ASSESSING RISKS AND CONTAINING OR MITIGATING GENE FLOW OF TRANSGENIC AND NON-TRANSGENIC PHYTOREMEDIATING PLANTS

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1. Introduction - needs for preventing gene flow in phytoremediating species

Plants have been used to correct human error over the ages. The few species capable of revegetating Roman lead and zinc mine tailings in Wales [2] 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) [3] or 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 are most efficient at dealing with surface wastes, and trees such as *Populus* spp., for dealing with deeper wastes [4]. *Brassica juncea* (Indian mustard) 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 some heavy metals. This ability has been enhanced by mutant selection (in tissue culture) for heavy metal resistance [5], from *Thlaspi* by protoplast fusion (along with many other genes) [6], but it was better yet to transgenically transfer genes leading to enhanced glutathione content [7, 8] 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, gene containment and/or mitigation seem necessary to prevent volunteers

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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 hybridising with related native or introduced 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 hybridising with other varieties of the same species or even other varieties of the same species. *Spartina* [9, 10] and *Populus* [11, 12] are often proposed for phytoremediation, yet they commonly form hybrids with other species in their genera. In the case of *Spartina,* the results were devastating when the new world *Spartina alterniflora* crossed with the European *S. maritime* around 1870, the hybrids massively displaced all other native species from the ecosystem [13]. Populus species easily form hybrids [14], and native species could easily be displaced by hybrids. 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 separately, molecular mitigation tools that can prevent 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 [15]. 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 [16]. The number 1 and 16th Worst Weeds of the World, *Cyperus* spp., are primarily spread asexually [17].

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

2. Assessing the likelihood of risks

Species used for phytoremediation are often no ordinary agricultural species, nor are they used in agricultural contexts. In their natural surroundings any species occupies a

specific niche that determines its occurrence in space, time and function of the ecosystem concerned. Genetic modification might alter a niche, and does certainly so when phytoremediation traits are attached to species formerly sensitive to the chemicals involved, and equally certain if genetic modification changes fitness. However, the basic biological traits will be the same as for any plant species.

In this section we examine the interaction between different factors that might produce an ecological risk situation. First the factors are indicated and briefly described as we did for the biotechnology derived herbicide resistant plants [18]. However, the situation is clearly more complicated since phytoremediation traits always operate under natural conditions compared to herbicide resistance, which requires that the herbicide be applied to be operational.

For contaminated sites it might be thought that there is no risk as the purpose of the use of phytoremediation plants is cleaning of the site by being grown there. However, there are many rare, sometimes endemic species that are specialised on growing on (heavy metal) containing soils. An example is *Viola calaminaria,* which is endemic in the Netherlands, Belgium and Western Germany, and completely restricted to zinc, lead, and/or cadmium containing soils [19]. Such a rare and endangered species may easily be out competed by an engineered if phytoremediating species are used on, or invade the *Viola* habitats, and in this way a loss of biodiversity may occur. Conversely, this species may be used to mine the genes for zinc phytoremediation, to be transformed into faster growing species, for use elsewhere.

The theoretical case of a remediation species becoming invasive on uncontaminated sites is currently a remote possibility but cannot completely ruled out, especially as they can flow via pollen to related species, forming hybrid swarms.

2.1. DECISION TREES FOR ASSESSING THE LEVEL OF RISK

Here we combine the different factors through the use of decision trees. We want to state clearly that a decision tree is not a quantitative tool producing a quantified risk. It is an aid in risk evaluation providing unbiased guidance in indicating hazards attached to a certain species. When a hazard is indicated, more detailed and quantified data acquisition will often be necessary.

Different levels of certainty apply to the various factors used. For instance: invasiveness is highly unpredictable whereas the presence or absence of vegetative propagation is not.

The keys are layered; first assessing the “biological hazard” (Key 1), which is equal for engineered and non-engineered species, and then we examine the extent to which measures aimed at containment and mitigation affect the total risk (Key 2). Finally, the keys are designed to assist in determining whether the growing conditions might trigger identified hazards into real risks, both on contaminated sites (Key 3) and in an uncontaminated environment (Key 4).

2.1.1 Key 1 Assessing basic hazards imposed by biology.

1. Invasiveness

1a. The plant has no known invasive characters:

Basic Biological Hazard low, go to Key 2A
1b. The plant is a known invasive, go to 2
2. The plant is a known invasive
2a. The plant has no sexual propagation (Most species have a sexual propagation pathway. However, some species may almost never propagate sexually, or when cultivated, propagation might be entirely vegetative), go to 3
2b. The plant has sexual propagation, go to 4
3. Known invasive, without sexual propagation
3a. The plant has a proven capacity for efficient natural long range dissemination:
Basic Biological Hazard high: go to Key 2C
3b. The plant has little capacity for efficient natural long range dissemination; dissemination takes place with the aid of people:
Basic Biological Hazard medium: go to Key 2B
4. Known invasive with sexual propagation
4a. The species is cross-pollinating - go to 5
4b. The species is self- pollinating - go to 6
5. Cross pollinating invasive species
5a. The plant has a proven capacity for efficient natural long range dissemination:
Basic Biological Hazard very high: go to Key 2D
5b. The plant has little capacity for efficient natural long range dissemination; dissemination takes place with the aid of people:
Basic Biological Hazard high: go to Key 2C
6. Self pollinating invasive species
6a. The plant has a proven capacity for efficient natural long range dissemination
Basic Biological Hazard high: go to Key 2C
6b. The plant has little capacity for efficient natural long range dissemination; dissemination takes place with the aid of people:
Basic Biological Hazard medium: go to Key 2B

2.1.2 Key 2A Assessing hazards imposed by containment and mitigation measures - Basic Biological hazard low.

1. Presence of added genetic containment and mitigation measures
1a. No measures new to the species added:
Biological hazard low, go to Key 3B and Key 4
1b. New measures have been added aimed at containment and/or mitigation:
Biological Hazard very low; go to Key 3A and Key 4

2.1.3 Key 2B Assessing hazards imposed by containment and mitigation measures - Basic Biological hazard medium.

1. Presence of added genetic containment and mitigation measures
1a. No measures new to the species added. (If containment or mitigation genes present in the species gene pool are used in cultivar breeding, no new possibilities are introduced in the species, and hence the hazard to the environment is estimated as equal to the basic biological hazard): Biological Hazard medium, go to Key 3C and Key 4

1b. New measures have been added aimed at containment and/or mitigation, go to 2
2. Decreasing gene flow
2a. The plant has added genes, not in the parent, enhancing containment, go to 3
2b. The plant has added genes, not present in the parent, enhancing mitigation, go to 5
3. New containment genes present
3a. Containment genes added at random, go to 4
3b. Containment genes present as tandem constructs:
Hazard very low, go to Key 3A and Key 4
4. Random containment genes present, presence of mitigation genes
4a. The plant has added genes engineered in tandem, enhancing mitigation:
Hazard very low, go to Key 3A and Key 4
4b. The plant has added genes incorporated at random, enhancing mitigation:
Hazard very low, go to Key 3A and Key 4
4c. The plant has no mitigation genes added: Hazard low, go to Key 3b and Key 4
5. No containment genes present, presence of mitigation genes
5a. The plant has added genes engineered in tandem, enhancing mitigation:
Hazard very low: go to Key 3A and Key 4
5b. The plant has added genes incorporated at random, enhancing mitigation:
Hazard low, go to Key 3B and Key 4

2.1.4 Key 2C Assessing hazards imposed by containment and mitigation measures - Basic Biological hazard high.

1. Presence of added genetic containment and mitigation measures
1a. No measures new to the species added:
Biological hazard high: go to Key 3D and Key 4
1b. New measures have been added aimed at containment and/or mitigation, go to 2
2. Decreasing gene flow
2a. The plant has added genes, not in the parent, enhancing containment, go to 3
2b. The plant has added genes, not in the parent species, enhancing mitigation, go to 6
3. New containment genes present
3a. Containment genes added at random, go to 4
3b. Containment genes present as tandem constructs, go to 5
4. Random containment genes present, presence of mitigation genes
4a. The plant has added genes engineered in tandem, enhancing mitigation:
Hazard very low, go to Key 3A and Key 4
4b. The plant has added genes incorporated at random, enhancing mitigation:
Hazard very low, go to Key 3A and Key 4
4c. The plant has no mitigation genes added. Hazard medium, go to Key 3C and Key 4
5. Tandem containment genes and mitigation genes present
5a. The plant has added genes engineered in tandem, enhancing mitigation:
Hazard very low, go to Key 3A and Key 4
5b. The plant has added genes incorporated at random, enhancing mitigation:
Hazard very low, go to Key 3A and Key 4
5c. No mitigation genes present: Hazard low, go to Key 3B and Key 4
6. No containment genes present, presence of mitigation genes

6a. The plant has added genes engineered in tandem, enhancing mitigation:
Hazard very low: go to Key 3A and Key 4
6b. The plant has added genes incorporated at random, enhancing mitigation:
Hazard medium, go to Key 3C and Key 4

2.1.5 Key 2D Assessing hazards imposed by containment and mitigation measures - Basic Biological hazard very high.

1. Presence of added genetic containment and mitigation measures
1a. No novel measures species added:
Biological hazard very high: go to Key 3E and Key 4
1b. New measures have been added aimed at containment and/or mitigation, go to 2
2. Decreasing gene flow
2a. The plant has added genes, not in the parent, enhancing containment, go to 3
2b. The plant has added genes, not in the parent species, enhancing mitigation, go to 6
3. New containment genes present
3a. Containment genes added at random, go to 4
3b. Containment genes present as tandem constructs, go to 5
4. Random containment genes present, presence of mitigation genes
4a. The plant has added genes engineered in tandem, enhancing mitigation:
Hazard very low, go to Key 3a and 4
4b. The plant has added genes incorporated at random, enhancing mitigation:
Hazard low, go to Key 3B and 4
4c. The plant has no mitigation genes added: Hazard high, go to Key 3D and Key 4
5. Tandem containment genes present, presence of mitigation genes
5a. The plant has added genes engineered in tandem, enhancing mitigation:
Hazard very low, go to Key 3A and Key 4
5b. The plant has added genes incorporated at random, enhancing mitigation:
Hazard very low, go to Key 3A and Key 4
6. No containment genes present, presence of mitigation genes
6a. The plant has added genes engineered in tandem, enhancing mitigation:
Hazard low: go to Key 3A and Key 4
6b. The plant has added genes incorporated at random, enhancing mitigation:
Hazard high, go to Key 3D and Key 4

2.1.6 Key 3A Assessing risks to contaminated sites - Biological hazard very low.

Contaminated site refers to all sites contaminated with the compound(s) for which the plant may be used in cleaning up. There still may be unwanted side-effects to some naturally occurring contaminated sites because they are inhabited by rare and protected wild species that evolved to withstand the contamination. These species may be out-competed by the “bioremediating” species.

1a. The species (transgenic or not) needs management to survive at the site, go to 2
1b. The species (transgenic or not) survives at a site without management, go to 4
2. Species only surviving under management
2a. The species poses a risk to higher trophic levels on the site, go to 3
2b. The species poses no risk to higher trophic levels on the site:

Risk very low for contaminated sites, go to Key 4
3. Species survives under management, posing risk to higher trophic levels on the site
3a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4
3b. To species not confined to this specific contaminated environment:
Risk low for contaminated sites, go to Key 4
4. Species survives without management
4a. Species unable to spontaneously invade a contaminated site, go to 5
4b. Species able to invade and dominate the contaminated site, go to 7
5. Species survives but unable to invade contaminated sites
5a. The species poses a risk to higher trophic levels on the site, go to 6
5b. The species poses no risk to higher trophic levels on the site:
Risk very low for contaminated sites, go to Key 4
6. Species survives, but unable to spontaneously invade, yet poses a risk to higher trophic levels on the site
6a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4
6b. To species not confined to this specific contaminated environment.
Risk low for contaminated sites, go to Key 4
7. Species survives and able to spontaneously invade contaminated sites
7a. The species poses a risk to higher trophic levels on the site, go to 8
7b. The species poses no risk to higher trophic levels on the site:
Risk very low for contaminated sites, go to Key 4
8. Species survives, can spontaneously invade, and poses a risk to higher trophic levels on the site
8a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4
8b. To species not confined to this specific contaminated environment:
Risk low for contaminated sites, go to Key 4

2.1.7 Key 3B Assessing risks to contaminated sites - Biological hazard low.

1a. The species (transgenic or not) needs management to survive at a site, go to 2
1b. The species (transgenic or not) survives at a site without management, go to 4
2. Species only survives under management
2a. The species poses a risk to higher trophic levels on the site, go to 3
2b. The species poses no risk to higher trophic levels on the site:
Risk very low for contaminated sites, go to Key 4
3. Species survives under management posing risk to higher trophic levels on the site
3a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4
3b. To species not confined to this specific contaminated environment:
Risk low for contaminated sites, go to Key 4
4. Species survives without management
4a. Species unable to spontaneously invade a contaminated site, go to 5
4b. Species can invade and dominate a contaminated site, go to 7

5. Species survives but unable to spontaneously invade contaminated sites
5a. The species poses a risk to higher trophic levels on the site, go to 6
5b. The species poses no risk to higher trophic levels on the site:
Risk very low for contaminated sites, go to Key 4
6. Species survives, but unable to spontaneously invade and poses risk to higher trophic levels on the site
6a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4
6b. To species not confined to this specific contaminated environment:
Risk low for contaminated sites, go to Key 4
7. Species survives and able to spontaneously invade contaminated sites
7a. The species poses a risk to higher trophic levels on the site, go to 8
7b. The species poses no risk to higher trophic levels on the site:
Risk very low for contaminated sites, go to Key 4
8. Species survives and can spontaneously invade, and poses a risk to higher trophic levels on the site
8a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4
8b. To species not confined to this specific contaminated environment.
Risk low for contaminated sites, go to Key 4

2.1.8 Key 3C Assessing risks to contaminated sites - Biological hazard medium.

1a. The species (transgenic or not) needs management to survive at a site, go to 2
1b. The species (transgenic or not) survives at a site without management, go to 4
2. Species only survives under management
2a. The species poses a risk to higher trophic levels on the site, go to 3
2b. The species poses no risk to higher trophic levels on the site:
Risk very low for contaminated sites, go to Key 4
3. Species survives only under management, but poses a risk to higher trophic levels on the site
3a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4
3b. To species not confined to this specific contaminated environment.
Risk low for contaminated sites, go to Key 4
4. Species survives without management
4a. Species unable to spontaneously invade contaminated site, go to 5
4b. Species can spontaneously invade and dominate a contaminated site, go to 7
5. Species survives but unable to spontaneously invade contaminated sites
5a. The species poses a risk to higher trophic levels on the site, go to 6
5b. The species poses no risk to higher trophic levels on the site:
Risk very low for contaminated sites, go to Key 4
6. Species survives, yet unable to spontaneously invade, but poses risk to higher trophic levels on the site
6a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4

6b. To species not confined to this specific contaminated environment:
Risk low for contaminated sites, go to Key 4
7. Species survives and is able to spontaneously invade contaminated sites
7a. The species poses a risk to higher trophic levels on the site, go to 8
7b. The species poses no risk to higher trophic levels on the site:
Risk very low for contaminated sites, go to Key 4
8. Species survives, can spontaneously invade and poses risk to higher trophic levels on the site
8a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4
8b. To species not confined to this specific contaminated environment:
Risk low for contaminated sites, go to Key 4

2.1.9 Key 3D Assessing risks to contaminated sites - Biological hazard high.

1a. The species (transgenic or not) needs management to survive at a site, go to 2
1b. The species (transgenic or not) survives at a site without management, go to 4
2. Species only survives under management
2a. The species poses a risk to higher trophic levels on the site, go to 3
2b. The species poses no risk to higher trophic levels on the site:
Risk very low for contaminated sites, go to Key 4
3. Species survives only under management yet poses a risk to higher trophic levels on the site
3a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4
3b. To species not confined to this specific contaminated environment:
Risk low for contaminated sites, go to Key 4
4. Species survives without management
4a. Species unable to spontaneously invade a contaminated site, go to 5
4b. Species spontaneously invades and dominates a contaminated site, go to 7
5. Species survives but unable to spontaneously invade contaminated sites
5a. The species poses a risk to higher trophic levels on the site, go to 6
5b. The species poses no risk to higher trophic levels on the site:
Risk very low for contaminated sites, go to Key 4
6. Species survives, is unable to spontaneously invade, yet poses risk to higher trophic levels on the site
6a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4
6b. To species not confined to this specific contaminated environment:
Risk low for contaminated sites, go to Key 4
7. Species survives and can spontaneously invade contaminated sites
7a. The species poses a risk to higher trophic levels on the site, go to 8
7b. The species poses no risk to higher trophic levels on the site:
Risk very low for contaminated sites, go to Key 4
8. Species survives, can spontaneously invade and poses a risk to higher trophic levels on the site

8a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4
8b. To species not confined to this specific contaminated environment:
Risk low for contaminated sites, go to Key 4

2.1.10 Key 3E Assessing risks to contaminated sites - Biological hazard very high.

1a. The species (transgenic or not) needs management to survive at a site, go to 2
1b. The species (engineered or not) survives at a site without management, go to 4
2. Species only survives under management
2a. The species poses a risk to higher trophic levels on the site, go to 3
2b. The species poses no risk to higher trophic levels on the site:
Risk very low for contaminated sites, go to Key 4
3. Species survives under management posing risk to higher trophic levels on the site
3a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4
3b. To species not confined to this specific contaminated environment:
Risk low for contaminated sites, go to Key 4
4. Species survives without management
4a. Species unable to spontaneously invade contaminated site, go to 5
4b. Species can spontaneously invade and dominate a contaminated site, go to 7
5. Species survives but unable to spontaneously invade contaminated sites
5a. The species poses a risk to higher trophic levels on the site, go to 6
5b. The species poses no risk to higher trophic levels on the site:
Risk very low for contaminated sites, go to Key 4
6. Species survives, is unable to spontaneously invade, yet poses a risk to higher trophic levels on the site
6a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4
6b. To species not confined to this specific contaminated environment:
Risk low for contaminated sites, go to Key 4
7. Species survives and can spontaneously invade contaminated sites
7a. The species poses a risk to higher trophic levels on the site, go to 8
7b. The species poses no risk to higher trophic levels on the site:
Risk very low for contaminated sites, go to Key 4
8. Species survives, can spontaneously invade, and poses a risk to higher trophic levels on the site
8a. To species confined to this specific contaminated environment:
Risk medium for contaminated sites, go to Key 4
8b. To species not confined to this specific contaminated environment:
Risk low for contaminated sites, go to Key 4

2.1.11 Key 4 Assessing risks to the natural, uncontaminated environment.

1. The basic biological hazard has been estimated in Key 2 as:
1a. biological hazard very low: non-invasive species, new containment and/or mitigation genes added: Risk very low

1b. Biological hazard low, go to 2
1c. Biological hazard medium, go to 3
1d. Biological hazard high, go to 5
1e. Biological hazard very high, go to 7
2. Biological hazard low – species can invade uncontaminated environment
2a. Species unable to spontaneously invade and dominate an uncontaminated site:
Risk to uncontaminated environment very low
2b. Species able to spontaneously invade and dominate an uncontaminated site:
Risk to uncontaminated environment low
3. Biological hazard medium - species can invade uncontaminated environment
3a. Species unable to spontaneously invade and dominate an uncontaminated site:
Risk to uncontaminated environment very low
3b. Species able to invade and dominate an uncontaminated site, go to 4
4. Invasive on uncontaminated sites: risk to trophic levels
4a. The species poses a risk to higher trophic levels
Risk to uncontaminated environment high
4b. The species poses no risk to higher trophic levels:
Risk to uncontaminated environment medium
5. Biological hazard high - ability to invade uncontaminated environment
5a. Species unable to spontaneously invade and dominate an uncontaminated site:
Risk to uncontaminated environment very low
5b. Species able to invade and dominate an uncontaminated site, go to 6
6. Invasive on uncontaminated sites: risk to trophic levels
6a. The species poses a risk to higher trophic levels:
Risk to uncontaminated environment very high
6b. The species poses no risk to higher trophic levels:
Risk to uncontaminated environment high
7. Biological hazard very high - ability to invade uncontaminated environment
7a. Species unable to spontaneously invade and dominate a uncontaminated site:
Risk to uncontaminated environment very low
7b. Species able to invade and dominate a uncontaminated site:
Risk to uncontaminated environment very high

3. Dealing with the risks

3.1. GENE FLOW

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 [20] 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 [21]. In agroecosystems, such inadvertent gene flow may be undesirable.

There are two general approaches to dealing with gene flow: (1) "contain" the transgenes in the novel variety so that gene inflow, gene outflow or both are precluded depending on the mechanism; (2) "mitigate" gene flow effects if there are inevitable "leaks" in the containment system, which should also prevent volunteer populations of the phytoremediation species from establishing and/or reaching maturity so that they cannot evolve into problems. Most discussions so far have dealt with "containing" gene flow from managed ecosystems to "natural" ecosystems with less on "mitigation" of the effects of gene flow after it has occurred [22-27]. Only recently has discussion begun dealing with gene flow within the agroecosystems, both on preventing and mitigating endo-feral (evolution within the biotype) and exo-feral (evolution of less domesticated forms by crossing with wild or weedy forms) dedomestication of species as volunteer weeds [28]. Containment and mitigation are discussed below in the general context of bi-directional containment as well as mitigation.

3.2. 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 phytoremediating species (i.e., to prevent outflow to related species), or to mitigate the effects of transgene flow once it has occurred [20, 22, 26, 29]. It is more important to prevent gene flow from the phyto-remediating species to outside the contaminated site than to prevent influx into the phytoremediation site, as the phytoremediating species should be most fit to live the contaminated 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.

3.2.1 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 [30-32]. 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 [33]. Indeed the bacterial genes *mer*A/*mer*B that convert organomercurials into elemental mercury (which is later volatilised) were successfully introduced into chloroplasts of tobacco [34]. 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 [35].

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 species. 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 than off site.

The claim of strict maternal inheritance of plastome-encoded traits [32, 36, 37] was not substantiated. Tobacco [38] and other species [39] 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 leaf marker) crossed with five different male sterile herbicide susceptible lines. Chloroplast-inherited resistance was pollen transmitted at a frequency of 3×10^{-4} in >780,000 hybrid offspring [40]. At this transmission frequency, the probability of transgene 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 [32] discounts the relevance of the findings with tobacco and *Setaria* as being due to an origin of the plastids from interspecific (closely related) cytoplasmic substitution, where pollen transmission barriers can break down [41]. *Setaria viridis,* the wild progenitor of *Setaria italica* is biologically con-specific with it [42]. 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 phytoremediating species and related species; 2) he [32] ignores the discussion in Darmency *et al*. [39] of cases of intraspecific transmission of plastomic traits by pollen at about the same frequency, within the same species, as reported above between species.

3.2.2 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 [40]. 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 interbreeding species adjacent to the phytoremediation sites. 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.

3.2.3 Genetic use restriction technologies and recoverable block of function

Other molecular approaches suggested for transgene containment include: seed sterility, utilizing the genetic use restriction technologies (GURT) (‘terminator gene’) [43, 44],

and recoverable block of function (RBF) [45] to prevent transgene flow. Such proposed technologies control both the gene influx of exo-ferality and endo-feral 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.

3.2.4 Repressible seed lethal technologies

An impractical technology has been proposed to use a "repressible seed-lethal system" [46]. The seed-lethal trait and its repressor must be simultaneously inserted at the same locus on homologous chromosomes in the hybrid used for phytoremediation (in our specific case), 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 containment mechanisms is absolute, 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.

3.2.5 Transient transgenics

It is possible to insert certain phytoremediation traits encoding transgenes on RNA viruses or in endomycorrhizae that are expressed in the plant, but are not carried through meiosis into reproductive cells, and thus there will be no gene flow via seeds or pollen. Attempts had been made to use endophytes to carry useful genes into plants by pressure-infiltrating the endophytes into seeds [47, 48]. The advantage of the technology was that it was not variety specific, such that indigenous species or varieties can be used. There are endophytes that naturally participate in phytoremediation processes, e.g. the *Methylobacterium* sp. that inhabits poplars and degrades explosives [49]. Genes from this or similar species can and have been engineered into other endophytic bacteria, with quite promising results [50, 51].

The same or other infection procedures could be used to introduce phytoremediation traits by disarmed plant disease viruses as the vector. The possibility that such a procedure might work was borne out in many cases with dicots showing that they express virus-encoded genes, e.g. [52]. It was possible to infect *Arabidopsis* with tobacco etch virus carrying the *bar* gene; the gene was fully expressed in the plants [53].

Cucurbits artificially infected with an attenuated zucchini yellow mosaic potyvirus containing the same transgene s were resistant in the field [54]. An NTPII carrying wheat streak mosaic virus was used to infect various grains, and the gene was expressed (immunologically) [55]. The virus carrying the genes was expressed in the roots following leaf infection, though not in all tissues.

Considerable technological obstacles of infection of the phytoremediating species will have to be worked out. While no gene flow from the plants is expected, endophytic bacteria are prone to horizontal gene transfer among themselves, an issue which bacterial biosafety experts will have to consider. There are biosafety issues relating to the mode of disarming to be considered, and it must be demonstrated that there is no gene introgression from the virus to the plant chromosomes, as well as no-extra-nuclear transmission of the virus through ovules or pollen in very large numbers of individuals. It is necessary to transfect the phytoremediating species every generation, which may be easier with perennials, such as poplar, and it may be more cumbersome with annual species.

3.3. PREVENTING ESTABLISHMENT BY TRANSGENIC MITIGATION

If a transgene confers even a small fitness disadvantage, the less fit transgenic 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 reproduce with considerably less success than its non-transgenic competitors. A concept of “transgenic mitigation” (TM) was proposed [22], 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. 1). TM could also be used as a stand-alone procedure with non-transgenic phytoremediating species to reduce the fitness advantage of hybrids and their rare progeny, and thus substantially reduce the risk of exo-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 favourable to phytoremediating species, 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 population [22].

Thus, it was predicted that if the primary gene(s) for phytoremediation advantage being engineered into a phytoremediating species or a crop will not persist in future generations if it is flanked by TM gene(s), such as genes (for crops) 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. With crops or phytoremediating species there is typically a small amount of 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 [20, 22].

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 phytoremediating species. The cost is even inconsequential in systems where biolistic co-transformation allows introducing genes into the same site such that the tandem construct is made by the plant.

3.3.1 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 Δ*gai* (gibberellic acid-insensitive) truncated gene as a mitigator [23].

Dwarfing would be disadvantageous to the rare weeds introgressing the TM construct, as they could no longer compete, but is desirable in many crops, preventing 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 reproductive than the wild type when cultivated alone (without herbicide). They formed many more flowers than the wild type when cultivated by themselves, which is indicative 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 of competition. The inability to achieve flowering on the TM plants in the competitive situation resulted in zero reproductive fitness of the TM plants grown in an equal mixture with the wild type at typical field spacing of plants resulting from seed rain of volunteer weeds [23].

From the data above it is clear that transgenic mitigation should be advantageous to a phytoremediation species growing alone, while disadvantageous to a hybrid with it living in the competitive environment of the phytoremediation site, or off 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. We have inserted the same construct into oilseed rape and have tested the selfed progeny, as well as hybrids with the weed *Brassica campestris*

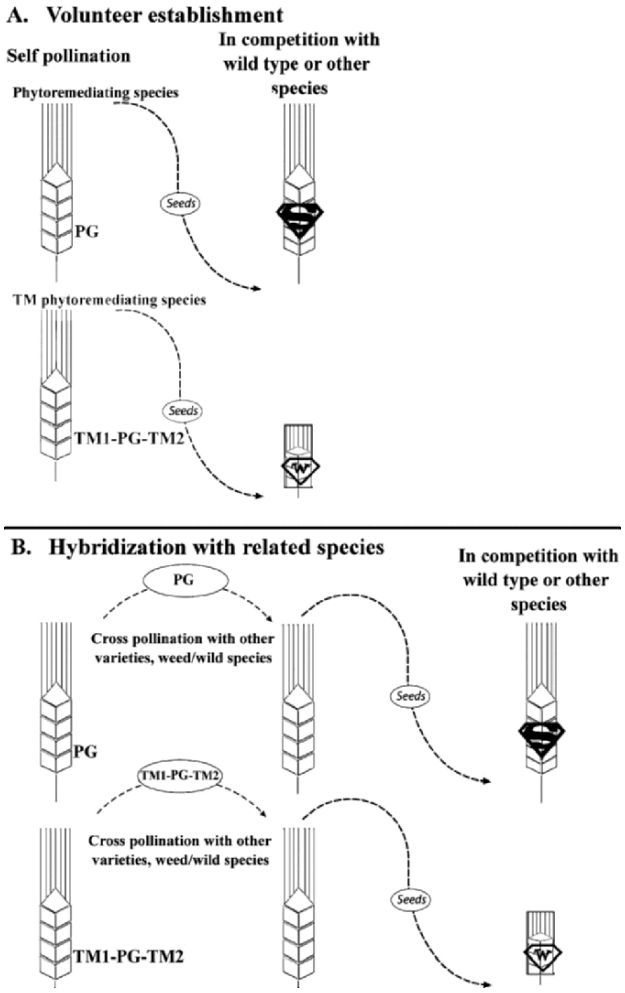

Fig. 1. Transgenic Mitigation to prevent establishment of (A) volunteers and (B) hybrids between phytoremediation species and relatives. The phytoremediation species bears desirable transgenes coupled in tandem with transgenes encoding traits that are neutral or positive for the phytoremediating species, but render volunteers or hybrids unfit to compete outside of cultivation. Source: From ref. [1], with permission of Springer Verlag.

x *B. rapa.* When cultivated alone, the dwarf transgenic oilseed rape grew at almost the same rate as the transgenic (Fig. 2A), but produced twice as much seed as the non-transgenic isoline (Fig. 2C). When the TM transgenic oilseed rape plants were co-cultivated in competition with the wild type, they were unable to grow normally (Fig. 2B), and hardly set seed (Fig. 2C) because they were so unfit to reproduce.

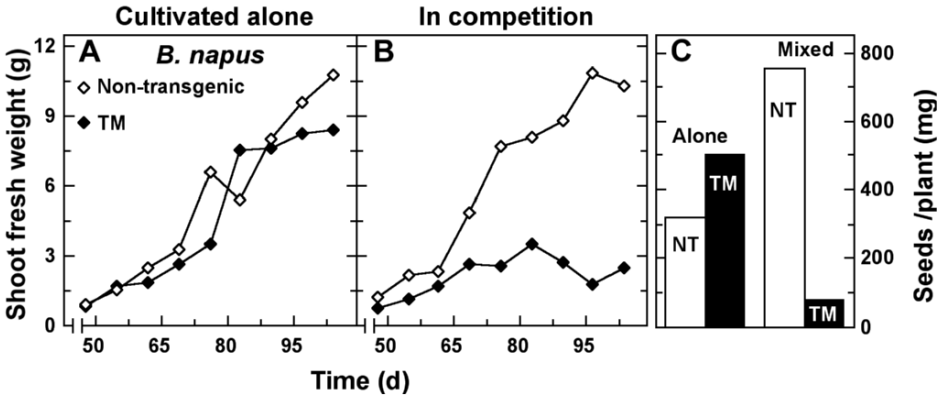

Fig. 2. Suppression of B. growth and C. seed yield of TM (transgenic mitigator) bearing oilseed rape plants carrying a dwarfing gene in tandem with a herbicide resistance gene (closed symbols and bars) when in competition with non-transgenic plants (open symbols and bars)), and A. near-normal growth of the transgenics and C. much higher seed yield of the transgenics when cultivated separately without herbicide at 3 cm spacing in a biocontainment screenhouse. (Unpublished data: Al-Ahmad and Gressel, 2005).

The rare hybrid offspring from escaped pollen bearing transgenic mitigator genes would not pose a dire threat, especially to wild species outside fields, as the amount of pollen reaching the pristine wild environment would only be at a minuscule fraction of the pollen from the wild type. This is dependent on the distance, source size, and on fertility barriers. Large-scale cultivation creates large pollen sources, and in theory a wild population having its niche on "the edge of agriculture" with coincident pollen shed could be swamped. There has been pollen flow, but no swamping with native DNA of wheat sporadically appearing in a ruderal *Aegilops* sp. [21]. Presently, there are no well documented cases where fertility barriers do not prevent more than the formation of a few infertile hybrids near the borders, as well as the rare introgressions, as have been happening for time immemorial. Any unfit hybrids and their rare backcross offspring containing transgenes linked to TM genes should still be eliminated. Further large-scale field studies will be needed with crop/weed pairs to continue to evaluate the positive implications of risk mitigation.

3.3.2 Risk that introgression of TM traits will affect relatives of the phytoremediating species

A model by Haygood *et al*. [56] claims to "prove" the premise that "demographic swamping" by transgenes would cause "migrational meltdown" of wild species related to the crop or phytoremediating species, especially if the introgressed genes confer unfitness. This proposition that recurrent gene flow from crops or phytoremediating species, even TM gene flow, could affect wild relatives deserves some discussion, as it negates the concept of transgenic mitigation.

They claim that their model demonstrates that recurrent gene flow from transgenic crops or phytoremediating species 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 x wild hybrids continually form, yet no evidence is presented that demographic swamping has occurred due to recurrent gene flow from the crops or phytoremediating species, nor 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.

There are other mundane yet fatal flaws in their model 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 or phytoremediating species/wild species systems, are discussed below:

- to get the level of swamping that they [56] discuss, the wild relative and the phytoremediating species would have to live in the same ecosystem. There are typically geographic separations between phytoremediation ecosystems and wild ecosystems, with the extent of pollen flow decreasing exponentially with distance between them – 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 hybridisation events in the wild from crop pollen will be rare, even with masses of pollen occurring within the agroecosystem. Thus their basic assumption of transgenic pollen swamping wild type pollen in the wild is invalid. Indeed, even when they assume an enormous 10% of hybridisations in the wild each generation coming from transgenic pollen, according to their model it will take about 20 generations of recurrent pollination for the unfit allele to become fixed in half the population, and 50 generations for an unfit gene to asymptotically reach 80% of the population. As discussed below, their other assumptions leading to these numbers are also off target, so it should actually take much longer;
- 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 pollen from one species to fertilize another species without any genetic barrier in the wild relative. Of the species mentioned in [57], 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 (as reviewed in [26]. The flow of genes between 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 is very low. Of course weedy rice is not a wild species (by definition), 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;
- 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 disfavoured crop allele….." [56] 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, transgenes from phytoremediating species 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.

Haygood *et al.* [56] further contend that their model would work if the phytoremediating species were heterozygous for the unfit gene (and many transgenic hybrids have the transgene in a single parent and are thus hemizygous). The data in Fig. 2 clearly demonstrate 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.* [56] (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.

In summary, 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 phytoremediating species. Weeds are man-made domesticated species (of a sort), and they are dependent on human controlled agro-ecosystems. These systems change continuously, which leads to continual shifting weed populations with an ever-changing composition. Over time new species invade, and old species go extinct, adapt, or are once more confined to their original natural environments. This is the nature of agriculture itself. It is likely that weeds that are evolutionarily threatened by the flow of unfit genes would evolve exclusionary mechanisms that block extinction;, e.g., they could evolve a shift to predominant self-fertilization that would protect them from transgenic pollen bearing unfit genes. The model of Haygood *et al*. [56] 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 so 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 [58]. Some pollen flowed, but the hybrid offspring cannot compete with wild-type offspring.

3.3.3 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 a uniform biobarcode™ system be used, where a small piece of non-coding DNA having uniform recognition sites are 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 [59].

4. Special transgenic mitigation genes for phytoremediation

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 metabolising halogenated hydrocarbons, as well as male sterility, and lack of fertility [60], but necessarily linked in tandem, so the traits can segregate. 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 [61]. 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 [62], 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 Univ., pers. comm. 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 [63], 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 [64], 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.

4.1. 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 species, where the vegetative material is harvested, and flowering (bolting) is detrimental. This could easily be effected by preventing gibberellic acid biosynthesis [65], either in a TM construct and/or by permanent mutation of the kaurene oxidase gene using a chimeraplastic gene conversion system [66], 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 relatives grow to prevent pollen transfer.

Delaying of bolting and flowering by using a different transgene has recently been demonstrated. Curtis *et al.* [67] 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.

4.2. SPECIAL CONTAINMENT/MITIGATION GENES FOR PHYTOREMEDIATING TREES

In forestry, the possibility of gene flow is especially problematic as the duration until long-term implications of gene movement become apparent can be longer than human lifetimes. The introgression of traits from these species to wild populations has been extensively discussed by [20, 68] and thus containment/mitigation requirements should be stringent. 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 barnase under the

T29 tapetum-specific promoter [69]. The ribonuclease is only produced in the tapetum and prevents pollen formation with no other ill effects.

If one has an important phytoremediating species 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 [60], 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 [70]. 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 2m)...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 sceptical, given that tall and straight trees is what they have been taught to seek all their careers (Steven Strauss, personal communication, 2004).

Another approach by scientists at Oji Paper Company in Japan for an analogous situation has been announced (in a news release) [71]. They engineered *Eucalyptus* to withstand very acid soils, and graft non-transgenic rapidly growing *Eucalyptus* on the transgenic acid-tolerant rootstock. There can be no transgene flow from these plants, unless suckers or shoots form on the rootstocks. Similar grafting approaches could be used with many bioremediating tree species.

5. 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. There is evidence that some of these systems are efficient in crops, and there is no reason they could not be used in phytoremediating species, where a risk of transgene flow is perceived. Thus, if a risk of establishment is discerned using the enabling decision tree proved above, such a risk 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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References

[1] Gressel, J and Al-Ahmad, H (2005) Assessing and managing biological risks of plants used for bioremediation, including risks of transgene flow. Z Naturforsch C, 60: 154-165

[2] Smith, RAH and Bradshaw, AD (1979) Use of metal tolerant plant populations for the reclamation of metalliferous wastes. J Appl Ecol 16: 595-603

[3] Kramer, U; Smith, RD; Wenzel, WW; Raskin, I and De, S (1997) The role of metal transport and tolerance in nickel hyperaccumulation by *Thlaspi goesingense* Halacsy. Plant Physiol 115: 1641-1650

[4] Pilon-Smits, E and Pilon, M (2002) Phytoremediation of metals using transgenic plants. Crit Rev Plant Sci 21: 439-456

[5] Schulman, RN; Salt, DE and Raskin, I (1999) Isolation and partial characterization of a lead-accumulating *Brassica juncea* mutant. Theor Appl Genet 99: 398-404

[6] Dushenkov, S; Skarzhinskaya, M; Glimelius, K; Gleba, D and Raskin, I (2002) Bioengineering of a phytoremediation plant by means of somatic hybridization. Intl J Phytoremed 4: 117-126

[7] Zhu, YL; Pilon-Smits, EAH; Tarun, AS; Weber, SU; Jouanin, L and Terry, N (1999) Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing gamma-glutamylcysteine synthetase. Plant Physiol 121: 1169-1177

[8] Bennett, LE; Burkhead, JL; Hale, KL; Terry, N; Pilon, M and Pilon-Smits, EAH (2003) Analysis of transgenic Indian mustard plants for phytoremediation of metal-contaminated mine tailings. J Envir Qual 32: 432-440

[9] Weis, JS and Weis, P (2004) Metal uptake, transport and release by wetland plants: implications for phytoremediation and restoration. Envir Intl 30: 685-700

[10] Rugh, CL; Gragson, GM; Meagher, RB and Merkle, SA (1998) Toxic mercury reduction and remediation using transgenic plants with a modified bacterial gene. Hortscience 33: 618-621

[11] Bittsanszky, A; Komives, T; Gullner, G; Gyulai, G; Kiss, J; Heszky, L; Radimszky, L and Rennenberg, H (2005) Ability of transgenic poplars with elevated glutathione content to tolerate zinc(2+) stress. Envir Intl 31: 251-254

[12] Schoenmuth, BW and Pestemer, W (2004) Dendroremediation of trinitrotoluene (TNT) - Part 2: Fate of radio-labelled TNT in trees. Envir Sci Pollution Res 11: 331-339

[13] Gray, AJ; Marshall, DF and Raybould, AF (1991) A century of evolution in *Spartina angelica*. Adv Ecol Res 21: 1-62

[14] Kowarik, I (2005) Urban Ornamentals Escaped from Cultivation, in J Gressel, Ed., Crop Ferality and Volunteerism, pp. 97-121, CRC Press, Boca-Raton

[15] Stankiewicz, M; Gadamski, G and Gawronski, SW (2001) Genetic variation and phylogenetic relationships of triazine-resistant and triazine-susceptible biotypes of *Solanum nigrum* - analysis using RAPD markers. Weed Res 41: 287-300

[16] Bervillé, A; Muller, M-H; Poinso, B and Serieys, H (2005) Ferality : risks of gene flow between sunflower and other *Helianthus* species, in J Gressel, Ed., Ferality and volunteerism in plants, pp. 209-230, CRC Press, Boca-Raton

[17] Holm, LG; Plucknett, JD; Pancho, LV and Herberger, JP (1977) The World's Worst Weeds, Distribution and Biology. University Press of Hawaii (Vol. pp. 609) Honolulu

[18] Gressel, J and Rotteveel, T (2000) Genetic and ecological risks from biotechnologically-derived herbicide resistant crops: decision trees for risk assessment. Plant Breeding Rev 18: 251-303

[19] Bizoux, JR; Brevers, F; Meerts, P; Graitson, E and Mahy, G (2004) Ecology and conservation of Belgian populations of *Viola calaminaria*, a metallophyte with a restricted geographic distribution. Belgian J Bot 137: 91-104

[20] Gressel, J (2002) Molecular biology of weed control, Taylor and Francis, London

[21] Weissmann, S; Feldman, M and Gressel, J (2003) Evidence for sporadic introgression of a DNA sequence from polyploid wheat into *Aegilops peregrina* (*Ae. variabilis*), in Proceedings - 10th International Wheat Genetics Symposium, Paestum (Italy), 539-542

[22] Gressel, J (1999) Tandem constructs: preventing the rise of superweeds. Trends Biotech 17: 361-366

[23] Al-Ahmad, H; Galili, S and Gressel, J (2004) Tandem constructs to mitigate transgene persistence: tobacco as a model. Mol Ecol 13: 697-710

[24] Al-Ahmad, H; Galili, S and Gressel, J (2005) Poor competitive fitness of transgenically mitigated tobacco in competition with the wild type in a replacement series. Planta (in press)

[25] Ellstrand, NC (2003) Dangerous liaisons – when cultivated plants mate with their wild relatives, Johns Hopkins University Press, Baltimore MD
[26] Stewart, CN; Halfhill, MD and Warwick, SI (2003) Transgene introgression from genetically modified crops to their wild relatives. Nature Rev Genet 4: 806-817
[27] Jenszewski, E; Ronfort, J and Chevre, AM (2003) Crop-to-wild gene flow, introgression, and possible fitness effects of transgenes. Envir Biosafety Res 2: 9-24
[28] Gressel, J and Al-Ahmad, H (2005) Molecular mitigation of ferality, in J Gressel, Ed., Crop Ferality and Volunteerism, pp. 371-388, CRC Press, Boca-Raton
[29] Daniell, H (2002) Molecular strategies for gene containment in transgenic crops. Nat Biotechnol 20: 581-586
[30] Khan, MS and Maliga, P (1999) Fluorescent antibiotic resistance marker for tracking plastid transformation in higher plants. Nat Biotechnol 17: 910-915
[31] Maliga, P (2002) Engineering the plastid genome of higher plants. Curr Opin Plant Biol 5: 164-172
[32] Maliga, P (2004) Plastid transformation in higher plants. Annu Rev Plant Biol 55: 289-313
[33] Tian, JL; Shen, RJ and He, YK (2002) Sequence modification of merB gene and high organomercurial resistance of transgenic tobacco plants. Chinese Sci Bull 47: 2084-2088
[34] Ruiz, ON; Hussein, HS; Terry, N and Daniell, H (2003) Phytoremediation of organomercurial compounds via chloroplast genetic engineering. Plant Physiol 132: 1344-1352
[35] Bizily, SP; Kim, T; Kandasamy, MK and Meagher, RB (2003) Subcellular targeting of methylmercury lyase enhances its specific activity for organic mercury detoxification in plants. Plant Physiol 131: 463-471
[36] Daniell, H; Datta, R; Varma, S; Gray, S and Lee, SB (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nat Biotechnol 16: 345-348
[37] Bock, R (2001) Transgenic plastids in basic research and plant biotechnology. J Mol Biol 312: 425-438
[38] Avni, A and Edelman, M (1991) Direct selection for paternal inheritance of chloroplasts in sexual progeny of *Nicotiana*. Mol Gen Genet 225: 273-277
[39] Darmency, H (1994) Genetics of herbicide resistance in weeds and crops, in SB Powles and JAM Holtum, Eds., Herbicide Resistance in Plants: Biology and Biochemistry, pp. 263-298, Lewis, Boca-Raton
[40] Wang, T; Li, Y; Shi, Y; Reboud, X; Darmency, H and Gressel, J (2004) Low frequency transmission of a plastid encoded trait in *Setaria italica*. Theor Appl Genet 108: 315-320
[41] Kiang, A-S; Connolly, V; McConnell, DJ and Kavanagh, TA (1994) Paternal inheritance of mitochondria and chloroplasts in *Festuca pratensis-Lolium perenne* intergeneric hybrids. Theor Appl Genet 87: 681-688
[42] Darmency, H (2005) Incestuous relations of foxtail millet (*Setaria italica*) with its parents and cousins, in J Gressel, Ed., Crop Ferality and Volunteerism, pp. 81-96, CRC Press, Boca-Raton
[43] Oliver, MJ; Quisenberry, JE; Trolinder, NLG and Keim, DL (1998) Control of plant gene expression. US Patent 5,723,765
[44] Crouch, ML (1998) How the terminator terminates: An explanation for the non-scientist of a remarkable patent for killing second generation seeds of crop plants http://www.bio.indiana.edu/people/terminator/html, The Edmonds Institute, Edmond WA, USA
[45] Kuvshinov, V; Koivu, K; Kanerva, A and Pehu, E (2001) Molecular control of transgene escape from genetically modified plants. Plant Sci 160: 517-522
[46] Schernthaner, JP; Fabijanski, SF; Arnison, PG; Racicot, M and Robert, LS (2003) Control of seed germination in transgenic plants based on the segregation of a two-component genetic system. Proc Natl Acad Sci USA 100: 6855-6859
[47] Fahey, JW and Anders, J (1995) Delivery of beneficial clavibacter microorganisms to seeds and plants. US Patent 5,415,672
[48] Tomasino, SF; Leister, RT; Dimock, MB; Beach, RM and Kelly, JL (1995) Field performance of *Clavibacter xyli* subsp. *cynodontis* expressing the insecticidal protein gene *cryIA(c)* of *Bacillus thuringiensis* against european corn borer in field corn. Biol Control 5: 442-448
[49] Van Aken, B; Yoon, JM and Schnoor, JL (2004) Biodegradation of nitro-substituted explosives 2,4, 6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, an octahydro-1,3,5,7-tetranitro-1,3, 5-tetrazocine by a phytosymbiotic *Methylobacterium* sp. associated with poplar tissues (*Populus deltoides* x *nigra* DN34). Appl Environ Microbiol 70: 508-517

[50] Barac, T; Taghavi, S; Borremans, B; Provoost, A; Oeyen, L; Colpaert, JV; Vangronsveld, J and van der Lelie, D (2004) Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. Nat Biotechnol 22: 583-588
[51] Newman, LA and Reynolds, CM (2005) Bacteria and phytoremediation: new uses for endophytic bacteria in plants. Trends Biotechnol 23: 6-8
[52] Koo, M; Bendahmane, M; Lettieri, GA; Paoletti, AD; Lane, TE; Fitchen, JH; Buchmeier, MJ and Beachy, RH (1999) Protective immunity against murine hepatitis virus (MHV) induced by intranasal or subcutaneous administration of hybrids of tobacco mosaic virus that carries an MHV epitope. Proc Natl Acad Sci USA 96: 7774-7779
[53] Whitham, SA; Yamamoto, ML and Carrington, JC (1999) Selectable viruses and altered susceptibility mutants in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 96: 772-777
[54] Shiboleth, YM; Arazi, T; Wang, Y and Gal-On, A (2001) A new approach for weed control in a cucurbit field employing an attenuated potyvirus-vector for herbicide resistance. J Biotech 92: 37-46
[55] Choi, I-R; Stenger, DC; Morris, TJ and French, R (2000) A plant virus vector for systemic expression of foreign genes in cereals. Plant J 23: 547-555
[56] Haygood, R; Ives, AR and Andow, DA (2003) Consequences of recurrent gene flow from crops to wild relatives. Proc Roy Soc London B 270: 1879-1886
[57] Gressel, J, Ed. (2005) Crop Ferality and Volunteerism, CRC Press
[58] Bradshaw, AD (1982) Evolution of heavy metal resistance - an analogy for herbicide resistance? In HM LeBaron and J Gressel, Eds., Herbicide Resistance in Plants, pp. 293-307, Wiley, New York
[59] Gressel, J and Ehrlich, G (2002) Universal inheritable barcodes for identifying organisms. Trends Plant Sci 7: 542-544
[60] USDA-APHIS (2004) Search Results of the Field Test Release Permits Database for the U.S., http://www.nbiap.vt.edu/cfdocs/fieldtests3.cfm
[61] Meilan, R; Brunner, AM; Skinner, JS and Strauss, SH (2001) Modification of flowering in transgenic trees, in N Morohoshi and A Komamine, Eds., Molecular breeding of woody plants, pp. 247-256, Elsevier, Amsterdam
[62] Skinner, JS; Meilan, R; Ma, CP and Strauss, SH (2003) The *Populus PTD* promoter imparts floral-predominant expression and enables high levels of floral-organ ablation in *Populus, Nicotiana* and *Arabidopsis*. Molec Breeding 12: 119-132
[63] Bilyeu, KD; Cole, JL; Laskey, JG; Riekhof, WR; Esparza, TJ; Kramer, MD and Morris, RO (2001) Molecular and biochemical characterization of a cytokinin oxidase from maize. Plant Physiol 125: 378-386
[64] Werner, T; Motyka, V; Laucou, V; Smets, R; Van Onckelen, H and Schmulling, T (2003) Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15: 2532-2550
[65] Hedden, P and Kamiya, Y (1997) Gibberellin biosynthesis: Enzymes, genes and their regulation. Annu Rev Plant Physiol Plant Molec Biol 48: 431-460
[66] Zhu, T; Mettenburg, K; Peterson, DJ; Tagliani, L and Baszczynski, CL (2000) Engineering herbicide-resistant maize using chimeric RNA/DNA oligonucleotides. Nat Biotechnol 18: 555-558
[67] Curtis, IS; Nam, HG; Yun, JY and Seo, K-H (2002) Expression of an antisense GIGANTEA (GI) gene fragment in transgenic radish causes delayed bolting and flowering. Transgenic Res 11: 249-256
[68] Llewellyn, DJ (2000) Herbicide tolerant forest trees, in SM Jain and SC Minocha, Eds., Molecular Biology of Woody Plants (Vol. 2), pp. 439-466, Kluwer, Dordrecht
[69] Mariani, C; Debeuckeleer, M; Truettner, J; Leemans, J and Goldberg, RB (1990) Induction of male sterility in plants by a chimeric ribonuclease gene Nature 347: 737-741
[70] Busov, VB; Meilan, R; Pearce, DW; Ma, C; Rood, SB and Strauss, SH (2003) Activation tagging of a dominant gibberellin catabolism gene (GA 2-oxidase) from poplar that regulates tree stature. Plant Physiol 132: 1283-1291
[71] Anonymous (2004) Oji grafts natural eucalyptus onto gene-altered eucalyptus. Nikkei Report, Aug 6, see: http://www.agbioworld.org/newsletter_wm/index.php?caseid=archive&newsid=2209