

Assessing and Managing Biological Risks of Plants Used for Bioremediation, Including Risks of Transgene Flow

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The plants used for phytoremediation pose special biological risks, whether transgenic or not, as most of the species: (a) are semi-domesticated; (b) are introduced from other habitats; (c) can become established in the contaminated site; (d) can spread and displace native species, and/or; (e) may introgress transgenes into related species. The addition of transgenes can reduce the risks, *e.g.* to sterilize or render the species and hybrid offspring hypersensitive to environmental effects (heat, cold), or to a chemical that will cull the species. Various measures can contain transgenes used in phytoremediation species to prevent gene flow, but most containment technologies are both uni-directional (prevent either outflow or influx), and are inherently leaky, even a concept specifically utilizable for phytoremediation – grafting non-transgenic scions on bioremediating transgenic rootstocks. Containment mechanisms should be either stacked with each other or with “mitigator” genes. Transgenic mitigation (TM) has mitigator genes added in tandem to the desired primary transgene (genetically linked) and the mitigator genes confer traits that are positive or neutral to the desired species but are deleterious to hybrids, keeping them at very low frequencies. The concept was demonstrated in tobacco and oilseed rape with a dwarfing mitigator gene that enhanced the reproductive productivity (harvest index) when cultured alone, but eliminated it from mixed populations. Besides the mitigator genes previously proposed for crop species (sterility, no seed shattering, dwarfing, no secondary dormancy) there are genes especially appropriate for phytoremediation, *e.g.* overexpression of cytokinin oxidase (reduces cytokinin levels) conferring reduced shoot systems (unfitness to compete) with a more extensive root system that is better for extracting toxic wastes as well as no-flowering for vegetatively propagated species. Thus, biotechnology can be harnessed to reduce risks from both non-transgenic and transgenic phytoremediation species.

Key words: Risk Assessment, Gene Containment, Mitigating Gene Flow

Introduction – Needs for preventing gene flow in phytoremediating species

The herbaceous plants, shrubs, and trees used for phytoremediation pose certain biological risks, whether transgenic or not. Many of the species are semi-domesticated and introduced from habitats far removed from the site requiring phytoremediation. Such species pose a risk of becoming established in the contaminated site after the contaminant is remediated, and also pose a risk of spread to adjacent areas, displacing native or other desirable species, or hybridizing with related native species or even other varieties of the same species. An added concern is that transgenes in the phytoremediation species may introgress into related species. If a non-transgenic species poses a risk, the addition of specific transgenes can actually reduce the risk. We describe below the molecular

tools that can be used to contain gene flow within the bioremediation site, and separate molecular mitigation tools that can prevent the establishment of such transgenes should they leak out of the phytoremediation site, which are appropriate for non-transgenic and transgenic bioremediating species alike. Molecular solutions to gene flow problems for non-transgenic phytoremediation species may sound oxymoronic in the present climate surrounding transgenics. Still, if the scientifically determined risk of spread of a phytoremediating species outweighs the utility of the species for phytoremediation, such molecular solutions should be sought to allow effective phytoremediation while preventing gene flow. Genes can flow from bioremediation sites in three forms – seeds carried by various vectors, vegetative propagules, and pollen. Typically pollen is thought of as the source of gene

movement, but even without human intervention seeds carrying an undesirable trait can move large distances; *e.g.* maternally inherited triazine resistance in *Solanum nigrum* has moved 20 km per year from a single site – the distance a bird flies from eating berries to defecating (Stankiewicz *et al.*, 2001). Some species can move long distances as vegetative propagules, *e.g.* feral forms of asexually propagated Jerusalem artichoke (*Helianthus tuberosus*) have become widely spread in Europe along riverbanks (Bervillé *et al.*, 2005). The number one worst weeds of the world, *Cyperus* spp. are primarily spread asexually (Holm *et al.*, 1977).

This review will not cover the toxicological risks of the pollutants sequestered in or vaporized from plants used for phytoremediation.

Molecular tools to prevent phytoremediation species and their genes from becoming established

Genes do flow in nature, not only within species, but also among related species that do not readily cross, in a process coined “diagonal” gene transfer (Gressel, 2002) to readily distinguish between vertical gene transfer in readily crossing species and horizontal gene transfer between totally unrelated species. For example, a DNA sequence typical of hexaploid wheat, found in modified form in some progenitors of wheat, was not found in > 90 accessions of *Aegilops peregrina* (syn. *Ae. variabilis*) but was found in two geographically distinct populations of that species with > 99% sequence identity to wheat (Weissmann *et al.*, 2003). In agroecosystems, such inadvertent gene flow may be undesirable.

Most discussions so far have dealt with ‘containing’ gene flow (preventing its movement) from managed ecosystems to ‘natural’ ecosystems (Gressel, 1999; Gressel, 2002; Gressel and Ehrlich, 2002; Ellstrand, 2003; Jenzewski *et al.*, 2003; Stewart *et al.*, 2003; Al-Ahmad *et al.*, 2004; Haygood *et al.*, 2004), with only some recent discussion on preventing and mitigating endo-feral (evolution within the biotype) and exo-feral (evolution of less domesticated forms by crossing with such forms) dedomestication of crops as volunteer weeds within the agroecosystem (Gressel and Al-Ahmad, 2005). There are two general approaches to dealing with gene flow: (1) *contain* the transgenes in the novel variety so that inflow is precluded; (2) *mitigate* mutational or gene flow effects if there

are inevitable “leaks” in the containment system, which should also prevent volunteer populations of the phytoremediation species from establishing/reaching maturity so that they cannot evolve into problems. Containment and mitigation are discussed below in the general context of bi-directional containment as well as mitigation.

Containing gene flow

Several molecular mechanisms have been suggested for containing gene flow (*i.e.*, to prevent gene flow between the phytoremediating species and relatives), especially by pollen, ignoring the other routes of sporophyte propagule (seeds and asexual parts) movement, especially transgenes within the crop (*i.e.*, to prevent outflow to related species), or to mitigate the effects of transgene flow once it has occurred (Gressel, 1999; Daniell, 2002; Gressel, 2002; Stewart *et al.*, 2003). In the case of phytoremediation species, it is more important to prevent gene flow from the crop than to prevent influx into the phytoremediation site, because of the comparatively much larger amount of the special population on the site. Even though the hybrids may be the same in either direction, the likelihood of such a hybrid establishing on a phytoremediation site is minimal.

Containment by targeting genes to a cytoplasmic genome

The most widely discussed containment possibility is to integrate the transgene of choice in the plastid or mitochondrial genomes (Khan and Maliga, 1999; Maliga, 2002, 2004). There are good reasons to engineer phytoremediating genes into chloroplasts besides the presumed biosafety. The chloroplasts are often the targets of environmental contaminants and need protection. Additionally, many genes of value come from bacteria with similar codon usage as chloroplasts. Such genes often need to be re-engineered to plant codon usage before inserting into the nuclear genome (Tian *et al.*, 2002). Indeed the bacterial genes *merA/merB* that convert organomercurials into elemental mercury (which is later volatilized) were successfully introduced into chloroplasts of tobacco (Ruiz *et al.*, 2003). Still, the same genes were active in *Arabidopsis* when the *merB* was augmented with a peptide that targeted the gene product into the endoplasmic reticulum, despite the bacterial codon usage differences (Bizily *et al.*, 2003).

The opportunity of gene outflow is limited due to the predominantly maternal inheritance of these genomes in many, but far from all species. This is presently an arduous technology, which so far is limited to a few crops. It does not preclude the outside species from pollinating the bioremediating species, and then acting as the recurrent pollen parent, but this is less of a problem on a bioremediation site.

The claim of strict maternal inheritance of plastome-encoded traits (Daniell *et al.*, 1998; Bock, 2001; Maliga, 2004) was not substantiated. Tobacco (Avni and Edelman, 1991) and other species (Darmency, 1994) often have between a 10^{-3} – 10^{-4} frequency of pollen transfer of plastid inherited traits. Pollen transmission of plastome traits can only be easily detected using both large samples and selectable genetic markers. A large-scale field experiment utilized a *Setaria italica* (foxtail or birdseed millet) with chloroplast-inherited atrazine resistance (bearing a nuclear dominant red leaf base marker) crossed with five different male sterile yellow- or green-leaved herbicide susceptible lines. Chloroplast-inherited resistance was pollen transmitted at a 3×10^{-4} frequency in > 780,000 hybrid offspring (Wang *et al.*, 2004). At this transmission frequency, the probability of herbicide resistance movement via plastomic gene flow is orders of magnitude greater than by spontaneous nuclear genome mutations. Thus, chloroplast transformation is probably unacceptable for preventing transgene outflow, unless stacked with additional mechanisms, and as noted above, will not at all impede gene inflow. Maliga (2004) discounts the relevance of the findings with tobacco and *Setaria* as being due to an origin of the plastids from inter-specific (closely related) cytoplasmic substitution, where pollen transmission barriers can break down (Kiang *et al.*, 1994). *Setaria viridis*, the wild progenitor of *Setaria italica* is basically con-specific with it (Darmency, 2005). There are two problems with this denigration of the relevance of pollen movement of plastome-encoded genes: 1) it is just such interspecific movement that could be a problem between crops and related species; 2) he (Maliga, 2004) ignores the discussion in Darmency (1994) of cases of intra-specific transmission of plastomic traits by pollen at about the same frequency, within the same species, as reported above between species.

Male sterility coupled with transplastomic traits

A novel additional combination that considerably lowers the risk of plastome gene outflow within a field (but not gene influx from related strains or species) can come from utilizing male sterility with transplastomic traits (Wang *et al.*, 2004). Introducing plastome-inherited traits into varieties with complete male sterility would vastly reduce the risk of transgene flow, except in the small isolated areas required for line maintenance. Such a double failsafe containment method might be considered sufficient where there are highly stringent requirements for preventing gene outflow to other varieties (*e.g.*, to organically cultivated ones), or where pharmaceutical or industrial traits are engineered into a species. Plastome-encoded transgenes for non-selectable traits (*e.g.* for phytoremediation) could be transformed into the chloroplasts together with a trait such as tentoxin or atrazine resistance as a selectable plastome marker. With such mechanisms to further reduce out-crossing risk, plastome transformation can possibly meet the initial expectations.

Genetic use restriction technologies (GURT) and recoverable block of function (RBF)

Other molecular approaches suggested for crop transgene containment include: seed sterility, utilizing the genetic use restriction technologies (GURT) ('terminator gene') (Crouch, 1998; Oliver *et al.*, 1998), and recoverable block of function (RBF) (Kuvshinov *et al.*, 2001) to prevent transgene flow. Such proposed technologies control both the gene influx of exo-fertility and endo-fertility volunteer seed dispersal, but theoretically if the controlling element of the transgene is silenced, expression would occur, rendering a critical defect in principle and practice. The frequency of loss of such controlling elements is yet unclear, as there have been no large-scale field trials to test this.

Repressible seed lethal technologies

An impractical technology has been proposed to use a "repressible seed lethal system" (Scherntaner *et al.*, 2003). The seed lethal trait and its repressor must be simultaneously inserted at the same locus on homologous chromosomes in the hybrid the farmer sows to prevent recombination (crossing over), a technology that is not yet workable in plants. The hemizygote transgenic seed lethal parent of the hybrid cannot reproduce by

itself, as its seeds are not viable. If the hybrid could be made, half the progeny would not carry the seed lethal trait (or the trait of interest linked to it) and they would have to be culled, which would not be easy without a marker gene. A containment technology should leave no viable volunteers with the transgene, but this complex technology would kill only 25% of the progeny and 50% would be like the hybrid parents and 25% would contain just the repressor. Thus, the repressor can cross from the volunteers to related weeds, and so can the trait of choice linked with the lethal, and viable hybrid plants could form. The death of a quarter of the seeds in all future generations is inconsequential to plants that copiously produce seed, as long as the transgenic trait provides some selective advantage.

In summary, none of the above unidirectional containment mechanisms is absolute (Fig. 1), but the risk could be reduced by stacking a combination of containment mechanisms, compounding the infrequency of gene introgression. Still, even at very low frequencies of gene transfer, once gene transfer occurs, the new bearer of the transgene

could disperse throughout the population if it has just a small fitness advantage.

Preventing establishment by transgenic mitigation

If a transgene confers even a small fitness disadvantage, the transgenic crop volunteers and their own or hybrid progeny should only be able to exist as a very small proportion of the population. Therefore, it should be possible to mitigate volunteer establishment and gene flow by lowering the fitness of transgene recipients below the fitness of competitors, so that the volunteer or hybrid offspring will not reproduce. A concept of “transgenic mitigation” (TM) was proposed (Gressel, 1999), in which mitigator genes are linked or fused to the desired primary transgene. Thus, a transgene with a desired trait is directly linked to a transgene that decreases fitness in volunteers (Fig. 2). TM could also be used as a stand-alone procedure with non-transgenic crops to reduce the fitness advantage of hybrids and their rare progeny, and thus substantially reduce the risk of ex-feral hybrid volunteer persistence.

This TM approach is based on the premises that: 1) tandem constructs act as tightly linked genes, and their segregation from each other is exceedingly rare; 2) the gain of function dominant or semi-dominant TM traits chosen are neutral or favorable to crops, but deleterious to volunteer progeny and their hybrids due to a negative selection pressure; and 3) individuals bearing even mildly harmful TM traits will be kept at very low frequencies in volunteer/hybrid populations because strong competition with their own wild type or with other species should eliminate even marginally unfit individuals, and prevent them from persisting in the field (Gressel, 1999).

Thus, it was predicted that if the primary gene of agronomic advantage being engineered into a crop will not persist in future generations if it is flanked by TM gene(s), such as genes encoding dwarfing, strong apical dominance to prevent tillering (in grains) or multi-heading (in crops like sunflowers), determinate growth, non-bolting genes, uniform seed ripening, non-shattering, anti-secondary dormancy. When they are in such a tandem construct, the overall effect would be deleterious to the volunteer progeny and to hybrids. Indeed a TM gene such as anti-shattering should decrease re-seeding, and thus the number of initial volunteers. (There is typically a small amount of

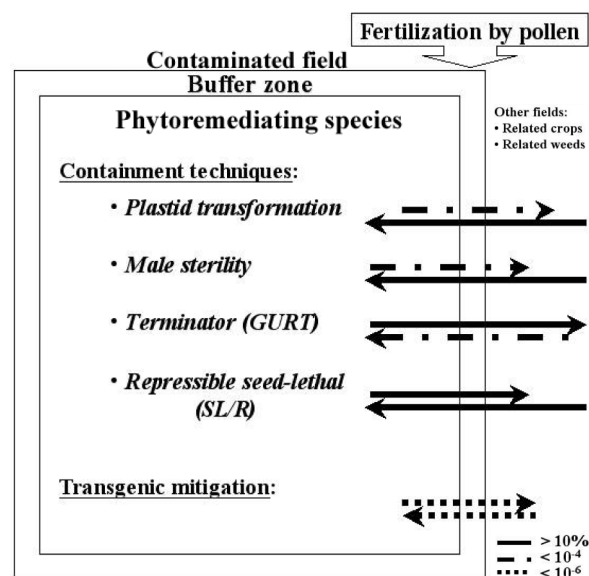
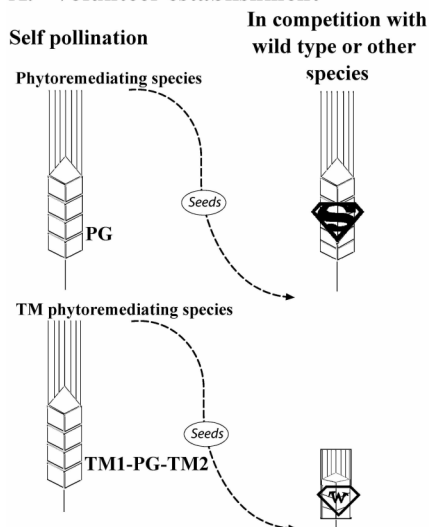


Fig. 1. Containment systems allowing gene flow in or out of a contaminated area being phytoremediated. Such systems indicate a need for stacking containment systems to contain gene flow bidirectionally, and to compound the safety factor, as well as to consider mitigation systems that reduce the effects of genes that leaked from containment.

A. Volunteer establishment



B. Hybridization with related species

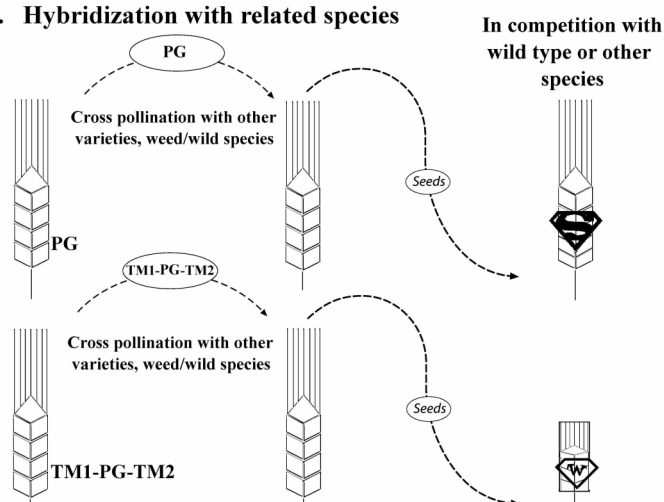


Fig. 2. Transgenic mitigation to prevent establishment of (A) volunteers and (B) hybrids between phytoremediation species and relatives. The phytoremediation species bears desirable transgenes (PG) coupled in tandem with transgenes that are neutral or positive for the crop (TM1 and TM2), but render volunteers or hybrids unfit to compete outside of cultivation.

shattering due to imperfect harvesting equipment, which may leave a few seeds behind). Because the TM genes will reduce the competitive ability of the rare hybrids, they should not be able to compete and persist in easily measurable or biologically significant frequencies in agroecosystems (Gressel, 1999, 2002).

Once TM genes are isolated, the actual cost of cloning them into TM constructs is minimal, compared to the total time and effort in producing a transgenic crop. The cost is even inconsequential in systems where co-transformation allows introducing genes into the same site such that the tandem construct is made by the plant

Demonstration of transgenic mitigation in tobacco and oilseed rape

We used tobacco (*Nicotiana tabacum*) as a model plant to test the TM concept: a tandem construct was made containing an *ahas*^R (acetohydroxy acid synthase) gene for herbicide resistance as the primary desirable gene of choice, and the dwarfing *Δgai* (gibberellic acid-insensitive) mutant gene as a mitigator (Al-Ahmad *et al.*, 2004). Dwarfing would be disadvantageous to the rare weeds introgressing the TM construct, as they could no longer compete with other crops or with fellow weeds, but is desirable in many crops, pre-

venting lodging and producing less stem with more leaves. The dwarf and herbicide resistant TM transgenic hybrid tobacco plants (simulating a TM introgressed hybrid) were more productive than the wild type when cultivated alone (without herbicide). They formed many more flowers than the wild type when cultivated by themselves, which is an indication of a higher harvest index. Conversely, the TM transgenics were weak competitors and highly unfit when co-cultivated with the wild type in ecological simulation competition experiments. The inability to achieve flowering on the TM plants in the competitive situation led to a zero reproductive fitness of the TM plants grown in a 1:1 mixture with the wild type at the typical field spacing used (Al-Ahmad *et al.*, 2004).

From the data above it is clear that transgenic mitigation should be advantageous to a phytoremediation species growing alone, while disadvantageous to a hybrid living in a competitive environment of the phytoremediation site. If a rare pollen grain bearing tandem transgenic traits bypasses containment, it must compete with multitudes of wild type pollen to produce a hybrid. Its rare progeny must then compete with more fit wild type cohorts during self-thinning and establishment. Even a small degree of unfitness encoded in the TM construct would bring about the elimination of the vast majority of progeny in all future generations, as long as the primary gene provides no selective advantage that counterbalances the unfitness of the linked TM gene. Most phytoremediating genes have a drag, not an increased fitness off the phytoremediation site.

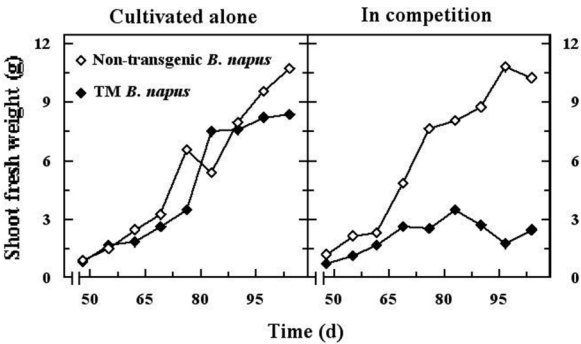


Fig. 3. Suppression of growth of TM (transgenic mitigator) bearing oilseed rape plants carrying a dwarfing gene in tandem with a herbicide resistance gene (closed symbols) when in competition with the wild type (open symbols) (right panel), and their normal growth when cultivated separately without herbicide (left panel) at 3 cm spacing in a screenhouse experiment (unpublished data: Al-Ahmad and Gressel, 2004).

We have inserted the same construct into oilseed rape and have tested the selfed progeny, as well as hybrids with the weed *Brassica campestris* = *B. rapa*. When cultivated alone, the dwarf transgenic oilseed rape grew at almost the same rate as the transgenic (Fig. 3), but produced twice as much seed at the expense of the stem tissue (Table I). When the TM transgenic oilseed rape plants were co-cultivated in competition with the wild type, they were unable to grow normally (Fig. 3), and hardly set seed (Table I) because they were so unfit to reproduce.

The rare hybrid offspring from escaped pollen bearing transgenic mitigator genes would not pose

Table I. Transgenic mitigated TM oilseed rape has high productivity and low relative competitive fitness in the screenhouse.

Biotype	Productivity grown alone	Relative fitness in competition with	
		<i>B. napus</i>	<i>B. rapa</i>
<i>B. napus</i> NT	320 ^b	—	4.32
<i>B. napus</i> TM	503 ^a	0.10	2.81
<i>B. rapa</i>	119 ^d	—	—
F ₂ hybrids (<i>B. rapa</i> x <i>B. napus</i> NT)	213 ^c	—	0.86
F ₂ hybrids (<i>B. rapa</i> x <i>B. napus</i> TM)	75 ^{e,d}	—	0.02
F ₂ BC ₁ [<i>B. rapa</i> x (F ₁ hybrids NT)]	63 ^{e,d}	—	0.21
F ₂ BC ₁ [<i>B. rapa</i> x (F ₁ hybrids TM)]	26 ^e	—	< 0.01

Productivity was measured as weight of seeds per plant (mg), and the relative fitness was calculated as the ratio of weight of seeds per transgenic to non transgenic biotype plants. Different letters indicate different LSD values at *P* ≥ 0.05 (unpublished data: Al-Ahmad and Gressel, 2004).

a dire threat, especially to wild species outside fields, as the amount of pollen reaching the pristine wild environment would only be a minuscule fraction of the pollen compared to the wild type. Further large-scale field studies will be needed with crop/weed pairs to continue to evaluate the positive implications of risk mitigation.

Risk that introgression of TM traits will affect wild relatives of the crop

Models by Haygood *et al.* (2003) claim to “prove” the premise that ‘demographic swamping’ by crop genes would cause ‘migrational meltdown’ of wild species related to the crop, especially if the introgressed genes confer unfitness. This proposition that recurrent gene flow from crops, even TM gene flow, could affect wild relatives deserves some discussion, as it negates the concept of mitigation.

They claim that their model demonstrates that recurrent gene flow from transgenic crops with less fit genes will cause wild populations to shrink. Firstly, conventional crops already belie this possibility. There are few if any major domesticated crops that are fit to live in a wild ecosystem, so their normal genes should confer a modicum of unfitness. Such crop \times wild hybrids continually form, yet they present no evidence that demographic swamping did occur from recurrent gene flow from the crops, or could we locate any published data to that effect. Indeed, considerable evidence has been presented that many crops exist near their wild or weedy progenitors, without causing the extinction of the progenitors, despite gene flow (Weissmann *et al.*, 2003).

There are other mundane yet fatal flaws in their models that are based on shaky premises and assumptions not borne out by plant biology. Three problematic issues that seem to invalidate the relevance of their model for the vast majority of conceivable crop/wild species systems, are discussed below:

1.) To get the level of swamping that they (Haygood *et al.*, 2003) discuss, the wild relative and the crop would have to live in the same ecosystem. There is typically geographic separation between phytoremediation ecosystems and wild ecosystems, with pollen flow decreasing exponentially with distance – usually to a low asymptote due to wind currents or insects not fully following simple physics. There should always be far more wild pollen in the wild ecosystems, so hybridization events

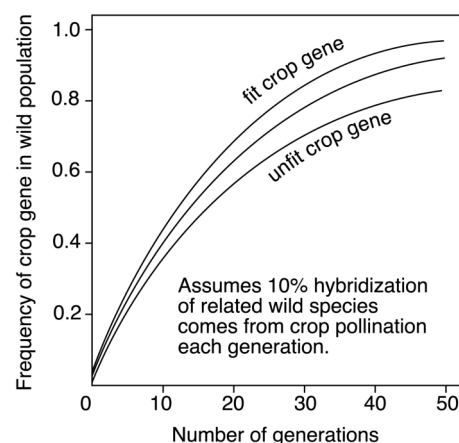


Fig. 4. Modeling of gene introgression under recurrent gene flow from crops to wild relatives, used by Haygood *et al.* (2003) to invalidate the use of transgenic mitigation as a failsafe mechanism to prevent transgene establishment in volunteers and related weeds in agroecosystems. Modified from Haygood *et al.* (2003).

in the wild from crop pollen will be rare, even with masses of pollen occurring within the agroecosystem. Thus their basic assumption of crop pollen swamping wild type pollen in the wild, is invalid. Indeed, even when they assume an enormous 10% of hybridizations in the wild each generation coming from crop pollen, according to their model it will take about 20 generations of recurrent pollination for the unfit crop allele to become fixed in half the population, and 50 generations for an unfit gene to asymptotically reach 80% of the population (Fig. 4). As discussed below, their other assumptions leading to these numbers are also off target, so it should actually take much longer.

2.) They assume synchronous flowering, no self-fertilization, and no genetic or other barriers to cross fertilization; indeed, this negates the definition of speciation. It is exceedingly rare for crop pollen to fertilize another species without any genetic barrier in the wild relative. Of the species mentioned in preceding chapters, this might only occur with con-specific wild sunflowers, which might fit this criterion, but even in this case there are genomic deterrents to introgression (reviewed in Stewart *et al.*, 2003). Con-specific rice and red (weedy)-rice does not fit their assumptions because they are cleistogamous, predominantly self-fertilizing before the flowers open, and the amount of outcrossing possible would be very low. Of course weedy rice is not a wild species (by defi-

nitition), so it too is not really relevant to their case. There are fertilization barriers of different chromosome numbers, non-homology etc, which limit fertilization of wild relatives of oilseed rape and wheat, so they are outside the models.

3.) Their models assume animal-type replacement rates – a few progeny per mating, where lower fitness can indeed become fixed. Most wild relatives of phytoremediating herbaceous or tree species produce copious amounts of seed to replace parents. Hundreds to thousands typically germinate in the area occupied by a parent and the process of self-thinning is ferociously competitive, eliminating less fit individuals. Our experimental data show that at realistic seed output and seeding rates, unfit individuals are eliminated or remain at a low frequency, just as unfit mutations are maintained in populations at some low frequency (the relative fitness multiplied by the mutation frequency).

Their conclusion that “the most striking implication of this model is the possibility of thresholds and hysteresis, such that a small increase in (unfit gene) immigration can lead to fixation of a disfavored crop allele ...” (Haygood *et al.*, 2003) flies in the face of evolutionary evidence, and decades of classic and contemporary field data showing that only near-neutral genes exist in pockets of the evolutionary landscape of plants, and blatantly unfit plant genes are not known to exist in such pockets unless all the fit genes are somehow removed. Just as endogenous unfavored gene mutations exist in the wild at a frequency lower than the mutation rate, crop transgenes that have a fitness penalty will exist in the wild at a rate lower than the immigration rate. As discussed above, the immigration rate to the wild is perforce very low. Unfit genes are eliminated from populations of plants that produce large numbers of seeds, whereas the genes could be fixed in populations of animals with few progeny. When a model contradicts reams of data, it is more likely than not that the model is invalid.

They further contend that their model would work if the crop were heterozygous for the unfit gene (and many transgenic hybrids have the transgene in a single parent and are thus hemizygous). The data in Figs. 3 and 4 clearly show that when even half of the backcross progeny contain a TM construct, they cannot compete with their non-transgenic sibs, let alone the wild type. Part of the problem may be that Haygood *et al.* (2003, p. 1880, column 2) “assume (that) the number of

plants surviving to maturity does not vary from one generation to the next”, a questionable assumption for unfit phenotypes when they must compete with fit cohorts and other species.

Where might their model have some validity? Even though, despite their claims, the model has limited validity for the ‘wild’ ecosystems, the model might be valid for a few weeds (not wild species) related to crops. When flowering weeds are at a low density in an agricultural ecosystem (and perhaps close by in ruderal systems) the model might be predictive, especially when the TM genes are introduced in multicopy transposons where all their progeny receive the transgene. If it were possible to so debilitate a weed population by “genetic self-biocontrol” instead of using aggressive cultivation or herbicides, would this be so bad? As weeds are man-made domesticated species (of a sort), should not people have the right to eliminate them? The nature of weeds is such that they do not go extinct, as much as the farmer would desire. It is far more likely that such evolutionarily threatened weeds would evolve exclusionary mechanisms that would block evolutionarily threatening gene flow, *e.g.*, they would evolve a shift to predominant self-fertilization that would protect them from crop pollen bearing unfit genes.

In summary, the model of Haygood *et al.* (2003) may be right for certain animal systems but irrelevant for the vast majority of plant systems. They fail to mention specific plant systems where their model might be valid. Indeed, the species that naturally phytoremediate mine sites (for the last 2000 years in the case of Roman sites) are too unfit to compete off of mine sites, that the heavy metal resistant genes are not found in the same species of wind-pollinated grasses a few cm from the edge of mine tailings (Bradshaw, 1982). The pollen flowed, but the offspring cannot compete with wild-type offspring.

Following transgene flow to volunteers and feral forms

Using the various containment and mitigation strategies it should be possible to keep transgene ‘leaks’ below risk thresholds, which have to be specified by science-based regulators on a case-to-case basis. As the numbers of transgenic species being released is increasing, and the problems of monitoring for such genes increases geometrically, we suggested that an uniform biobarcodeTM sys-

tem should be used, where a small piece of non-coding DNA having uniform recognition sites at the ends (for single PCR primer pair amplification) with an assigned variable region in between. Thus, PCR-automated sequencing could be used to determine the origin of 'leaks', contamination, liability, as well as intellectual property violations (Gressel and Ehrlich, 2002).

Special transgenic containment and mitigation genes for phytoremediating plants

Plants have been used to correct human error over the ages. The few species capable of revegetating Roman lead and zinc mine tailing in Wales (Smith and Bradshaw, 1979) taught us that there are a limited number of species that can withstand toxicants: some by exclusion, and others that can withstand toxic wastes after they have been taken up. Plants with the latter type mechanism are of interest for phytoremediation. Ideally, one might consider that it is best to use the species that naturally take up particular toxic wastes, but these are often slow growing (e.g. mosses, lichens, or the *Thlaspi* species that take up heavy metals) (Kramer *et al.*, 1997) and may have a potential to be weedy. If the desired wild species do not exist locally, there may be a reticence or legal issues about introducing them into the ecosystem, toxic as it may be, due to fear that the plants or their genes may spread to other areas. Two types of multi-cut species are used, with the cut material burnt to extract the heavy metals or to oxidize the organic wastes: herbaceous species such as *Brassica juncea* and *Spartina* spp. (cord grasses), which most efficient are dealing with surface wastes, and trees such as *Populus* spp., for dealing with deeper wastes (Pilon-Smits and Pilon, 2002). Thus, heavy metal tolerance has been brought into *Brassica juncea* (Indian mustard) from *Thlaspi* by protoplast fusion (along with many other genes) (Dushenkov *et al.*, 2002). *Brassica juncea* wild type had been used commercially, because it grows rapidly, and is easy to cultivate as a crop, but especially because of its inherent ability to take up heavy metals. This ability has been enhanced by mutant selection (in tissue culture) for heavy metal resistance (Schulman *et al.*, 1999), but it was better yet to transgenically transfer genes leading to enhanced glutathione content (Zhu *et al.*, 1999; Bennett *et al.*, 2003) to make the necessary phytochelatins. A single cropping of *B. juncea* does not clean

up a toxic site; many growth cycles are required, with multiple harvests and natural reseeding. *B. juncea*, even more than its close relative *B. napus* (oilseed rape) is not fully domesticated, and the multiple cycles of cropping would allow the possibility of selecting for feral forms that may persist or crossing the genes into related *Brassica* species, or cultivated varieties of Indian mustard. Thus, mitigation seems necessary to prevent volunteers from becoming feral and to prevent crossing into related species. Similarly, many oppose introducing transgenic or non-transgenic phytoremediating tree species such as poplars unless they can be prevented from establishing outside of the contaminated area or from hybridizing with related native or introduced species. Human plantings of trees and other ornamental species have often displaced native species either by competition or by hybridization (Kowarik, 2005).

Containment and mitigation genes

As more genes become isolated and their properties elucidated, it appears that many might be specifically utilizable to contain and mitigate gene flow in plants used for phytoremediation. Some genes that can be used for containment might be better used for mitigation. For example, various *Populus* species have been genetically engineered and field tested out of doors for heavy metal tolerance or for metabolizing halogenated hydrocarbons, as well as male sterility, and lack of fertility (USDA-APHIS, 2004). Male sterility and lack of fertility can prevent gene outflow, albeit typically leaky. Thus, some pollen bearing the phytoremediation traits can escape to the wild, and some pollen from the wild can fertilize the few flowers appearing on a tree. In the case of vegetatively propagated species such as poplars, male sterility can be coupled with female sterility, which will prevent pollen from nearby related species from effectively pollinating the phytoremediating poplar. Additionally, floral ablation can be used (no pollination in either direction) can be used, as described in a review of the earlier literature (Meilan *et al.*, 2001). A presently used cytotoxin gene under the control, of a PTD flower promoter imparts "high levels" of floral ablation in poplar, a species commonly used for phytoremediation (Skinner *et al.*, 2003), with complete loss of flower buds in some lines tested in the greenhouse, in plants also engineered for early flowering. Whether they are

leaky and allow some flowering as plants mature is being tested in field trials now in progress (S. H. Strauss, Oregon State University, personal communication 2004). If the infertility is not 100% and the genes are just used for containment, *i.e.* not engineered in a tandem construct with the phytoremediation genes, the infertility genes can segregate from the phytoremediation genes in further generations, giving fertile plants with the phytoremediation traits. If the same infertility genes are engineered in a tandem construct or in such a way that they will be linked *in planta* (as happens with most biolistic co-transformants), the two sets of traits will remain linked, and the rare escapee bearing infertility and phytoremediation will remain “mitigated”, *i.e.* in a perennially low proportion of the population.

Some traits are appropriate containing/mitigating both tree, shrub, and herbaceous phytoremediating plants. For example: the overexpression of a cytokinin oxidase (Bilyeu *et al.*, 2001), which reduces the levels of isopentenyl and zeatin type cytokinins. This in turn leads to phenotypes with far reduced shoot systems (unfitness to compete) but with faster growing more extensive root systems (Werner *et al.*, 2003), all the better for extracting toxic wastes.

Irreversible sterility is best for trees and shrubs that can be vegetatively propagated, reversible male sterility is better for herbaceous species, as it allows seed production, as described below.

Special containment/mitigation for herbaceous phytoremediation agents

Mitigating genes should easily prevent or delay flowering in rosette type herbaceous species such as the *Brassica* spp. that are two phase crops, where the vegetative material is harvested, and flowering (bolting) is detrimental. This could easily be effected by preventing gibberellic acid biosynthesis (Hedden and Kamiya, 1997), either in a TM construct and/or by permanent mutation of the kaurene oxidase gene using a chimera-plastic gene conversion system (Zhu *et al.*, 2000), a system that as yet is hard to use in plants. Kaurene oxidase suppression would require the use of gibberellic acid to ‘force’ flowering for seed production. There should be a concomitant biosafety requirement that seed production areas be far removed from areas where weedy or other feral or wild beets grow to prevent pollen transfer.

Delaying of bolting and flowering by using a different transgene has recently been demonstrated. Curtis *et al.* (2002) engineered a fragment of the *GIGANTEA* gene, the gene encoding a protein that is part of the photoperiod recognition system, into radish using an antisense approach. Bolting was considerably delayed, and thus seed production could come about without reversal mechanisms if seed producers waited long enough. If despite all isolation distances, a TM construct or a mutant in a seed production area introgresses with a wild species, the progeny will also be delayed, *i.e.*, the transgenic hybrid would be non-competitive with cohorts.

Special containment/mitigation genes for phytoremediating trees

In forestry, the possibility of gene flow is especially problematic as the long-term implications of gene movement are longer than human lifetimes. The introgression of traits from these species to wild populations has been extensively discussed by Llewellyn (2000) and Gressel (2002), and thus containment/mitigation should be tight. Some phytoremediating species such as the poplars are vegetatively propagated and thus flowers and seeds are not important – indeed may provide a metabolic/genetic drag. Such phytoremediating trees can be vegetatively propagated, and if sterile, besides possibly higher yield and biosafety, allergy-causing pollen clouds and messy fruits would be prevented. An ideal gene for doing this is *bar-nase* under the T29 tapetum-specific promoter (Mariani *et al.*, 1990). The ribonuclease is only produced in the tapetum and prevents pollen formation with no other ill effects.

If one has an important crop in which transgenics are exceedingly worthwhile, yet the risks of cultivation too great, one could envisage using a pollen sterility system coupled with flower drop, as described above and the crop could be propagated by artificial seed, *e.g.*, artificially encased somatic embryos produced in mechanized tissue culture systems. As noted above, such genes are being tested (USDA-APHIS, 2004), but whether in tandem with phytoremediation traits, or separate is not clear.

Poplar height is under control of gibberellic acid, just as it is with herbaceous species (Busov *et al.*, 2003). The GAI and related dwarfism genes are thus being tested in poplar to ascertain whether the shorter, fatter trees concept cited will

grow any faster and be less competitive under competition. So far a field trial has been growing for one year and the researches at Oregon State University have many short, fattish trees (size varies from 1/3 to 2 m), but it will take several more years to ascertain the capacity to mitigate (Steven Strauss, personal communication, 2004). They believe that better genes or more specific promoters may be needed to really make the concept work. The professional foresters are quite skeptical, given that tall and straight trees is what they have been taught to seek all their careers (Steven Strauss, personal communication, 2004).

Another approach recently announced by Oji Paper Company in Japan for an analogous situation has been announced (in a news release). They engineered *Eucalyptus* to withstand very acid soils, and graft non-transgenic rapidly growing *Eucalyptus* on the transgenic stock. There can be no transgene flow from these plants, unless suckers or shoots form on the rootstocks. Similar grafting approaches could be used with bioremediating tree species.

Concluding remarks

Systems exist that can theoretically preclude a phytoremediating species from becoming established outside the contaminated area being treated, whether by containing gene flow or by preventing the establishment of hybrids by mitigation. Thus, if a risk of establishment is discerned, it should not preclude developing transgenic phytoremediation species – it should stimulate the imagination to devise and test systems to deal with the potential problems.

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