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Genetically Modified Food Crops: Current Concerns and Solutions for Next Generation Crops

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Introduction

The United States has a $500 billion annual market for food; nearly 50% of corn, cotton or soybean planted in 1999 has been genetically modified (GM) for producing either an insecticidal protein from *Bacillus thuringiensis* or resistance to certain herbicides. Benefits derived from these genetically modified crops include increase in productivity, conservation of topsoil and a decrease in the use of toxic herbicides/insecticides that would otherwise contaminate soil and water. For example, U.S. National Center for Food and Agricultural Policy reported that GM corn increased yield by 47 million bushels on 4 million acres in 1997, a year of high corn borer infestations and by 60 million bushels on 14 million acres in 1998. Around two million fewer acres of corn were sprayed with insecticides as a result. For cotton, yields were up 85 million pounds and 5 million fewer acres were sprayed with insecticides (Brower *et al.*, 1999). However, several environmental concerns have led to wariness and lack of public acceptance of genetically modified food crops around the world.

There are several concerns among the scientists as well as the public regarding genetically modified food crops. One of the primary concerns is the presence of clinically important antibiotic resistance genes in transgenic plants that could inactivate oral doses of the antibiotic, or such genes could be transferred to pathogenic microbes in the gastrointestinal tract or in soil, rendering them resistant to treatment with such antibiotics. In addition, there are several environmental concerns. In the case of

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Abbreviations: GM, genetically modified; *Bt* crops, crops modified with genes from *Bacillus thuringiensis*; CRY, insecticidal protein encoded by *Bt*; ATP, adenosine triphosphate; hpt, hygromycin phosphotransferase; Ac/Ds, transposable elements; ipt, isopentenyl transferase; MAT, multi-auto transformation system; T-DNA, transfer DNA from the tumour inducing plasmid of *Agrobacterium tumefaciens*; EPSPS, 5-enolpyruvyl shikimate-3-phosphate synthase; ORF, open reading frame; FLARE-S; Fluorescent Antibiotic Resistance Enzyme conferring resistance to spectinomycin/streptomycin.

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plants genetically engineered for herbicide resistance, primary concern is the escape of foreign genes through pollen or seed dispersal from transgenic crop plants to their weedy relatives, creating super weeds or causing gene pollution among other crops. Such dispersal of pollen from transgenic plants to surrounding non-transgenic plants has been well documented. The high rate of such gene flow from crops to wild relatives (as high as 38% in sunflower and 50% in strawberries) is certainly a serious concern.

Hoyle (1999) describes a number of situations where out-cross of nuclear transgenic plants with other crops have created serious problems. For example, organic crop production is a significant segment of Canadian agri-food industry, approaching one billion dollars in sales annually with 20% increase in sales each year in the recent past. However, Canadian farmers have lost European markets from 83 tons in 1994/95 to 20 tons in 1997/98 because of uncertainty of genetic purity and inability of farmers to guarantee that their produce is free of genetically engineered traits. At the same time, farmers who cultivate genetically engineered varieties also claim to be affected by genetic pollution. A canola farmer in Canada cultivated a glyphosate (Round-up) resistant cultivar (Quest) and a glufosinate (Liberty) resistant cultivar (Innovator) 30 metres away across an intervening road that exceeds the standard buffer zone of 6 metres. Two applications of Round-up herbicide in 1998 to the field sown with glufosinate resistant cultivar killed all the weeds but revealed glyphosate resistant canola in the field sown with other cultivars. This population was thickest near the road, where airborne dispersal of pollen from glyphosate resistant canola could occur. Meanwhile, a Canadian farmer is being sued by Monsanto for possessing and growing glyphosate resistant canola without a license. However, the farmer claims that his crops were contaminated by resistance genes via wind or bee pollination. Because of all these concerns, Canadian National Farmers Union is lobbying the Canadian Federal Government to legislate industry compensation for unintended genetic alteration of crops.

The use of commercial, nuclear transgenic crops for insect resistance, expressing *Bacillus thuringiensis* (*Bt*) toxins has escalated in recent years due to their advantages over traditional chemical insecticides. However, in crops with several target pests with varying degrees of susceptibility to *Bt* (eg cotton), there is concern regarding the sub-optimal production of toxin, resulting in reduced efficacy and increased risk of *Bt* resistance. Additionally, reliance on a single (or similar) *Bt* protein(s) (also known as CRY proteins) for insect control increases the likelihood of *Bt* resistance development. Additionally, because many CRY genes share over 90% protein homology, resistance to one CRY protein may confer resistance to another CRY protein. Nowhere is this of a greater concern than with the cotton bollworm/corn earworm, which usually feeds on corn in the spring and early summer, then migrates over to cotton to complete several more generations. The primary strategy currently used to delay development of insect resistance to *Bt* plants is providing refuges of host plants that do not produce *Bt* toxins. Such refuges should provide susceptible insects for mating with resistant insects. In order for such random mating to occur, resistant adults from non-*Bt* plants and susceptible adults from *Bt* plants must emerge synchronously. Rate of survival of hybrid insects (F1) created by such mating is less (2%) than resistant insects (37%), indicating that *Bt* resistance developed is recessive (Liu *et al.*, 1999). However, a recent study by Liu *et al.* (1999) of a resistant strain of pink

bollworm larvae on *Bt* cotton shows that resistant insects take a week longer to develop than susceptible insects on non-*Bt* cotton. Median longevity of the male pink bollworm is less than a week, and 80% of moths mate within three days of emergence. This developmental asynchrony favours assortative mating among resistant moths emerging from *Bt* plants. Such assortative mating would generate a disproportionately high number of homozygous resistant insects, accelerating the evolution of *Bt* resistance. This developmental asynchrony favours mating that could reduce the expected benefits of refuge strategy. Clearly, different insecticidal proteins should be produced in lethal quantities in order to decrease the development of resistance. There is also concern that pollen from *Bt* corn may be toxic to monarch butterflies (Losey *et al.*, 1999). This review explores the scientific validity of these concerns, and possible solutions to address valid concerns.

Genetic modification of plants free of antibiotic resistance genes

Antibiotic resistance genes have been routinely used to distinguish transgenic plants from untransformed plants. Once transgenic plants are generated, antibiotic resistance genes serve no useful purpose, but they continue to produce their gene products. The primary concern is the presence of clinically important antibiotic resistance genes in transgenic plants that could inactivate oral doses of the antibiotic, or such genes could be transferred to pathogenic microbes in the gastrointestinal tract or in soil, rendering them resistant to treatment with such antibiotics. Food & Drug Administration of the United States recently evaluated these questions for the use of kanamycin resistance in tomato, cotton and canola; they found that kanamycin and neomycin are very toxic antibiotics and, therefore, have very limited oral clinical use and that they are used only in situations where patients are not consuming food. FDA also observed that the co-factor ATP was not present in adequate quantities in food to degrade a significant amount of the antibiotic. FDA further observed that there is no known mechanism by which genes could be transferred from a plant chromosome to a microbe. However, the use of marker genes that encode resistance to other clinically useful antibiotics will be evaluated by FDA using the aforementioned criteria. Therefore, techniques are required for genetic engineering plants without the use of antibiotic resistance genes.

The assertion by the FDA that genes could not be transferred from a plant chromosome to a microbe has stimulated a number of investigations (Syvanen, 1999). When transgenic plants were fed to mice and the coliform bacteria isolated from faeces were examined for the presence of antibiotic resistance genes, none could be detected. When Erwinia, which causes spoilage of vegetables, was grown on transgenic plants, no transformants containing the antibiotic resistance gene were detected (Syvanen, 1999). In another investigation, the fate of plasmid DNA was monitored after being fed to mice. Although most DNA was rapidly degraded, a fraction was detected in faeces, lymphocytes and foetuses of pregnant females. However, transformation of the gut bacteria was not detected, and naturally occurring horizontal gene transfer has not yet been demonstrated (Syvanen, 1999). If such horizontal gene transfer were ever to occur, then several approaches are currently available to avoid the introduction of antibiotic resistance genes.

Several strategies have been tested to eliminate the presence of the antibiotic

resistance genes in transgenic plants. These strategies include excision of selectable marker genes via Cre/lox recombination (Dale and Ow, 1991), use of herbicide resistance as alternative selectable markers instead of the antibiotic resistance (De Block *et al.*, 1987), altered metabolic pathways (Perl *et al.*, 1993; Rathinasabapathy *et al.*, 1994) or co-transformation of plants with two vectors, one carrying the marker gene and the other carrying the gene of interest. By co-transformation approach, genes are integrated at different sites on the chromosome. Following the selection of transformed plants, traditional breeding techniques can be used to eliminate the selectable marker gene. In yet another approach, the selectable marker gene is cloned between plant transposable elements (Ds elements) and introduced along with a transposase gene that excises between the Ds elements and integrates into a site away from the gene of interest (Goldsbrough *et al.*, 1993). Once it is moved to a distant site, the selectable marker gene is eliminated via breeding. Thus, several new approaches are now in place to generate markerless transgenic plants. A few such examples are reviewed in this section.

Dale and Ow (1991) described a strategy for engineering plants free of selectable markers. A luciferase gene was introduced into the tobacco nuclear genome by using the hygromycin phosphotransferase gene (hpt) as a selectable marker. Flanked by recombinant sites from the bacteriophage P1 Cre/lox recombination system, the hpt gene was subsequently excised from the plant genome by the Cre recombinase. The Cre-catalyzed excision event in the plant nuclear genome was precise and did not alter nucleotides at the recombination site. After removal of the Cre-encoding locus by genetic segregation, transgenic plants were obtained that had incorporated only the gene of interest. Accomplishing gene transfer without the incorporation of antibiotic resistance markers in the host genome should ease public concerns, as well as obviate the need for different selectable markers in subsequent steps of genetic manipulation into the same host.

Scientists from Nippon Paper Industries, Japan (Ebinuma *et al.*, 1997) developed the multi auto-transformation system (MAT) in which the selectable marker is composed of a chimeric isopentenyl transferase gene inserted into the maize transposable element Ac. The ipt gene encodes the enzyme isopentenyl transferase and is located on the Ti plasmids of *Agrobacterium tumefaciens*. This enzyme catalyzes the condensation of isopentenyl pyrophosphate with AMP to produce isopentenyl AMP, a precursor of several cytokinins (Ebinuma *et al.*, 1997). Therefore, ipt genes are used to manipulate endogenous cytokinin levels and produce prolific shoots/roots in hormone free medium. However, ipt genes are not commonly used as selectable markers because the transgenic plants lose apical dominance and are unable to root due to over production of cytokinins. Ebinuma *et al.* (1997) used the maize transposable element Ac, which has the ability to move to new locations within a genome to remove the ipt gene. Ac elements that excise sometimes (about 10%) do not reinsert or reinsert into a sister chromatid and are lost subsequently during segregation. Thus, the MAT vector system provides yet another approach to produce marker-free transgenic plants, particularly without sexual crosses or seed production. This method could be particularly valuable for fruit and forest trees, for which long generation times are a more significant barrier to breeding and genetic analysis. The drawbacks of this system are the low frequency of the somatic loss of the Ac-element (0.5–1%), longer time for regeneration of plants, and variability in the morphological criteria

used to identify transgenic plants. In order to resolve this, Kunkel *et al.* (1999) have recently developed a dexamethasone inducible system to tightly regulate expression of isopentenyl transferase. Such a combined system allows introduction of multiple genes without the use of antibiotic resistance markers (Kunkel *et al.*, 1999).

Japan Tobacco Company scientists (Komari *et al.*, 1996) developed novel 'Super-binary' vectors that contained two separate T-DNAs. One T-DNA contained a drug resistance selectable marker gene, while the other contained the gene of interest. A large number of tobacco and rice transgenic plants were produced via *Agrobacterium* mediated transformation that carried the 'Super-binary' vectors. Frequency of co-transformation was about 50%; progeny that are drug sensitive, but containing the gene of interest, were obtained from more than half of the co-transformants. Therefore, non-selectable T-DNA was genetically separable from the selectable marker gene. Because several DNA fragments could be inserted into the non-selectable T-DNA, their Super-binary vectors may be useful in the production of marker-free transgenic plants of diverse plant species.

Co-transformation has been used to introduce a selectable marker gene and a gene of interest from separate T-DNAs into the plant nuclear genome. In transgenic plants in which transgenes are inserted at sufficiently unlinked loci, the gene of interest can be segregated from the selectable marker gene in the subsequent generation. Co-transformation has been accomplished by using a single plasmid with multiple T-DNAs, or separate plasmids with different T-DNAs that are contained in one or more *Agrobacterium* strains (Daley *et al.*, 1998). For example, transgenic rape seed and tobacco plants which do not contain a selectable marker gene were obtained using a single *Agrobacterium* strain containing two binary plasmids by Calgene scientists (Daley *et al.*, 1998). Genes from both plasmids were expressed in about 50% of the primary transgenic plants. Progeny expressing only one of the transgenes were observed in about 50% of the co-transformed lines, confirming that the genes were inserted at different loci. Therefore, by the single strain co-transformation method, one could use the selectable marker gene during regeneration of transgenic plants and subsequently recover marker-free progeny.

Out-cross concerns about herbicide resistant crops and possible solutions

Crops genetically engineered for herbicide resistance were the first to be field tested for an introduced novel trait. Several hundred such field tests have been done, or are currently in progress. These field trials clearly demonstrate the power of this gene technology in protecting crops to survive, while killing weeds very effectively. However, one serious limitation of this strategy is the creation of herbicide resistant weeds from widespread use of herbicide resistant crops. Unfortunately, such herbicide resistant weeds are harder to control because they develop resistance against most potent herbicides that are currently used for their control. Herbicide resistance confers survival in the presence of herbicides that otherwise cause death or severe reduction in growth.

Keeler *et al.* (1996) have broadly defined weeds in this context as follows: 'If biotechnology produces a plant that interferes with someone or something, it has produced a weed, regardless of taxonomic identity of the plant species'. By this definition, if pollen from genetically engineered crops out-crossed with crops of

neighbouring fields, thereby modifying their genotype, the resultant seeds would generate weeds. Therefore, the pollen mediated out-cross would generate weeds among crop plants. Such out-cross could have a deleterious effect on non-transgenic crops if pollen carries genes that would cause sterility in plants, such as the 'terminator' gene currently in development.

IS GENE POLLUTION A SERIOUS ENVIRONMENTAL CONCERN?

Creation of herbicide resistant weeds is no longer a theoretical prediction. Herbicide resistant populations of weeds have already reduced the utility of several herbicides among major crops (Keeler *et al.*, 1996). With only rare exceptions, all cultivated crops have wild relatives; therefore, escapes of transgenes are a strong possibility somewhere in the world. Dispersal of pollen from transgenic cotton plants to surrounding non-transgenic plants has been observed (Llewellyn and Fitt, 1996; Umbeck *et al.*, 1991). The escape of foreign genes through pollen is especially a serious environmental concern, in the case of herbicide resistance genes, because of the high rates of gene flow from crops to wild relatives. For example, the frequencies of marker genes in wild sunflowers averaged about 28 to 38%; in wild strawberries growing within 50 metres of a strawberry field, more than 50% of the wild plants contained marker genes from cultivated strawberries (King, 1996). Similarly, transgenic oil seed rape, genetically engineered for herbicide resistance out-crossed with a weedy relative, *Brassica campestris* (field mustard) and conferred herbicide resistance even in the first back-cross generation under field conditions (Mikkelsen *et al.*, 1996).

Keeler *et al.* (1996) have summarized valuable data on the weedy wild relatives of sixty important crop plants and potential hybridization between crops and wild relatives. This table is reproduced in this article (*Table 12.1*). Scientists reading this article are encouraged to fill data gaps in this table and expand this list by providing such information to original authors. Among sixty crops, only eleven do not have congeners (belonging to the same genus), and the rest of the crops have wild relatives somewhere in the world. A majority of these crops have wild relatives in the U.S.A. Authors also discuss examples of crops for which problems with herbicide resistance gene escape are highly probable, and warn against genetic engineering of such crops via the nuclear genome including rice, oats, sorghum, canola, sunflower, lettuce, artichoke, radish, etc. These crops are a challenge, and significant ingenuity will be required to provide crops with foreign genes that do not exasperate weediness in weedy, compatible wild relatives.

A number of situations where nuclear transgenic crops out-crossed with other crops have resulted in serious consequences (Hoyle, 1999), including loss of markets for organic crops because of uncertainty of genetic purity and contamination of genetically pure varieties with herbicide resistance genes, as pointed out in the introduction. Such situations have led to lawsuits against farmers, regulatory agencies and biotech companies. As pointed out by Crawley (1999), the important point is whether or not the product of cross-pollination poses a threat. If the hybrid plant is a problem, then genetic modification should not be introduced unless there is a method to contain pollen transfer. Distance will not protect from cross-pollination. For example, canola pollen can move up to 8 kilometres; pollen from corn and potatoes move about one

kilometre (Hoyle, 1999). Wind is only one of the common methods of pollen dispersal; insects are far more effective pollinators than wind. Therefore, gene pollution is indeed a serious environmental concern.

METHODS OF GENE ESCAPE FROM TRANSGENIC PLANTS

Escape of herbicide resistance genes to wild relatives occurs predominantly via dispersal of viable pollen. Keeler *et al.* (1996) focus on the role of gene flow to weedy wild relatives as a potential problem because, in their opinion, 'this is a far greater concern than any other mode of escape of transgenes'. These authors further point out that 'transgenes can only reach weed populations if carried to weeds on viable pollen; if the crop produces no pollen or viable pollen, there will be no gene flow'. The potential for gene flow via pollen depends on several factors, including the amount of pollen produced, longevity of pollen, dispersal of pollen (via wind, animal), plant/weed density, dormancy/rehydration of pollen, survival of pollen from toxic substances secreted by pollinators, and distance between crops and weeds. Keeler *et al.* (1996) point out that it is impractical to prevent out-cross between weeds and wind pollinated crops because of the large pollen clouds produced and distance travelled by viable pollen.

However, it is possible, under exceptional circumstances, for the herbicide resistant crop to be fertilized by pollen from wild relatives and serve as a female parent for a hybrid seed. If this happens, the hybrid seed may germinate and establish a resistant population. However, for this to happen, the herbicide resistant crop that served as the female parent must escape harvesting, and the hybrid seeds must survive to germinate, grow and reproduce. Alternatively, dispersal of seeds from transgenic plants may occur among weedy relatives during harvest, transportation, planting, and harvest. This can give rise to mixed populations. Introgressive hybridization could result in super weeds. This again would depend on the persistence of the crop among weeds and probability of forming mixed strands.

METHODS TO CONTAIN GENE ESCAPE

Genetic containment methods include suicide genes, infertility barriers, male sterility, and maternal inheritance. The latter two have been experimentally tested. Anthers, the male reproductive organs, are composed of several cell and tissue types and contain anther specific mRNAs (Mariani *et al.*, 1990). Anthers produce pollen grains that contain sperm cells. A specialized anther tissue called the tapetum plays an important role in the formation of pollen. The tapetum generally surrounds the pollen sac in early development and is not present as an organized tissue in the mature anther. The tapetum synthesizes a number of proteins that aid in pollen development or become components of pollen. Many male sterility mutations interfere with the tapetal cell differentiation and/or function, indicating that this tissue is essential for the production of functional pollen. Mariani *et al.* (1990) have shown that the 5'-region of a tobacco tapetum-specific gene (TA29) can activate the expression of β-glucuronidase and ribonuclease genes (RNase T1 and barnase) within the tapetal cells of transgenic tobacco and oil seed rape plants. Expression of RNase genes selectively destroyed the tapetum during anther development, prevented pollen

Table 12.1. Distribution of weedy wild relative of 60 herbaceous crop plants and potential for hybridization between crop and wild relatives

Species	No. of congeneric species reported as weeds	If so, how weedy, where?[a,b]	Compatibility of crop and relatives?
Apiaceae			
Apium graveolens	4	*A. leptophyllum S: HA; P:* 1 SAm, 1 Af; C: 5; X: 6 more) 2 spp. C in 3 countries, 1 X	Hybrids?
Daucus carota	9	6 C in 8 countries	Hybrids?
Pastinaca sativa	0		Does not hybridize with wild congeners
Petroselinum sativum	1	*P. segetum* C: Eu	Does not hybridize with wild congeners
Asteraceae			
Carthamus tinctorius	8	*C. lanatus* S: Aus, C 2 countries X 5 C. *oxyacantha* S: 2A, X 2 countries; 6 more C-X weeds	Not compatible with weedy wild relatives, will hybridize with wild relatives that are not considered weedy
Cichorium endivia	3	*C. intybus* S: 1 SAm, 3 Eu 1 A; P: 2, Eu 1 A, 2 Med; C: 9 incl. USA; X 12 C. *pumilum* P: Isr; C	Hybrids? 3 spp.
Cynara scolymus	3	C. cardunculus S: Arg, Aus; p: 1 Eu, X 4 2 C	Free hybridization between *C. scolymus* and *C. cardunculus*
Helianthus annuus	6	S: *H. ciliaris* USA; X: H, *californicus, Ciliaris, grosseserratus, maximiliani, Petiolaris*, and *tuberosus*	1 hybridizes freely with *H. annuus* (*H. petiolaris*)
H. tuberosus	6	Only *H. annuus* (c.f.) S: Mex; P: Arg, USA	Does not hybridize with any weedy congeners
Lactuca sativa virosa,	19	*L.capensis* P: 1 Af; *L. floridana* C: USA; *L. scariola* . P: 1 Med, *L serriola P: USA;* C: 1 Af, Aus, Can, ; Med 1 SAm L. taraxacifoliaP: 1 Af	Hybridizes with some wild relatives (*L. Serriola, L. L. saligna;* C: 1 A, Aus)
Brassicaceae			
Brassica campestris	13	*B. juncea P*: Can; C: Arg, Aus; X: 1 Oc, 1 CAm, USA; *B. kaber* P: Can; C: 1 Med, USA; *B. rapa* P: SAm; C: 1 Af, 2 U, USA; *B. tournefortii P*: Aus; C 1 Med; X 1 Oc; *B. nigra (=Sinapis nigra)* S USA, C: 10 countries, X: 7 countries	*B. campestris* hybridizes with *B. nigra* and *B. napus*
B. napus	14	See *B. Campestris*	Hybridizes readily with *B campestris*
B. oleracea	14	See *B. campestris*	Hybridizes with European wild congeners that are not considered weedy
Rorippa nasturtium-aquatica	1	*Rorippa sp.* P: A; C: 6 in; 8 X (incl. 2 in USA) 2 *Nasturtium* spp. X: 1 country each	Watercress is incompatible with wild relatives
Raphanus sativus	2	*R. raphanistrum* S: A, Af, Eu, Oc, SAm, USA; *R. microcarpus* C: Eu	Hybridizes with *R. raphanistrum, R. maritimus*, and *R. Landra*
Chenopodiaceae			
Beta vulgaris	0	No weedy congeners; incompatible with wild congeners	
Spinacia oleracea	0	No weedy congeners; incompatible with wild congeners	

Convolvulaceae			
Ipomoea batatas	57	*I. cordofana* P: Af; *I. hederacea* S: USA; *I. lacunosa* P: Sam; *I. panduata* S: USA; *I. purpurea* S: USA; *I. tiliacea* P: SAm; *I. triloba* P: Oc; S: CAm, HA, USA; *I. wrightii* P: USA	*I. batatas* is not very fertile, but is compatible with *I. triloba* and others of the *I. batatas* complex
Cucurbitaceae			
Citrullus lanatus	1	C: Aus	Incompatible with weedy relatives
Cucumis melo	8	*C. myriocarpus* P: Aus; *C. anguria* P: USA	Hybridizes with wild relatives that are not considered weeds
C. sativus	8	See *Cucumis melo;* also *C. melo* P: SAm	Hybridizes with *C. hardwickii* in Asia, which is not considered very weedy
Cucurbitra maxima	4	*C. texana* P: USA; *C. pepo* X: USA	Hybridizes with nonweedy congeners in SAm
C. moschata	4	*C. texana* P: USA; *C. pepo* X: USA	Hybridizes with nonweedy congeners in SAm
C. pepo	3	See *C. maxima*	Hybridizes with nonweedy congeners in SAm
Euphorbiaceae			
Ricinus communis	0	No other species in genus	No compatible relatives
Fabaceae			
Arachis hypogaea	0	No weedy congeners	Hybridizes with nonweedy congeners, including *A. monticola*
Cicer arietinum	0	No weedy congeners	No hybrids with other members of this genus of 39 southern Asian species
Glycine max	2	*G. soya* C: Jpn, another sp. X: Af	Hybridizes with *G. soya* to produce weedy *G. gracilis*
Lens culinaris	0	No *Lens* sp. are weedy	Interfertile with *I. orientalis*, which is not considered weedy
Medicago sativa	20	*M. lupulina* P: A; C: USA; *M. polymorpha* C: USA; 10 other spp. C (total of 17 countries).	Hybridizes with *M. sativa* var. *falcata*, to make *'M. varia'* populations
Phaseolus lunatus	7	*P. lathyroides* P: Aus; C: HA, A; *P. trilobus* P: A	Hybrids?
P. vulgaris	7	See *P. lunatus*	Hybrids occur, although in general there is little geographical overlap between wild and cultivated species; the hybrids are hard to classify, leading to taxonomic problems defining the species.
Pisum sativum	1	*P. elatius* C: NAf, Por	*p. arvense* and *p. elatius* (the latter is weedy)
Vicia faba	25	*V. sativa* S: A, Eu USA; P: A, Oc; C, X: 27 countries; *V. cracca* P: I Eu, USA; *V. hirsuta* P: A; *V. narbonensis* P: NAf; *V. villosa* P: USA; 13 more spp. C somewhere in the world	Does not hybridize with wild congeners (Hancock, 1992)
Liliaceae			
Allium ampeloprasm	13	*A. macrostemon* P: A; *A. nigrum* P: NAf; *A vineale* P: Aus, Tur, USA; *A. canadense* P: USA; 4 spp. C somewhere	
A. cepa	13	See *C. ampeloprasm*	
A. sativum	13	See *C. ampeloprasm*	
Asparagus officinalis	1	1 *A. lucidus* C: A	Long history in cultivation, wild relatives in southern Asia

Table 12.1. *cont.*

Species	No. of congeneric species reported as weeds	If so, how weedy, where?[a,b]	Compatibility of crop and relatives?
Linaceae			
Linum usitatissimum	5	*L. peyroni* C: NAf	Forms fertile hybrids with *L. africanum* and *L. angustifolium,* and 5 others, of which *L. angustifolium* is considered a weed
Malvaceae			
Gossyium hirsutum	0	No *Gossypium spp.* are weeds	Hybridizes with wild congeners that are not weedy
Abelmoschus esculentus	0	No *Abelmoschus* spp. are weedy	Okra is incompatible with its wild relatives
Poaceae			
Avena sativa	10	*a. barbata* p: Eu X: USA; *a. byzantina* P: Arg, Af; *A. fatua* S: Arg, Aus, Can, Eu, SAf, USA; P. CAm, SAm; C, X: 34 more countries; A. *sterilis* spp. *ludoviciona* S: Aus, Eu; P: Af. A; *A. sterilis* S: Aus Med; A *strigosa* S: Af; 2 others C in 3 countries	Hybridizes with very weedy *A. fatua* despite ploidy differences
Hordeum vulgare	13	*H. jubatum* S: AL; C: Can, USA; *H. murinum* S: Aus, Oc; P: Med; C X 24 countries, including USA (X); *H. leporinum* P: Aus; X: Arg, USA; 7 others are C in 8 countries (*H. pusillum* USA)	Chiefly hybridizes with *H. spontaneum* which is sometimes considered part of *H. vulgare*
Oryza sativa	6	*O. punciata* S: Af; *O. barthii* P: Af; *O. perennis* P: 1 Af; 1 other is C in 1 country	Taxonomic debates put red rice (variously *O. rufipogon* and *O. Sativa* var. *rufipogon*) in and out of the same species as cultivated rice.
Panicum miliaceum	59	*P. fasciculatum* S: CAm, SAm; P: SAm; C: As; X: USA; *P. maximum* S: Aus, SAm, CAm, Af, HA; P: 1 Car, SAm, Af, CAm; C: 3 countries X: 22, incl. USA; *P. repens* S: Af, Eu, HA, Oc, As; P: 2 Oc Af, C: 5 countries, incl. USA	Hybrids?
Pennisetum glaucum	13	*P. japonicum* P: Jpn; *P. macrourum* P: Aus; *P. pedicellatum* S: Af, As; P: Aus; *P. polystachyon* S: As; P: As; *P. purpureum* S: SAm, Af; C: HA, Oc, CAm; X: 14 countries, incl. USA	Hybrids?
Saccharum officinarum	4	*S. behghalense* P: As; *S. spontaneum* S: As, Oc;P: As, CAm; X: 20 countries; 2 spp. X in 2 countries	All *Sacchrum* sp. are interfertile
Secale cereale	1	*S. montanum* X: Tur	Weedy annual *Secale* sp. are close enough to rye to be considered *S. creale* by some Authors
Sorghum bicolor	11	*S. bicolor* ssp. *arundinacuum* P: SAm; X: Af; *S. halepense* P: Arg, Aus, SAm, CAm, Oc, Eu, HA, As, Med, USA; S: Oc, SAm, Af, Eu, CAm; C: 18 other countries; *S. bicolor* spp. *arundiancuum* S: Af; P: Af, USA; C: 3 other countries;	*S. bicolor* (2×) hybridizes freely with the serious weed *S. halepense* (4×)

		S. vulgare, *S. bicolor* ssp. *bicolor* P: As, SAm; C: countries, 2spp. C in 3 countries 5 spp. X (2 in USA)	
Triticum aestivum	1	*T. ramosum* X: Eu, As	Hybridizes infrequently with *Aegilops (Triticum) cylindrica*, jointed goatgrass (a P weed in incl. USA)
T. turgidum	2	*T. aestivum* X: As; *T. ramosum* X: Eu, As	Hybrids form with *Secale*, *Hordeum*
Zea mays	0	No members of Zea are weeds	Hybridizes with teosinte (*Zea* spp.), but teosintes not considered weedy
Polygonaceae			
Rheum rhaponiticum	0	No weeds in Rheum	Propagated asexually by dividing clumps, seeds Infertile; does not hybridize with congeners
Solanaceae			
Capsicum annuum	2	*C. frutescens* X: As, CAm, Oc, Med; *C. baccatum* X: Med	Hybridizes with congeners that are not considered weeds
C. annum var. frutescens	2	See *C. annuum*	Hybridizes with congeners that are not considered weeds
Lycopersicon esculentum	2	2 spp. ×: both in Per	Yes?
Nicotiana tabacum	7	*N. glauca* C: Aus, HA, Af; X: SAm, Oc, USA; *N. longiflora* C: Arg; *N. suavelolus* C: Aus; 4 spp. X in 1 country each (*N. trigonophylla* USA)	Cultivated tobacco is the hybrid of two semiweeds of SAm
Solanum melongena	66	*S. alatum* P: Med; *S. americanum* P: USA; *S. auriculatum* P: Aus; *S. carolinense* P: USA; C: Can; *S. dubium* P: Af; *S. dulcamara* C: USA; S. *elaegnifolium* S: Af, USA; P: Aus; C: Arg, Af; X: SAm, CAm, USA; *S. glaucophyllum* P: Arg; *S. gracilius* P: SAm; *S. grossedentatum* P: Af; *S. hystrix* P: Aus; *S. nigrum* S: A, Aus, HA, Med, Oc, USA; P: Af, Can, CAm, Eu, SAm; C 14 other countries, incl. USA; X: 32 others); *S. nodiflorum* S: HA; C: Af; *S. ptycanthum* P: USA; *S. rostratum* S: Mx, USA; C: 1 Af, Aus; *S. saccaroides* P: USA; *S. torvum* S: Oc; P: A, Af, Aus, Oc; C: 3 other countries, incl. USA; *S. triflorm* P: Aus, USA; *S. tuberosum* P: USA; *S. villosum* S: Med; C: 1 Af; X: USA; *S. xanthocarpum* P: 1 A, As; 23 spp. C weeds somewhere	Hybridize?A
S. tuberosum	66	See *S. melongena*	Hybridizes with relatives in the subsection *Potato* which are not considered weeds

Table reproduced from Keeler *et al.*, 1996, with kind permission from CRC press.

[a] Locations: NAm = North America, Cam = Central America, SAm = South America, A = Asia, Af = Africa, Eu = Europe, Oc = Oceania, USA = United States, Sov = former Soviet Union, AL = Alaska, Per = Peru, Can = Canada, HA = Hawaii, Med = Countries around the Mediterranean Sea, Mx = Mexico, Arg = Argentina, Aus = Australia, NZ = New Zealand, Jpn = Japan, Car = countries around the Caribbean Ocean, Sir = Israel, NAf = North Africa, Por = Portugal, Tur = Turkey, SAf = South Africa.

[b] Severity of weed problem: S = serious, P = principal, C = common, X = present and weedy, but importance uncertain (after Holm *et al.*, 1979), S = troublesome weed of several crops and states, P = troublesome weed of a few or one crop or state, C = common weed, as listed in Bridges (1992).

[c] Major sources: Holm *et al.*, 1979; Schery, 1972; Bridges, 1992; Hancock, 1992.

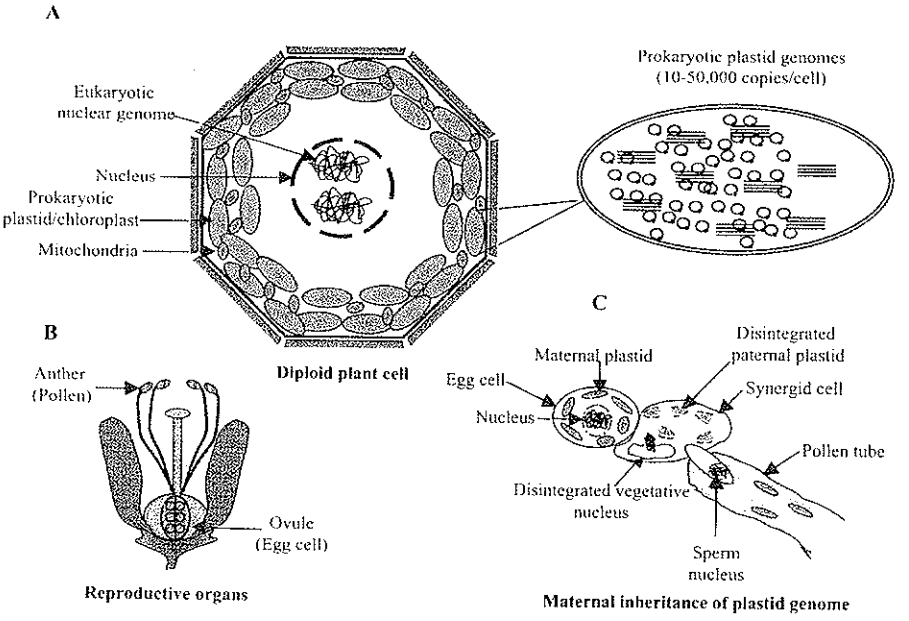

Figure 12.1. Transformation of cellular organelles. A. Foreign DNA is most commonly introduced into the nuclear genome resulting in integration of a few copies of the foreign gene. However, when introduced via the plastid genome, as many as 10,000 copies of the foreign gene per cell are stably integrated. B. Reproductive organs: After meiosis, haploid egg and sperm cells are formed. C. Yet another advantage of introducing foreign gene via the chloroplast genome is that the zygote contains only maternal plastids because the paternal plastids disintegrate in the synergid cell. This results in biocontainment of the introduced genes based on lack of gene flow (out-cross) through pollen.

formation and produced male sterile plants. This approach could be used to contain any out-cross of transgene with other crops or weeds. However, male sterility is possible only in crops where the product is not a seed or fruit requiring fertilization (like lettuce, carrot, or cabbage).

Maternal inheritance of a herbicide resistance gene and prevention of escape via pollen has been successfully demonstrated recently (Daniell *et al.*, 1998). Engineering foreign genes through chloroplast genomes (which are maternally inherited in most of the crops, see *Figure 12.1*) is a practical solution to this problem. In addition, the target enzymes or proteins for most herbicides (of the amino acid/fatty acid biosynthetic pathways or photosynthesis) are compartmentalized within the chloroplast. An example of genetic engineering herbicide resistance via chloroplast genome to overcome out-cross and gene pollution problems is discussed below.

MATERNAL INHERITANCE OF CHLOROPLAST GENOMES

Scott and Wilkinson (1999) have recently analysed several factors that would influence the transgene movement of chloroplast genes from crops to wild relatives under natural conditions. They studied the mode of inheritance of plastids, incidence of sympatry to quantify opportunities for forming mixed populations, and persistence of crops outside agriculture limits for introgression. They studied plastid inheritance

in natural hybrids collected from two wild populations growing next to oilseed rape along 34 km of the Thames River and assessed the persistence of 18 feral oil seed rape populations over a period of three years. These studies concluded that there would be no pollen-mediated transgene movement from oilseed rape. A low incidence of sympatry (0.6–0.7%) between the crop and weed species occurred; however, mixed strands showed a strong tendency towards rapid decline in plant number, seed return and, ultimately, extinction within three years. Thus, they concluded that gene flow would be rare if plants are genetically engineered via the chloroplast genome.

The prevalent pattern of plastid inheritance found in the majority of angiosperms is uniparental maternal, and chloroplast genomes are maternally inherited for most of the crops (*Figure 12.1*). However, there are always exceptions to most observations and maternal inheritance of chloroplast genomes is certainly not without exception. It is known that in pines (gymnosperms) plastids are transmitted in a biparental mode. Paternal transmission *via* pollen in tobacco has been reported, but with provisos. In transmission of paternal chloroplasts in tobacco, authors mention that there is occasional (0.07–2.5%) paternal transmission in a species typically exhibiting strict maternal inheritance. In rapeseed, paternal mitochondrial DNA is transferred to the egg but not the chloroplast DNA (Daniell and Varma, 1998).

AN EXAMPLE OF GENETIC ENGINEERING OF HERBICIDE RESISTANCE VIA CHLOROPLAST GENOME TO OVERCOME GENE POLLUTION

Glyphosate is a potent, broad-spectrum herbicide, which is highly effective against a majority of grasses and broad leaf weeds. Glyphosate works by competitive inhibition of the enzyme 5-enol-pyruvyl shikimate-3-phosphate synthase (EPSPS) of the aromatic amino acid biosynthetic pathway. Synthesis of EPSP from shikimate 3-phosphate and inorganic phosphate is catalyzed by EPSPS. This particular reaction occurs only in plants and microorganisms, which explains the non-toxicity of glyphosate to other living forms. Use of glyphosate is environmentally safe as it is inactivated rapidly in soil, has minimum soil mobility, and degrades to natural products, with little toxicity to non-plant life forms. However, glyphosate lacks selectivity and does not distinguish crops from weeds, thereby restricting its use. EPSPS based glyphosate resistance has been genetically engineered by the overproduction of the wild type EPSPS (Shah *et al.*, 1986) or by the expression of a mutant gene (aroA) encoding glyphosate resistant EPSPS (Cioppa *et al.*, 1987). In all of the aforementioned examples, without exception, herbicide resistance genes have been introduced into the nuclear genome, thereby increasing the risk of out-cross to weedy relatives. In this section, an example of genetic engineering plants for herbicide resistance via the chloroplast genome is discussed. For details not provided in this review, the reader is referred to Daniell *et al* (1998).

Chloroplast integration and expression vectors with EPSPS

The chloroplast vector pZS-RD-EPSPS contains the 16S rRNA promoter (Prrn) driving the *aadA* (aminoglycoside adenyl transferase) and EPSPS genes with the *psbA* 3' region (the terminator from a gene coding for photosystem II reaction centre components) from the tobacco chloroplast genome. This construct integrates the

EPSPS and *aadA* genes into the spacer region between the *rbcL* (the gene for the large sub-unit of RuBisCO) and *orf*512 genes (code for the *accD* gene) of the tobacco chloroplast genome. This vector is useful to integrate foreign genes specifically into the tobacco chloroplast genome; this gene order is not conserved among other plant chloroplast genomes (Maier *et al.*, 1995). On the other hand, the universal chloroplast expression and integration vector pSBL-RD-EPSPS can be used to transform chloroplast genomes of several other plant species because the flanking sequences are highly conserved among higher plants; the universal vector uses *trnA* and *trnI* genes (chloroplast transfer RNAs coding for Alanine and Isoleucine) from the inverted repeat region of the tobacco chloroplast genome as flanking sequences for homologous recombination.

Characterization of chloroplast transgenic plants expressing EPSPS

Transgenic plants were obtained within 3–5 months after bombardment, as described by Daniell (1993, 1997). Typically, out of 16 bombarded leaves, 10 independently transformed shoots were identified. The integration of the aroA gene into the chloroplast was confirmed by PCR and Southern analyses. In addition, the high level of resistance to glyphosate observed was confirmed by determination of the copy number of the foreign gene in the transgenic plants. The copy number of the integrated gene was determined by establishing homoplasmy for the transgenic chloroplast genome. Tobacco Chloroplasts contain 5,000–10,000 copies of their genome per cell (McBride *et al.*, 1995). If only a fraction of the genomes are actually transformed, the copy number, by default, must be less than 10,000. By establishing that in the transgenics the EPSPS transformed genome is the only one present, one could establish that the copy number is 5,000–10,000 per cell. This proves that only the transgenic chloroplast genome is present in the cell and there is no native, untransformed, chloroplast genome, without the EPSPS gene present. This establishes the homoplasmic nature of transformants, simultaneously providing an estimate of about 10,000 copies of the foreign EPSPS gene per cell. This would explain the high levels of tolerance to glyphosate observed in transgenic plants.

Seeds collected from transgenic plants after the first self-cross were germinated in the presence of spectinomycin. All of the seeds germinated remained green and grew normally (*Figure 12.2B*). The 100% resistance to spectinomycin in all of the clones examined shows maternal inheritance of the introduced genes. A heteroplasmic condition would have given rise to variegated progeny on spectinomycin (Svab and Maliga, 1993); lack of such variegated progeny also confirms homoplasmy, as confirmed by Southern blot analysis. All of the untransformed seedlings were bleached and did not grow in the presence of spectinomycin (*Figure 12.2A*). The lack of variation in chlorophyll pigmentation among the progeny also underscores the absence of position effect, an artifact of nuclear transformation (*Figure 12.2B*).

Eighteen-week-old control and transgenic plants were sprayed with equal volumes of different concentrations (0.5 to 5 mM) of glyphosate. Untransformed control tobacco plants were extremely sensitive to glyphosate; they died within seven days even at 0.5 mM glyphosate (*Figure 12.3B*). On the other hand, the chloroplast transgenic plants survived concentrations as high as 5mM glyphosate (*Figure 12.3A*). These results are intriguing, considering the fact that the EPSPS gene from petunia

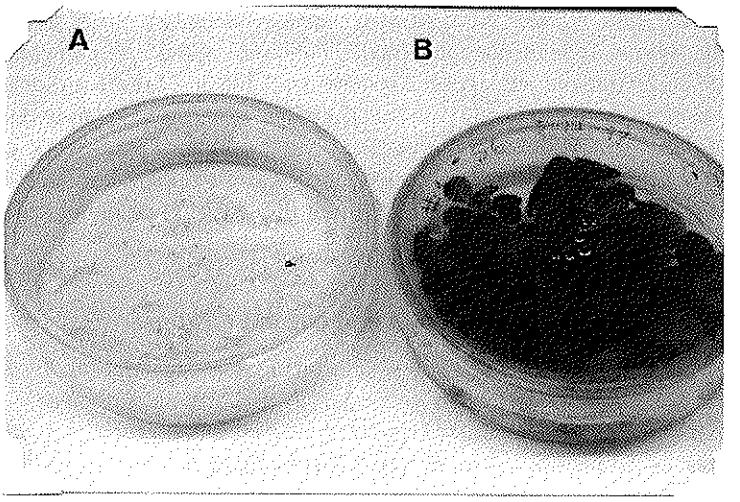

Figure 12.2. Analysis of maternal inheritance in the seed progeny of chloroplast transgenic plants. The control and transgenic seeds were germinated in MSO medium containing spectinomycin (500μg/ml). (A) Control seedlings. (B) Transgenic seedlings.

Figure 12.3. Herbicide resistance in the progeny of chloroplast transgenic plants. Eighteen week old plants sprayed with 5mM glyphosate. (A) Transgenic Plants. (B) Control plants.

used in these chloroplast vectors has a low level of tolerance to glyphosate. Sensitivity to glyphosate by EPSPS should have been compensated by overproduction of the enzyme by thousands of copies of the EPSPS gene, present in each cell of the transgenic plants. Also, this is the first report of expressing an eukaryotic nuclear gene within the prokaryotic chloroplast compartment. It is well known that the codon preference is significantly different between the prokaryotic chloroplast compartment

and the eukaryotic nuclear compartment. Ideally, a mutant aroA gene from a prokaryotic system (which does not bind glyphosate) should be expressed in the chloroplast compartment; such genes are now available and exhibit a thousand-fold higher level of resistance to glyphosate than the petunia gene used in this investigation. In light of these observations, it is possible that integration of prokaryotic herbicide resistance genes into the chloroplast genome could result in incredibly high levels of resistance to herbicides, while still maintaining the efficacy of biological containment.

Concerns about insect resistant crops and possible solutions

The use of commercial, nuclear transgenic crops expressing *Bacillus thuringiensis* (*Bt*) toxins has escalated in recent years due to their advantages over traditional chemical insecticides. However, in crops with several target pests, each with varying degrees of susceptibility to *Bt* (eg cotton), there is concern regarding the sub-optimal production of toxin, resulting in reduced efficacy and increased risk of *Bt* resistance. Additionally, reliance on a single (or similar) *Bt* protein(s) for insect control increases the likelihood of *Bt*-resistance development (Tabashnik *et al.*, 1990). Most current commercial transgenic plants that target lepidopteran pests contain either Cry1Ab (corn) or Cry1Ac (cotton) (Koziel *et al.*, 1993; Perlak *et al.*, 1990). *Bt* corn is targeted primarily against European corn borer, although other pests such as the corn earworm or cotton bollworm may be affected. *Bt* cotton is targeted primarily against the tobacco budworm. However, other pests such as armyworms and cotton bollworm are economically damaging but have only limited susceptibility to Cry1Ac. Use of single *Bt* proteins to control insects such as tobacco budworm and cotton bollworm could lead to relatively rapid *Bt* resistance development (Gould, 1998; Gould *et al.*, 1992). Additionally, because Cry1Ab and Cry1Ac share over 90% protein homology, resistance to one Cry1A protein would most likely impart resistance to another Cry1A protein, as has been observed in tobacco budworm (Gould *et al.*, 1995, 1992). Nowhere is this more of a concern than with cotton bollworm/corn earworm, which usually feeds on corn in the spring and early summer, then migrates over to cotton to complete several more generations (Gould, 1998). Clearly, different *Bt* proteins are needed in order to decrease the development of resistance. Plant-specific recommendations to reduce *Bt* resistance development include increasing *Bt* expression levels (high dose strategy), expressing multiple toxins (gene pyramiding), or expressing the protein only in tissues highly sensitive to damage (tissue specific expression).

Evolving levels of *Bt* resistance in insects should be dramatically reduced through the genetic engineering of the chloroplast genome in transgenic plants. When transgenic tobacco leaves expressing Cry2Aa2 protoxin in chloroplasts were fed to susceptible, Cry1A resistant (20,000–40,000-fold), and Cry2Aa2 resistant (330–393-fold) tobacco budworm, cotton bollworm and the beet armyworm, 100% mortality was observed against all insect species and strains (Kota *et al.*, 1999). Also, multiple *Bt*, as well as other insecticidal proteins, could be expressed in a single operon in transgenic plants because chloroplasts, unlike the nucleus, process polycistronic transcripts (Daniell *et al.*, 1994). Therefore, one such example of chloroplast genetic engineering is discussed below.

AN EXAMPLE OF GENETIC ENGINEERING CROPS FOR INSECT RESISTANCE TO OVERCOME *BT* RESISTANCE AND TOXICITY OF TRANSGENIC POLLEN TO NON-TARGET INSECTS

The tobacco chloroplast expression vector described above was also used to introduce a novel *Bt* coding sequence into the chloroplast genome. This class of *Bt* proteins, Cry2A, is toxic to many caterpillars, such as the European corn borer and tobacco budworm, and is quite different in structure/function from the Cry1A proteins (resulting in less cross-resistance). Cry2A proteins are about half the size of Cry1A proteins, and therefore should be expressed at higher levels. Because of similar protein synthetic machinery between chloroplasts and *E. coli* (Brixey *et al.*, 1997; Guda *et al.*, 1999), *cry*2Aa2 expression of the tobacco vector in *E. coli* was analysed by western blots using Cry2A antibodies. This showed the presence of the 65 kDa Cry2Aa2 protein. Tobacco leaves then were bombarded with DNA-coated tungsten particles, as described elsewhere (Daniell, 1993, 1997). As many as thirteen putative transformants were obtained out of 13 bombarded leaves. The positive clones were analysed by PCR and Southern hybridization to confirm the site-specific integration of *cry*2Aa2, and to establish copy number, as explained before. Insect bioassays were done as described (Kota *et al.*, 1999). There was 100% mortality of tobacco budworm

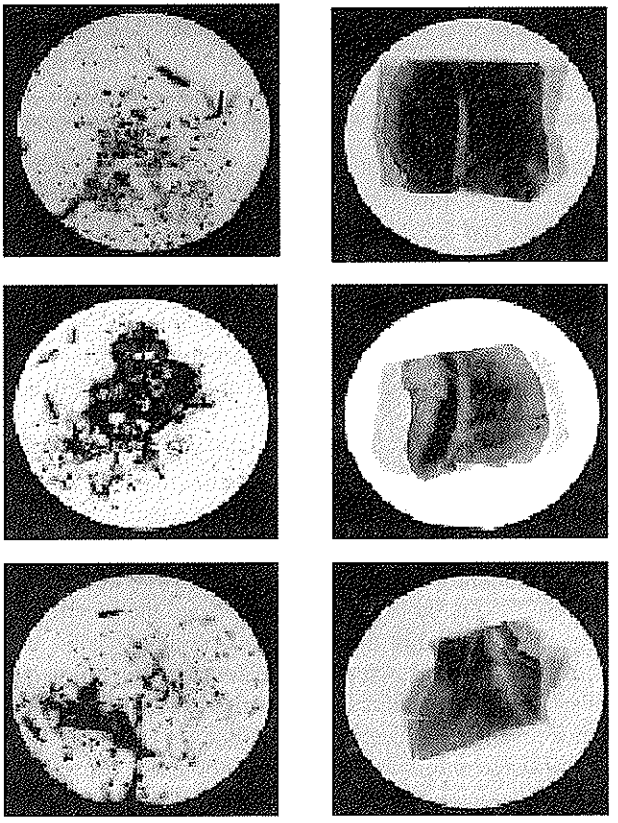

Figure 12.4. Leaf bioassay of control (left) and Cry2Aa2 chloroplast transgenic tobacco leaves (right) assayed against various tobacco budworm strains. Susceptible (top), Cry1Ac-resistant (middle), Cry2Aa2-resistant (bottom).

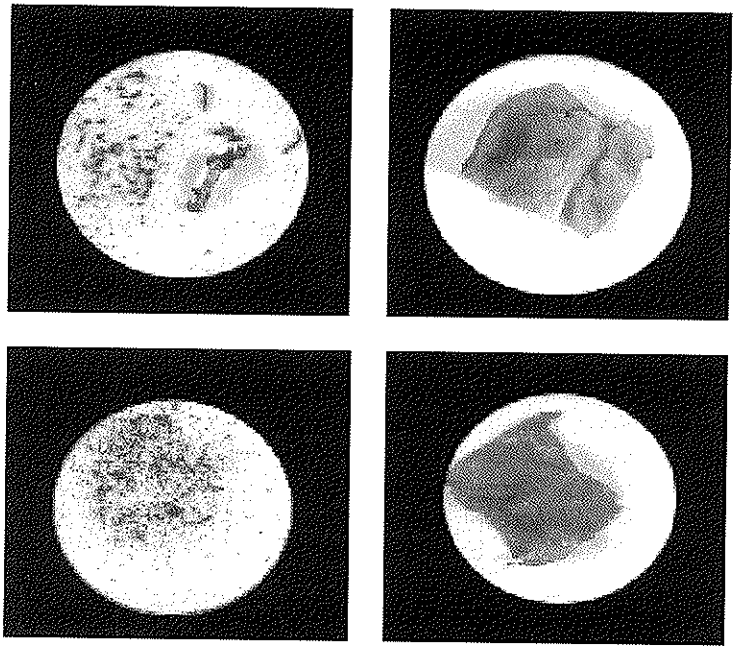

Figure 12.5. Leaf bioassay of control (left) and Cry2Aa2 chloroplast transgenic tobacco leaves (right) assayed against cotton bollworm (top) and beet armyworm (bottom).

feeding on transgenic leaves and the leaf pieces were essentially intact, while the control leaf pieces were completely devoured (*Figure 12.4*). Similar results were obtained with Cry1Ac and Cry2A resistant insects. Bioassays were also conducted using insects that were reared on control leaves or artificial diet for 5 days (ca. 2^{nd}-3^{rd} instar), and then moved to transgenic leaves. Even these older larvae that are more tolerant than neonates showed 100% mortality. When transgenic leaves were fed to cotton bollworm and beet armyworm, 100% mortality was observed, whereas there was no mortality observed in the control, and the entire leaf piece was devoured (*Figure 12.5*).

Several reasons should have contributed to such high levels of Cry2A expression in chloroplasts in comparison to those observed using nuclear expression, including the prokaryotic nature of chloroplasts, the prokaryotic codon composition of *cry*2Aa2, high copy number of *cry*2Aa2 genes per cell, and the small size of the protoxin gene, as suggested earlier (Daniell *et al.*, 1994). This level could be further increased with the doubling of gene dosage by inserting *cry*2Aa2 into the inverted repeat region of the chloroplast genome instead of the single copy region. Studies in *E. coli* suggest that ORF's upstream of *cry*2Aa2 (within the *cry*2Aa2 operon) are required for folding Cry2Aa2 proteins to form cuboidal crystals (Daniell *et al.*, 1994). Such crystals are far more stable than soluble Cry2A protein (Daniell *et al.*, 1994). If crystals are desired for enhanced stability, the entire *cry*2Aa2 operon should be expressed in chloroplasts because chloroplasts routinely express and process polycistrons (Daniell *et al.*, 1994).

With the successful introduction of *cry*2Aa2 into the chloroplast genome, the high-dose strategy should be attainable. This study shows 100% mortality of both *Bt* susceptible and Cry1Ac-resistant and Cry2Aa2-resistant tobacco budworm. This is

the first report where neonate insects, highly resistant to *Bt*, were killed using *Bt* transgenic leaf material, even though tobacco budworm is less sensitive to Cry2Aa2 than Cry1Ac. These results are promising when related to reports showing marginal to high levels of cross-resistance to Cry2Aa2 (Gould *et al.*, 1992, 1995). This study also shows 100% mortality of cotton bollworm that contrasts with *Bt* cotton (Cry1Ac) efficacy against cotton bollworm. The inefficient control of cotton bollworm might also result in faster development of *Bt* resistance because a moderate level of suppression (25–50% mortality) can increase the probability of resistance development (Gould, 1998; Tabashnik *et al.*, 1997). In this context, plants expressing *cry*2Aa2 through the chloroplast, either singly, or as part of a gene-pyramid with other insecticidal proteins (preferably non-*Bt* proteins with different modes of action), could become an invaluable tool for resistance management.

Chloroplast genetic engineering: past, present and future

When we developed the concept of chloroplast genetic engineering (Daniell and McFadden, 1988), it was possible to introduce isolated intact chloroplasts into protoplasts and regenerate transgenic plants (Carlson, 1973). Therefore, early investigations on chloroplast transformation focused on the development of *in organello* systems using intact chloroplasts capable of efficient and prolonged transcription and translation (Daniell *et al.*, 1983, 1986) and expression of foreign genes in isolated chloroplasts (Daniell and McFadden, 1987). However, after the discovery of the gene gun as a transformation device by John Sanford (Daniell, 1993), it was possible to transform plant chloroplasts without the use of isolated plastids and protoplasts. Knoblauch *et al.* (1999) recently describe a novel galinstan expansion femtosyringe method that allows microinjection of foreign DNA and other substances into prokaryotic cells and eukaryotic organelles. The essence of this method is the heat induced expansion of a liquid metal called galinstan (alloy of gallium, indium and tin) within a glass syringe to expel samples through a capillary tip with a diameter of about 0.1 μm. Injection of a plasmid containing the *bla* gene (coding for β-lactamase) into the filamentous cyanobacterium *Phormidium laminosum* resulted in stable transformation. This demonstrates ability of the cell to survive damage during DNA delivery. The most common drawback of microinjection is the release of cellular contents into the needle, resulting in damage to the recipient cell. Similarly, injection of the *gfp* gene in attached leaves of tobacco and *Vicia faba* resulted in the production of green fluorescent protein within chloroplasts. The usefulness of the galinstan technique for transformation of eukaryotic organelles will ultimately depend upon successful demonstration of stable transformation in chloroplasts of higher plants. Polyethylene glycol is yet another method for DNA delivery into chloroplasts using protoplasts but this may not be as efficient as particle bombardment (Golds *et al.*, 1993).

Similar to nuclear transformation, chloroplast genetic engineering was accomplished in several phases. Transient expression of foreign genes in plastids of dicots (Daniell *et al.*, 1990; Ye *et al.*, 1990) was followed by such studies in monocots (Daniell *et al.*, 1991). Unique to chloroplast genetic engineering is the development of a foreign gene expression system using autonomously replicating chloroplast expression vectors (Daniell *et al.*, 1990). Stable integration of a selectable marker gene into the tobacco chloroplast genome (Svab and Maliga, 1993) was also

accomplished using the gene gun. However, useful genes conferring valuable traits via chloroplast genetic engineering have been demonstrated only recently. For example, plants resistant to *Bt* sensitive insects were obtained by integrating the *cry1Ac* gene into the tobacco chloroplast genome (McBride *et al.*, 1995). Plants resistant to *Bt* resistant insects (up to 40,000-fold) were obtained by hyper-expression of the *cry2Aa2* gene within the tobacco chloroplast genome (Kota *et al.*, 1999). Plants have also been genetically engineered via the chloroplast genome to confer herbicide resistance and the introduced foreign genes were maternally inherited, overcoming the problem of out-cross with weeds (Daniell *et al.*, 1998). Chloroplast genetic engineering has also been used to produce pharmaceutical products that are not used by plants (Guda *et al.*, 1999). Chloroplast genetic engineering technology is currently being applied to other useful crops (Daniell, 1999c).

ADVANTAGES OF CHLOROPLAST GENETIC ENGINEERING

A remarkable feature of chloroplast genetic engineering is the observation of an exceptionally large accumulation of foreign proteins in transgenic plants, including 18% of FLARE-S ('Fluorescent Antibiotic Resistance Enzyme conferring resistance to spectinomycin/streptomycin') in total soluble protein (Khan and Maliga, 1999) and 30% of GUS protein in total soluble protein (McBride *et al.*, 1994). Recent stable expression in chloroplasts of GVGVP, a protein based polymer with varied medical applications (such as the prevention of post-surgical adhesions and scars, wound coverings, artificial pericardia, tissue reconstruction and programmed drug delivery), a hundred times higher than nuclear expression, makes chloroplast genetic engineering a valuable tool to produce pharmaceutical proteins in plants (Guda *et al.*, 1999). It is well known that the level of foreign gene expression is not adequate for commercial feasibility of several pharmaceutical proteins when expressed via the nuclear genome; levels of expression of pharmaceutical proteins vary over three orders of magnitude, 0.001 to 1% of total cellular protein (May *et al.*, 1996). Therefore, it is wise to exploit this major advantage by engineering foreign genes via the chloroplast genome instead of the nuclear genome.

Besides extremely high protein levels, chloroplast gene expression also results in tissue specificity, occurring predominantly where functional plastids are present. This may be important in engineering insect resistant plants wherein most worms predominantly feed on leaves where plastids are abundantly present, thereby consuming the highest level of the insecticidal protein. An added advantage is that the insecticidal protein will not be expressed in edible parts of the plant or pollen. This is especially important in the light of recent reports that pollen from *Bt* corn is highly toxic to Monarch butterflies (Losey *et al.*, 1999). Since there is no chloroplast DNA in pollen of most crops, toxic insecticidal proteins will not be expressed in pollen of chloroplast transgenic plants. Similarly, protein synthesis is almost shut down in chromoplasts that are present in fruits. If desired, chromoplast specific promoters should be used to engineer insect resistance in fruits. As explained earlier, chloroplast transformation utilizes two flanking sequences that, through homologous recombination, insert foreign DNA into the spacer region between the functional genes of the chloroplast genome, thus targeting the foreign genes to a precise location. This eliminates the 'position effect' frequently observed in nuclear transgenic plants. The

maternal inheritance of the chloroplast genome in most crops also reduces the potential for out-crossing of foreign genes to other plants (especially weedy species).

CHALLENGES IN CHLOROPLAST GENETIC ENGINEERING

While there are several reports of genetic engineering of the chloroplast genome in tobacco, other major crops (including cereals) have been transformed only recently (Daniell, 1999c). Potato plastid transformation has been accomplished recently (Sidorov *et al.*, 1999). One of the major limitations has been the lack of knowledge of chloroplast genome sequences to locate spacer regions and transcriptional units to target site-specific integration of foreign genes. In order to overcome this limitation, Daniell *et al.* (1998) have recently developed a universal vector that can transform any chloroplast genome because it integrates into a highly conserved region. Experiments are in progress to transform a variety of crops using this universal vector.

Another limitation has been the ability to regenerate plants only from embryonic tissues in cereals and not from mesophyll cells. Cells from embryogenic tissues contain only proplastids and not mature plastids. It has been suggested that these plastids are smaller than the size of microprojectiles used for DNA delivery and, therefore, may pose problems in transformation experiments. Successful expression of chloramphenicol acetyl transferase in proplastids of NT1 cells (Daniell *et al.*, 1990) and β-glucuronidase in proplastids of wheat embryos (Daniell *et al.*, 1991) and other non-green plastids (Hibberd *et al.*, 1998; Khan and Maliga, 1999) via particle bombardment suggest that particle size may not be a problem in transforming proplastids. Khan and Maliga (1999) describe the use of a novel Fluorescent Antibiotic Resistance Enzyme conferring resistance to spectinomycin/streptomycin (FLARE-S) to detect chloroplast transformation, especially from non-green plastids. FLARE-S was obtained by translational fusion of aminoglycoside adenylyl transferase (*aadA*) with the green fluorescent protein (GFP) from the jellyfish *Aeqorea victoria*. Because of the presence of thousands of copies of chloroplast genomes per cell, transformation unavoidably yields chimeric tissues in which transformed cells should be selected and regenerated into plants. FLARE-S facilitates distinction of transformed and wild-type sectors in the chimeric tissue, thereby significantly reducing the time and effort required to obtain stably transformed plants. This is especially important while transforming non-green plastids because the green/bleached phenotype observed under streptomycin/spectinomycin selection while transforming green plastids is not available. Demonstration of GFP expression in rice, which lacks visually identifiable tissue culture phenotype, is a significant observation. The fact that FLARE-S is expressed from the chloroplast genome is evident from PCR studies and the observation of a mixed population of untransformed and transformed plastids. Unfortunately, only a very small fraction of chloroplasts expressed FLARE-S. Obtaining stably transformed rice plants exhibiting homoplasmy still appears to be a distant accomplishment. Some of the challenges in transforming agronomically useful crops include optimization of tissue culture techniques, and the selection process to obtain transgenic plants via particle bombardment, especially from non-green tissues. Even if homoplasmy is not obtained in the 1st generation, it could be accomplished in subsequent generations by germination of T1 seeds under appropriate selection. Heteroplasmy of chloroplast genomes has been observed in nature

(Frey, 1999) and accomplishing homoplasy for the introduced trait may not be always necessary.

Yet another concern is the possibility of yield drag in transgenic crops because of the hyper-expression of foreign genes via the chloroplast genome (at times as high as 30% of the total soluble protein). High levels of expression of several foreign proteins in transgenic tobacco has not affected growth rates, photosynthesis, chlorophyll content, flowering, or seed setting (Daniell, 1999b). Chloroplasts are used to handling such abundant proteins without deleterious effect on productivity. For example, the Calvin cycle enzyme, ribulose bis-phosphate carboxylase/oxygenase (RubisCO), is synthesized as much as 50% of the total soluble protein; such high levels of synthesis have not affected the productivity of crop plants. Indeed, excess RubisCO is constantly made and degraded in chloroplasts. However, long-term tests using agronomically important crops grown under field conditions are needed to confirm this observation. Recent success in accomplishing potato plastid transformation should pave the way for studies on such agronomic traits (Sidorov *et al.*, 1999). All of these findings augur well for chloroplast genetic engineering of economically useful crops. Thus, several environmentally friendly approaches have been opened for new advances in plant biotechnology and genetic engineering.

Epilogue

There is serious concern among scientists, policy makers, regulatory agencies, and the public regarding widespread release of genetically modified food crops. The relationship between the scientific community and the general public has never been worse in living memory (Haerlin and Parr, 1999). Such negative public perception prohibits advancement of this technology and prevents realization of its full potential. Most of these concerns could be alleviated by employing some of the solutions discussed in this review. Sincere efforts to address public concerns should expedite consumer acceptance of genetically engineered crops. Acknowledgement of public concerns and the availability of solutions for their concerns by the biotech industry are important steps in the right direction. While it may be profitable to market genetically modified crops on hand, in the near future such an approach would seriously damage the long-term vitality and survival of the plant biotech industry. However, as pointed out by Haerlin and Parr (1999), instead of rethinking their research and development strategies, most companies and governments still treat public acceptance as just an additional challenge to be overcome by asserting the safety of their technology. Biotech companies are out of touch with the values of society that cannot be overcome by means of any scientific risk assessment.

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