene flow among transgenic events of *B. napus* into *B. rapa*. If differential hybridization rates are found, these "genomic safe spots" may

be useful to curtail potentially disruptive gene flow. The second part is to examine the consequence of gene flow (discussed later). This safe spot hypothesis is borne from the genomic relationship between *B. napus* and *B. rapa*. These two species share one genome (A genome), but *B. napus* possesses the C genome as well. *Brassica rapa* possesses the A genome only. Therefore, it seems reasonable that if a transgene were located on the C genome, then perhaps gene flow to *B. rapa* would be attenuated for those events. There is approximately 50% chance that a random transgene insertion would occur in either the A or C genome in *B. napus*. So, our strategy has been to create a number of transgenic events that will probably consist of equal number of events each in A and C genomes. Subsequently, the transgenic *B. napus* events are handcrossed and crossed in the field to form hybrids and backcrossed hybrids (BC_1) with *B. rapa*. If there were safe spots in the C genome, then, *a priori*, we would expect significantly less BC_1 hybridization rate with half the events. *Brassica napus* and *B. rapa* F_1 hybrids would putatively consist of AAC genome status. In BC_1 and subsequent events, the genomic status will move toward AA as *B. napus* chromosomes are lost. It is important to note in all our experiments, we are selecting for positive transgenic events in the progeny after each crossing stage.

HYBRIDIZATION EXPERIMENTS

The interested reader can find more details about the following experiments elsewhere (Halfhill *et al.* 2001ab & 2002) Nine T_2 GFP/Bt events (GT 1-9) and three GFP events (GFP 1-3) were hand-crossed with three lines of *B. rapa* (from Irvine, California, USA, courtesy of Art Weiss; Milby, Quebec, Canada, and Waterville, Quebec, Canada. Parental canola lines were germinated on moist filter paper, and fluorescent individuals were selected for the hybridization experiment. The *B. rapa* lines were used as the pollen recipients. Both species were allowed to flower, and hand-crossing was performed by removing a canola flower and pollinating the *B. rapa* plants. The hand crossing continued as long as both plants continued to flower. All seeds were collected from the *B. rapa* parent, and were germinated on moist filter paper and screened by visual assay for GFP fluorescence. Plants that expressed GFP were backcrossed in the same fashion as above. The hybrids were used as pollen donors to produce backcrosses with their respective *B. rapa* accession.

All GT and GFP events generated F_1 transgenic hybrids through the hand-crossing experiment. Plants in a portion of the canola lines (GT 1, GT 5, GT 7, GT 8, GFP 1, and GFP 2) were homozygous for GFP, while the other lines were still segregating (1:2:1). The hybrids were characterized in the same manner as the parental canola events, and demonstrated identical GFP macroscopic fluorescence patterns as the parent canola events. The hybrid lines backcrossed with *B. rapa* resulted in a backcrossed generation (BC_1) for each line crossed. The frequency of transgenic hybrids (F_1) recovered from each cross ranged from 25% to 86%, and the percent of BC_1 plants recovered ranged from 15% to 34%. Due to differences in canola zygosity, the F_1 hybridization frequency varied to an expectedly high degree with the variety of homozygous and hemizygous canola parents. The BC_1 hybridization frequency was similar between all crossing types with the hemizygous F_1 hybrid donating pollen to the maternal weedy parent.

The genomic location of transgene integration into canola, whether on the A or C genome, has been suggested to play a role in the ability of transgenic events to pass fitness-enhancing transgenes to *B. rapa* (Metz *et al.* 1997). In this model, hybrid plants were putatively triploid (AAC), and the chromosomes on the C genome were unstably passed or lost during meiotic divisions. If the transgene is on the C genome, the gene could be lost to the

next generation leading to no transgenic backcrosses. By this model, the location of transgene insertion would result in different backcross frequencies between transgenic events, and certain lines would be "safer" in regards to gene flow and integration. This assumption has been challenged by the fact that the A and C genomes share a significant degree of homology, and recombination rates may be high and allow for increased rates of transgene integration into *B. rapa* (Tomiuk *et al.* 2000). The backcross frequencies presented in this study support the hypothesis that there are likely few "safe" locations in the canola genome with regards to gene flow. In this study, twelve independent canola events generated backcrosses at similar rates. This is in contrast to the findings of Metz *et al.* (1997), in which two independent herbicide tolerant canola events produced BC_1 plants at significantly different rates. The sample size of 12 transgenic events presented in this study is the largest analyzed, and adds data to an argument that has been historically theoretical. Further studies utilizing GFP canola and introgressed relatives in the *Brassiceae* family will expand the knowledge of gene flow in this agriculturally important group of crops and weeds.

CONSEQUENCE OF GENE FLOW

We assume that gene flow from *B. napus* into *R. raphanistrum* will be an extremely rare occurrence. But ecological history is replete with examples of rare but immensely important events. Transgenes have almost certainly been transferred from *B napus* to free-living *B. rapa* plants into the F_1, BC_1, BC_2 generations and beyond. This occurrence does not signify a risk in and of itself. Despite the arguments from certain environmental groups and dogmatic regulators, there is no such thing as zero tolerance when it comes to gene flow. Genes have been flowing back and forth between crops and weeds since crops have existed as such. Furthermore, there are much greater imminent risks in agricultural systems compared with gene flow and its results (e.g., pesticide residues, soil erosion, land use). So the appropriate question to be asked is not "will gene flow happen?" It most certainly will, especially from *B. napus* into *B. rapa*. But will transgenes persist in unintended hosts and will there be any detrimental consequences?

There are two different types of transgenes in commercial production in canola. The most prominent are herbicide tolerance genes to allow plants to survive otherwise lethal doses of glyphosate (Roundup), glufosinate (Liberty), and bromoxynil. The second class has oil-modification traits expressed in the seeds, and there are no obvious advantages of these traits residing in either volunteers or wild relatives. Most of the canola in Canada is planted to exploit herbicide tolerance (75% is transgenic HT, but 25% is nontransgenic imidazolinone-HT), and therefore many opportunities exist for interspecific gene flow resulting in HT hybrids. However, the biggest problem associated with herbicide tolerant canola is volunteerism in follow-up years that will require alternative and/or additional control measures (Hall *et al.* 2000, Légère *et al.* 2001, Beckie *et al.*, unpublished data). Volunteers can emerge from the seed bank over multiple years and serve as a potential pollen source for dispersal of transgenes to weedy relatives and canola crops that follow in rotation or are located in nearby fields. Herbicide tolerant wild relatives would be another variation of this problem, but less severe, because of significantly fewer numbers. Transgenic wild relatives would have no selective advantage outside of agriculture, and would therefore not constitute a special problem.

There are several transgenic traits that must be assessed for ecological consequence; traits that might confer some fitness advantage to a free-living host, or weed in an agricultural sense. The trait that we have used is insect resistance conferred by Bt cry1Ac, which kills lepidopteran herbivores on crucifers such as the diamondback moth (*Plutella xylostella*). These

plants have been field tested by commercial entities as well as academic labs. However, other insect resistance genes such as Bt Vip3A, *Photorhabdus luminescens* toxins, Cholesterol oxidase (CO) from *Streptomyces* culture filtrate, proteinase inhibitors, lectins and chitinases represent the future of insect resistance traits (reviewed by Stewart, 1999). Other transgenic phenotypes such as disease resistance and tolerance to various stresses such aluminium will also need special assessment.

Our approach for testing ecological performance is to perform competition experiments between crops and transgenic weedy hybrids (in progress for Bt canola and *B. rapa*). We are growing winter wheat that has interspecific competitors (WT or Bt *B. rapa* hybrids) under varying insect pressures. Therefore, if there is greater competition from transgenic wild relative plants in agriculture, we should be able to assay the increment of biomass or yield penalty of the crop within those particular plots. We have data from a greenhouse study with soybean that shows that there might be greater competitive ability from Bt *B.rapa* when cruciferous herbivores are present. Future research will expand from the current understanding that transgene flow will occur, to making predictions of the potential ecological and agricultural consequences of this outflow.

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Gene flow in forest trees: From empirical estimates to transgenic risk assessment

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ABSTRACT

The extent to which transgene flow from plantations can be effectively predicted, managed, and monitored, will be a critical biological and social factor influencing the adoption of transgenic plantations. Studies of historical and contemporary gene flow levels, via genetic structure surveys and parentage analyses, demonstrate that gene flow is generally extensive in both wind- and animal-pollinated forest tree species. Organelle genome studies have shown that pollen is by far the greatest source of gene dispersal, particularly for tree species with limited seed movement.

Marker studies, however, give little insight into the extent and consequences of gene flow in actual deployment scenarios. For example, despite the potential for extensive gene flow, there appears to be extremely low levels of "genetic pollution" of wild poplar stands by plantations of hybrids. Factors such as dilution by regional seed and pollen clouds, deleterious impacts of genotype backgrounds, engineered sterility genes, and the effects of transgenes on competitiveness in the wild, can greatly influence observed levels. Case-by-case analyses are required for useful predictions.

We introduce a spatial simulation model called STEVE that we have used to estimate the levels of future transgene flow from poplar plantations in the United States Pacific Northwest. It synthesizes data from a variety of ecological and genetic processes, and permits virtual experiments that investigate how diverse genetic, ecological, and management factors might influence the magnitude and variance of gene flow over a 50 to 100 year period. Similar approaches could be used elsewhere to help identify priority research needs, suggest means for mitigation where warranted, and to aid in design of monitoring programs for large-scale research and commercial applications.

INTRODUCTION

Any child who has played outdoors in the temperate zone knows that there is copious pollen production from most species of forest trees. They could probably also tell you, at least if asked, that wind can move pollen considerable distances. Naturalists observing the flights of pollinators have known for centuries that—intermixed with intensive local foraging—most pollinators also engage in long distance flights (Levin and Kerster 1974; Loveless and Hamrick 1984). It is also widely known that for some trees, seeds can move great distances due to wind, water, or animal vectors (Cain et al. 2000; Clark et al. 1998). For example, in western Oregon a "snow" of cottonwood seeds often forms near the rivers in June due to their abundant black cottonwood (*Populus trichocarpa*) populations.

The consequent "genetic unity" of widely dispersed tree populations is therefore no secret and no surprise. However, the magnitude and variation in gene flow is beginning to be well understood, primarily due to extensive molecular marker studies over the last few decades (e.g., Loveless and Hamrick 1984). Most of these studies have employed allozymes, which provide low cost means for studying patterns of genetic differentiation, and by inference, gene flow. However, recent studies, particularly using highly polymorphic DNA markers, have enabled more precise and more direct inferences, particularly of within-population mating patterns and immigration (reviewed below).

Until recently, interest in gene flow was largely restricted to a modest subset of evolutionary and population biologists, and to plant breeders, for whom it was important to interpret patterns of phylogeny, adaptation, and degree of contamination in seed production populations. With the advent of genetic engineering, and the biopolitical and legal controversies it has generated, gene flow has moved onto the public stage (e.g., Ellstrand 2001). "Genetic pollution" due to inadvertent mixing of seeds and pollen flow was the cause of "Tacogate," which caused Star-Link corn to be withdrawn from the market and the responsible company to pay compensatory damages in excess of US $100 million (O'Reilly 2001). The legal and financial repercussions of the case are likely to continue for several years. Likewise, the "genetic pollution" of rural, native stocks of corn in Mexico due to gene flow from genetically engineered corn has caused recent headlines (Quist and Chapela 2001), as have ongoing lawsuits brought by organic farmers due to "contamination" and consequent loss of organic certification as a result of gene flow from nearby genetically engineered crops (Saskatchewan Organic Directorate 2001). Because of the strong sentiments against genetic engineering by some sectors of the public, even small amounts of contamination appear capable of generating great concern (Thompson 2001). Labeling regulations for GMO crops that are under development in many countries appear to be requiring strict limits on the extent of transgene flow and crop mixing. The extent to which we understand, can predict, and can efficiently monitor gene flow may therefore be the most important biological determinant of whether transgenic crops are adopted and publicly accepted.

Because of the extensive gene flow possible from trees, and their very limited history of domestication, it has long been known that genes from intensively bred or engineered trees are highly likely to enter wild populations unless very special measures—such as use of sterile trees—are taken to avoid it (Strauss et al. 1995). In the southern hemisphere, where some of the most intensive plantation forestry in the world occurs, most of the trees grown are exotics, thus greatly reducing concerns over "pollution" of wild populations. However, in many of these places the planted trees have feral, "naturalized" forms, and in some cases these have given rise to invasive weeds that, because of their size, can have substantial impacts on local ecosystems (Richardson 1998). Thus, gene dispersal of exotics may present even more of an ecological hazard than that from native trees—where there is often a large "buffer" provided by wild populations, and where the trees already occupy defined niches (Strauss 1999). Although forestry crops are mostly not consumed as food, trees—even when they are farmed like row crops—tend to be considered symbols of durability and wildness by the public (Thompson 2001). And while it is true that people tend to worry more about the quality of their food than they do about the quality of their paper, it is also true that people are far more passionate about their trees than they are about soybean or broccoli crops. Perceived "genetic adulteration" via gene flow, even at a low level, may thus be a significant impediment to public acceptance of transgenic plantations.

Transgene flow, and means to predict its frequency and consequences, are therefore globally important issues for the future of plantation forestry. Unfortunately, we have a limited

knowledge base for risk assessments. There have been very few studies of gene flow from conventional plantations to wild populations. Due to the long lifespans and generation times of trees, predictions of impacts must consider very large temporal and spatial scales—and thus embrace high levels of uncertainty about future ecological conditions (James et al. 1998). New studies, and new tools, will be needed to make rational inferences about possible impacts of transgenic plantations.

We review in detail what is known about gene flow in forest trees, and then consider landscape simulation methods that might be employed for analyzing, predicting, and monitoring gene flow from tree plantations. As a case study, we focus on a recent analysis we undertook for poplars (genus *Populus*) in the Pacific Northwest United States. We suggest that by the use of simulation models to guide research—and to aid in prediction and monitoring during commercial development—society will be in a better position to make informed choices about the circumstances under which transgenic plantations might be acceptable.

DIRECT TRACKING OF SEED AND POLLEN DISPERSAL

Early studies of gene flow in forest trees focused on the physical dispersal potential of pollen and seeds. As early as 1919, pollen grains of *Pinus sylvestris* had been observed far beyond the range of the species (Lindgren et al. 1995). Later studies confirmed long-distance movements for both pollen and seed (Levin and Kerster 1974). However, tracking of propagules provides estimates of *potential* gene flow; *realized* gene flow also depends on fertilization and establishment success. Factors such as pollen competition, phenological synchrony, and seed predation can result in levels of gene flow far below those predicted by propagule movement alone (Adams 1992). Nonetheless, these studies demonstrated the astonishing mobility of pollen, and to a lesser extent, of light seeds (Di-Giovanni et al. 1996).

MEASURING HISTORICAL LEVELS OF GENE FLOW

The advent of genetic markers revolutionized methods of measuring gene flow. Over the last three decades, rates of migration have typically been inferred from the degree of genetic differentiation among populations as measured by the fixation index F_{ST} (Wright 1931, 1965), or its many extensions and analogs (e.g., G_{ST}, representing the interpopulation component of total gene diversity: Nei 1973). A common approach has been to estimate the fixation index from allele frequency data and then convert this to the mean number of migrants per generation ($N_e m$) using the relationship

$$N_e m \approx \frac{1 - F_{ST}}{4 F_{ST}} \text{ (Wright 1931).}$$

There are many methods for estimating the fixation index, each with strengths and weaknesses (see Cockerham and Weir 1993 and Neigel 1997 for discussion). However, the most common approach in forest trees has been to use allozyme allele frequencies to calculate G_{ST}, and derive $N_e m$ using Crow and Aoki's (1984) correction for the number of populations (or subpopulations) in the sample.

Other approaches for measuring historical gene flow include the "rare allele method" and coalescent methods. The rare allele method is based on the approximately negative linear relationship between the logarithm of $N_e m$ and the average frequency of private alleles in the demes of a genetically subdivided population (Slatkin 1985). The large sample sizes needed for

application of this method and the required availability of rare alleles have limited the use of this approach in tree population studies (see Govindaraju 1989 for comparison of $N_e m$ estimated through F_{ST} and through the rare allele method). Gene coalescence-based methods (reviewed in Neigel 1997) are based on genealogical relationships among alleles. They appear to be superior to other methods when variation within populations exceeds variation among populations (Beerli and Felsenstein 1999). They should therefore be highly suitable to forest trees. However, we are aware of no studies that have applied this method to any forest tree taxa.

All such methods of measuring gene flow are 'indirect', because they apply genetic models (and underlying assumptions) to infer long-term levels of gene flow (Neigel 1997; Sork et al. 1998). For example, the correlation between F_{ST} and $N_e m$ is often unreliable because the assumptions of the underlying population structure models are rarely met in real populations (e.g., equilibrium between genetic drift and migration, negligible selection and mutation, equal contributions of migrants from all populations: Bossart and Prowell 1998; Whitlock and McCauley 1999). Indirect measures reflect the complex interactions of all demographic parameters and evolutionary forces acting on a population, and the resulting gene flow estimates should be taken as long-term averages estimated over a large number of populations (Sork et al 1999).

Despite these shortcomings for estimating contemporary gene flow, indirect approaches have provided a number of valuable insights about historical forces shaping forest tree genetic structure. There have been a large number of studies of allozyme gene diversity, geographic structure, and gene flow among populations of forest trees (reviewed in Govindaraju 1989; El-Kassaby 1991; Hamrick et al. 1992; Müller-Starck et al. 1992; Hamrick and Nason 2000), and a few generalizations have emerged:

1. *Trees are characterized by higher genetic diversity and lower levels of differentiation compared to other plant groups (Hamrick et al. 1992).* The interpopulation component of total gene diversity (based on the fixation index) of woody species rarely exceeds 10-15% (Table 1). This low differentiation suggests extensive gene flow among tree populations. However, some authors have hypothesized that the observed patterns of genetic variation may also be due to the long pre-reproductive phase of most tree species mitigating founder effects, and trees' long generation span retarding differentiation through genetic drift (e.g., Kremer 1994; Austerliz et al. 2000).
2. *Wind-pollinated tree species typically have interpopulation differentiation levels of less than 10%, which translates to more than 2 successfully established migrants per population in each generation.* Hamrick et al. (1992) reported an average interpopulation differentiation of 8% for wind-pollinated trees based on 146 data sets. In species with large and continuous ranges, interpopulation differentiation is often below 3% (e.g., *Pinus sylvestris*, *Picea abies, Quercus petraea, Fagus sylvatica*: Müller-Starck et al. 1992; *Pinus ponderosa, P. contorta*: El-Kassaby 1991; *Quercus chrysolepis*: Montalvo et al. 1997). On the opposite extreme, species with small and fragmented populations can have interpopulation differentiation in the range of 15 to 30%. (e.g., *Pinus cembra, P. halepensis, P. nigra*, *Castanea sativa*: Müller-Starck et al. 1992; *Pinus torreyana, P. muricata*: Hamrick et al. 1992). This observation suggests that even though long-distance pollen dispersal is possible, its effect may be insufficient for genetic homogenization of spatially isolated populations.
3. *Outcrossed animal-pollinated tree species have a detectably (but not significantly) higher degree of interpopulation genetic differentiation compared to wind-pollinated trees.* The

average interpopulation differentiation for animal-pollinated trees with mixed mating systems was 10% based on 37 data sets (Hamrick et al. 1992). As in wind-pollinated species, spatial distribution seemed to be a good predictor of the degree of differentiation. Moran (1992) reviewed interpopulation differentiation in Australian eucalypts and cited values in the range of 8 to 12% for widespread species (e.g., *Eucalyptus saligna*, *E. cloeziana*, *E. delegatensis*); 30% for the highly disjunct *E. nitens* (widespread, but with a highly discontinuous distribution); and 61% for isolated populations of *E. caesia*. However, 67 scattered and putatively isolated low-density populations of the insect-pollinated, and presumably bird-dispersed, *Sorbus torminalis* had interpopulation differentiation of only 15% (Demesure et al. 2000), suggesting that gene flow rates can in some cases be high among spatially isolated populations (but see Austerlitz et al. 2000).

DIRECT METHODS OF MEASURING GENE FLOW THROUGH PARENTAGE ANALYSIS

Direct observations of gene dispersal obviate the need for tenuous assumptions about historical conditions. Instead, they provide short-term "immigration snapshots." Generally, direct methods require the genotyping of all potential parents in a population and estimation of the proportion of progeny that could *not* have been produced by within-population mating. One approach employs simple paternity exclusion, feasible where the maternal genotype can be readily determined (Smith and Adams 1983; Devlin and Ellstrand 1990). However, the low variability of allozyme markers greatly limits the ability to distinguish between local and immigrant genotypes (Adams 1992). To help overcome this problem, a number of methods employ maximum likelihood to either assign parentage (Meagher 1986; Adams et al. 1992; Smouse and Meagher 1994; Kaufman et al. 1998), or to estimate mating parameters that provide the best fit to observed progeny genotypes (Devlin et al. 1988; Roeder et al. 1989; Adams and Birkes 1991).

In the early 1990s, highly variable DNA-based markers began to become affordable for parentage analyses. The high genetic resolution provided by microsatellite and AFLP data allowed scientists to conduct paternity analyses based on genotypic exclusion with acceptable levels of discrimination (Dow and Ashley 1998; Streiff et al. 1999; Lian et al. 2001), as well as to apply maximum likelihood assignments with higher confidence (Gerber et al. 2000; Kameyama et al. 2000).

Seed orchards. There have been a number of studies of genetic contamination of forestry "seed orchards." Seed orchards are plantations in which selected genotypes are placed to allow cross-pollination for production of seeds to be used for reforestation. Most of these orchards are within the range of native or planted populations, and distinct orchard blocks (that service distinct ecogeographic regions) are in many cases planted in proximity for management efficiency. Thus, there are often high levels of unwanted pollen immigration into the orchard blocks. This can result in substantial loss of genetic gain compared to expectations based on selection theory, and can compromise adaptation of seed orchard crops to their intended plantation environments (Adams and Burczyk 2000).

The tracking of rare allozyme alleles has been used to measure orchard contamination (Friedman and Adams 1982), and to study mating patterns within seed orchards (Prat 1995). However, such alleles are often difficult to identify, and force inferences to be based on a very limited number of genotypes. Instead, pollen contamination of seed orchards is usually estimated via simple paternity exclusion, adjusting the observed proportion of immigrants by the probability that an immigrant gamete will be distinguishable from the potential orchard

gamete pool (Adams and Burczyk 2000). Other statistical procedures have also been implemented (El-Kassaby and Ritland 1986; Plomion et al. 2001). These studies have revealed great variation in pollen contamination, even when the same analytical approaches and tree species are considered (Table 2; Adams and Burczyk 2000). Nonetheless, it is clear that pollen contamination is often very large, commonly exceeding 40%, even when the closest stands of the same species are several hundred meters away.

Less is known about pollen contamination in seed orchards of animal-pollinated tree species such as eucalypts compared to that of the wind-pollinated conifers. However, these studies also suggest that contamination can be considerable, implying that pollinators can travel long distances from outside orchards (Campinhos et al. 1998; Junghans et al. 1998)—as well as move pollen extensively within orchards (Burczyk, unpublished data).

Commercial plantations. We know of very few studies of the effects of gene flow from forest plantations on wild populations. The best-studied cases are from interspecific hybrids in the genus *Populus*, which are often planted in proximity to wild populations. In Europe, most of the planted cottonwood hybrids include the native *Populus nigra* as a parent. These hybrids are interfertile with wild populations of *Populus nigra*, whose populations are greatly reduced in extent due to destruction of riparian habitat by farming and human habitations. Nonetheless, several studies have reported extremely low levels of gene flow to wild *P. nigra* stands (Benetka *et* al*.* 1999; Legionnet and Lefevre 1996). Likewise, in the Pacific Northwest United States gene flow from hybrid poplar plantations into wild black cottonwood populations was extremely low, despite the presence of large male plantations in close proximity to native female trees (DiFazio 2002).

Wild populations. Due to restrictions on marker resolution and the high cost of genotyping, spatially distinct forest stands are usually chosen for study, and parentage analysis then applied to a sample of progeny. Typically, seeds are collected from mother trees of known genotype, and paternity estimated by comparing inferred paternal genotypes with those of all potential fathers in the analyzed population (Schnabel and Hamrick 1995; Kaufman et al. 1998; Dow and Ashley 1998; Streiff et al. 1999; Schuster and Mitton 2000). Some researchers have also sampled seedlings and/or saplings, attempting to estimate both pollen- and seed-mediated gene flow (Dow and Ashley 1996; Isagi et al 2000; Konuma et al. 2000). However, such analyses require considerably more exclusion power than paternity analysis (Marshall et al. 1998).

Results from paternity analyses in wind-pollinated species generally agree with predictions from studies of genetic structure (Table 3). In most cases, the frequency of immigrant pollinations was over 30% (e.g., 31% in *Pinus densiflora*, Lian et al. 2001; 57% in *Quercus macrocarpa*, Dow and Ashley 1998; 65% in *Q. robur* and 69% in *Q. petraea,* Streiff et al. 1999), but appeared rather low (6.5%) in a spatially isolated population of *Pinus flexilis* (Schuster and Mitton 2000). Remarkably, Kaufman et al. (1998) reported extensive pollen-mediated gene flow (a minimum of 37%) in a population of the tropical pioneer *Cecropia obtusifolia*, even though the closest population of the same species was at least 1 km away. Kaufman et al. (1998) suggested that successful pollen traveled as far as 10 km. Similarly, Leonardi, et al. (unpublished) observed extensive pollen immigration into stands of *Populus trichocarpa* that were isolated by up to 16 km from the nearest ungenotyped pollen source (Table 4).

As with indirect methods, gene flow estimates based on parentage analysis in animal-pollinated tree species were somewhat lower, but generally similar to immigration rates in wind-pollinated trees (e.g., 17 to 30% in *Gleditsia triacanthos*, Schnabel and Hamrick 1995; 20 to 30% in *Rhododendron metternichii,* Kameyama et al. 2000; 74% in *Magnolia obovata*, Isagi et

al. 2000; and 21 to 69% in *Neobalanocarpus heimii*, Konuma et al. 2000). These results support the notion that animals can be effective agents of long-distance pollen and seed dispersal. However, there is also considerable variance in pollen immigration between species, even when isolation distances are similar (Stacy et al. 1996), cautioning against broad generalizations.

Although paternity analysis based on highly variable markers appears to be the most effective current method for measuring gene dispersal in ecological time, estimates can be greatly affected by the presence of null alleles, and the misgenotyping of complex microsatellite and AFLP phenotypes. Both of these types of errors will cause overestimates of gene flow (Marshall et al. 1998). Despite high reported exclusion probabilities, it is therefore important to treat many of the published estimates with caution; their accuracy will improve over the next several years as applications of molecular technology for gene flow studies mature.

THE PROMISE OF ORGANELLE DNA MARKERS

One of the limitations of parentage analysis using nuclear loci is that it is often difficult to distinguish the contributions from the different agents of gene flow. The predominant uniparental inheritance of organelle DNA (Corriveau and Coleman 1988; Birky 1995), however, provides a means to differentiate pollen- from seed-mediated migration. Chloroplasts are inherited maternally in most angiosperms, and paternally inherited in most gymnosperms. Plant mitochondria are generally passed on maternally, except for some gymnosperms (see Wagner 1992 for a review of organelle genome inheritance in trees). The high amounts of length variability detected in some regions of the chloroplast genome (reviewed in Provan et al. 2001) facilitate its use for gene flow studies. Chloroplast microsatellites can increase efficiency of paternity analysis (Ziegenhagen et al. 1998), and aid the estimation of pollen contamination in conifer seed orchards (Stoehr et al. 1998, Plomion et al. 2001).

Applied in combination with nuclear loci, plastid DNA markers are also valuable for inferring historical levels of gene flow. For example, Ennos (1994) compared F_{ST} values calculated over nuclear and organelle DNA markers and concluded that pollen-mediated gene flow in wind-pollinated species may exceed seed-mediated gene flow by a factor of 18 to 68 (in the light-seeded pines), and up to 196-fold in the acorn-bearing oaks. Similar studies have been conducted in other tree species (El Mousadik and Petit 1996; Latta et al. 1998; Oddou-Muratorio et al. 2001). Analysis of the spatial structure of cpDNA enabled estimates of postglacial seed dispersal rates, and recolonization routes, of European oaks (Petit et al. 1997). However, because of the lack of independence of polymorphic loci in organelle genomes, differences in mutation rates among genomes, and difficulties in assuming homology among repeat "alleles," organelle-based inferences should be treated cautiously (cf. Hong et al. 1993; Strauss et al. 1993).

SPATIAL SIMULATION MODELING

Extrapolation of short-term or historical gene flow observations to spatial and temporal scales that are relevant for management and ecological policy remains a major challenge (Levin 1992; Turner et al. 2001). Because of the time and expense required for a typical parentage analysis study, only a limited number of populations and years can be examined (Ouborg et al. 1999; Cain et al. 2000). However, ecologically significant levels of establishment may occur only once or twice per generation (i.e., on a decadal scale) (James et al. 1998), and in particular habitats. An emerging solution is the use of spatial simulation models to extrapolate

results of short-term gene flow studies with knowledge of ecological processes (Dunning et al. 1995; King 1991). They provide an extensible framework for integrating data from disparate demographic and genetic field studies with landscape-scale analyses of ecosystem dynamics (Sork et al. 1998; Higgins et al. 2000). In addition, such models allow 'virtual experiments' through sensitivity analyses in which selected components of the system are manipulated to determine their importance in determining long-term outcomes (Turner et al. 2001).

CASE STUDY: GENE FLOW IN POPLAR

We analyzed gene flow in wild black cottonwood populations, and from hybrid poplar plantations, in the northwestern United States. The primary objective of these studies was to provide data for assessing the extent of transgene dispersal that is likely to occur should transgenic hybrid poplars be cultivated in the region. We studied gene flow using parentage analysis in three wild populations with contrasting ecological characteristics (Leonardi et al., unpublished), and gathered data on seedling establishment and survival in experimental plots and in the wild (DiFazio et al. 1999). We also inferred landscape-level spatial and temporal dynamics of black cottonwood establishment from a chronosequence of GIS layers encompassing some of the same populations included in the field studies (Figure 1).

The model, called STEVE (Simulation of Transgene Effects in a Variable Environment—but also named to reflect that four Steves contributed to its development!), provides a spatially explicit representation of gene flow (DiFazio 2002; Figure 2). It operates on a landscape grid (23 km x 37 km, 100 m^2 cells) containing information about elevation, habitat type, and poplar populations. The simulation has an annual time step, with modules to simulate creation and conversion of poplar patches, growth, reproduction, dispersal, and competition within poplar cohorts. The simulations track two genotypes, transgenic and conventional. Transgenic trees originate in plantations and may spread to the wild through pollen, seed, and/or vegetative propagules. The relative amounts of propagules produced in each location are proportional to basal area (i.e., trunk cross-sectional area) of each genotype, modulated by a fecundity factor.

We structured and parameterized the model based on results of our field studies of gene flow. They indicated that long-distance dispersal is considerable for *Populus* (Table 4; Leonardi et al., unpublished), with the tail of the distribution quite 'fat' (*sensu* Kot et al. 1996). We therefore chose to model gene dispersal as a two-stage process, with local dispersal modeled explicitly by a negative exponential distribution, and long-distance dispersal modeled as if a portion of the pollen and seeds were panmictic at the landscape scale. This is analogous to a mixed model approach (Clark et al. 1996). The biological basis for this approach is that locally dispersed pollen and seed is subject to local air flows and eddies, and follows predictable patterns of dispersal in which probability of deposition declines exponentially with distance from the source (e.g., Di-Giovanni et al. 1996). However, a portion of the pollen can be caught in updrafts and escape from local air flows, potentially traveling great distances (e.g., Lanner 1965). Seed dispersal was modeled in the same way, though based on more limited field studies of movement, and assumed much less local and long-distance movement than for pollen. *Populus* seeds are very light and contain cotton appendages that facilitate wind and water dispersal; therefore, a portion of the seeds is expected to attain stochastic long-distance dispersal (Wright 1952).

This method of modeling pollen and seed dispersal had major implications for gene flow from transgenic plantations. Modeled gene flow was highly sensitive to changes in the proportion of pollen and seed dispersed long-distances (Figures 3A and B), but relatively insensitive to the slope of local dispersal curves (Figures 3C and D). This was primarily because

poplars require very intense disturbance, abundant moisture, and freedom from most competition by other plants for successful establishment. These conditions are rarely met in space and time. The majority of establishment sites therefore occurred beyond the local seed and pollen shadows of the plantations (Figure 3E). Also, because long-distance dispersal was insensitive to wind in this model (pollen and seed were assumed to be panmictic at the landscape scale), wind speed had no detectable effect on gene flow from plantations (Figure 3F). Long-distance dispersal ensured that a proportion of plantation-derived propagules would encounter stochastic establishment sites regardless of distance from plantations, which explains why this portion of the dispersal function was overwhelmingly important in determining gene flow. One implication of this result is that future research on gene flow in *Populus* would benefit most from better definition of the dynamics of long-distance dispersal, rather from studies of local pollen movement and mating between trees within stands.

Sensitivity analyses allowed us to study the consequences for gene flow of many ecological conditions and transgenic deployment scenarios over a 50 to 100 year time frame. For example, we studied (DiFazio 2002) the consequences of:

1. Transgenes that imparted herbicide resistance with respect to various scenarios of herbicide use and disturbance on the landscape
2. Transgenic trees with insect resistance, with varying levels of insect attack in wild populations
3. Reductions in fertility due to transgenic or other sources, and implications of various levels of efficiency and stability
4. The effects of transgenes with positive or negative effects under natural selection
5. Effects of transgenic vs. non-transgenic plantation area, plantation gender, and rotation length (time to harvest)

Most of these simulations also included stochastic variation, so that natural environmental variances, and uncertainty in parameter estimates, could be reflected in model outputs. Ideally, the model structure and parameters would be continually revised based on research results, and by results of monitoring programs during commercial deployment. The most important contribution of spatial simulation models such as STEVE is that they provide a comprehensive, explicit logical framework for thinking about the long-term consequences of different options for deploying transgenic, as well as conventionally bred, plants. It therefore helps to reduce the immense ecological complexity of tree gene flow to a set of specific, testable predictions that can guide further research, and inform business plans, regulatory decisions, and ultimately public views about transgenic technology in plantation forestry.

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Table 1. Neighborhood sizes and per-generation migration events estimated by two indirect methods for some tree genera.

Genus	G_{ST}	N_em	N_em*	N_a
Abies	0.063	3.72	8.71	260
Picea	0.055	4.30	-	133-260
Pseudotsuga	0.074	3.13	-	-
Pinus	0.065	3.60	1.51-21.83	158-534
Quercus	0.107	2.09	2.24-6.74	-
Populus	0.041	5.85	-	-
Eucalyptus	0.169	1.23	0.76-6.51	-

G_{ST} - averages from Hamrick et al. (1992)
N_em - estimated based on the G_{ST} values
N_em*- estimated for some species using the method of Slatkin (1985); from Govindaraju (1989)
N_a - neighborhood area in m² (area from which the parents of some central individual may be treated as randomly drawn) estimated for some species; from Govindaraju (1988)

Table 2. Estimated pollen contamination in clonal seed orchards of forest trees.

Tree species	S	Di	b	m
Picea abies	13.2	none	-	0.55-0.81 [1]
		-	0.10-0.17	
Picea glauca	-	1000	-	0.01
Pseudotsuga menziesii	1.8-3.3	none	-	0.29-0.91
	20	500	-	0.11
Pinus sylvestris	22.9	2000	-	0.48
	3	1000	0.15	-
	6	500	0.38	-
	12.5	>100	-	0.72
Pinus taeda	2	100	-	0.36
Pinus maritima	-	none	0.36 [2]	-
Eucalyptus grandis x *E. urophylla*	7.4	400	0.14 [3]	-
	8	800	0.03 [4]	-

S - area in which all potential parents have been genotyped (ha)

D_i - distance to the nearest population or tree of the same species (m)

b - observed proportion of immigrant pollen gametes

m - pollen contamination adjusted for the probability to distinguish local and migrant gametes

References from Adams and Burczyk 2000, unless otherwise indicated:

[1] Pakkanen et al. 2000

[2] Plomion et al. 2001

[3] Campinhos et al. 1998

[4] Junghans et al. 1998

Table 3. Mean pollination distance and gene flow estimates from parentage analyses in wind- and animal-pollinated trees.

Tree species	S	D_i	d_{wp}	d_{op}	m	Reference
A. Wind-pollinated						
Pinus flexilis	-	>2000	133-140	155-265	0.07	Schuster and Mitton 2000
Pinus densiflora	9.1	N/A	68	-	0.31	Lian et al. 2001
Quercus macrocarpa	5	>100	75	-	0.57	Dow and Ashley 1998
Quercus robur	5.8	>100	22-58	333	0.65	Streiff et al. 1999
Quercus petraea	5.8	>100	18-65	287	0.69	Streiff et al. 1999
Cecropia obtusifolia	8.6	>1000	-	-	0.37	Kaufman et al. 1998
B. Animal-pollinated						
Gleditsia triacanthos	3	>85	-	-	0.17-0.30	Schnabel and Hamrick 1995
Ficus (from 3 diff. species)	-	>1000	-	-	>0.90	from Hamrick and Nason 2000
Rhododendron metternichii	1	>50	-	-	0.20-0.30	Kameyama et al. 2000
Magnolia obovata	69	N/A	131	-	0.74*	Isagi et al. 2000
Neobalanocarpus heimii	42	None	188-196	-	0.21-0.69*	Konuma et al. 2000

S - area in which all potential parents have been genotyped (ha)
D_i - distance to the nearest population of the same species (m)
d_{wp} - average pollination distance within the reference stand (m)
d_{op} - mean pollination distance from assumed dispersal curve (m)
m - proportion of offspring with immigrant paternal gametes
* Offspring having one or both parents located outside the reference stand

Table 4. Population and gene flow statistics from three microsatellite-based studies of pollen dispersal in Oregon, USA (Leonardi, et al., unpublished).

Site	r[1]	Mothers[2]	Fathers[3]	N[4]	D_i *	d_{wp} *	d_{op}*	P[5]	M[6]	G[7]
Willamette	0.25	5	221	235	100-300	138	809	103	32	43
Luckiamute	1	5	57	423	1000-1100	128	-	98	5	76
Vinson	10	28	54	849	2680-9760	1093	4157	355	29	58

[1] Radius of sampled area (km)
[2] Number of trees from which seeds were collected
[3] Number of reproductively mature male trees within sampled area
[4] Number of progeny genotyped
[5] Number of seeds for which a single putative father was compatible within the sampled area
[6] Number of seeds for which multiple putative fathers were compatible within the sampled area.
[7] Percentage of seeds for which no compatible fathers were identified within the sampled area
*Defined in Table 3

Figure 1. GIS representation of modeled area in northwestern Oregon (37 km x 23 km: 845 km^2). White shows main channels of the Columbia River, black, areas of hybrid cottonwood plantations, dark grey, wild black cottonwood stands, and light grey is non-poplar land (mostly farms, coniferous uplands, and wetlands; DiFazio et al. 2002).

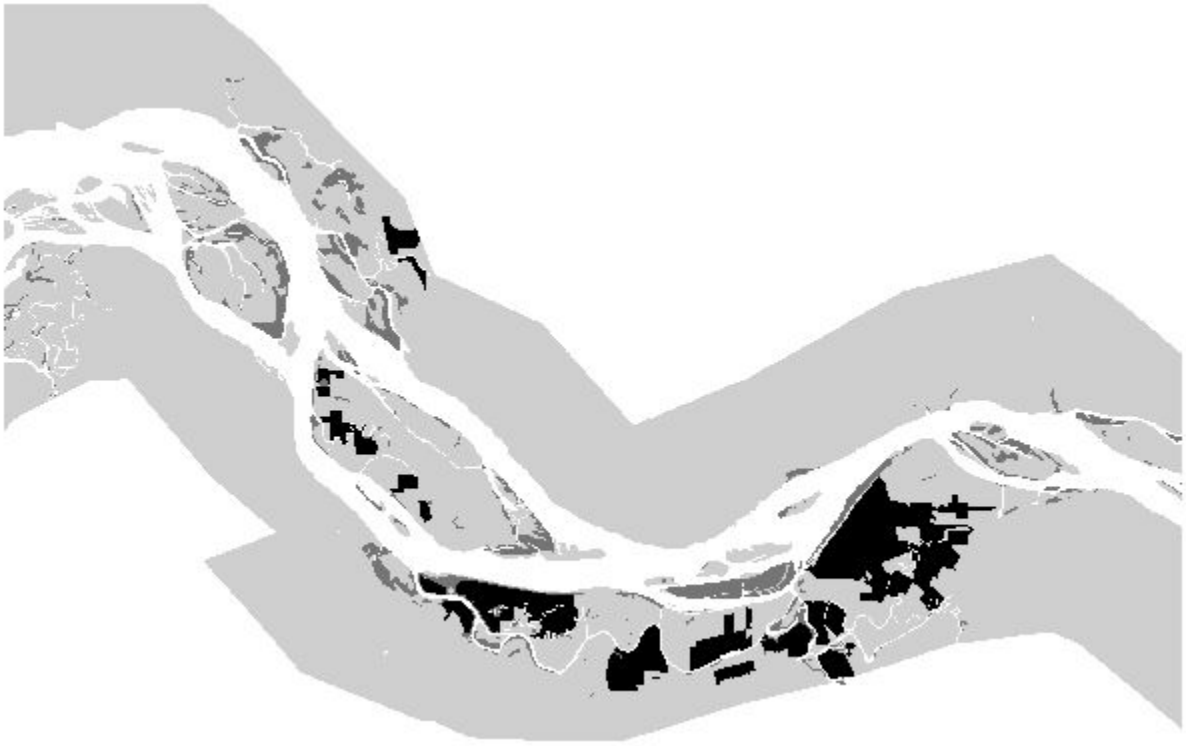


Figure 2. The STEVE model. Model begins with preprocessing of GIS layers representing initial simulation conditions. Data are stored in a spatial database containing information about elevation, cover type, poplar populations, plantations, and agricultural fields. Simulation begins with management activities such as plantation harvesting and herbicide spraying. Poplar establishment and mortality is simulated in the disturbance function. Seed, pollen, and vegetative propagules are produced proportional to basal area of each genotype, followed by dispersal, establishment, growth and mortality. Outputs are text files and spatial data layers**.**

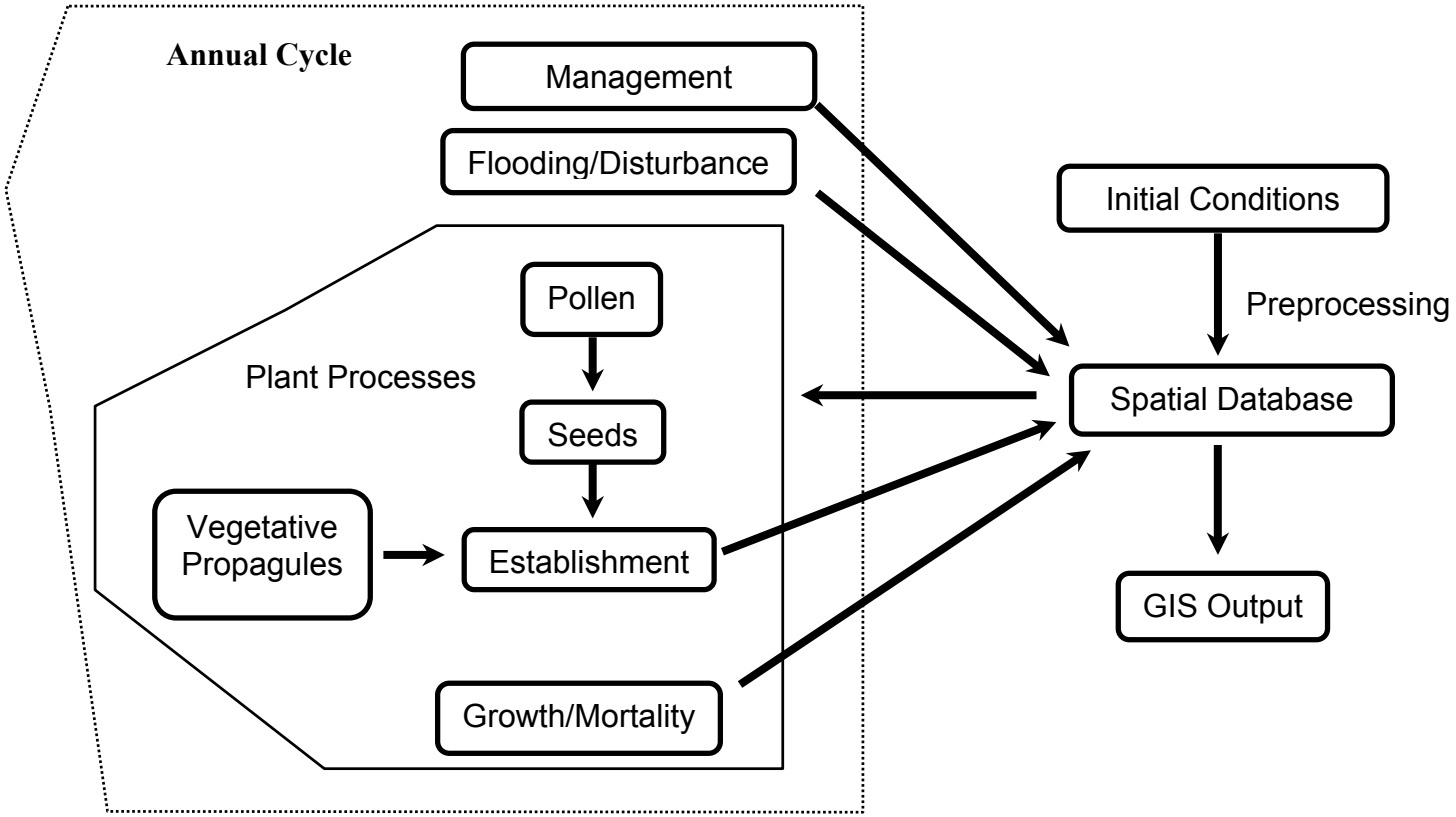
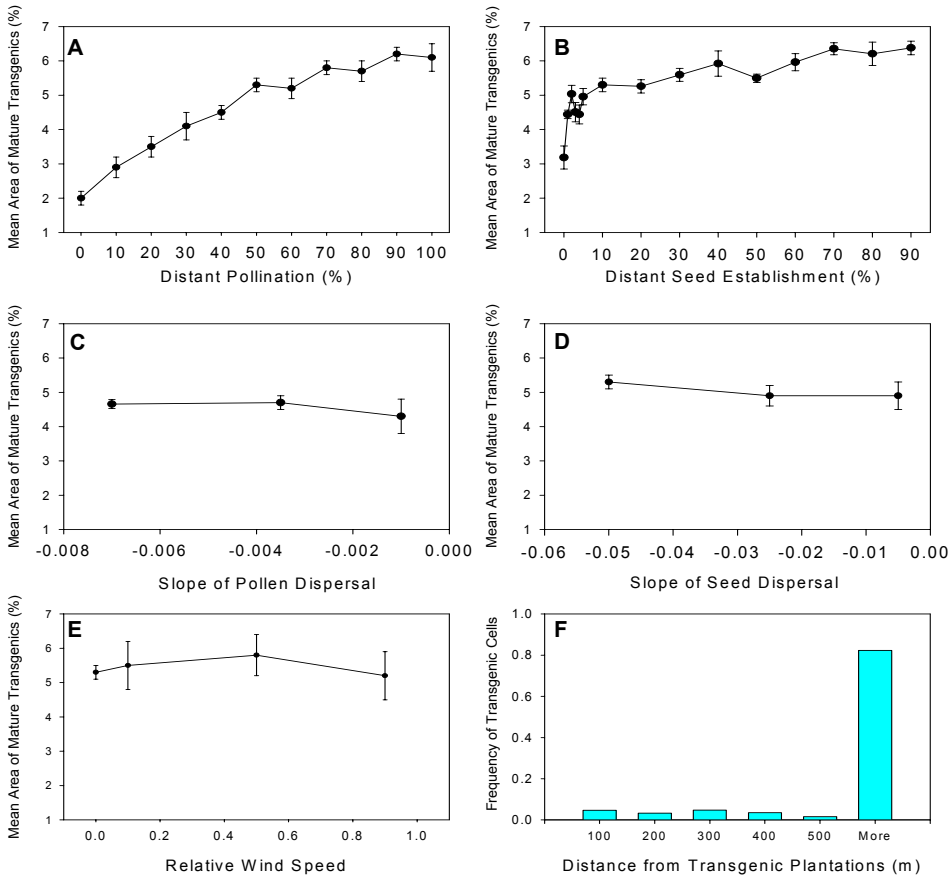


Figure 3. Effects of dispersal and wind on simulated gene flow. Error bars are 1 standard error from 10 repetitions with each set of parameter values.
A. Effects of distant pollination on transgene flow. Distant pollination is the proportion of seeds that are fathered by trees that do not occur in the local population. This parameter has a strong effect on transgene flow, reflecting the importance of long distance pollen dispersal.
B. Effects of distant seed establishment on transgene flow. Distant seed establishment had minor effects except at very low levels.
C and **D.** Effects of varying the slope of the negative exponential distributions depicting local pollen and seed dispersal, respectively. Varying this slope had little effect on gene flow.
E. Effect of relative wind speed, with wind direction set at 90 degrees.
F. Distance of transgenic cohorts from mature transgenic plantations. The local pollen and seed shadows end at 440 m and 220 m respectively.



U.S. Regulatory Oversight for the Safe Development and Commercialization of Plant Biotechnology

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Biotechnology is an enabling technology with broad application to many different areas of industry and commerce. Broadly defined, biotechnology "includes any technique that uses living organisms (or parts of organisms) to make or modify products to improve plants or animals or to develop microorganisms for specific use." Products of biotechnology have entered mainstream American commerce in the human health care arena (pharmaceuticals) and now in agriculture. For American agriculture, biotechnology has the potential to increase productivity, enhance the environment, improve food safety and quality, and bolster American agricultural competitiveness. Its incorporation into agriculture and its ability to have a significant beneficial impact on agriculture in the U.S. have been aided by an oversight structure put in place to assure safety and facilitate transfer of the technology.

BASIC STRUCTURE: Roles and Jurisdictional Responsibilities of the 3 Federal Agencies Providing Regulatory Oversight for Products of Biotechnology

A. The U.S. Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) exercises oversight for the importation, interstate movement, and the field testing of certain genetically engineered organisms, primarily new plant varieties and plant products, and assures that these new varieties are as safe to use in agriculture as traditional varieties.

1. The APHIS rules for regulating genetically modified organisms (GMOs) engineered using components from plant pathogenic material are found at 7 CFR Part 340 and were issued under authority of the Federal Plant Pest Act of 1957 (7 U.S.C. 150aa-150jj) and the Plant Quarantine Act of 1912 (7 U.S.C. 151-167). These rules allow the agency to regulate a new plant variety if there is reason to believe the plant may cause injury disease or damage to other plants.

2. USDA's regulatory role over agricultural biotechnology products starts at an early stage in the R&D process. Our role allows us to determine during early development of a new variety that it may be safely used in agriculture, so that it can enter into further agronomic testing or breeding without unnecessary scrutiny from us, while additional reviews regarding food or pesticidal safety are completed in consultation with the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA).

B. The FDA has authority under the Federal Food, Drug, and Cosmetic Act (FFDCA; 21 U.S.C. 321 et seq.) to ensure that genetically engineered food (except meat and poultry) is not adulterated or misbranded.

1. The Agency published on May 29, 1992, a policy statement concerning "Foods Developed from New Plant Varieties (57 FR 22984-23005) which advises developers of new plant varieties under what circumstances to consult with FDA when plant breeding may raise safety concerns based on the host plant, donor organisms, and new substances that have been introduced into the food.

2. FDA does not consider that recombinant derived products require a special or unique review procedure based on process. FDA notes in its policy statement that if the genetic modification does not result in the introduction of toxicants to the food supply, does not alter the level of toxicants already present, does not alter the composition of bioavailability of nutrients in new varieties, and does not result in the transfer of allergenic compounds to new varieties, then the new variety can be considered equivalent to traditional varieties.

3. Approved products that may have altered nutritional composition or that contain compounds that are potentially allergenic or require new practices for their preparation would need to be labeled with that information. This policy is not new regulation or deregulation, but rather how FDA has traditionally regulated such foods.

C. The EPA, under authority of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA 7 U.S.C. 136-136y and the FFDCA (21 U.S.C. 346a and 371), assures the safe use in the environment of novel plant-pesticidal substances introduced into plants or new uses of herbicides in conjunction with newly developed plant varieties. This includes registration and labeling of new pesticidal substances and setting out conditions for their use. Under authority of the FFDCA the EPA may also exempt from tolerance-setting procedures pesticidal substances which the Agency believes presents no risk to public health.

EPA finalized (59 FR) the use of substances in crop plants with traits introduced specifically for pest resistance (called plant incorporated protectants (PIPs) from outside the range of sexual compatibility of that plant prior to conducting any field test greater than 10 acres.

MAJOR FEATURES OF THE U.S. REGULATORY PROGRAM FOR PRODUCTS OF BIOTECHNOLOGY: FOSTERING THE SAFE DEVELOPMENT OF BIOTECHNOLOGY

A. U.S. regulatory oversight is a risk-based system that uses existing statutory authorities.

1. The types of risks that may be posed by new varieties are the same in kind as those that may be posed by other varieties (e.g., increased weediness of a new variety or the presence of increased levels of toxic substances that are known and inherent parts of the foods we consume).

2. Extensive scientific evaluations by USDA, FDA, and EPA have identified no safety problems unique to the biotechnological development process. Note that this is the first time new plant varieties have been subject to Federal oversight; plants developed through classical techniques are not subject to such oversight.

B. The regulatory procedures have been adjusted based on our experience and reflecting new knowledge based on advancements in the technology. USDA regulations addressing biotechnology products, which originally went into effect in 1987, have been significantly amended three times, in 1989, 1993, and 1997, so as to keep pace with this accelerating growth of knowledge.

1. Major modifications have been made to our regulations on March 31, 1993 (58 FR 17044-17059) established petition procedures to confer nonregulated status to those engineered crops determined to be as safe to grow as crops produced through classical means and allowing field testing to occur for six listed crops to occur through a simplified process referred to as "notification".

2. The May 2, 1997, amendments (62 FR 23945-23958) further expand notification beyond the six listed crops as well as simplifying the petition process for similar classes of products.

C. Our USDA regulatory procedures are designed to be as flexible as possible focusing on end results via performance standards and self-certification rather than specifying design requirements. We have built in requirements that seek to make our system as transparent as possible.

STATUS OF RESEARCH, DEVELOPMENT, INTEGRATION OF NEW AGRICULTURAL BIOTECHNOLOGY PRODUCTS IN THE UNITED STATES

A. There has been a rapid growth of the number and kinds of organisms tested under APHIS oversight.

1. From 1987 to present, we have evaluated in excess of 8,000 field trials at more than 24,000 field sites in most of 50 States and territories involving different plant species (as diverse as sugar cane, poplar trees, turfgrass, rice, and sunflower).

2. Derivatives of all the major U.S. crops (corn, soybeans, potatoes, tomatoes, cotton, and tobacco) have had a large number of trials; only wheat has lagged behind, for technical reasons. Ability to conduct these trials has been facilitated by the adoption of notification procedures.

3. The most common traits engineered into crops are herbicide tolerance, insect resistance, disease resistance, and product quality.

One of the most commonly perceived risks associated with GM crops concerns the possibility that transgenes will escape from confines of agriculture with serious environmental consequences. Indeed, the National Research Council (USA) in its landmark 1989 report considered that “the potential enhanced weediness is the major environmental risk perceived for the introduction of GM plants”.

The two questions often asked:

* Will engineered crop plants become invasive?

* Will the transfer of genes from transgenic crops to their wild relatives increase their "invasiveness"?

There has been a great deal of discussion by ecologists and population geneticists on the above two issues.

WILL ENGINEERED CROP PLANTS BECOME INVASIVE?

To address this issue we will first look at the invasive potential of the recipient plant and then discuss the role of new traits (phenotypes).

WHAT IS THE INVASIVE POTENTIAL OF THE PLANTS BEING ENGINEERED?

The likelihood that GE plants will become invasive and constitute serious weed problems is often considered remote (Royal Society of Canada, 2001, http://www.rsc.ca/foodbiotechnology/indexEN.html). This is because most of today"s major crop species (e.g. soybean, rice, wheat, beans) have been subjected to intense artificial selection over centuries for traits (phenotypes) with low survival value under most natural conditions. Traits such as non-shattering of grain in cereals, lack of seed dormancy, and requirement of high fertilizer inputs, restrict the ability of most domesticated species to thrive outside the agroecosystem. Although crops are grown over vast areas of the world today and they are generally alien introduction in those environments, there are relatively few cases in which they persist without deliberate human intervention for more than a few growing seasons. Such volunteer plants are usually confined to agroecosytems and rarely if ever invade undisturbed natural communities. Domesticated crop plants are not represented among the world's serious plant invaders. This is because persistence in wild communities results from the combined effects of many genes working in cooperation to produce a functioning phenotype adapted to local ecological conditions. Therefore, in most cases insertion of highly specific transgenes into a crop species possessing a plethora of domesticated traits is unlikely to alter its ecology so that it becomes converted into an aggressive invading species. Such targeted genetic modifications are unlikely to nullify many generations of human selection involving countless loci.

Most engineered plants that have been commercialized to date (beet, corn, cotton, flax, melon, papaya, potato, raddicho, rice, soybean, squash, and tomato) have few weedy characteristics and would not be considered invasive plants by any reasonable standard. We realize that the certain plants that have been commercialized, e.g., canola, have been domesticated relatively recently as compared to maize and possess two fitness traits of its parent plant: weak seed dormancy and some seed shattering. In our opinion, most plants that are likely to be commercialized in the next decade are likely to be major crop plants because of the cost of developing the plants, intellectual properties right issues, and the cost of obtaining the numerous international reviews necessary for these products to enter commercial production will require support from companies. Genetically engineered turfgrasses and trees are two nonfood plants that might be commercialized in the next 5 years.

HOW DID THE IDEA THAT GMOs MIGHT BE INVASIVE PLANTS ORIGINATE?

In the absence of experience with GMOs, some scientists in late 1980s argued that experience with "exotic" (i.e., nonindigenous) species might help provide guidance (29,32). One of the most extensive discussions on this issue was in the Office of Technology Assessment report "Harmful Non-indigenous Species (NIS) in the United States" which devoted a chapter to this issue. However, the comparison of GMOs to NIS itself provoked a debate. The approach was criticized because GMOs introduced in to the same environment as the parent non-engineered organism differ by only a few genes. Effects of the gene changes in GMOs are well characterized, allowing better prediction of how they affect the organism's ecology. In addition, most NIS differ from indigenous organisms by man genes that generally are not well characterized.

Thus, many scientists and the Council for Agricultural Science and Technology (CAST) have suggested that more appropriate comparison GMOs and invasive plants is that GMOs should be compared to traditionally improved counterparts that have one or a few new traits introduced.

WHAT IS THE SCIENTIFIC BASIS OF USDA'S BIOTECHNOLOGY EVALUATION (BE) COMPARING TRADITIONALLY BRED PLANTS TO ENGINEERED PLANTS?

In our risk assessment processes, we focus on the key concept of familiarity. In 1989 National Research Council, an arm of the U.S. National Academy of Sciences, published its report entitled, "Field Testing Genetically Modified Organisms." One conclusion reached was that the use of plants modified by classical breeding techniques for field testing has a history of safe use. A similar conclusion was reached by Ecology Society of America in their 1989 paper authored by Tiedje et al. That crops modified by engineering should pose risks no different from those modified by classical genetic methods for **similar traits**. Thus, if the genetically modified plant is phenotypically similar to a plant that has been (or could be) bred by traditional breeding techniques this parallel association is called familiarity. The concept of familiarity allows regulators to draw on past experience with introduction of modified plants into the environment. Familiar does not necessarily mean safe. It does mean that the level of risks associated with the introduction of new pest resistance genes into plants by classical methods and the evaluation of new cultivars by national variety registration agencies, has made the introduction into the environment of these types of modified plants of negligible risk. Other important familiarity factors are whether the plant is new to the particular environment where it is intended to grow, the nature of the trait (gene), and that the evaluations should be made on a case-by-case basis. One important point of this conclusion is that it is more important to evaluate the phenotype produced than the process/technique used to produce it. All engineered crop plants that have been commercialized in the U.S. to date have been grown in the same environment that their nonmodified progenitors were grown in.

HOW DO THE TYPES OF ENGINEERED TRAITS COMPARE TO TRADITIONAL TRAITS?

Below is a comparison for traditional sources of pest resistant/tolerance genes that exist in melon. Those in italics have had engineered resistance genes field tested under APHIS oversight.

Acremonium hypocotyl rot, Alternaria cucumerina Leaf blight, Aphid, Anthracnose, spider mites, Cladosporium cucumerinum, cucumber scab, *cucumber mosaic virus*, Colletotrichum lagenarium, Corynespora melonis, Corynespora cassiicola, Cucumber beetle, Cucumber green mottle mosaic virus, Diabrotica, Downy mildew, Erwinia tracheiphila, fruit fly, Fusarium oxysporum cucumerinum, Gummy stem blight, Leaf miner, Melon necrotic spot virus, mites tolerance, Pickle worm, *Powdery mildew*, papaya ringspot virus, Pseudomonas lachrymans, Root knot nematode Meloidogyne hapla, *squash mosaic virus, watermelon mosaic virus 2, and zucchini yellow mosaic virus*.

WHAT EVIDENCE TO DATE DO WE HAVE THAT GMOS ARE LONGER-LIVED THAN THEIR NONENGINEERED PARENTS?

The most publicized reports on this issue are from Crawley (1993, 2001)**.** He reported for 4 different crops (canola, potato, maize and beet) that were engineered to be resistant to herbicide or insects that in no case were the engineered plants found to be more invasive or more persistent than their conventional counterparts in a 10 year study. Many others, including data submitted to BE as part of the petition process, have published similar results although other have seen changes.

GENE FLOW FROM ENGINEERED CROPS AND WILD PLANTS

A major environmental concern associated with agricultural biotechnology is that gene flow from engineered crops to related weeds will result in formation of novel weed phenotypes that have the potential to become invasive. It is worthy to note that gene flow between crop and weeds has been known for over a century and is not a unique characteristic of engineered plants. Crops can be divided into three classes with respect to likelihood of gene flow in the US.

1. No possibility - where there are no wild relatives in the country - soybean and corn in the US.
2. Low probability - crops that are with predominantly autogamous (many cereals) or propagated largely by asexual reproduction and flower infrequently (sweet potato and sugar cane).
3. Moderate to high probability - where the crop is an out crosser and is being grown in a region where sexually compatible relatives occur (canola in some areas of the US).

There are many examples of hybridization and introgression between domesticated plants and their wild relatives. Many of these involve hybridization that has been implicated in weed evolution. One of the best examples is Johnsongrass (*Sorghum halepense*) which arose from the hybridization of cultivated sorghum (*S. bicolor*) and the wild *S. propinquum*. Some of the ecological traits thought to have been acquired from the crop include earlier flowering, greater seed production, larger individual seed weight, and earlier emergence, traits that are often associated with weediness. But there is no evidence that any pest resistance genes from cultivated sorghum have enhanced the invasiveness of Johnsongrass.

WHAT IS THE BIOTECHNOLOGY EVALUATION (BE) STAFF APPROACH TO ADDRESS INVASIVENESS?

BE staff believes that most domesticated crops have low potential for invasiveness. However, we believe that if data is submitted to show that there has been no change in fitness related characteristics that there is a reasonable certainty that engineered plant is comparable to nonengineered parent plant.

The fitness characteristic we evaluate in deciding whether to approve for deregulation can be found in the Appendix. It is obvious that many of the fitness characteristic BE evaluates are similar to those mentioned in Baker's weed list (1965). It is not our intent to suggest that engineered plants are weeds or invasive but the similarity arises because in developing our list we looked at fitness characteristics identified by weed scientists and ecologists in study of weeds/invasive plants. Using a precautionary approach, BE staff believe that the lack of change in any of these characters strongly support our conclusion that any engineered plant we evaluate poses no more risk than a comparable traditionally bred crop plant.

ARE THERE ENGINEERED TRAITS THAT RAISE CONCERNS?

Yes. Most of these traits deal with stress tolerance, e.g. heat or cold tolerance, or salt tolerance might allow a plant to grow in a new environment. However, this is the where the biology of the plant is a critical factor. For example, cold tolerant citrus developed for use in Florida that can survive a few hours in cold temperatures probably raise no concerns since these plants do not have any invasive characteristics. However, increasing the heat tolerance of a canola (a cool climate crop) that is sexually compatible with several species that have weedy characteristics may require an extensive environmental review prior to deregulation.

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APPENDIX: Fitness characteristics used to compare the transgenic plant to its counterpart with respect to the characteristics which influence reproductive and survival biology:

a. **Growth Habit** - e.g., note any changes in basic morphology of the plant

b. **Life-span** - e.g., plants can be categorized as annual, biennial, perennial. Would the presence of the introduced trait produce a change?

c. **Vegetative vigor**.

d. **Ability to overwinter (or overseason)**.

e. **Number of days to onset of flowering; number of days for flowering**.

f. **Number of days until maturity** - depending on the plant species, this could be defined as the time to the production of mature fruit or seed (suitable for harvesting). In many species this characteristic is dependent upon factors such as day length and/or degree days.

g. **Seed Parameters**

Seed production - This might be measured as either yield (number of seeds orfruit per cultivated area) or the number of viable seeds per plant.

Continuous seed/ fruit production - Length of time (days) of seed/fruit production. This might include but is not limited to changes between determinant and indeterminant flowering.

Seed dormancy - For example, characterize any changes in the ability of the seed to remain viable after remaining in the soil.

Seedling emergence - Proportion of seeds planted that emerge as seedlings under field conditions and a description of the various environmental conditions, to evaluate emergence in more variable environments, especially those of unmanaged ecosystems.

h. **Seedling survival to reproduction**

I. **Outcrossing frequency within species** (0-1, 2-20, 21-100%).

j. **Pollinator species (if appropriate) -** This might be addressed through information on whether the same pollinator species have been seen in the field or have there been changes in pollinator species visiting the flowers. Data on changes in flower morphology, color, fragrance, etc. might also indicate interactions with pollinators may have been altered.

k. **Pollen Parameters**

Amount of pollen produced, proportion of viable pollen, the longevity of pollen under varying environmental conditions.

Physical parameters such as stickiness, shape, and weight that might affect the viability or performance of the pollen in leading to successful pollination.

l. **Fertility or infertility** - consider both male and female aspects.

m. **Self-compatibility or –incompatibility**

n. **Asexual reproduction**, i.e. vegetative reproduction; ability to root; parthenocarpy

o. **Seed Dispersal Factors-** This might be addressed by considering characteristics such as seed shattering or dispersal by animals, if appropriate.

Gene flow in turf and forage grasses (Poaceae)

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ABSTRACT

Currently, over 117 species of grasses are used for turf, forage and erosion control, with new species being evaluated every year. Only a relatively few number of these species have been genetically transformed through biotechnology. With DNA biotechnological enhancement being considered for virtually all commercially important plants, the number of field-tests for transgenic plants are increasing exponentially. This trend is also happening with turf and forage grasses, with an increase in the number of new species being tested each year. Currently the following species have permit/notifications through USDA/APHIS to conduct field tests: creeping bentgrass (*Agrostis stolonifera* L.); Kentucky bluegrass (*Poa pratensis* L.); perennial ryegrass (*Lolium perenne* L.); tall fescue [*Festuca arundinacea* Schreber or *Lolium arundinaceum* (Schreber) Darbyshire]; Bermuda grass [*Cynodon dactylon* (L.) Pers.]; Russian wildrye [*Psathrostachys juncea* (Fischer) Nevski]; Bahiagrass (*Paspalum notatum* Flügge var. *saurae* Parodi); Kentucky bluegrass x Texas bluegrass (*Poa pratensis* x *P. arachnifera* Torrey); St. Augustine grass [*Stenotaphrum secundatum* (Walters) O. Kuntze]; and velvet bentgrass (*Agrostis canina* L.). The significance of gene escape will vary with the biology of the plant donor, the wild recipient, and the introduced gene(s). In regards to the crop donor, primary risks will probably come from crops: 1) that have undergone little domestication because there would be minimal ecological and reproductive divergence from the wild progenitors (Ellstrand and Hoffman 1990); 2) grown sympatric with wild relatives (e.g. centers of origin) or cross-compatible species (or genera); 3) that have biotypes or related taxa that are already aggressive weeds; 4) that can also be weeds themselves; and 5) outcross with some degree of self-incompatibility. Because of the large number of grass species used for turf and forage, and since the potential for gene escape and its consequences will be different for each species or species complex, only those taxa currently in the APHIS Field Test program will be discussed at this time. What is known biologically and systematically about each taxon, and the complexes they form will be presented.

There are currently over 20 cool season and 14 warm season grass species used for turf, and over 30 species of cool season and 53 warm season species used for forage; with new species being evaluated every year. Only a relatively few number of these species have been genetically transformed through biotechnology. With DNA biotechnological enhancement being considered for virtually all commercially important plants, the number of field-tests for transgenic plants are increasing exponentially. This trend is also happening with turf and forage grasses, with an increase in the number of new species being tested each year. The following is the number of permits/notifications have been issued or pending (the number in parentheses is the total number submitted) by USDA/APHIS (http://www.nbiap.vt.edu/cfdocs/fieldtests1.cfm):

SPECIES	1993-1997	1998	1999	2000	2001	2002
Creeping bent grass	11 (12)	16 (19)	25 (26)	20 (24)	22 (23)	1 (1)
Kentucky blue grass	---	1 (2)	8 (8)	7 (7)	7 (7)	---
Perennial Ryegrass	---	---	1 (1)	1 (1)	1 (1)	---
Tall Fescue	---	---	2 (3)	1 (1)	5 (6)	---
Bermuda grass	---	---	2 (2)	2 (2)	3 (3)	---
Russian Wildrye	---	---	---	1 (1)	1 (1)	---
Paspalum notatum	---	---	---	---	1 (1)	---
Kentucky blue grass x Texas blue grass	---	---	---	---	1 (1)	---
St. Augustine	---	---	---	---	2 (2)	---
Velvet bent grass	---	---	---	---	1 (1)	---

Because of the large number of grass species used for turf and forage and since the potential for gene escape and its consequences will be different for each species or species complex, only those taxa currently in the APHIS Field Test program will be discussed at this time. What is known biologically and systematically about each taxon, and the complexes they form will be presented.

Bentgrasses (*Agrostis* spp.)

Currently there are five species of bentgrass grown commercially for turf or forage: *Agrostis canina* L., *A. capillaris* L., *A. castellana* Boiss. & Reuter, *A. gigantea* Roth, and *A. stolonifera* L.. Creeping bentgrass (*Agrostis stolonifera* L.) has the potential to be the first perennial (stoloniferous), wind pollinated, outcrossing transgenic crop to be grown adjacent to feral and cultivated populations of creeping bentgrass, as well as, cross-compatible perennial relatives and native species. These are traits that can increase the risk of outcrossing, persistence, and introgression of alien genes into an adjacent population. As the release of a genetically engineered bentgrass cultivar draws closer, questions regarding pollen flow and gene introgression into local populations must be answered to insure environmentally and agriculturally safe introduction of transformed products.

Agrostis stolonifera has been documented as forming natural hybrids with the following species: 1) *A. canina*; 2*) A. capillaris*; 3) *A. castellana*; 4) *A. gigantea*; 5) *A. mertensii* Trinius; and 6) *A. vinealis* Schreber (Murbeck 1898; Weber 1920; Fouillade 1932; Philipson 1937:

Stuckey and Banfield 1946; Paunero 1947; Davies 1953a,b; Jones 1953, 1956a,b,c; Bradshaw 1958; Bradshaw 1959; Björkman 1960; Widén 1971; Suckling and Forde 1978; Bradshaw 1975; Tutin 1980; Edgar and Forde 1991; and Forde 1991). Some of the interspecific hybrids are so common in nature that they have been given scientific names. The following interspecific hybrids have been formally named: 1) *A.* x *murbeckii* Fouillade (*A. stolonifera* x *A. capillaris*); and 2) *A.* x *bjoerkmanii* Widén (*A. capillaris* x *A. gigantea*).

A few studies have been published on artificial interspecific hybridization in *Agrostis.* The following experimental interspecific crosses have been made:

1) creeping bentgrass x *A. vinealis* (Björkman, 1960; Jones 1956a, 1956b). The seed set and pollen fertility were poor. At least 7 chromosomes remained unpaired in all cells; the maximum pairing was 1_{IV} 5 $_{III}$ 1 $_{II}$ 7 $_{I}$. One genome of *A. stolonifera* showed partial homology with those of *A. vinealis*.

2) creeping bentgrass x *A. capillaris* (syn =*A. tenuis* Sibth.) (Björkman 1960; Edgar and Forde 1991; Davies 1953a,b; Jones 1956b, 1956c). The seed set and pollen fertility were poor in one study, but *Agrostis capillaris* x *A. stolonifera* hybrids had 41.0% pollen fertility in another study. These hybrids were stoloniferous, vigorous, and apparently long-lived in pastures (Forde 1991). *Agrostis stolonifera* and *Agrostis capillaris* probably have one ancestral diploid type in common and show good homology in one pair of their genomes and partial homology between the other one. The inter-genome pairing of *A. capillaris* is responsible for the fluctuation in pairing in the hybrids, which was also responsible for the fluctuation in pollen fertility.

3) creeping bentgrass x *A. gigantea* Roth (Davies 1953a,b; Jones 1956c). The seed set was 19.9 seeds/panicle and pollen fertility was 0 to 30% (Jones 1956c). The stable pairing and constant occurrence of seven univalents suggested that they constituted two pairs of highly homologous genomes, and the third show little homology with the others. Davies (1953a) reported that the F_1 hybrids were quite fertile.

4) *A. capillaris* x *A. gigantea* (Davies 1953a,b; Jones 1956c). The seed set of the hybrids was 39.3/panicle and pollen fertility was 41 to 55%. The F_1 hybrids were as fertile as their parents on the basis of number of seeds per panicle under conditions of open pollination. The stable pairing and constant occurrence of seven univalents suggested that they constituted two pairs of highly homologous genomes, and the third showed little homology with the others; the latter genome was derived from an unknown ancestor. The F_2 hybrids (inter-pollination of F_1's) had a pollen fertility of 53.3 to 62.8%. Backcrosses of the F_1 with A. capillaris [(*A. capillaris* x *A. gigantea*) x *A. capillaris*] had a pollen fertility of 20.6 to 75% (Jones 1956c). Davies (1953a) reported that the F_1 hybrids were quite fertile.

5) *A. capillaris* x *A. vinealis* and (*A. capillaris* x *A. vinealis*) x *A. vinealis* (Jones 1956b). The F_1 hybrids between *A. capillaris* and *A. vinealis*, the F_2 generation, and backcrosses of the F_1's with *A. vinealis* were as fertile as the parents and were indistinguishable from *A. vinealis* at meiosis. The quadrivalent pairing sheds light not only on the interspecific relationships but also on the nature of *A. capillaris* itself. The purely bivalent pairing in *A. capillaris* disguised the fact that its genomes showed appreciable homology.

6) *A. castellana* x *A. capillaris* (Forde 1991). The hybrids were as fertile as the parents. The pollen fertility was 92.3%. The 11 F_1 hybrids were allowed to interpollinate with *A. capillaris* and *A. castellana* collections. The resulting progenies were vigorous, highly fertile, and displayed various combinations of the parental characters.

The interspecific hybrids had varying degrees of fertility, from sterile to as fertile as the parents. As would be expected, various genotypes differed in fertility, which mandated the use of multiple genotypes in crossing experiments. Most of the hybrids were vigorous regardless of fertility. The hybridization studies of Davies (1953a,b), Jones (1956a,b,c), Björkman (1960),

and Forde (1991) clearly demonstrated that *A. canina*, *A. capillaris*, *A. castellana*, *A. gigantea*, *A. stolonifera*, and *A. vinealis* form a complex of interpollinating, cross-compatible species that readily cross when the species are sympatric (FIG. 1). *Agrostis stolonifera* (A_2A_2SS) is a allotetraploid, in which both of its genomes are highly homologous with two genomes of *A. gigantea* ($A_1A_1A_2A_2SS$). *Agrostis stolonifera* and *A. capillaris* ($A_1A_1A_2A_{2)}$ have one genome in common. *Agrostis capillaris* is a segmental allotetraploid, in which both of its genomes are highly homologous with two genomes of *A. gigantea* ($A_1A_1A_2A_2SS$). Davies (1953b) concluded that both *A. capillaris* and *A. stolonifera* have something in common with *A. gigantea*, and they were involved in its evolution, or they at least have common origins with it. Widén (1971) also hypothesized that *A. gigantea* may have arisen from the fusion of A_1A_2S gametes of *A.* x *murbeckii* (the interspecific hybrid between *A. stolonifera* and *A. capillaris*). *Agrostis capillaris* also has a close chromosomal relationship with *A. vinealis* and *A. castellana*. *Agrostis vinealis* possibly evolved through introgression of a non-rhizomatous autotetraploid of *A. canina* with a rhizomatous plant of an *A. capillaris* type.

Intergeneric hybrids have also be reported between *Agrostis* and *Polypogon* (Björkman, 1960). The following *Polypogon* species have documented as hybridizing with creeping bentgrass: 1) *Polypogon monspeliensis* (L.) R. Desfontaines, the hybrid between these two taxa occurs frequently enough that taxonomists have given it a name, x *Agropogon littoralis* (J.E. Smith) C.E. Hubbard; 2) *Polypogon viridis* (Gouan) Breistr., named x *Agropogon robinsonii* (Druce) Meldris & D. C. McClint.; 3) *P. fugax*; (Björkman, 1960). Tutin (1980) hypothesized that *Agrostis parlatorei* Breistr. and *A. moldavica* Dobrescu & Beldie are perhaps derived through the hybridization of *A. castellana* and *Polypogon viridis*.

This information is relevant, because several of these *Agrostis* species occur in the Willamette Valley, where the bulk of bentgrass production occurs. "Information on the amount of out-crossing among *Agrostis* species does not exist for turfgrass seed production and managed turfgrass areas" (Johnson et al. 1999). A total of 19 species have been documented in Oregon (Harvey, In Prep.). Of the 19 species, 12 are reported to occur in the Willamette Valley and can be found in bentgrass seed production areas. Six of the 12 are naturalized species: *A. canina*, *A. capillaris, A. castellana*, *A. stolonifera*, *A. gigantea*, and *A. vinealis*. These six species are not only used commercially, but also are considered weeds in other crops. Of the remaining 12 species that are native to the area, the following six are known to occur in the Willamette Valley: *A. diegoensis* Vasey, *A. exarata* Trinius, *A. hallii* Vasey, *A. longiligula* A. Hitchcock, *A. oregonesis* Vasey, and *A. scabra* Willdenow. Carlbom (1966) in a biosystematic study of nineteen species of *Agrostis*, native to the Pacific states, all belonging to sect. *Trichodium* (Michx.) Trinius, reported on the reproductive biology of some of these species by conducting experiments on seed set in controlled self-pollinations. He reported that *A. exarata* was autogamous (self-compatible); *A. diegoensis*, *A. hallii*, and *A. oregonesis* were allogamous (self-incompatible or cross fertilized); *A. scabra* was both, depending on ecotype; and *A. longiligula* was not tested. Putative natural hybridization and introgression were observed between *A. diegoensis* and *A. hallii*; *A. diegoensis* and *A. pallens*. The outcrossing of these species with the six naturalized species was not studied.

Three species of *Polypogon* are also documented in Oregon: *P. monspeliensis* (L.) Desfontaines, *P. interruptus* Kunth, and *P. viridis* (Gouan) Breistroffer.

Wipff and Fricker (2001) studied the: 1) intraspecific gene movement; and 2) interspecific gene introgression and stability of the hybrids in creeping bentgrass transformed with the 'bar' gene which confers glufosinate resistance. Pollen movement was determined by placing transects of non-transgenic creeping bentgrass around a nursery of 286 transgenic plants genetically engineered for tolerance to the herbicide glufosinate. Approximately 250

non-transgenic creeping bentgrass plants were planted in transects around the transgenic nursery in 1998 and 1999 near Hubbard, OR. In 1998, the following transects were established: 1) two circles around the nursery at 109 (33.2 m) and 272.5 ft (83.1 m) with plants spaced at 50 ft (15.24 m) and 100 ft (30.48 m), respectively; and 2) two line transects aligned with prevailing winds (NE) with one transect NE 244 ft (74.4 m) and the SW transect 370 ft (112.8 m) from the edge of the nursery. These initial transect lengths were based on Oregon Seed Certification isolation distances, which is 165-300 ft (50.3-91.4 m) (depending upon field size) for certified seed production. In 1999, the length of the line transects were increased to the following: 1) SW transect, 978 ft (298.1 m); 2) NE transect, 268 ft (81.7 m); 3) SE transect, 612 ft (186.5 m); and 5) NW transect, 319 ft (97.2 m).

In 1998, >0.02 % transgenic plants were recovered at the ends of both the NE [244 ft (74.4 m)] and SW [300 ft (91.4 m)] transects, as well as, from most of the points along the circle transects (Table 3, Figure 4). In 1999, >0.02 % transgenic plants were recovered at the following distances: 1) NE transect, 268 ft (end of transect); 2) SW transect, 958 ft; 3) NW transect, 319 ft; and 4) SE transect, 612 ft from edge of transgenic nursery (Table 1). Using non-linear regression (exponential decay model), the following distances were predicted for transgenic pollen introgression to the 0.02% level. In 1998, along the SW transect, transgenic pollen traveled 3,500 ft (1,066.8 m) and along the NE transect it traveled 4,296 ft (1,309,4 m). In 1999, the transgenic pollen was estimated to have traveled 1,022 ft (331.5 m) to the SW, 1,892 ft (576.7 m) to the NE, 861 ft (262.4 m) to the NW, and 1,022 ft (331.5 m) to the SE.

The higher pollen flow in 1998 was also seen in adjacent non-transgenic bentgrass nurseries. Seed was sampled from a nursery (designated OVN) 638 ft S of the transgenic nursery. Approximately two million seeds (160 g) were planted and three applications of Finale® were applied to the seedlings. Three hundred fifty-four seedlings were found to be resistant. Five plants were confirmed by Southern Blot analysis to contain the bar gene. Seeds were also sampled from a 0.1 ac (0.04 ha) Penn A-1 breeder seed field 1,417 ft (432 m) SW of the transgenic nursery, and a Penn A-2 field just to the SE of the A-1 field. Two million seeds were planted of each cultivar and two to three applications of Finale® were applied to the seedlings. After the second application of herbicide, no Penn A-2 seedlings survived; however Penn A-1 had survivors. Three plants survived and Southern Blot analysis confirmed the presence of the bar gene.

The same two nurseries (Penn A-1 and OVN) were retested in 1999. Penn A-2 was not tested, because the nursery was destroyed in the fall 1998. Again, 160 g of seed was planted from each nursery and sprayed with 2 to 3 applications of Finale®. No seedlings survived from the Penn A-1 nursery and 459 seedlings were recovered from the OVN nursery. Southern Blot analysis confirmed the presence of the bar gene in five of the plants tested. This not only demonstrated that pollen was viable for at least 1,400 ft (426 m), but also established the fact that transgenic pollen can successfully compete with non-transgenic pollen in a field situation.

The second part of the study was to evaluate interspecific hybridization in creeping bentgrass. Twelve species of bentgrass have been documented as occurring in the area of bentgrass seed production. Six are naturalized species and are part of a complex that freely hybridize: *A. canina*, *A. capillaris*, *A. castellana*, *A. gigantea*, *A. stolonifera*, and *A. vinealis*. Accessions of *A. canina*, *A. capillaris*, *A. castellana*, *A. curtissii*, *A. gigantea*, *A. pallens*, and an *A.* sp. were placed in the transgenic nursery prior to flowering and allowed to interpollinate. The crossing experiments resulted in the introgression of the bar gene from creeping bentgrass into *A. canina*, *A. capillaris*, *A. castellana*, *A. gigantea*, *A. pallens,* and *A.* sp. The results from the this study showed: **1**) the transgenic bar gene can flow to other species of *Agrostis* (i.e. interspecific gene flow); **2**) intraspecific gene flow in creeping bentgrass is possible for much

longer distances than traditionally theorized; and **3**) the transgenic bentgrass plants were fertile and stable.

Devlin and Ellstrand (1990), using a method of paternity analysis, reported a gene flow greater than 1 % at 8000 m distance in *Agrostis capillaris* (syn = A. tenuis).

RYEGRASS AND TALL FESCUE

Ryegrass

The genus *Lolium* L., native to Europe, temperate Asia and North Africa (Terrell 1968), has been introduced into most temperate areas of the world. Traditionally, the seven or eight species are recognized in two reproductive groups: self-pollinated and cross-pollinated. The cross-pollinated group consists of three taxa: *L. perenne* L. (Perennial Ryegrass); *L. multiflorum* Lamarck (= *L. perenne* var. *aristatum* Willdenow) (Italian or Annual Ryegrass); and *L. rigidum* Gaudin. (Stiff Ryegrass). *Lolium rigidum* is polymorphic, with the morphological limits and taxonomic nature of this taxon being uncertain and complex, and intergrading with *L. perenne* and *L. multiflorum* (Terrell 1966, 1968). Terrell (1966) regarded the reproductive data inconclusive due the uncertainty about the morphological limits of *L. rigidum*. *Lolium rigidum* will cross with *L. perenne* and *L. multiflorum* and the resulting progeny will be fertile.

Lolium perenne and *L. multiflorum* are wind and cross pollinated, and are highly, but not completely, self-sterile (Nilsson 1930a; Jenkin 1931a, 1931b; Terrell 1968). All reported chromosome counts for these species have been diploid ($2n$=14), except for tetraploid ($2n$ = 28) cultivars of perennial and Italian ryegrass that are a result of chromosome doubling in the laboratory. Numerous crossing experiments between *L. perenne* and *L. multiflorum* have demonstrated that the two taxa hybridize readily, the crosses have good seed set and seed germination, produce vigorous and fertile F_1 progeny, and are completely interfertile (e.g. Jenken 1924, 1931c, 1933, 1954a, 1954b; Nilsson 1930b; Jenkin and Thomas 1938; Hertzsch 1938; Corkhill 1945; and Jahuar 1975). Griffiths (1950) reported finding natural hybrids between the two taxa when they are grown in proximity to one another. Manner (1960) gave an extensive literature review publications relating to both artificial and natural hybrids between these taxa. Naylor (1960) in a detailed study of the two taxa and their progenies did not find any evidence of structural differences in their chromosomes nor any reduction in bivalent pairing of the hybrids and concluded that the two taxa were not distinct species. Biologically, the two taxa belong to the same species. Jahuar (1975, 1976) reported that the chromosomes of *L. perenne* paired with those of *L. multiflorum* despite the presence of their homologous partners and concluded the these two species are closely related and their recognition as separate species is not warranted.

Numerous plant-breeding experiments have also demonstrated a close relationship between *Festuca pratensis* (Meadow Fescue), *F. arundinacea* (Tall Fescue), *Lolium perenne* and *L. multiflorum* (Terrell 1979). This close relationship was further confirmed in a serological study of seed proteins by Jaworski et al. (1975). Darbyshire (1993) moved species in *Festuca* subg. *Schedonorus* (which includes *Festuca arundinacea*, *F. pratensis*, *F. gigantea*, and *F. mazzettiana*) into *Lolium* and provided a literature review of the data supporting this classification. Jahuar (1975) concluded that *Festuca pratensis*, *Lolium perenne*, and *L. multiflorum* have little structural differentiation among the chromosomes and no effective barriers to gene exchange existed between the three taxa, except that the hybrids between *F. pratensis* and *Lolium perenne* (including *L. multiflorum*) are usually sterile. Berg et al. (1979) discussed the cytogenetics and genetics of tall fescue and its hybridization with *Lolium* species.

Giddings et al. (1997a,b) conducted a series of experiments on pollen dispersal for determining the risk of introducing genetically modified wind-pollinated forage grasses. Pollen dispersal was studied from a perennial ryegrass (*Lolium perenne* L.) source using pollen traps. Giddings et al. (1997a) reported that the most striking feature reoccurring throughout their analysis was the high variability of pollen deposition. They found a large amount of variation in pollen dispersal over time and to traps of different orientations. Twelve data sets were collected and were used to test Bateman's equations (Bateman 1947) for the wind dispersal of pollen. Batman's equations were found not to be useful for describing dispersal over distance and clearly needed to be modified to take factors such as wind direction into account. Giddings et al. (1997b) studying the influence of wind direction on pollen dispersal found that, for 11 of the 12 data sets, the new equation fit significantly better than Bateman's original equations. They also produced 15 data subsets from the mean wind directions to test Bateman's equations for dispersal downwind from a pollen source. These equations fit only four of the data subsets. They found that the amount of pollen deposited does not always decrease smoothly with increasing distance from the source. Three of the 12 data sets they analyzed had an increase in pollen deposition with distance; more pollen was collected at 80 m than at 60 m. This highlights the danger of assuming that deposition decreases smoothly over distance varying only with wind direction. Pollen clouds are taken high into the atmosphere, moved, and deposited during times of calm. So, pollen could be transported considerable distances from the source. Variable wind speeds and turbulence important factors affecting pollen movement, however, models involving such additional parameters would be complex and would probably show chaotic dynamics, making prediction difficult.

Giddings (2000) predicted *Lolium perenne* pollen dispersal using a Gaussian plume model, which takes into account distance and wind direction. The model was used to calculate, using integration, possible pollen deposition onto small conspecific populations one kilometer from the source. Initially the source and recipient populations were 2-m in radius (the same size that the source data is based). The percentage of immigrant pollen was compared for six different sets of parameter values previously estimated from pollen-dispersal experiments (Giddings et al. 1997 a, b). In the 2-m radius plots, 0.008 to 3.52 % of the pollen from the donor "transgenic" population would be deposited on the downwind "recipient" population. The source size was then scaled up to 10.24 ha (25.3 ac) to simulate what might happen if transgenic ryegrass was grown on a large scale When the source size was scaled up to 10.24 ha, 29.74 to 99.64 % of the pollen from the donor "transgenic" population would be deposited on the downwind "recipient" population. Recipient populations centered 90° to the mean wind direction received 4.90 to 51.12 % of the pollen from the source population. Giddings (2000) concluded that *Lolium perenne* pollen can easily travel a kilometer. Because of the potential for long-distance pollen movement, the use of 'guard rows' around transgenic crops to prevent gene flow out of the field seems unlikely to be effective at preventing pollen dispersal at some distance from the crop.

Giddings et al. (1997a) reported that the forage ryegrasses [*Lolium perenne* L. and *L. perenne* var. *aristata* C. von Willdenow (syn = *L. multiflorum* J. de Lamarck)] are widely cultivated cross pollinating forage crops in the U.K., which readily outcross with wild and feral populations and occasionally with fescue species (*Festuca* spp.). *Lolium-Festuca* hybrids (x *Festulolium* Ascherson & Graebner) can be fertile and have an ability to backcross with either of the parents.

Tall Fescue

Tall fescue [*Festuca arundinacea* Schreber or *Lolium arundinaceum* (Schreber) Darbyshire] has a wide native distribution in temperate and cool climates throughout Europe, North Africa, and in west and central Asia and Siberia, and has been introduced into North and South America, Australia, New Zealand and in south and east Africa (Terrell 1979). The species has tetraploid to decaploid cytotypes, but the most widely grown ecotypes and cultivars of tall fescue, for turf and forage, are bivalent forming allohexaploids, $2n = 42$ (Berg et al. 1979).

The allohexaploid tall fescue has three closely related genomes designated AABBCC (Jahuar 1975a). The A genome was contributed by diploid *F. pratensis* (meadow fescue) (Nilsson 1940; Malik and Thomas 1966, 1967). Although the karyotypes developed by Malik and Thomas (1966) do not support the proposal of Carnahan and Hill (1961) that *Lolium* donated a genome to tall fescue, the crossibility of *Lolium* to tall fescue and chiasmatic chromosome pairing in the hybrids ($2n = 28$) strongly suggests a close relationship. A similar relationship exists between *Lolium* and red fescue (*Festuca rubra* L.) (Jahuar 1975a). Perhaps the donor B genome is extinct or it was a progenitors of one or more of the modern species of *Lolium* (Berg et al. 1979). Malik and Thomas (1967), Malik and Tripathi (1970), and Malik and Mary (1971) considered the B and C (designated them as B and B') genomes to be very similar and described tall fescue as an autoallololohexaploid. Jahuar (1975, 1975a, 1977b) and Clarke et al. 1976) also considered the genomes of tall fescue are closely related and their meiotic integrity is maintained by the genetic control of chromosome pairing, like hexaploid wheat (*Triticum aestivum* L.). The identification of the donor of the C genome is of particular importance because it is believed that the regulatory gene(s) which confer diploid-like meiotic behavior to tall fescue are located in this genome (Jahuar 1975b, 1977b).

Interspecific hybrids between *F. arundinacea*, *F. pratensis* and *F. gigantea* occur (see Berg et al. 1979 for lit. review). Genetic information can probably be transferred from one species of *Festuca* to another, but the frequency of success in obtaining hybrids is probably dependent of the genotypes of the parents. The high incidence of interspecific pairing in the hybrids suggests that genetic information could be exchanged. However, the frequent occurrence of male sterility and sometimes female sterility limits the success of transferring genetic information from one species to another (Berg et al. 1979).

Taxonomic implications of crosses between *Festuca* and *Lolium* are reviewed by Terrell (1966), but the closest relationships are among the four agricultural taxa: *F. pratensis, F. arundinacea*, *L. perenne*, and *L. multiflorum*, as proved by the numerous plant breeding experiments over many years. *Lolium-Festuca* hybrids (x *Festulolium* Ascherson & Graebner) can be fertile and have an ability to backcross with either of the parents.

KENTUCKY BLUEGRASS

Poa is a very diverse genus of widely adapted, cool season grasses containing approximately ± 300 species. Interspecific hybridization results in many *Poa* species being introgressed with each other to such an extent that species classification is often difficult (Stebbins 1950). *Poa* contains a wide variety of breeding systems such as: apospory; displospory; self-incompatible outcrossing; self-compatible, gynomonecy; dioecious; and incompletely dioecious (Wedin and Huff 1996).

Kentucky bluegrass (*Poa pratensis* L.) is extensively cultivated worldwide as a forage, turfgrass, and for conservation purposes. This species is found between about 30°N latitude to above 83°N latitude, from sea level to elevations over 4,000 m in alpine habitats (Clausen

1961). Its highly variable chromosome number (x = 7; 2*n* = 28-154), its ability to hybridize with and absorb entire genomes of other species of *Poa*, and the ability of apomictic (facultative aposporous apomixis) reproduction to restore fertility to superior hybrid genotypes help explain the widespread distribution and great genetic diversity in this species (Clausen 1961; Dale et al. 1975; Hiesey and Nobs 1982).

One of the fascinating aspects of facultative apomictic species is the fact that they have the potential to produce three basic types of progeny (Nogler 1984): 1.) maternal clones (apomictic reproduction); 2.) hybrids which result from the fertilization of the sexual megagametophyte (n + n); and 3.) hybrids which result from the fertilization of an unreduced egg (2n + n or occasionally 2n+2n) or the fertilization of a reduced (or unreduced) egg by an unreduced male gamete (n+2n). The fertilization of an unreduced egg allows the incorporation of a genome into a organism with an unreduced maternal genome. The hybrids are stabilized by apomixis and this leads to an agamic complex (or "hybrid swarms") occupying large geographical areas. In apomictic bluegrasses, five types of progeny are known to be produced (Grazi et al. 1961): 1.) maternal clones (apomictic reproduction); 2.) hybrids which result from the fertilization of the sexual megagametophyte (n + n), called BII hybrids; 3.) hybrids which result from the fertilization of an unreduced egg (2n + n or occasionally 2n+2n) or the fertilization of a reduced (or unreduced) egg by an unreduced male gamete (n+2n), often called BIII hybrids; 4.) both gametes unreduced (2n + 2n); and 5.) haploid, from parthenogenic development of reduced gametes. Bashaw and Funk (1987) state that Kentucky bluegrass generally behaves as a cross-pollinated species whenever sexual reproduction is involved. Progeny arising from self-fertilization can show substantial inbreeding depression. Progeny from cross fertilization differ greatly in size, vigor, morphological characteristics, maturity, pest resistance, stress tolerance, and mode of reproduction.

Facultative apomixis is an efficient reproductive system in which the products of wide hybridization can escape the penalties of sterility and it tends more to break down species barriers than to evolve new ones (Harlan and de Wet 1963). The removal of the strong selection against sterile or partially sterile hybrids allows them to persist, reproduce, and backcross or outcross. In hybrids from sexual reproduction, apomixis effectively increases the generation time for each genotype, which increases the time available for gene flow due to pollen transfer and seed dispersal. The plants persist long enough and disperse widely enough that they can backcross and hybridize again. Thus, the group of plants potentially exchanging genetic material becomes quite large. In hybrids resulting from unreduced gametes, fertility is generally maintained and whole genomes from other species can be successfully incorporated. The continuity of variation in morphological characters is similar to that seen in a hybrid zone between sexual species, but spread out over vast geographical areas. Thus the morphological variation in a facultative apomictic complex is usually continuous and usually obscuring whatever discrete lineages that went into its formation (Kellogg 1990).

The following interspecific hybridizations in Kentucky bluegrass have been documented:

P. pratensis x *alpina* (Akerberg 1942; Akerberg and Bingefors 1953)
P. ampla x *P. pratensis* (Clausen et al. 1947)
P. scabrella x *P. pratensis* (Clausen et al. 1947)
P. longiflora x *P. pratensis* (Almgard 1966; Williamson and Watson 1980; van Dijk and Winkelhorst 1982)
P. arachnifera x *P. pratensis* (Oliver 1910)
P. caespitosa x *P. pratensis* (Clausen et al. 1962).
P. pratensis x *P. compressa* (Dale et al. 1975).

Muntzing (1940) found that interspecific hybridization often resulted in a breakdown of apomixis resulting in partially or completely sexual F1 hybrids. However, apomictic recombinants often appear in succeeding F_2 and F_3 generations (Akerberg and Bingfors 1953). The whole Poa genus combines an ability to produce polyploids with an ability to reproduce either sexually or by apomixis, thereby enabling its species to absorb heredities from other species and to develop highly diverse forms, some of which have been named species. The *Poa pratensis* group has been able to do this on a grander scale than any other species, and as it spread across the Northern Hemisphere it absorbed genomes from many different sources, so that at present it is not possible to trace its ancestors, many of which have disappeared (Clausen 1961).

BERMUDA GRASS

In much of the southeastern U.S., Bermuda grass [*Cynodon dactylon* (L.) Pers.] is an important forage and turf grass, but it also can be an aggressive weed. Bermuda grass commonly has both rhizomes and stolons, and is adapted to a wide range of climatic and edaphic conditions. It is the one species in the genus with a near cosmopolitan distribution and has the greatest genetic diversity. It occurs worldwide between approximate latitudes 45° N and 45° S. The reproductive mode in the genus is sexual and predominantly outcrossing (Taliaferro 1997). One of the strong barriers to gene flow between certain taxa appears to be the failure of hybrid seed to germinate (Harlan et al. 1969).

Cynodon transvaalensis Burtt-Davy, a diploid species, will cross with tetraploid Bermudagrass to form a sterile, triploid hybrid called *C.* x *magennisii* Hurcombe, which is used for turf (de Wet and Harlan 1970). *Cynodon nlemfuensis* and Bermudagrass hybridize readily, though seed set in the F1 hybrids is poor (de Wet et al. 1969). Bermudagrass and its hybrids are easily propagated vegetatively and many cultivars are propagated this way. There are some seeded cultivars available.

ST. AUGUSTINE GRASS

St. Augustine grass [*Stenotaphrum secundatum* (Walt.) O. Kuntze] is found, in the U.S., from the Carolinas south to Florida, west along the Gulf Coast to Texas; and in southern and central California. Because of its lack of winter hardiness, it is restricted to areas with mild winter temperatures. St. Augustine grass is an aggressive, stoloniferous species that is widely used for turf and pastures.

There are two predominant races known in the U.S. The 'breviflorus race' is diploid ($2n = 20$), with white stigmas, is capable of seed production, and is found throughout the range of the species in the U.S. The other race is the South African "Cape" race, which is a 'sterile' triploid ($2n = 30$) with purple stigmas that is thought to be a hybrid between *S. secundatum* and *S. dimidiatum* (L.) Brongn. , and has become established in Florida and California. This race was reported to be sterile by Sauer (1972), but Busey (1997) reported that this race was able to produce some seed. The perennial *Stenotaphrum* species may all be complexes of normal sexual populations and diverse clones, some of which are merely self-sterile genotypes that are isolated spatially. Sterile or not, the plants produce little seed and propagate themselves vegetatively with great vigor" (Sauer 1972).

BAHIAGRASS

Pensacola Bahiagrass (*Paspalum notatum* Flügge var. *saurae* Parodi) is a sexual, diploid (2n = 20) taxon introduced into the U.S. from South America that has become one of the major forage grasses in the southeastern U.S. (Burton 1967). A tetraploid (2*n* = 40) variety (*P. notatum* var. *latiflorum* Döll) also occurs in the southeastern U.S., but is an obligate apomict and the most common variety of this species in tropical and subtropical America (Parodi 1948). There is little information regarding the hybridization of the cytotypes.

Hodgson (1948) reported a pollen flow half distance of 8.3 m. Half distance is the distance required to reduce the pollen amount by 50 percent.

RUSSIAN WILDRYE

Russian wildrye [*Psathyrostachys juncea* (Fischer) Nevski] is a cool season, perennial bunchgrass, native to the steppe and desert regions of Russia and China, introduced into the U.S. in 1927. This species is diploid (2*n* = 14), outcrossing and reproduces sexually. Once established, it is one of the best sources of grazing the semi-arid rangelands of the Intermountain West and the Northern Great Plains (Jensen 1997).

CONCLUSION

Grass species used for turf, forages, rangeland, and bioremediation grasses that have undergone relatively little domestication and often have wild relatives growing sympatric (Arriola and Ellstrand 1996; Giddings et al. 1997a; Ellstrand and Hoffman 1990) and/or can be weeds outside of cultivation and in other crops, are particularly at risk of spreading transgenic DNA. Bermuda grass [*Cynodon dactylon* (L.) C. Persoon] is an important perennial forage and turfgrass, but it is also, in many areas of the United States, is one of the worst weeds. If a gene that would confer a selective advantage were introduced into a cultivar (by either biotechnology or conventional breeding) and the gene escaped into the weedy populations, the immediate economic and ecological impact could be significant (Ellstrand and Hoffman 1990).

These problems are not just theoretical, because even within highly domesticated crops, crop-weed hybridizations have occurred, leading to the evolution of aggressive weeds (Barrett 1983), and demonstrating that the hazards created by these hybridizations occur in nature and are not unique to genetic engineering. These weeds are difficult to control because they share so many traits with the crop. Examples of this can be found in cultivated sorghum [*Sorghum bicolor* (L.) C. Moench], pearl millet (*Pennisetum glaucum* L.), radish (*Raphanus sativus* L.), and sugar beets (*Beta vulagaris* L. subsp. *vulgaris*). Johnsongrass [*Sorghum halepense* (L.) C. Persoon] is known to be an interspecific hybrid descendant of *S. bicolor* and *S. propinquum* (K. Kunth) A. Hitchcock (Paterson et al. 1995) and is considered to be a primary noxious weed worldwide (Holm et al. 1977). Its hybridization with cultivated sorghum resulted in the evolution of invasive weedy biotypes (Baker 1972; Arriola and Ellstand 1996). A similar case happened with pearl millet. A common noxious weed [*P. sieberanum* (Schlecht.) Stapf & Hubbard] of pearl millet in Africa is also a biotype of pearl millet. This weedy biotype, which mimics pearl millet, evolved through the hybridization of pearl millet with its wild progenitor [*P. violaceum* (Lam.) Rich.] (Brunken et al. 1977). In California during the nineteenth century, cultivated radish hybridized with an introduced weed, *Raphanus raphanistrum* L.) to create a new weed known as wild radish (Panetsos and Baker 1967). More recently, a new weed

evolved in France when sugar beets (*Beta vulgaris* L. subsp. *vulgaris*) hybridized with a subspecies from the Mediterranean [*B. vulgaris* ssp. *maritima* (L.) Archang.] (Boudry et al. 1993). These examples also demonstrate that new weeds can evolve quickly.

Since many of these problems begin with gene escarpment through the pollen, the logical first step in a biotechnology risk assessment is the quantifications of pollen movement and the intraspecific, interspecific and intergeneric hybridization possibilities between transgenic crops and non-transgenic populations. Pollen viability is one of the most important traits, and determining how far viable pollen travels under various environmental conditions must be assessed, because the longer the pollen is viable the longer distance it can travel, and the more time it has to potentially introgress into surround populations.

Absolute containment of transgenes is undoubtedly impossible. Genes cannot only escape via the pollen, but also through seeds that are left in fields and lost during handling, and via vegetative propagules. Instead of asking whether absolute containment is possible, approaches that will maximize the level of containment should be the focus. Which will be different for each species or species complex.

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FIGURE 1. Genomic phylogeny of the *Agrostis stolonifera* complex.

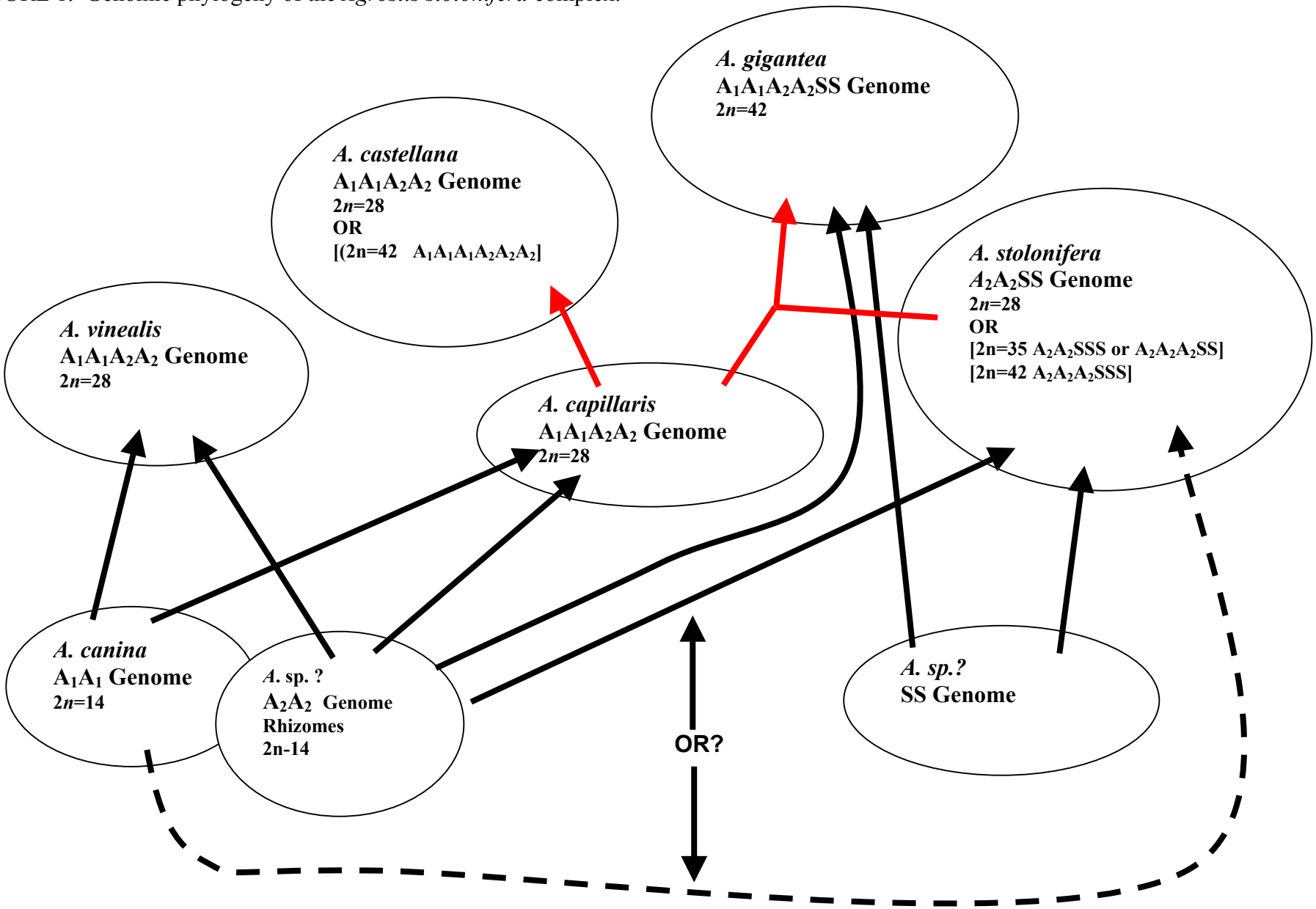

TABLE 1
POLLEN FLOW DISTANCES (ft)
FROM EDGE OF NURSERY

	1998			1999		
DIRECTION	OBS.	0.10%	0.02%	OBS.	0.10%	0.02%
NE	247.5	3,457	4,296	268	1504	1,892
SW	369.5	2,796	3,500	958	821	1,022
SE	----	----	----	612	821	1,022
NW	----	----	----	319	687	861

POLLEN FLOW DISTANCES (m)
FROM EDGE OF NURSERY

	1998			1999		
DIRECTION	OBS.	0.10%	0.02%	OBS.	0.10%	0.02%
NE	75	1,054	1,309	82	459	577
SW	113	852	1,067	292	250	312
SE	----	----	----	187	250	312
NW	----	----	----	97	209	263

Gene Flow Assessment for Plant-Incorporated Protectants by the Biopesticide and Pollution Prevention Division, U.S. EPA

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ABSTRACT

The Federal Insecticide, Fungicide and Rodenticide Act directs the U.S. EPA to examine all potentially adverse environmental impacts, including those which may arise from gene flow of plant-incorporated protectants (PIPs) to wild or feral populations of sexually compatible plants. In addition to this mandate, the Federal Food Drug and Cosmetic Act requires the issuance of a food tolerance or exemption from the requirement of a tolerance for all pesticidal substances that may enter the food supply whether through seed mixing or cross pollination. To date, three crop species have been registered by the Agency as PIPs and all have received exemptions from the requirement of a tolerance. Maize, cotton and potato were reviewed for their potential to hybridize with wild and feral relatives of sexually compatible plants in the U.S., its territories and possessions. For *Zea mays* ssp. *mays* it was concluded that there was no biologically plausible avenue for gene flow to other plants within these geographical boundaries since there are no sexually compatible species present, other than in special plantings (*e.g.*, herbaria, research plots, demonstration plots). The conclusion for Bt-potato (*Solanum tuberosum* ssp. *tuberosum*) was largely the same since the only compatible relatives are found at higher elevations in the southwest and are not found in areas where commercial potato production occurs. Upland cotton, *Gossypium hirsutum*, does have the potential to hybridize with Hawaiian cotton, *G. tomentosum*, and feral populations of *G. hirsutum* in the Florida Keys, and of *G. hirsutum* / *G. barbadense* on the U.S. Virgin Islands and Puerto Rico. For these reasons, restrictions on field plot experimental use permits and commercial planting of Bt-cotton has been instituted in these areas.

INTRODUCTION

Regulatory Authority

In the United States, the regulation of plants or their components produced through recombinant DNA techniques has been performed by the U.S. Department of Agriculture (USDA), the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA). Each of these government entities utilizes different statutes and regulations to provide oversight for plants, pesticides and plant products which fall under their domain based upon what the intent and function of the novel plant types are. As the field of biotechnology expands and provides us with new challenges from a regulatory perspective, the regulations and guidelines directing scientific review are re-examined and updated.

The subject of this presentation is the regulation of transgenic plant-incorporated protectants designed for enhanced pest or disease resistance and their potential for gene flow. These plant-incorporated protectants are subject to oversight under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). The U.S. EPA implements and enforces the provisions of this act to regulate all types of pesticides, including, for example, those plant-incorporated protectants (formerly 'plant-pesticides') produced in genetically modified crops with insect or pathogen resistance. In addition, any pesticide which is applied to food or feed requires a food tolerance for residue on the crop or food product as mandated by the Federal Food, Drug and Cosmetic Act.(FFDCA). The FFDCA authorizes EPA to establish a tolerance (maximum residue level) for a pesticide if the 'residue in or on food is safe'. Similarly, EPA may establish an exemption from the requirement of a tolerance if the Administrator determines that the residue is 'safe'. The Food Quality Protection Act modifies and joins the other two statutes to provide for a thorough assessment of the risks of all pesticides. Through FIFRA, FFDCA, FQPA, the Plant-Incorporated Protectant Rule, and the guidelines published in Title 40, Code of Federal Regulations, Parts 150 to 189, the EPA registers and regulates the field testing and commercial application of crop species meeting the definition of Plant-Incorporated Protectant (*i.e.*, a pesticidal substance produced in a plant and the genetic material necessary for its production).

EPA Oversight of Plant-incorporated Protectants

FIFRA defines a 'pesticide' as "any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest . . . ." (FIFRA 2(u)). Unless it is exempted or falls within certain minor exceptions, under FIFRA, a pesticide may be sold or distributed in commerce only if EPA has issued either an experimental use permit or a registration for the product (7 USC.136). Plants themselves are exempted from FIFRA oversight (40 CFR 152). In general, EPA may approve the sale and distribution of a pesticide only if the Agency determines that use of the product will not cause "unreasonable adverse effects on the environment". FIFRA defines "unreasonable adverse effects on the environment" to mean (1) any unreasonable risk to man or the environment, taking into account the economic, social, and environmental costs and benefits of the use of a pesticide, or (2) in the case of a pesticide that requires approval under FFDCA, a human dietary risk from residues from a use which causes a pesticide residue in food that is not 'safe'. (FIFRA 2(bb)) The latter portion of the standard is a "risk-only" standard, while the first part of the FIFRA standard involves balancing risk and benefits.

Once a product is registered, FIFRA requires the registrant to report to EPA any information concerning the unreasonable adverse effects of the pesticide on the environment (FIFRA 6(a)2). FIFRA also authorizes EPA to issue "data call-in notices", which require the registrants of a pesticide to develop and submit any additional information the Agency needs to evaluate the pesticide to determine whether the registration may remain in effect (FIFRA 3(c)(2)(B).

Following the publication of the proposed Plant-Pesticide Rule (2) in 1994, a significant volume of comments were received regarding the scope of the proposed regulation and what the ultimate effects might be on the regulated community (*e.g.*, biotechnology corporations, researchers), international trade, the environment and the American consumer. Not surprisingly, the arguments for changing the proposed Plant-Pesticide Rule (hereafter, the Rule) often centered upon the Rule not providing sufficient protection for the environment or the consumer of agricultural products, to being too severe in terms of regulatory burden on the scientific community and potential registrants of plant-pesticides. Other suggestions included a name change to plant-incorporated protectants to distinguish these from conventional pesticides.

Within the context of plants genetically modified to repel, destroy or in any way prevent the action of a plant pest, a plant-pesticide / plant-incorporated protectant is a pesticidal substance that is intended to be produced and used in a living plant and the genetic material necessary for the production of such a substance. A pesticidal substance, when in reference to plant-pesticides only, means a substance that is intended to be produced and used for a pesticidal purpose during any part of a plant's life cycle. Given these definitions, plants which are used to prevent insect feeding, reduce or eliminate pathogens, are termed plant-incorporated protectants (PIPs) under the Rule, which was finalized in 2001. Plants designed for tolerance to chemical herbicides (*e.g.*, Roundup-Ready™ , Liberty-Link™ genotypes) do not meet the definition proposed since these herbicide resistant plants do not themselves manufacture an herbicidal product and, therefore, are not registered by EPA as pesticides. The chemical herbicide itself (*e.g.*, Roundup™ - glyphosate, Liberty™ - glufosinate ammonium), however, requires Section 3 registration (FIFRA) and review of tolerances (FFDCA, Sec. 408) if proposed for use on a plant not already within the scope of the product label. Plants developed to express herbicide tolerance only are regulated by the USDA-APHIS.

GENE FLOW ISSUES OF PLANT-INCORPORATED PROTECTANTS

Under FIFRA, EPA has reviewed the potential for gene capture and expression of the *B.t.* endotoxins by wild or weedy relatives of corn, cotton and potatoes in the U.S., its possessions or territories. *B.t.* plant-pesticides that have been registered to date have been expressed in agronomic plant species that, for the most part, do not have a reasonable possibility of passing their traits to wild native plants. Feral species related to these crops, as found within the United States, cannot be pollinated by these crops (corn, potato and cotton) due to differences in chromosome number, phenology and habitat. The only exception, however, is the possibility of gene transfer from *B.t.* cotton to wild or feral cotton relatives in Hawaii, Florida and the Caribbean.

Maize - *Zea mays* ssp. *mays*

To date, all of the maize Plant-incorporated Protectants (PIPs) registered by the U.S. EPA (the 'Agency') have been based upon the use of the delta-endotoxin from the bacterium *Bacillus*

thuringiensis as a means of insect control. While they vary in their spatial and temporal expression in the plant, as well in the genetic makeup of the transforming constructs, for purposes of the discussion of gene flow they can be dealt with as a unit.

Zea mays is a wind-pollinated, monoecious, annual species with imperfect flowers. This means that spatially separate tassels (male flowers) and silks (female flowers) are found on the same plant, a feature which limits inbreeding. A large variety of types are known to exist (*e.g.*, dent, flint, flour, pop, sweet) and have been selected for specific seed characteristics through standard breeding techniques. Maize cultivars and landraces are known to be diploid (2n = 20) and interfertile to a large degree. However, some evidence for genetic incompatibility exists within the species (*e.g.*, popcorn x dent crosses; Mexican maize landraces x Chalco teosinte). *Zea mays* has been domesticated for its current use by selection of key agronomic characters, such as a non-shattering rachis, grain yield and resistance to pests. The origin of corn is thought to be in Mexico or Central America, based largely on archaeological evidence of early cob-like maize in indigenous cultures approximately 7200 years ago.

A close relative of corn or maize is the genus *Tripsacum.* Sixteen species of *Tripsacum* are known worldwide and generally recognized by taxonomists and agrostologists; most of the 16 different *Tripsacum* species recognized are native to Mexico, Central and South America, but three occur within the U.S. In the Manual of Grasses of the United States, A. S. Hitchcock (revisions by Agnes Chase; 1971) reports the presence of three species of *Tripsacum* in the continental United States: *T. dactyloides*, *T. floridanum* and *T. lanceolatum*. Of these, *T. dactyloides*, Eastern Gama Grass, is the only species of widespread occurrence and of any agricultural importance. It is commonly grown as a forage grass and has been the subject of some agronomic improvement (*i.e.*, selection and classical breeding). *T. floridanum* is known from southern Florida and *T. lanceolatum* is present in the Mule Mountains of Arizona and possibly southern New Mexico.

For the species occurring in the United States, *T. floridanum* has a diploid chromosome number of 2n = 36 and is native to Southern Florida; *T. dactyloides* includes 2n = 36 forms which are native to the central and western U.S., and 2n = 72 forms which extend along the Eastern seaboard and along the Gulf Coast from Florida to Texas, but which have also been found in IL and KS; these latter forms may represent tetraploids (x = 9 or 18; Lambert, personal communication, 1999); and *T. lanceolatum* (2n = 72) which occurs in the Southwestern U.S. *Tripsacum* differs from corn in many respects, including chromosome number (*T. dactyloides* n = 18; *Zea mays* n = 10). Many species of *Tripsacum* can cross with *Zea*, or at least some accessions of each species can cross, but only with difficulty and the resulting hybrids are primarily male and female sterile (Duvick, personal communication, 1999; Galinat, 1988; Wilkes, 1967). *Tripsacum*/maize hybrids have not been observed in the field, but have been accomplished in the laboratory using special techniques under highly controlled conditions.

The potential for pollen-directed gene flow from maize to Eastern Gama Grass is extremely remote. This is evidenced by the difficulty with which *Tripsacum dactyloides* x *Zea mays* hybrids are produced in structured breeding programs. Additionally, the genus does not represent any species considered as serious or pernicious weeds in the United States or its territories. Any introgression of genes into this species as a result of cross fertilization with

genetically-modified maize is not expected to result in a species that is weedy or difficult to control. In many instances where hybridization has been directed between these two species, the resultant genome is lacking in most or all of the maize chromosomal complement in subsequent generations.

Teosintes, specifically *Z. mays* ssp. *mexicana* (Schrader) Iltis, *Z. mays* ssp. *parviglumis* Iltis and Doebley*, Z. mays* ssp. *huehuetenangensis* (Iltis and Doebley) Doebley*, Z. luxurians* (Durieu and Ascherson) Bird*, Z. perennis* (Hitchc.) Reeves and Mangelsdorf and *Z. diploperennis* Iltis, Doebley and Guzman*,* have co-existed and co-evolved in close proximity to maize in the Americas over thousands of years; however, maize and teosinte maintain distinct genetic constitutions despite sporadic introgression (Doebley, 1990).

The teosintes retain a reduced cob-like fruit / inflorescence that shatters more than cultivated maize, but still restricts the movement of seeds as compared to more widely dispersed weedy species. Hence, the dispersal of large numbers of seeds, as is typical of weeds, is not characteristic of teosintes or maize. In their native habitat, some teosintes have been observed to be spread by animals feeding on the plants. Teosintes and teosinte-maize hybrids do not survive even mild winters and could not propagate in the U.S. corn belt. Additionally, some types have strict day length requirements that preclude flowering within a normal season (*i.e.*, they would be induced to flower in November or December) and, hence, seed production under our temperate climate (Beadle, 1980; Iltis, personal communication; 2000; Wilkes, personal communication; 2000; Wilkes, 1967).
Since both teosinte and *Tripsacum* are included in botanical gardens in the U.S., the possibility exists (although unlikely) that exchange of genes could occur between corn and its wild relatives. EPA is not aware, however, of any such case being reported in the United States. Gene exchange between cultivated corn and transformed corn would be similar to what naturally occurs at the present time within cultivated corn hybrids and landraces. Plant architecture and reproductive capacity of the intercrossed plants will be similar to normal corn, and the chance that a weedy type of corn will result from gene flow with cultivated corn is extremely remote.

Given the cultural and biological relationships of various teosinte species and cultivated maize over the previous two millennia, it would appear that significant gene exchange has occurred (based upon morphological characters) between these two groups of plants and that no weedy types have successfully evolved as a result. More recent cytogenetic, biochemical and molecular analyses have indicated that the degree of gene exchange is far less than previously thought (Doebley, 1984; Doebley *et al.*, 1987; Kato, 1997a, 1997b; Smith *et al.*, 1985). Partial and complete gametophytic incompatibility has been documented among cultivated maize, landraces and teosinte (Kermicle, 1997). The former is demonstrated by differential pollen growth and a skewed recovery of alleles linked to incompatibility genes. Complete incompatibility mechanisms serve to isolate a species or subspecies and are evidenced as pollen exclusion or non-functioning of pollen types on certain genotypes. Attempts to cross six collections of *Zea mays* ssp. *mexicana* with U.S. maize cultivars (W22, W23) yielded no or few seeds in five of the six groups (Kermicle and Allen, 1990).

Based on the ability of maize to hybridize with some teosintes, the suggestion of previous genetic exchange amongst these species over centuries, and their general growth habits, any introgression of genes into wild teosinte from *Zea mays* is not considered to be a significant

agricultural or environmental risk. The growth habits of teosintes are such that the potential for serious weedy propagation and development is not biologically plausible in the United States.

Many of the *Zea* species loosely referred to as "teosintes" will produce viable offspring when crossed with *Zea mays* ssp. *mays*. None of these plants are known to harbor weedy characteristics and none of the native teosinte species, subspecies or races are considered to be aggressive weeds in their native or introduced habitats. In fact, many are on the brink of extinction where they are indigenous and will be lost without human intervention (*i.e.*, conservation measures). Further, none of the landraces or cultivated lines of *Zea mays* are considered to have weedy potential and are generally considered to be incapable of survival in the wild as a result of breeding practices (*e.g.*, selection for a non-shattering rachis) during domestication of the crop.

Upland Cotton - ***Gossypium hirsutum***

EPA has reviewed the potential for gene capture and expression of the Cry1Ac endotoxin in cotton by wild or weedy relatives of cotton in the United States, its possessions or territories. EPA has concluded that there is a possibility for gene transfer in limited geographic locations where wild or feral cotton relatives exist.

Domesticated *Gossypium* species often exist as feral populations that are self-sustaining in their native or introduced habitats in the tropics and subtropics. Although capable of persisting in disturbed areas, such as beaches or adjacent areas along the coast, this group does not contain any species considered to be noxious or problematic weeds in the U.S., its possessions or territories (Wendel, 2000[B]). Cotton and related congenerics do not withstand cold temperatures and would not overwinter in the temperate areas of the United States. A review of the weeds of the world list for *Gossypium*, notes only *G. tomentosum* of Hawaii as a weed (Holm *et al*. 1979). This species is considered as on the decline, however (Meredith, 2000).

There are five species of New World allotetraploids (2n = 4x = 52) which share the A-D genome complement: *G. barbadense*, *G. darwinii*, *G. hirsutum*, *G. mustellinum*, *G. tomentosum*. Of these, *G. barbadense*, *G. tomentosum*, and, of course, *G. hirsutum* are found in the United States or its possessions and territories. All are interfertile to some degree.

***Gossypium hirsutum* / *G. barbadense* - General Biology**

G. hirsutum, or Upland Cotton, grows as an annual or perennial herb or shrub, typically 5 ft in height, but occasionally taller in its perennial habit. Seeds are produced in an ovoid, beaked capsule, 3 to 5 celled, which splits in a loculocidal manner and contains copious lint (Hortus Third, 1976). Upland Cotton is grown as an annual across much of the southern U.S. and has been the subject of numerous agronomic and genetic studies aimed at varietal improvement. Fibers of Upland Cotton are well suited to textile applications and the species is the most widely grown crop for fiber and is also an important source of food oils and seed meal / hulls.

Seeds of Upland Cotton and Pima Cotton, *G. barbadense*, typically require some form of treatment to ensure adequate germination. This may take the form of heat treatment, particularly in hard-seeded Pima types, and a sulfuric acid de-linting treatment to remove fuzz or linters from the seed coat. De-linting can also be done mechanically, but is most often performed chemically; failure to remove residual lint or fuzz can complicate the mechanics of

planting as seed will aggregate. Additionally, those seeds that may escape from cultivation in the U.S., during transport of cotton at harvest for example, do not give rise to persistent populations due to the seed treatment requirements and the competition of multiple plants from seed that is heavy and not commonly dispersed by animals or wind. The requirement for significant moisture also prohibits growth of escapes in many locations and those that do survive set few or no seeds (Bassett, 2000). Even in areas of significant rainfall (*e.g.*, Mississippi), escaped Upland Cotton has not been able to establish itself due to its poor colonizing ability (Percy, 2000).

Gossypium spp. in the United States, Territories, and Possessions

There are four species of cotton, *Gossypium*, in the United States. Two of them, *Gossypium hirsutum* (upland cotton) and *Gossypium barbadense* (sea island cotton, pulpulu haole, Pima), are used commercially and escaped plants can be found growing in the wild in climates where they can survive the winter (*e.g.*, Mexico, Caribbean basin) and have access to adequate water supply (*e.g.*, in or near creek beds). In addition, two native wild species of *Gossypium* occur in the United States: *G. thurberi* Todaro and *G. tomentosum* Nuttall ex Seeman.

Upland cotton, *G. hirsutum*, is cultivated throughout the world and is present in many southern U.S. locales from Virginia across the Gulf states as far north as Missouri and west to California. Feral populations of *G. hirsutum* exist in southern Florida, U.S. Virgin Islands, and possibly Puerto Rico, but are not known to persist elsewhere in the U.S. or its possessions and territories. Pima cotton, *G. barbadense*, is also found in the Carribean, including the Virgin Islands. The semi-wild cotton of the Virgin Islands may constitute an introgression of genetic components from *G. hirsutum* and *G. barbadense* (Wendel, 2000[A]). Upland Cotton is genetically compatible with *G. barbadense* or Pima Cotton, also a tetraploid, and will produce viable, fertile progeny when crossed. Alleles specific to *G. barbadense* were found at a low frequency in feral *G. hirsutum* populations in the tropics and subtropics in areas where they are sympatric (Ellstrand *et al.*, 1999).

G. thurberi Todaro (*Thurberia thespesiodes* Gray) occurs in the mountains of Southern Arizona and northern Mexico at 2,500 to 5,000 feet (rarely at 7000 feet), and is rather common on rocky slopes and sides of canyons in late summer and autumn. The diploid species *G. thurberi* is not found in the areas where cotton is grown (*i.e.*, desert valleys) and the progeny would be sterile due to their triploid state if gene flow did occur with Upland or Pima Cottons. Attempts to cross the two deliberately (*G. hirsutum*) with *G. thurberi* as the female parent were unsuccessful (Stewart, 1992). Additionally, the flowering periods of the commercial cotton and *G. thurberi* are primarily incongruous. Any gene exchange between plants of *Gossypium hirsutum* and *Gossypium thurberi*, if it did occur, would result in triploid (3x = 39 chromosomes), sterile plants because *G. hirsutum* is an allotetraploid (4x = 52 chromosomes), and *G. thurberi* is a diploid (2x = 26 chromosomes). Such sterile hybrids have been produced under controlled conditions, but they would not persist in the wild; in addition, fertile allohexaploids (6x = 78 chromosomes) have not been reported in the wild.

The second wild native species, *Gossypium tomentosum*, occurs in Hawaii on the six islands of Kahoolawe, Lanai, Maui, Molokai, Nihau and Oahu (Stephens, 1964). Upland, Hawaiian and Pima cotton are all tetraploids (4x = 52) that can crossbreed. Introgression has been claimed for what one author considered hybrid swarms of *G. barbadense* x *G. tomentosum*, but conclusive proof of this is lacking. *G. tomentosum* is a tetraploid capable of forming fertile

hybrids with *G. hirsutum* despite some fertility or compatibility factors (Stelly, 2000). Winter nursery seed increases on any of these islands could result in further exposure of wild *G. tomentosum* to cultivated species which will cross readily as all are tetraploids of the A-D genome type. It has been the policy of this Agency to preclude the culture of *B.t.*-cotton in Hawaii for this reason. Unfortunately, the culture of non-modified cotton poses a threat to the biological diversity of this species and introgression of sequences from *G. barbadense* and *G. hirsutum* have likely occurred previously. As *G. tomentosum* may bloom at the same time as domestic cotton, there is no guarantee of either geographic or temporal isolation. In *G. tomentosum* pollination is thought to be nocturnal and although flowers may open during the day, stigmas are typically not receptive during the day. The pollination biology of this species remains to be elucidated, however. For these reasons, EPA imposed stringent sales and distribution restrictions on the registration for cotton expressing the Cry1Ac □-endotoxin grown in Hawaii. During registration, the Agency required the following labeling statement to mitigate the potential for the *cry1Ac* gene to move from cultivated cotton to *G. tomentosum*:

> "Not for commercial sale or use in Hawaii. Test plots or breeding nurseries established in Hawaii must be surrounded by either 12 border rows of non-*B.t.* cotton if the plot size is less than 10 acres or 24 border rows if the plot is over 10 acres and must not be planted within 1/4 mile of *Gossypium tomentosum*."

With respect to gene flow between varieties and species of *Gossypium*, four conditions need to exist: (1) sexual compatibility between the parents, (2) the periods of fecundity or style receptiveness / anthesis must coincide, (3) a vector capable of moving the pollen between the parents must be present, (4) the progeny of the cross must be fertile and viable in the environment in which they develop (Stewart, 1992). Although all species of *Gossypium* are self-fertile, they require an insect vector for cross-pollination as wind dissemination of pollen is not a factor.

There are only five areas in the United States, and its territories and possessions wherein cultivated cotton has the opportunity to outcross to wild or feral species which are genetically compatible: (1) southern Arizona, (2) Hawaiian islands, (3) southern Florida (Stewart, 2000), (4) U.S. Virgin Islands, and (5) Puerto Rico.

G. thurberi (Arizona Wild Cotton), is present in the elevated regions of Arizona and does not grow in areas of commercial cotton production. *G. thurberi* is a diploid and produces sterile, triploid progeny when crossed with the tetraploids *G. hirsutum* or *G. barbadense* (Percival, 2000).

In the very south of Florida, feral *G. hirsutum* exists in apparently self-sustaining populations (Percival, 2000; Wendel, 2000[A]). Since these would readily cross with cultivated cotton, sale of *B.t.*-Cotton is restricted south of Interstate 60. There is currently no commercial cotton production in the southern part of Florida.

Evidence from germplasm collections indicates that feral *G. barbadense* and possibly *G. hirsutum* exist in the Carribean, including Puerto Rico and the U.S. Virgin Islands (Meredith, 2000; Percy, 2000). There is presently no production of commercial cotton in either of these places, hence, outcrossing is not an issue at this time.

Pollination of Upland and Pima Cotton

The presence of these feral populations representing sexually compatible recipients of insect vectored pollen from commercial plantings of cotton may be mitigated by instituting isolation distance requirements in conjunction with border rows of non-*B.t.* cotton. Isolation distances applied to commercial seed production (*i.e.*, Foundation, Registered or Certified seed) vary with region and are designed to minimize cross-pollination between types, but not to absolutely exclude it.

G. hirsutum is self-pollinated in the absence of insects, but is readily cross-pollinated in the presence of appropriate insect vectors, such as the bumble bees of the genus *Bombus*, *Mellisodes* bees or the honey bee, *Apis mellifera* (McGregor, 1976). The potential for cross pollination between *G. hirsutum* and other relatives in the immediate vicinity is dependent on a variety of factors including ploidy, presence of insect vectors, use of broad-spectrum insecticides, temporal synchrony of anthesis, and distance between plants. Many species of *Gossypium* are interfertile, but some are predominantly inbreeding species by design and would not readily outcross with other species in a natural setting (Wendel, 2000[A]).

During the cultivation of cotton, *G. hirsutum* and *G. barbadense*, for commercial seed production, various states have instituted requirements and standards to preclude pollen-directed gene flow between species and varieties. For example, in Arizona the minimum distance mandated by the Arizona Crop Improvement Association (Simons, 2000) between fields containing different species (*i.e.*, Pima vs. Upland), or between varieties differing substantially in leaf type, is 1320 feet (Simons, 2000). For varieties which differ in lint color (all classes), the distance increases to 5280 feet and requires a buffer zone of at least 100 feet of border rows or an intervening field.

The inability of plants or seeds of either of *G. hirsutum* or *G. barbadense* to survive freezing temperatures restricts their persistence as perennials or recurrent annuals to tropical and sub-tropical areas. Feral *G. hirsutum* occurs in parts of southern Florida in the Everglades National Park and the Florida Keys. Cotton is not grown commercially in these areas at this time (*i.e.*, cultivated cottons are found in the northernmost portions of the state), but the containment provisions of the initial registration must continue for areas in Florida where feral cotton occurs. EPA imposed sale and distribution restrictions on *B.t.* cotton in Florida, restricting its use to those sites North of Tampa (Route 60). The Agency is satisfied that the planting restrictions on *B.t.* cotton (*i.e.*, no *B.t.* cotton south of Tampa) will mitigate concerns for gene transfer to wild cotton:

"In Florida do not plant south of Tampa, (Florida Route 60)."

As noted in the FIFRA SAP report, the isolation distances typically required for seed production may not be adequate for mitigating pollen flow between crop types or between crops and their wild relatives. Additionally, the use of non-*B.t.* cotton as a trap crop surrounding *B.t.* cotton fields may not be sufficient in itself to preclude gene flow due to the insect vectors associated with pollination of this genus.

Studies underway at the University of California Davis, Biotechnology Seed Center, indicate that outcrossing between cotton plots was detectable at 5,475 feet, the longest distance measured in this seed production area (Sundstrom, 2001). A continuation of this study with

added design to simulate areas of low and high pollinator activity will provide greater information on pollen flow as this study progresses.

Potential Recipients of Gene Flow in Cotton

A wild relative of *Gossypium hirsutum* (Upland cotton) and *G. barbadense* (Pima cotton) exists in Hawaii on at least six of the islands (Stephens, 1964). The pollinators for this species are not known and may be the same or different from those insects pollinating cultivated *G. hirsutum* on the islands. Pollination is thought to be nocturnal, while flowers may open during the day, stigmas are typically not receptive during the day. Although it is presumed that the insect vectors for cultivated cotton are the same in the continental U.S. and in Hawaii, this has not been established (Wendel, 2001). Much of the basic biology of *G. tomentosum* remains to be discovered.

G. barbadense is found in the Carribean, including the Virgin Islands. The semi-wild cotton of the Virgin Islands may constitute an introgression of genetic components from *G. hirsutum* and *G. barbadense* (Wendel, 2000[B]). Upland Cotton is genetically compatible with *G. barbadense* or Pima Cotton, also a tetraploid, and will produce viable, fertile progeny when crossed. Alleles specific to *G. barbadense* were found at a low frequency in feral *G. hirsutum* populations in the tropics and subtropics in areas where they are sympatric (Ellstrand *et al.*, 1999). Currently there does not appear to be any commercial cultivation of cotton on these islands. Puerto Rico is also known to contain feral populations of *G. hirsutum* and *G. barbadense*, or possibly hybrid swarms of the two species (Wendel, 2001). Although the precise distribution of these feral populations is not known with certainty, they are apparently common in this territory.

Gene Flow in Cotton - Mitigation

Current Agency restrictions in Hawaii preclude the sale and use of *B.t.* cotton for commercial planting in this state: "Not for commercial sale or use in Hawaii. Test plots or breeding nurseries established in Hawaii must be surrounded by either 12 border rows of non-*B.t.* cotton if the plot size is less than 10 acres or 24 border rows if the plot is over 10 acres and must not be planted within 1/4 mile of *Gossypium tomentosum*." The use of test plots for gathering field data following issuance of an experimental use permit or permission for a seed increase in the vicinity of *G. tomentosum* populations could have the same net effect (*i.e.*, gene flow). In light of the lack of basic biological data on *G. tomentosum* (*e.g.*, pollinator ecology, compatibility / sterility factors, potential impact of *B.t.* on herbivores, distribution of native populations), conservative measures may be needed to mitigate hybridization with cultivated cotton. If complete isolation and prevention of gene flow is desired, then test plot plantings of *B.t.* cotton in Hawaii may require a minimum 3 mile distance from *G. tomentosum* with 24 border rows of non-*B.t.* cotton surrounding the plots or exclusion from planting in this state. In situations where breeding nurseries and genetic purity are at stake, border rows surrounding the test plots consisting of a suitable floriferous malvaceous species which utilizes similar pollinators as *G. hirsutum* would lessen the possibility of cotton pollen moving out of these test plots and nurseries. These border plants would serve as a pollinator 'trap' to reduce long range pollen dissemination by insects and must include a species genetically incompatible with upland cotton. Additionally, the flowering period of the border trap crop must by congruous with that of the breeding nursery in order to be effective. However, if these nurseries and test plots are frequently treated with chemical insecticides likely to

eliminate all pollinators in the immediate area, the possibility of pollen movement would be negligible.

Presently there are no restrictions on planting of *B.t.* cotton in the Virgin Islands or Puerto Rico, both of which are known to have extant populations of wild *G. hirsutum* or *G. barbadense*. In the former case, no commercial plantings of cotton or test plots of *B.t.* cotton are planned for the U.S. Virgin Islands, hence, gene flow is not a concern there. Planting of *B.t.* cotton should be restricted, however, if prevention of gene flow is desired. Puerto Rico has seen use as a winter nursery for some commercial cotton breeding and the populations of wild *Gossypium* are apparently commonly distributed throughout the island. Similar restrictions, as imposed for Hawaii, would be appropriate in order to reduce the possibility of cotton pollen moving from the nurseries to wild cotton plants in this situation based upon the distribution of wild populations around the island and the propensity of insect pollinators to move substantial distances,. In both cases, monitoring of native populations of established *Gossypium* spp. may be necessary to assess the efficacy of this isolation procedure for *B.t.* cotton. This would entail monitoring of wild populations for evidence of gene introgression through PCR or similar sensitive methods. Alternatively, the absolute restriction of planting *B.t.* cotton in Puerto Rico and the U.S. Virgin Islands, would of course, alleviate any concerns over gene flow.

Potato - *Solanum tuberosum* ssp. *tuberosum*

EPA has reviewed the potential for gene capture and expression of the *B.t.* Cry3A plant-pesticide by wild or weedy relatives of cultivated potato in the United States, its possessions and territories. Based on data submitted by the registrant and a review of the scientific literature, EPA concluded that there is no foreseeable risk of unplanned pesticide production through gene capture and expression of the *Bacillus thuringiensis* subsp. *tenebrionis (B.t.t)* Colorado potato beetle control protein gene (Cry3A) in wild relatives of the transformed plant, *Solanum tuberosum* L in the U.S. or its possessions or territories.

***Solanum* spp. in the United States, Territories and Possessions**

Tuber-bearing *Solanum* species, including *S. tuberosum*, cannot hybridize naturally with the non-tuber bearing *Solanum* species in the U.S. Three species of tuber-bearing (section *Petota*) wild species of *Solanum* occur in the United States: *Solanum fendleri, Solanum jamesii,* and *Solanum pinnatisectum*. Successful gene introgression into these tuber-bearing *Solanum* species is virtually excluded due to constraints of geographical isolation and other biological barriers to natural hybridization (USDA/APHIS, March 1995). These barriers include incompatible (unequal) endosperm balance numbers (EBN) that lead to endosperm failure and embryo abortion, multiple ploidy levels, and incompatibility mechanisms that do not express reciprocal genes to allow fertilization to proceed. No natural hybrids have been observed between these species and cultivated potatoes in the U.S.

In the U.S., *S. fendleri* and *S. jamesii* are restricted to high elevation habitats in the continental Southwest, far removed from the centers of commercial potato production. Their distribution has been described by Hawkes (1999):

> 1) *S. fendleri* subsp. *fendleri* Asa Gray. Arizona, Colorado, New Mexico and Texas at 1,600 to 2800 meters in dry oak-pine forest, but not under dense shade.

2) *S. fendleri* subsp. *arizonicum* Hawkes. Arizona in pine forest clearings and roadsides from about 2000-2550 meters.

3) *S. jamesii* Torr. Arizona, Colorado, New Mexico, Texas, and Utah.

S. pinnatisectum is reported to be found in Arizona, though it is considered primarily a Mexican species (USDA/NRCS, 1999). While somatic hybrids (protoplast fusion) can be made and some of these fusions produced plants that can be backcrossed with potato, it cannot naturally cross with *S. tuberosum* because of abortion of hybrid endosperm (Thieme *et al.*, 1997).

Solanum spp. - Gene Flow

If plants of *Solanum tuberosum* (commercial potato) and either of the three native tuber-bearing species were to grow contiguously, cytological differences in ploidy level and/or endosperm balance number between the wild and cultivated species would bar successful hybridization and gene introgression (Johnston *et al.*, 1980). Controlled crosses between *S. fendleri* and *S. tuberosum*, for example, have been successful only with intermediate bridging crosses and have produced hybrids incapable of further sexual reproduction (Soest, 1986). This does not present a risk of spread because intermediate bridging crosses do not occur in nature.

All cultivated potatoes in the U.S. belong to the species, *S. tuberosum*. Although it is possible to produce potatoes sexually from true seed (Martin, 1987), commercial production of *S. tuberosum* in the United States is done asexually through the use of tubers. The production of fruits by the crop, when it occurs, is only incidental to plant growth necessary for tuber maturation. Therefore, even in cases where non-*B.t.* potato fields are in close proximity to *B.t.* potato fields, cross-pollination would not result in the tubers containing the *B.t.* gene since they are vegetatively propagated. Seed potato (*i.e.*, cut tuber pieces) production from such tubers would also be *B.t.* gene free.

Many barriers exist for gene transfer from CPB-resistant potatoes to other potato cultivars or free-living relatives. The widely planted cultivar, Russet Burbank, is male sterile. Other cultivars range from Shepody with "almost nil" pollen shed (Young *et al.*, 1983), and Atlantic, which is also largely male sterile (Schneider, 1995), to the self-fertile variety, Superior. Lack of floral nectaries and paucity of pollen production in many cultivars restrict insect-mediated (primarily bumblebees) cross pollination (Arndt *et al.*, 1990). Cross pollination drops to very low levels within a few meters of the pollen source (USDA/APHIS, 1995).

Berries produced by self- or cross-fertilization within potato fields have been reported to result in volunteer potato weeds in subsequent crops (Lawson and Wiseman, 1983). Factors reducing the probability of this event include: low self and / or cross fertility among many of the potato cultivars being grown in the United States, critical environmental conditions necessary for fruit set, even with fertile cultivars (Burton, 1989), and competitive disadvantage of seed-produced potatoes in tuber-produced fields. Therefore, CPB-resistant potatoes are unable to outcross to male-fertile potato cultivars, and the chances for successful cross-pollination of CPB-resistant potatoes by male-fertile potato cultivars and subsequent

seed production will be minuscule. The potential for the CPB-resistant potatoes to become an aggressive weed in the U.S. is negligible.

CONCLUSIONS

With most of the major food crops, wild relatives are not a major concern in the United States as many of these crop species had their centers of origin in Europe, Asia, Africa or South America . Exceptions to this include sunflower, squash, blueberry, cranberry and several trees used for pulp or wood. There are also cases wherein the crop species also has an introduced or naturally weedy relatives known to be problematic (*e.g.*, canola - mustards, sorghum - Johnson grass, oats - wild oats, creeping bentgrass - various *Agrostis* spp.). In these instances cross-pollination is assumed to occur unless the registrant can show data that this is not the case. This can be performed by demonstration of non-overlapping geographic distribution of species concerned or through the use of genetic or agronomic mechanisms to preclude transfer of fertile pollen. Data concerning pollen movement and hybridization potential will be critical for many plants since wild and feral populations of sexually compatible species exist within the U.S., its territories and possessions.

The Agency will look toward FIFRA-sanctioned scientific advisory panels and the academic community at large for input into the assessment of the risk involved with regulatory approval of PIPs.

DISCLAIMER: The content and opinions expressed in this analysis are those of the author and do not in any way constitute the policy or endorsement of the U.S. Environmental Protection Agency, the U.S. Department of Agriculture or the Food and Drug Administration.

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The Evolution of a Biological Risk Program: Gene flow between Wheat (*Triticum aestivum* L.) and Jointed Goatgrass (*Aegilops cylindrica* Host)

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ABSTRACT

The development of a biological risk program to determine the potential for gene flow between a cultivated species to a wild species involves a series of steps that lead from determining if there is a problem to developing ways to minimize the potential for undesirable gene flow. In the case of wheat and jointed goatgrass the presence of seed on hybrids between the two species in the field led to the initial studies to determine if there was the potential for gene flow by backcrossing to jointed goatgrass. Greenhouse and field studies confirmed that not only was backcrossing possible but that partial self-fertility could be restored in the second backcross generation. This indicated that gene movement between the two species would be possible in the field with the hybrid acting as a bridge for gene transfer. Once the potential for gene flow was confirmed, studies were then initiated to determining ways to minimize gene movement. In the case of wheat and jointed goatgrass it may be possible to reduce the transfer and retention of wheat genes in a jointed goatgrass background by locating the gene in wheat on a genome not shared with jointed goatgrass, and by using cultural practices to prevent the hybrid or BC_1 generation reducing the potential for restoration of self-fertility. These methods may reduce the potential for gene flow but it would still need to be determined if they reduce the risk to 'an acceptable level'.

INTRODUCTION

There is much concern today with the biological risk of genes moving from cultivated species to weedy relatives. One example would be the concern of genes moving from wheat to its weedy relative jointed goatgrass. What is of interest is why the concern is so much greater today than previously, even though jointed goatgrass and wheat have been cohabitating the same fields for many years. What will be presented is how research into the scientific curiosity of viable seed on a wheat x jointed goatgrass hybrid developed into a research program whose goal is to determine the potential for gene flow between the two species and how to minimize the risk of such gene flow occurring.

Jointed goatgrass has been a weed in wheat in the United States since the early 1900's (Mayfield 1927; Johnston and Parker 1929). The fact that hybrids could occur in the field has been known for almost as long. In fact, the ability to produce hybrids has been used by wheat genetics and breeding programs to transfer desirable genes from jointed goatgrass to wheat. The assumption was made that while seed could be produced on hybrids with controlled crosses in a greenhouse, seed production by backcrossing in the field would not occur. That is why in the early 1990's it was a surprise to find seed on wheat x jointed goatgrass hybrids in a foundation field of winter wheat in the Pacific Northwest.

Concerned more that a new hybrid weed species had developed rather than concerned with genes moving from wheat to jointed goatgrass, the viable seed off these hybrids were germinated and the mitotic chromosome number was determined. Based on the chromosome number it was determined that the seed was most likely the result of a backcross to wheat and if the backcross plant showed some fertility and backcrossed again that a plant similar to wheat and not jointed goatgrass would be produced. This eliminated the concern of a new hybrid species developing but it did raise two interesting questions; first, could a backcross with jointed goatgrass also occur and second, could this be a way for a gene to move from wheat to jointed goatgrass. The initial questions for a biological risk assessment program had been asked.

BIOLOGY OF WHEAT AND JOINTED GOATGRASS

To understand how hybridization could occur so readily between the two species one must understand the genetics and growth patterns of the two species. Wheat is an allohexaploid with genomes from three related diploid species. The three genomes are designated A, B, and D (Kimber and Sears 1987). Jointed goatgrass on the other hand is an allotetraploid with genomes from two related diploid species (C and D). The fact that the two species share the same genome from the donor species *Aegilops tauschii* allows for the production of hybrids and can explain the successful backcrossing that was observed in the field since the two D genomes in the hybrids pair and undergo normal chromosome separation during meiosis (Zemetra et al. 1998) which allows for the potential for viable gametes, especially female gametes.

The close genetic relationship also means that the two species will have similar growth patterns. Winter wheat is planted in the fall and requires a low temperature vernalization period to move from vegetative to the reproductive phase. Depending on temperatures in the late spring/early summer anthesis can be as short as 2-3 days or as long as 1-2 weeks. The more extended period of anthesis is found in areas of the Pacific Northwest such as the Palouse region of northern Idaho and eastern Washington where temperatures are moderate in the day and relatively cool at night. Differences in flowering date between different cultivars can extend this period to 1-3 weeks. Jointed goatgrass is also a winter annual that first emerges in the fall (Morishita 1996) and undergoes a vernalization period before flowering; but the requirement for vernalization may not be as strong in jointed goatgrass as it is in wheat. Flowering time for jointed goatgrass is similar to that found in wheat and in a small study the length of flowering period of jointed goatgrass covered the start of flowering of the earliest wheat cultivar to the end of flowering of the latest wheat cultivar. This complete overlap of flowering maximizes the time for cross-pollination and hybrid production to occur.

The shared genetic background and similar growth pattern is the reason that jointed goatgrass is a major weed species of wheat in the western United States (Donald and Ogg 1991). Because it emerges at the same time as wheat and is physiologically similar to wheat it is very difficult to control using conventional tillage practices and there no herbicides that can differentiate between jointed goatgrass and wheat. In the 1990's with the advent of successful genetic transformation of wheat, an alternative method was proposed: herbicide resistant wheat (Newhouse et al. 1992; Ball et al. 1999). This would allow for the elimination of jointed goatgrass from wheat fields without harming the wheat. Concurrently, herbicide

resistance in wheat was being developed using conventional chemical mutation techniques. The potential for the availability of herbicide resistance genes in wheat gave increased importance to the question whether genes could move from wheat to jointed goatgrass. While herbicide resistant wheat gave new hope for the control of jointed goatgrass, the specter of a more difficult to control herbicide resistant jointed goatgrass raised questions as to whether the technology should be used at all.

To answer the questions on whether gene flow could occur between wheat and jointed goatgrass more detailed studies needed to be done on fertility of hybrids and backcrosses, rate of restoration of self-fertility in backcross generations and the effect of environment (greenhouse versus field) on seed set. Without the ability to restore self-fertility by natural backcrossing in the field, hybrids would not serve as a conduit for gene flow and the concern for gene movement would be unfounded.

RESTORATION OF SELF-FERTILITY

Studies were conducted in both the greenhouse and field to study the fertility of wheat x jointed goatgrass hybrids and their backcross progeny. Initial studies were done in the greenhouse using both wheat and jointed goatgrass as the recurrent parents to determine the rate of self-fertility restoration for both directions of backcrossing (Zemetra et al. 1998). A second study was done to determine the difference in male and female fertility in backcrosses with jointed goatgrass as the recurrent parent (Wang et al. 2001). In the first study it was found that the percent seed set was the same regardless of the pollen donor species and that female fertility increased with each subsequent backcross generation. With jointed goatgrass as the recurrent parent, partial self-fertility was restored after two backcrosses while restoration of self-fertility with wheat backcrosses took an extra backcross generation (Table 1). In the study of male and female fertility of the hybrids and subsequent backcross generations, similar results were found. Female fertility increased with each backcross with initial male fertility lagging one generation behind (Table 2). The male fertility observed explains why hybrids were first thought to be sterile (no viable pollen) and that partial self-fertility could be restored in the BC_2 generation. The restoration of fertility followed the restoration of the recurrent parent's chromosome number (Table 1). As the number of homologous pairs increased and the number of univalents decreased, percent gamete viability would be expected to increase. While the restoration of self-fertility after only two backcrosses raised the concern for the movement and retention of wheat genes in jointed goatgrass, the question still remained whether such backcrossing would occur in the field.

For backcrossing to occur in the field pollen must be available for backcrossing and seed set must occur on hybrids and the various backcross generations in the field. Several studies have documented that wheat pollen can move anywhere from 60 m to 1000 m (Khan et al. 1973; Virmani and Edward, 1983). Pollen movement of jointed goatgrass has not been studied but it should be similar to wheat and the more open flower morphology of jointed goatgrass would indicate a greater potential for pollen movement. The production of hybrids between the two species is not surprising since out-crossing rates of up to 10% for wheat have been observed (Enjalbert et al. 1998) depending on both cultivar and year. The male sterility in the wheat x jointed goatgrass hybrids results in a very open floret allowing for pollination by either wheat or jointed goatgrass.

To determine if backcrossing did occur in the field hybrids and subsequent BC_1 plants were planted in the field with jointed goatgrass and, in the case of the hybrids, jointed goatgrass and wheat (Snyder et al. 2000). Seed was set on both the hybrids and the BC_1 plants demonstrating that backcrossing could occur under natural conditions. Since a BC_2 generation was produced, restoration of self-fertility would also occur in the field. Based on Wang et al. (2001) results in the greenhouse, the partially self-fertile BC_2 generation could also serve as a pollen source for out-crossing to jointed goatgrass. In a study with herbicide resistant wheat and jointed goatgrass, hybrids with seed were found in the control plots the second year (Seefeldt et al. 1998). Interestingly, six of the seven BC_1 plants proved to be herbicide resistant though that was not a surprise once it was determined that the backcrosses most likely involved the herbicide resistant wheat as the recurrent parent.

Based on these results it became apparent that there was a low possibility for backcrossing to occur in the field and with partial self-fertility restored after only two backcrosses, it was possible for genes to move from wheat to jointed goatgrass. While it could occur, wheat gene movement and retention through crossing and backcrossing to jointed goatgrass had not been observed or demonstrated. Also, while it may occur, were there genetic differences or crossing bottlenecks that could be exploited to minimize the potential for movement thus reducing the biological risk of gene flow.

RETENTION OF WHEAT GENES IN JOINTED GOATGRASS BACKCROSSES

If it is conceded that genes from wheat could move to jointed goatgrass, the next question is would these genes be retained over a series of backcrosses to jointed goatgrass? With the presence of shared and unshared genomes the question can be further refined to differentiate between retention of genes/chromatin on the shared genome compared to retention of genes/chromatin from the unshared genome. In studying retention of wheat genes from the shared genome there would be a high expectation of retention due to the homology between the D genomes of the two species as indicated by the formation of bivalents during meiosis in the hybrids. It has been proposed in other species that there may be a preference for the recurrent parent's chromatin that would reduce the retention of the cultivated species genes (Rieseberg et al. 1995). Two approaches were used to study this in wheat and jointed goatgrass backcrosses. The first was to use an herbicide resistance gene on the D genome in wheat and determine its retention in a BC_2 population that had not undergone any herbicide selection. The second method was to use molecular markers that differentiated between the D genomes of wheat and jointed goatgrass on the same BC_2

Table 1. Chromosome number, backcross seed set and self-fertility of wheat x jointed goatgrass hybrids and BC_1, BC_2, and BC_3 plants where jointed goatgrass or wheat were used as recurrent parents.

Cross		No. of	Female parent chromosome no.		Backcross percent seed set		Female parent percent self-fertility	
Female	Male	plants	mean	range	mean	range	mean	range
hybrid x jointed goatgrass		---	35	35	2.2	---	0.0	---
hybrid x wheat		---	35	35	2.0	---	0.0	---
BC_1 x jointed goatgrass		15	34	30 - 49	5.1	0.0 - 20.3	0.0	---
BC_1 x wheat		11	44	39 - 56	4.6	0.0 - 14.6	0.0	---
BC_2 x jointed goatgrass		9	33	31 - 36	37.4	6.7 - 77.0	20.9	0.0 - 73.2
BC_2 x wheat		5	43	40 - 46	13.7	6.9 - 27.8	0.0	---
BC_3 x wheat		3	43	41 - 44	55.8	48.7 - 65.5	61.3	55.1 - 70.1

From: Zemetra et al. (1998)

Table 2. The fertility of *Triticum aestivum* x *Aegilops cylindrica* F_1 hybrids and their backcross progenies when backcrossed to *A. cylindrica* (JGG).

Cross combinations	no. of plants	% seed set	range
Male fertility			
JGG x F_1	bulked	0.0	(/)
JGG x BC_1	12	1.8	(0 – 9.5)
JGG x BC_2	13	8.9	(0 – 38.5)
JGG x BC_2S_1	11	31.3	(0 – 78.6)
JGG x BC_2S_2	12	56.0	(27.8 – 88.5)
Female fertility			
F_1 x JGG	bulked	0.9	(/)
BC_1 x JGG	11	4.4	(0 – 13.2)
BC_2 x JGG	12	18.0	(0 – 81.0)
Self-fertility			
F_1	bulked	0.0	(/)
BC_1	11	0.06	(0 – 1.6)
BC_2	12	6.9	(0 – 24.8)
BC_2S_1	11	55.6	(0.6 – 90.2)
BC_2S_2	12	78.6	(48.1 – 94.3)

From: Wang et al. (2001)

population to determine if the wheat D genome markers were retained at the expected Mendelian frequencies (Kroiss 2001). In both cases the herbicide resistance gene and the majority of the wheat molecular markers were present at the expected frequencies. This indicated that if a gene is on the D genome of wheat it has the potential to be retained in the jointed goatgrass backcrosses after self-fertility has been restored. If herbicide selection pressure had been present (such as herbicide application in the field) the frequency of retention of the wheat gene would most likely be higher.

The presence of unshared genomes between wheat and jointed goatgrass may provide an avenue to reduce the potential for retention of wheat genes in the jointed goatgrass backcrosses. It has been observed that with each backcross the chromosome number is reduced and approaches that of the recurrent parent jointed goatgrass (Zemetra et al. 1998). Further, once partial self-fertility is restored, the extra chromosomes in the backcross continue to be lost with each subsequent generation of selfing. In a study using genomic in situ hybridization (GISH), Wang (2000) demonstrated that the extra chromosomes were from the A and B genomes of wheat and that they were present as univalents, making them susceptible to loss during meiosis. In a study of two BC_2S_2 families, one originated from a BC_2S_1 with 29 chromosomes and the other from a BC_2S_1 with 30 chromosomes, both cases displayed a

continued loss of the chromosomes from the unshared genomes. In the case of the BC_2S_1 with 29 chromosomes only 16% of the progeny retained the extra chromosome. With the BC_2S_1 plant with 30 chromosomes, 40% percent retained one extra chromosome and of that 40%, 37% (or 15% of the total) retained two extra chromosomes. GISH meiotic analysis of the BC_2S_2 plants with 30 chromosomes showed that the two chromosomes were univalents so they were non-homologous and susceptible to loss in subsequent generations of selfing. Based on these results, it appears that placement of a gene on an unshared genome could reduce the potential for retention in the jointed goatgrass backcrosses.

There are two possible ways that the chance of retention would increase for wheat genes found on unshared genomes with jointed goatgrass. The first would be if a translocation occurred between the A or B genome of wheat and either the C or D genome of jointed goatgrass. In his analysis of jointed goatgrass backcrosses using GISH, Wang et al. (2000) did find a few translocations between an A or B genome chromosome and a C genome chromosome in both a BC_1 plant and a BC_2S_2 plant. In the case of the BC_2S_2 plant it was selfed and its progeny checked for retention of the translocation. The translocation was not detected in 20 BC_2S_3 individuals and the normal jointed goatgrass chromosome constitution was restored (Wang 2000). While this demonstrates that retention due to translocation may not occur at a high frequency, this was only one case and further study would be needed to determine the actual frequency of chromosome rearrangement in the backcrosses.

The second way that the frequency of retention of genes from unshared genomes may be increased is by selection pressure. If there was selection pressure for the gene on the unshared genome it would remain at a higher frequency in survivors of each generation, increasing the potential for restoration of homology or for chromosome rearrangement. Research is currently underway using an herbicide resistance gene on either the A, B or D genome to study the effect of selection pressure on retention of genes on shared and unshared genomes. The results of this study should help determine if placement of a transgene on a specific genome can reduce the potential for movement and retention of the gene into a wild relative. This will have implications with wheat and other allopolyploids such as canola (*Brassica napus* L.).

REDUCING THE RISK OF GENE FLOW

Based on the assumptions that herbicide resistant wheat will go into commercial production and that there is the potential for genes to move between wheat and jointed goatgrass, the key question will be how can the risk of gene movement be reduced to an acceptable level? The first approach that most companies are taking is to place the gene on an unshared genome. As stated previously, with the loss of univalents with each backcross or self-generation, the potential for retention of a wheat gene is reduced compared to that of a gene on the shared D genome. While questions still remain on the effect of selection on the rate of retention and chromosome arrangement, locating a transgene on an unshared genome is a proactive move that can help reduce the potential for gene flow.

The other method that can be used to reduce the risk of gene movement is proper management of the transgenic crop to eliminate the generations necessary to allow for gene flow to occur (Wang et al. 2001). What is meant by that statement is that by eliminating the early generations (hybrid and BC_1) from the field the potential for the development of partially

fertile BC_2 plants that could self or cross to jointed goatgrass would be greatly reduced (Wang et al. 2001). While complete elimination of the weedy parent, jointed goatgrass, from the field would be the goal of the producer in using a herbicide resistant wheat, it is not realistic to believe that all the goatgrass would be eliminated. Therefore it should be expected that hybrids would be produced in the field, with either wheat or jointed goatgrass as the female parent. To prevent hybrids from being produced in a subsequent field from a cross to wheat as the female parent the seed from the field should not be replanted and sold for commercial use. Since jointed goatgrass spikelets easily disarticulate it is possible that hybrids resulting from a cross to jointed goatgrass as the parent would not be harvested and would remain in the field. The use of crop rotation and herbicides with different modes of action in subsequent years could be used to eliminate the hybrids that remained in the field. A question can be raised concerning the dormancy of the hybrid seed since jointed goatgrass is known to have some seed dormancy. Studies are currently underway to determine the level of dormancy in wheat x jointed goatgrass hybrids and how dormancy would affect a management plan to reduce the risk of gene movement.

If hybrids are found in a field, a similar approach can be taken to eliminate the production of a BC_1 generation. Insuring that the seed from that field is not replanted and crop and herbicide rotations are used would reduce the possibility of a BC_1 plant growing in a subsequent year, serving as a progenitor for BC_2 plants that would have the potential for restoration of self-fertility. The actual production of BC_1 plants that had jointed goatgrass as the recurrent parent is currently under investigation. Backcrossing to wheat in the field would slow the restoration of fertility and the transfer of the gene into a genetic background that could easily cross to jointed goatgrass. Research is currently under way using BC_1 seed collected from hybrids in fields in Washington, Idaho and Oregon to determine the rate of backcrossing to jointed goatgrass using GISH for the number of C genome chromosomes in the BC_1 plants (Wang et al. 2002). Such information would also give an indication of the level of risk for the production of partially self-fertile BC_2 plants if the management plans fail to eliminate hybrids and BC_1 plants from the field.

SUMMARY

In summary, the development of a biological risk assessment program involves several steps, with the conclusion of each step leading to one or more additional avenues of investigation. In the case of wheat and jointed goatgrass, what began as a scientific curiosity evolved into a study of the potential risk of gene movement based on the results of initial studies on restoration of self-fertility and the development of wheat that carried genes that could give a competitive advantage to jointed goatgrass. The evidence that genes could move between the two species and the potential for commercial development of herbicide resistant wheat then lead to a series of studies on how to minimize the potential for gene flow between the two species. The final step of such a risk assessment program is to determine if it is possible to develop a management plan for the herbicide resistant wheat that would minimize the potential for gene movement into jointed goatgrass to an acceptable level, allowing the use of the Herbicide resistant wheat to reduce the losses caused by weeds such as jointed goatgrass while reducing the risks of losing the technology due to creation of a herbicide resistant weed due to gene flow. The most difficult question to answer will be what is meant by 'an acceptable level'.

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