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John Davison

Risk mitigation of genetically modified bacteria and plants designed for bioremediation

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Abstract While the possible advantages of bioremediation and phytoremediation, by both recombinant microbes and plants, have been extensively reviewed, the biosafety concerns have been less extensively treated. This article reviews the possible risks associated with the use of recombinant bacteria and plants for bioremediation, with particular emphasis on ways in which molecular genetics could contribute to risk mitigation. For example, genetic techniques exist that permit the site-specific excision of unnecessary DNA, so that only the transgenes of interest remain. Other mechanisms exist whereby the recombinant plants or bacteria contain conditional suicide genes that may be activated under certain conditions. These methods act to prevent the spread and survival of the transgenic bacteria or plants in the environment, and to prevent horizontal gene flow to wild or cultivated relatives. Ways in which these genetic technologies may be applied to risk mitigation in bioremediation and phytoremediation are discussed.

Keywords Phytoremediation · Genetically modified organisms · Biosafety · Horizantal gene transfer · Risk-assessment

Introduction

A recent report on "the top 10 biotechnologies for improving human health" gave high priority to bioremediation by plants and microorganisms but, while emphasizing the potential of these techniques, it concluded by commenting on the potential environmental risks [11]. Sites contaminated by metals (e.g., Zn^{2+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Cr^{2+}) and xenobiotics (e.g., trichloroethylene, polychlorinated biphenyls, trinitrotoluene, polycyclic aromatic hydrocarbons, nitroglycerine) pose enormous health and environmental problems. At present, contaminated sites are treated by physical, chemical and thermal processes following excavation and transportation. The cost of removal of 1 m^3 soil from a 1-acre contaminated site is estimated at US $0.6–2.5 million. In contrast, the cost of phytoremediation of a 1-acre site is estimated at US $2,000–5,000 [49]. In addition, phytoremediation causes minimum site disruption, stabilizes the soil against erosion, and concentrates heavy metals.

Despite these advantages, microbial bioremediation in the field has advanced little over the past 10 years, and almost no true field-releases of transgenic bacteria have been performed. This slow progress can be attributed chiefly to the potential risks, as perceived by the general public and politicians, resulting in tight regulations and reducing incentives for in situ bioremediation research. Phytoremediation (bioremediation by plants) is a relatively new field but again most research is being done in academic laboratories under confined conditions. In this short review on transgenic risk mitigation, the advantages and potentials of bioremediation by plants and microbes will be treated lightly, since these have been the subjects of several excellent reviews (cited below). Many of the potential risks associated with microbial bioremediation are shared by bacteria used for agricultural purposes (nitrogen fixation, *Rhizobium*, *Bradyrhizobium*; biocontrol of phytopathogens, plant growth stimulation, *Pseudomonas*, *Bacillus*, *Agrobacterium*, *Azospirillum* [2, 55, 56]. Similarly, for phytoremediation, many of the potential problems are shared with transgenic plants used for agricultural purposes, and for the production of industrial and pharmaceutical products [10, 47]. This short review will discuss the potential risks associated with the environmental use of genetically modified microorganisms and plants for bioremediation and phytoremediation, and ways in which these risks could be mitigated. Particular attention will be paid to ways in which molecular genetics can be used for risk

J. Davison
Laboratory of Cellular Biology,
Institut National de la Recherche Agronomique,
Route de St Cyr, Versailles, 78026, France
E-mail: davison@versailles.inra.fr

mitigation, since this area is often neglected by legislators [35].

Bioremediation potential of naturally occurring microorganisms

Several microorganisms (*Pseudomonas*, *Burkholderia*, *Sphingomonas*, *Ralstonia*, *Comamonas*, *Achromobacter*, *Alcaligenes*, *Rhodococcus*, *Dehalococcoides*) are known to degrade xenobiotics, or to accumulate or detoxify heavy metals and, for more than 30 years, it has been hoped that these bacteria might be used in the clean-up of toxic xenobiotics (phenol, trichloroethylene, trinitrotoluene, dioxins [60, 72]) and heavy metal pollutants (cadmium, mercury, lead, zinc, uranium [13, 43]). An important difference exists between bioremediation of toxic metals and bioremediation of xenobiotics. Metals are elements and can change only their elemental state (e.g. in the conversion of Hg^{2+} to the volatile Hg^0, thus moving the metal from the soil to the atmosphere). In contrast, bioremediation of xenobiotics can result in the complete mineralization of the toxic substance. In situ bioremediation often uses naturally occurring microorganisms (bioattenuation), but may be improved by the addition of nutrients, such as nitrogen and phosphorus, surfactants and oxygen (biostimulation [85]). In such treatments, the nature of the microbial ecosystem is unknown or may be determined during the event. A third possibility is bioaugmentation, where bacteria, usually isolated from the same site, may be injected into the site. Transgenic bacteria may be included in this category, though in real life situations this has rarely been the case. A major difficulty with in situ biodegradation is the difficulty in predicting the end result due to the myriad of environmental factors that may intervene [83].

Potential risks of bioremediation using naturally occurring bacteria

It should be noted that, from the biosafety viewpoint, not all naturally occurring soil bacteria are ideal as bioremediation agents. For example, *Burkholderia cepacia* has potential as an agent for bioremediation, and for biological control of phytopathogens. However, it is a human pathogen known to be involved in cystic fibrosis and it is resistant to multiple antibiotics [38]. This has led to rejection by the Environmental Protection Agency (EPA) of its use as an environmental agent. Similarly, *Pseudomonas aeruginosa* is a nosocomial pathogen and indeed the name *Pseudomonas* may have a "public image problem" in that *P. aeruginosa* could be confused with other Pseudomonads such as *P. fluorescens* or *P. putida*. With the availability of bacterial genome sequences, it is becoming clear that characteristic differences exist between pathogens (plant and animal) and non-pathogens. These include the absence, in the latter, of the type-III protein secretion systems responsible for injecting specific proteins into eukaryotic cells, and of a variety of other virulence determinants. The genomes of several other environmentally important bacteria have also been completed or are underway and such studies may enable better science-based predictions of the biosafety of these microorganisms.

One potential risk is that the special conditions of bioremediation might select for bacteria with undesirable properties. It is known, for example, that toluene tolerance in Pseudomonads (a condition advantageous for environmental toluene degradation) is accomplished via deregulation of efflux pumps, and that these same efflux pumps are responsible for the pumping out of various antibiotics and biocides [31]. Thus, the possibility that in situ remediation of a toluene-contaminated site could simultaneously select for antibiotic- and biocide-resistant bacteria must be considered.

An incident due to accidental biostimulation occurred in the French Vaucluse region. In accordance with its much-criticized European Common Agricultural Policy, French farmers were subsidized to produce apples that had no market and 80,000 t (corresponding to half of the production for the region) was subsequently dumped. Following a flood, the rotting apples infiltrated the water table and probably stimulated the growth of bacteria such as *Shewanella putrefaciens* that reduce Mn(IV) to Mn(II) [43]. The former is insoluble, but the latter is soluble in the absence of oxygen and reached the drinking supplies of a nearby village, at more than 60 times the permitted level. On contact with the air, it precipitated and formed stalactites of MnO_2 on faucets (Fig. 1). Water had to be shipped into the village by road-tanker.

Construction of genetically improved biodegradation strains

Considerable effort has gone into understanding the genetics and biochemistry of xenobiotic degradation and heavy metal mobilization as well as into the construction of bacteria with improved biodegradation properties. This subject is covered in many recent reviews [13, 32, 59–61, 80, 83] and is outside the scope of the present article.

Risk mitigation in genetically modified bacteria

Monitoring of recombinant strains

The fate of environmentally released recombinant bacteria needs to be monitored by marking them genetically with easily detectable markers [86]. Early experiments used genes such as *lacZ*, *xylE* and *gusA*. More recently, these have been replaced by the green fluorescent protein

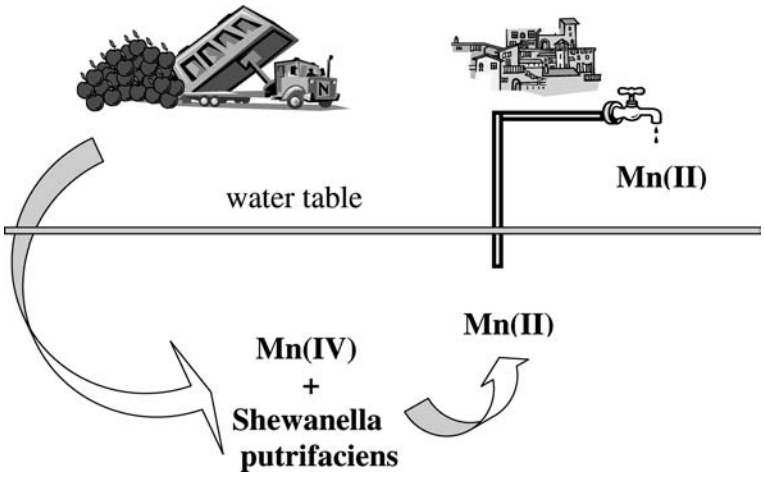

Fig. 1 Accidental manganese pollution. Sugars and organic acids from rotting apples penetrate the water table and act as carbon sources for Mn(IV)-reducing bacteria, resulting in Mn(II) contamination of drinking water

gfp genes [46] and *lux* genes for bioluminescence [64], which do not exist in soil bacteria and can be detected with greater sensitivity.

Genuine bioremediation field-trials using living genetically modified bacteria have not been performed frequently due to the difficulty of complying with environmental regulations, both in the United States and Europe. One particularly interesting trial, performed in large lysimeters, used genetically engineered *P. fluorescens* able to degrade naphthalene. A special feature of the design of this bacterium was the presence of *lux* genes giving a bioluminescent signal when degrading naphthalene and salicylate, thus permitting on-line monitoring of the degradation process [68].

Horizontal transfer of recombinant genes to other microorganisms

One frequently posed criticism is the idea that, once liberated into the environment, recombinant genes may be transferred from their host to other bacteria. The horizontal transfer (by transformation, transduction and conjugation) of a variety of genes (antibiotic resistance, heavy metal resistance, symbiotic and degradative) in a variety of environmental situations has been reviewed [15]. It was concluded that inter-specific horizontal gene transfer has been observed for many different bacteria, for many genes, and in many environmental situations. It would thus be a mistake to suppose that recombinant genes introduced into the environment will not spread to other related bacteria unless special precautions are taken. In particular, estimates of low, or non-detectable, DNA transfer in laboratory experiments (e.g., $< 10^{-9}$ cell^{-1} generation^{-1}) may be irrelevant given the huge numbers of bacteria that could be released, together with their replication over a long time period.

Conversely, a number of different techniques have been devised to reduce horizontal gene flow to other bacteria.

Use of defective transposons

Genetic engineering typically relies on plasmid vectors, but there is general agreement that these are unsuitable for environmental release due to their propensity for horizontal transfer [15–17, 20, 78]. In fact, non-conjugative plasmids (such as RSF1010), typically used for genetic engineering with *Pseudomonas*, retain the *oriT* region that permits them to be transferred efficiently in the presence of large self-transmissible plasmids (such as RP4). Even if the *oriT* region is removed, the plasmid can still be transmitted by co-integrate formation using the homology of common DNA sequences. Most attempts to build vectors for biodegradation field-release purposes focus on defective transposons that, once integrated into the chromosome, cannot do so again due to lack of the transposase gene. Genes introduced into mini-transposons showed extremely low levels of horizontal transfer ($< 10^{-9}$ cell^{-1} generation^{-1} [78]).

Elimination of antibiotic resistance genes

Transposition is a rare event, so that transposition vectors require a strong selective marker—usually antibiotic resistance. However, for environmental release, an antibiotic marker is undesirable for biosafety reasons. A number of transposons carrying resistance to zinc, mercury or tellurite, have been proposed [16, 17], but these may allow a selective advantage under certain conditions. Since the selective markers are used only to detect the transposition event, the simplest solution is their excision at a later stage by a site-specific recombination event [17, 70]. Such modern methods allow the excision of DNA sequences contained between two site-specific recombination sites (*loxP* or FRT) following transient expression of the recombinase (*cre* or FLP). An example (Fig. 2) shows the elimination of an antibiotic resistance marker by the site-specific ResA resolvase, so that the recombinant bacterium contains only the *lux* genes necessary for environmental monitoring. In addition to alleviating biosafety concerns, this type of technique

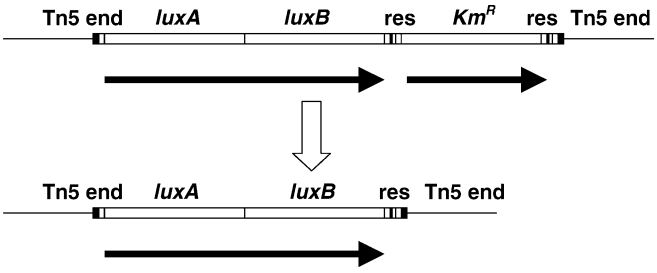

Fig. 2 Excision of unnecessary DNA. An unnecessary DNA segment containing an antibiotic resistance gene and located between two *res* sites, is excised by transient expression of the ResA resolvase

allows recycling of selective markers for serial strain constructions.

Suicide mechanisms preventing escape of recombinant bacteria

Even the use of defective transposons leaves open the possibility that horizontal transfer could occur by recombination with a conjugative plasmid or by bacterial conjugation. Thus, many vectors that contain additional safeguards using a wide variety of suicide mechanisms have been constructed, not all of which will be considered here [16]. In its simplest case, suicide may be repressed by an environmental signal (e.g., the pollutant to be degraded), allowing cell survival. The lack of this signal results in the expression of the suicide gene and triggers cell death. In one of the best-investigated examples, the lethal *gef* gene, carried by a mini-Tn5 transposon, was placed under the control of the LacI repressor. Thus, cell survival depends on the continued presence of this negative control factor. On the other hand, transcription of the *lacI* gene itself depended on the positive-activator XylS (from the *meta*-cleavage pathway), which is active only in the presence of 3-methylbenzoate. Thus, the cells are viable as long as 3-methylbenzoate is present. Cells lacking 3-methylbenzoate, either due to its degradation, or due to escape to the environment, would die due to Gef expression. The rate of escape from killing in the laboratory was about 10^{-8} cell^{-1} generation^{-1}. The system was tested and shown to be effective in outdoor experiments involving bulk soil or rhizosphere soil. No evidence of spread outside of the experimental plots was found [78].

To avoid loss of lethal function due to mutation, doubly contained strains were constructed that carry an additional function based on the *asd* gene for diaminopimellic acid synthesis. This gene is essential for cell wall synthesis and strains lacking it have an absolute requirement for external diaminopimellic acid, which is unavailable in soil environments. A *P. putida* strain carrying a deletion of the *asd* gene was provided with an alternative *asd* gene positively controlled by the XylS activator. The same XylS activator also negatively controlled *gef* expression via transcription of the *lacI* gene as described above. Thus, a strain deprived of 3-methylbenzoate would die in an environmental situation due to killing by Gef protein and also due to diaminopimellic acid deprivation (Fig. 3). The level of survival of this strain in the absence 3-methylbenzoate was below the limit of detection ($< 10^{-9}$ cell^{-1} generation^{-1} [65]).

Suicide mechanisms preventing horizontal gene transfer

The above suicide system is designed to permit fail-safe killing of recombinant strains escaping into the wild. Similar systems could prevent the transfer of recombinant genes to other bacteria via conjugation, transduction or transformation. A different conditional-lethality system was based on the colicin E3 (*colE3*) gene that kills many different bacteria by cleavage of 16S ribosomal RNA [79]. In the natural situation, this lethality function is counteracted by an immunity function (encoded by the *immE3* gene), giving a poison/antidote effect. To examine whether the killing function could be used to prevent horizontal transfer of plasmid DNA to other bacteria, the *colE3* gene was placed on a plasmid and the *immE3* gene was placed on the chro-

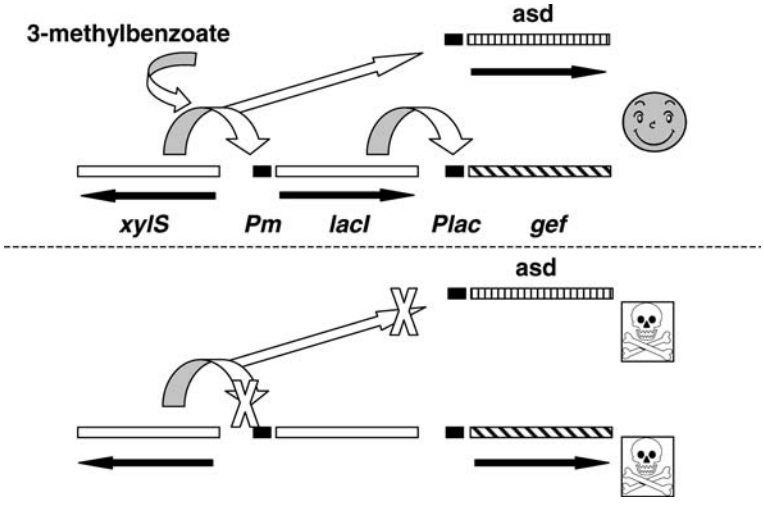

Fig. 3 In the presence of 3-methylbenzoate, the XylS activator positively controls transcription of both the *asd* gene and the *lacI* gene. The Asd enzyme catalyzes the synthesis of the essential diaminopimellic acid, and the LacI repressor prevents transcription of the Gef toxin. In the absence of 3-methylbenzoate, neither of these proteins is produced and the cell dies due to toxin production and diaminopimellic acid deprivation

mosome of *Escherichia coli* or *P. putida*. Thus, horizontal transfer of the plasmid to another bacterium would result in cell death in the recipient, since the *colE3* gene would be transferred in the absence of the chromosomal *immE3* gene. This system was able to prevent horizontal transfer to a wide variety of Gram-negative bacteria [78].

A major problem with this type of containment system is that the killing mechanism can easily be circumvented by a mutation that inactivates the killing function. A solution to this is the use of two independent killing systems, so that mutation in one system does not circumvent the other. Such a dual system used a plasmid carrying two lethal genes coding for the colE3 toxin and the *Eco*RI restriction endonuclease. These kill by completely different mechanisms (inhibition of protein synthesis, and degradation of DNA). In a real-life degradation trial, these functions would flank, for example, the biodegradation genes, so that a deletion removing both *colE3* and the *ecoRIR* genes would also remove the gene to be contained. The respective "antidote" genes *immE3* and *ecoRM* (*Eco*RI methylase) were placed on the bacterial chromosome so that they would not be transferred along with the plasmid (Fig. 4). This dual containment system showed enhanced containment of gene transfer compared to the individual lethality functions alone [78].

Phytoremediation

Certain plants show potential for the bioremediation of both metal and xenobiotic sites. For example, the brake fern (*Pteris vittata*) is able to accumulate large quantities of arsenic in the fronds [44] and the hybrid poplar is able to degrade trichloroethylene to CO_2 [25, 34]. However, phytoremediation is a relatively new discipline and much remains to be achieved. Recent advances in the phytoremediation of xenobiotic and of heavy-metal pollution have been recently reviewed and will be mentioned only briefly here (Table 1) [9, 27, 41, 48–52, 62, 67, 73].

Genetic engineering techniques have been used to add new phenotypic characters to model plants (*Arabidopsis thaliana*, *Nicotiana tabacum*) with the aim of understanding and improving their phytoremediation properties. The bacterial gene *merA* (coding for mercuric reductase) was expressed in *A. thaliana*, which then showed enhanced resistance to $HgCl_2$ accompanied with atmospheric volatilization. This technique was later applied to the construction of transgenic yellow poplar, which volatilized elemental mercury at ten-times the rate of the untransformed plant [50]. *A. thaliana* plants expressing the *merB* gene (coding for organomercuric lyase) showed enhanced resistance to methylmercury and this resistance was improved by targeting the enzyme to the endoplasmic reticulum, thus improving access to its hydrophobic substrate [7]. Some other examples of genetically engineered plants for enhanced bioremediation are given in Table 1. Curiously, very few reviewers mention biosafety concerns (a notable exception being [49]) and none mentions ways to improve biosafety using genetic mitigation techniques.

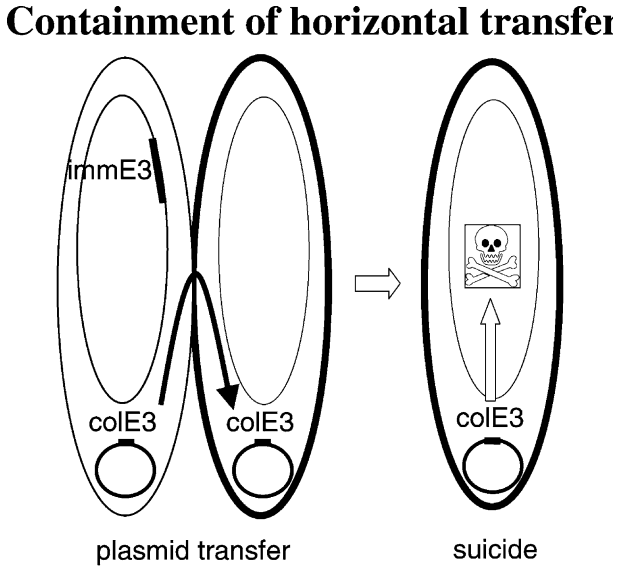

Fig. 4 Cell suicide following plasmid transfer. A bacterium (*thin cell wall*) carries two suicide genes coding for the colE3 toxin and the *Eco*RI endonuclease, carried by a plasmid. The lethality of these two suicide genes is prevented by their appropriate antidote proteins, coded by the *immE3* and the *ecoRIM* genes, which are located on the chromosome. During plasmid transfer to another bacterium, the antidote genes are not transferred, resulting in death of the recipient bacterium (*thick cell wall*)

Potential risks associated with transgenic plants

A great deal has been written about the potential and imagined risks of transgenic plants for agricultural use [10, 12, 28, 29, 40, 57, 58, 74, 75] and much, but not all, of it applies to transgenic plants for use for phytoremediation. It seems unlikely, at least in the short-term, that transgenic phytoremediation plants will contain herbicide resistance, insect resistance and virus resistance genes, which have been major subjects of biosafety discussions. In addition, phytoremediation plants will not be intended as human or animal foods, so that food safety, allergenicity, and labeling are not relevant issues.

Transgene flow

Many crop plants are capable of cross-pollination of, and by, wild relatives. There are some exceptions, for example potatoes, tomatoes and maize have no wild relatives in Europe. However, the seriousness of the situation is well illustrated by the cross pollination of European cultivated sugar beets (*Beta vulgaris* ssp. *vulgaris*) by the wild sea-beet *B. vulgaris* ssp. *maritima* [28, 29]. From this example, it should be noted that the problem of cross-pollination is not specific to transgenic

Table 1 Examples of genetic modification of plants for enhanced phytoremediation

Host plant	Transgenic protein expressed	Plant phenotype	Reference
Brassica juncea (Indian mustard)	γ -Glutamylcysteine synthetase	Cadmium accumulation	[87]
B. juncea	ATP sulfurylase	Accumulation of selenium and other metals	[84]
B. juncea	Gamma-glutamylcysteine synthetase, glutathione synthetase	Accumulation of cadmium and other metals	[3]
B. juncea	Cystathionine-it *c*-synthase	Selenium volatilization	[81]
Arabidopsis thaliana (Thale cress)	Selenocysteine lyase	Selenium accumulation	[62]
A. thaliana	Nitroreductase	TNT degradation	[36]
A. thaliana	Yeast cadmium factor (YFC1)	Cd(II) and Sb(II)	[76]
A. thaliana	Mercuric reductase	Hg(II) volatilization	[66]
A. thaliana	Organomercurial lyase	Methylmercury detoxification	[5]
A. thaliana	Arsenate reductase and γ -glutamylcysteine synthetase	Arsenic accumulation	[23]
Liriodendron tulipifera (Yellow poplar)	Organomercurial lyase	Methylmercury detoxification	[6, 7]
Nicotiana tabacum (Tobacco)	Human cytochrome P450 2IE1	Trichloroethylene degradation	[25]
N. tabacum	Citrate synthetase	Al(III) tolerance	[19]
N. tabacum	Metallothionein	Cadmium partitioning	[24]
Oryza sativa (Rice)	Mercuric reductase	Mercury volatilization	[37]

plants. Gene flow from cultivated plants to wild relatives is also a problem since it could result in loss of genetic diversity, and genetic extinction of the wild plants. It is important to consider whether the transgene flow to wild relatives is likely to confer a selective advantage, and whether it could lead to more invasive weedy plants that are likely to cause environmental or agricultural problems [28, 29, 75]. It seems possible that plants designed to grow in polluted sites could indeed donate a selective advantage to relatives growing on similar sites, and the situation would thus need to be monitored. Whether such an advantage could extend to non-polluted agricultural environments is less evident, but needs investigation.

Insertion into chloroplast DNA

Plant geneticists have developed mechanisms to mitigate the risks of gene flow to wild relatives. For example, transgenic flow from plants to wild relatives can be restricted if the transgene is targeted to the chloroplast DNA, rather than to the nucleus, since chloroplast DNA is almost entirely maternally inherited [45]. Others have criticized this approach since, although transmission of plastid DNA via pollen is rare, it is not zero in all plants [1]. There is also the possibility of gene flow from the chloroplast to the nucleus, though genetic constructs designed for chloroplast expression would not normally function if transferred to the nucleus. Selection for chloroplast insertion generally uses antibiotic selection (spectinomycin), though the marker gene may subsequently be removed by homologous recombination [14]. However, a new selection method has been devised to obviate the use of antibiotic selection in chloroplast transformation. This uses the betaine aldehyde dehydrogenase gene of spinach, which converts the toxic betaine aldehyde into non-toxic betaine glycine, an osmoprotectant [14]. A special feature of chloroplast engineering is that chloroplasts are present in large numbers in leaves, but not in roots. Thus, it is unsure how effective the insertion of transgenes into chloroplast DNA would be for phytoremediation purposes.

Conditional lethality in transgenic plants

Several constructions that confer conditional lethality on transgenic plants, thus reducing or eliminating gene flow, have been proposed. It has been suggested that the transgene-of-interest could be part of a tandem construct containing a second gene that is beneficial, or neutral, under agricultural conditions, but disadvantageous in the wild. Examples include genes that prevent seed-shatter or secondary dormancy [35]. Proof of the principle was obtained using a cassette consisting of a semi-dominant gibberellic-acid-insensitive gene that causes dwarfing, and a model desired-trait for herbicide resistance. Greenhouse experiments showed that plants containing the cassette were unable to compete with normal plants when sown in close spacing in the absence of herbicide treatment to mimic competition in the wild [1]. For phytoremediation purposes, this technique is inapplicable in its present form, since competition with wild plants may be a desired trait.

A different technique, called recoverable block of function [42], is again based on the poison/antidote idea. The *Bacillus amyloliquefaciens* ribonuclease (barnase) gene is expressed from a sulphydryl endopeptidase promoter, active at the time of seed-pod development, thus preventing seed germination. The "antidote" to barnase is the expression of the barstar gene, which is

placed under the control of a heat-shock promoter. Prolonged heating of the developing seeds to 40°C in a greenhouse results in barstar production and removal of barnase inhibition, thus permitting correct seed development and germination. High-temperature-treated seeds would germinate correctly and grow in the field, but progeny seeds would not encounter the prolonged high temperatures necessary for removal of the barnase block, and so would fail to germinate.

Genetic Use Restriction Tecnology (GURT; often referred to as "Terminator") provides a way to protect the intellectual property of the inventor, though it has received extensive criticism, since it prevents the farmer from re-sowing saved seeds. However, it has the advantage that it causes seed death at a late stage in germination and thus prevents escape and perpetuation of transgenic crops. Terminator could be revived in one form or another as a gene containment strategy [39, 74]. It could be particularly useful in phytoremediation and in biopharming where replanting saved seeds is not a priority, in situations where the seeds are not intended for human and animal consumption, and where environmental dissemination is to be avoided. While the definitive version of the terminator technology has been published only as a patent application, the principle is clear (Fig. 5). Plants are constructed to contain three genetic elements:

1. A bacterial tetracycline-responsive Tet repressor gene (from *Tn*10) under the control of a constitutive promoter. The repressor is inactivated in the presence of tetracycline.
2. A phage P1 site-specific recombinase gene (*cre*) under the control of a promoter that is subject to repression by the Tet repressor.
3. A gene coding for a protein toxin (a ribosomal inhibitor protein from *Saponaria officinalis*) expressed from a seed-specific plant promoter (late embryogenesis abundant, LEA), active only at a very late stage of seed maturation. The expression of this toxin gene is prevented by a DNA sequence blocking its transcription. This latter sequence is bounded by the sites of action (*loxP*) of the site-specific recombinase Cre.

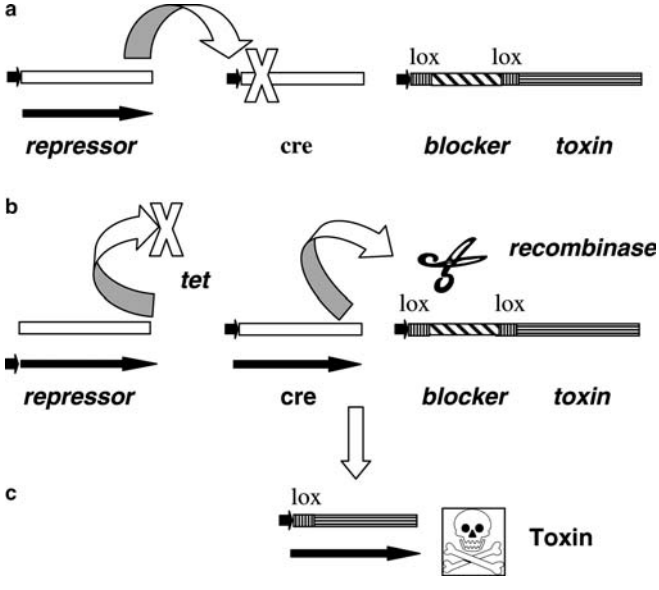

Fig. 5a–c GURT technology. **a** The site specific recombinase gene *cre* is repressed by the presence of the Tet repressor, while expression of a toxin from a seed-specific promoter is prevented by a transcriptional blocker bounded by two lox sites (the substrate of the Cre recombinase). **b** Soaking the seeds in tetracycline inactivates the Tet repressor, thereby permitting production of Cre, which excises the transcriptional blocker. **c** At the time of seed maturation the seed-specific promoter is active and the toxin gene is transcribed, resulting in seed death

Thus, viable plants and seeds can be grown under normal conditions when the recombinase is not produced. However, soaking the seeds in tetracycline inactivates the Tet repressor and allows transcription of the recombinase, which then excises the transcription-blocking DNA region. Thus, tetracycline-treated seeds will germinate and produce normal plants, but toxin production will be initiated in late seed development, thus killing the almost mature seed. When used for phytoremediation, seeds produced by GURT technology would be dead and have no possibility of environmental escape, thereby eliminating one biosafety concern. On the other hand, seeds of related plants that were cross-fertilized by the GURT pollen would also be sterile, and natural populations might suffer genetic extinction. GURT has also been criticized since it uses large quantities of tetracycline for seed treatment. However, this point may be trivial, since tetracycline may simply be the example used for patent purposes, and the same end-point could probably be reached using different promoters inducible by other stimuli, such as ethanol. Thus, a modified form of GURT to prevent the escape of transgenes used for phytoremediation could be interesting.

A somewhat similar technique also uses genetic control of seed sterility but has a different outcome in that the seeds only become sterile upon outcrossing to other plants [69]. Seed sterility is achieved by using two genes (*iaaM* and *iaaH*), from the Ti plasmid of *Agrobacterium tumefaciens*, that encode the enzymes for the production of the plant hormone indole-3-acetic acid, expression of which permits normal plant and seed development but inhibits seed germination. The *iaaM* gene is expressed under the control of an embryo-specific phaseolin promoter genetically modified to contain a binding site (*tetO*) for the Tet repressor (from *Tn10*). Thus, binding of the Tet repressor prevents transcription of *iaaM* and permits seed germination. The Tet repressor is supplied on the homologous chromosome, under the control of the cauliflower mosaic virus (CaMV) 35S promoter, following crossing to a homozygous plant carrying the *tetR* gene on both homologous chromosomes. Thus, the resulting hybrid plant for field use is hemizygous for both the Tet-repressed *iaaM* gene and the *tetR* gene, and

may be perpetuated by selfing to produce seeds for agriculture or phytoremediation. However, if the hybrid plants were to outcross to agricultural or wild relatives, then repression would be lost, resulting in sterile seeds. With this system, the gene-of-interest (e.g., mercury volatization), would be part of the cassette containing the *iaaM* gene, although it would have its own tetracycline-independent promoter. The technique still needs considerable optimization in terms of the level of repression achieved. In addition, to prevent recombination and facilitate seed production, the seed-lethal cassette and the tetracycline repressor would need to be at the same site on homologous chromosomes. This has not yet been achieved and, with the present state-of-the-art, may be technically difficult.

Still another technique to alleviate cross-pollination has been described by Ceres [47]. A male-sterile line that carries cassettes containing several genes is used, e.g., (1) a gene of interest (for phytoremediation, or pharmaceutical production), (2) a gene (such as LEAFY COTYLEDON 2 of *A. thaliana*) that is seed-lethal when expressed, and (3) a pigment gene for easy identification of hybrid plants. These genes are all located downstream of yeast upstream activator sequences (UAS) and are not expressed in the original plant, since the yeast transcription factor, responsible for the transcriptional activation of the UAS, is absent (Fig. 6). However, on cross-pollination by a transgenic male-fertile plant producing the yeast transcription factor, the three genes are expressed in the hybrid seeds, ensuring expression of the protein of interest, the color-identification gene, and the seed-lethality gene. The promoter controlling the production of the yeast transcription factor may be constitutive or chosen for its temporal or tissue-specific expression. The system is highly versatile due to its modular construction and could easily be adapted for use in phytoremediation.

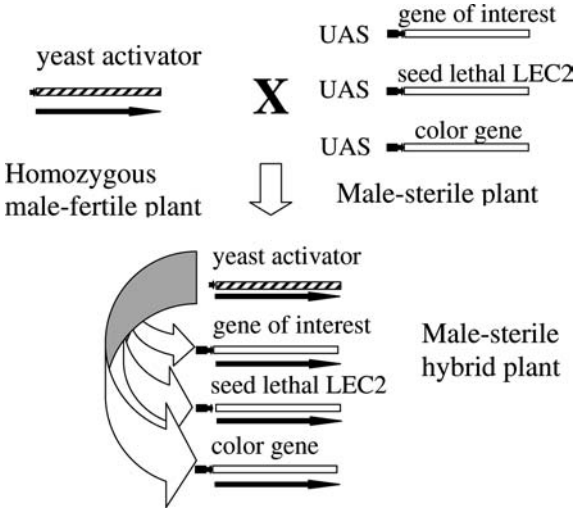

Fig. 6 Activation of seed lethality by a yeast transcriptional activator. A first plant line (*left*) contains a gene coding for a yeast transcriptional activator. A second male-sterile plant line (*right*) contains a series of genes located downstream of yeast upstream activator sequences (*UAS*). Among these latter genes is a seed-lethal gene expressed from an embryo-specific promoter, but this gene is not expressed since its activation depends on the yeast transcriptional activator, present only in line 1. Crossing these plants generates a viable hybrid plant in which the lethality function will be expressed at seed maturation

Finally, it should be noted that the above mitigation techniques have been tested only in model plants under contained conditions. None have been tested in large-scale field-trials. Thus, while promising, they will not be ready for application to phytoremediation in the near future.

Antibiotic resistance markers in transgenic plants

The use of antibiotic resistance markers during the selection of transgenic plants has also been much criticized, on the grounds that these markers could be transferred, by transformation, to soil bacteria and eventually to pathogens. Still wilder suggestions have even foreseen their transfer, via intestinal bacteria, to humans, with the result that "humans become resistant to antibiotics". Firstly, it must be remembered that these antibiotic resistance markers are already present in the environment, since they were originally obtained from highly mobile genetic elements in bacteria, where they evolved as a consequence of misuse of antibiotics in medicine and animal feed [15, 18]. They present a serious (perhaps, in the long-term, insurmountable) medical problem in hospitals, though curiously, we find little public outcry against antibiotic misuse. Secondly, the reality of gene transfer from transgenic DNA to bacteria has been deeply investigated using worst-case scenarios with the highly transformable bacteria *Acinetobacter* ssp BD413 and *Pseudomonas stutzeri*. It is clear that such transformation takes place only when the recipient bacterium already contains sequences homologous to the antibiotic resistance gene. However, under these circumstances, genetic transformation of the recipient bacterium may be obtained from transgenic leaves, roots, and even pollen, which can travel a considerable distance [22, 77]. A recent working party of the British Society for Antimicrobial Chemotherapy essentially dismissed the possibility of a health hazard as a consequence of transfer of antibiotic resistance genes from transgenic plants to bacteria [5].

The use of antibiotic resistance genes as selective markers in transgenic plants may also be dismissed for another reason, since it represents an old-fashioned method of transgenic crop production. Future transgenic crops are likely to be derived using selective markers other than antibiotics. A recent method uses the non-antibiotic gene *dao1* (D-amino acid oxidase), which provides for both positive and negative selection [30]. Another method of avoiding antibiotic resistance markers employs a virulent strain of *A. tumefaciens*, thus increasing transformation frequency to a point where PCR screening enables the identification of transformed

plants in the absence of selection [21]. Another system, multi-auto-transformation, termed MAT, uses an isopentyl transferase gene (for the synthesis of a cytokinin precursor) that permits visual selection of transformed plants; the cytokinin gene is carried by the self-excisable transposon *Ac*, so that marker-free segregants can then be obtained [26].

Alternatively, new techniques that excise the antibiotic genes may be used. Site-specific mechanisms using Cre recombinase (or the yeast FLP recombinase) to excise the DNA carrying the antibiotic or other markers sandwiched between two *loxP* (or FRT) sites can be used. An improved version expresses the site-specific recombinase from a chemically inducible promoter (Fig. 7) [71, 88, 89] and this may prevent possible complications due to the constitutive production of the Cre recombinase, which may act undesirably at pseudo-lox sites. Techniques for the removal of genetic markers have the additional advantage that the plants can subsequently be retransformed using the same selective marker.

Cross contamination of human and animal foods

In principle, plants used for xenobiotic and heavy metal bioremediation should not be destined for human or animal consumption, and preferably should not be food plants at all [examples include the use of the brake fern to accumulate arsenic or the yellow poplar for Hg(II) remediation]. In this respect, bioremediation plants share this problem with plants designed to produce pharmaceuticals, but have the advantage that they do not use prime farmland. In addition, they will not be harvested in the classical sense, and could even be harvested prior to seed maturity, so that physical segregation will be easier. This is an important matter, as is illustrated by the Starlink corn incident, where GM-corn, destined for animal consumption, contaminated corn destined for human consumption, as a result of a combination of cross-pollination and physical mixing after harvesting.

Another problem will be to prevent wild animals from grazing on phytoremediation plants. Polluted sites are in principle always protected by fences, at least from entry by humans and large animals. Most phytoremediation, particularly for heavy metals, relies on accumulation in the above-surface parts of the plant. The plants must then be harvested and processed, or the metals will simply return to the soil after plant death.

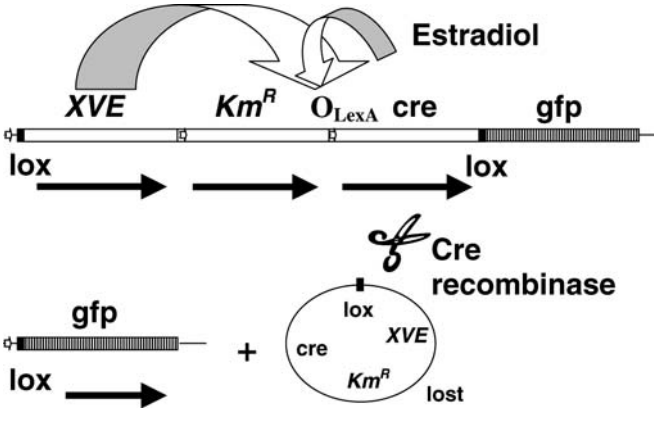

Fig. 7 Chemical-regulated site-specific excision. A hybrid transcriptional regulator gene contains an estradiol-responsive domain and a lexA-operator binding domain. In the absence of estradiol, this activator does not function, but, in its presence, it activates transcription of the *cre* gene, via interaction with the lexA-operator sites. Production of the Cre site-specific recombinase causes excision of the DNA segment between the two *lox* sites, leaving only the *gfp* gene

Effect of transgenic plants on rhizosphere ecology

A great deal of research has been devoted to the effects of transgenic plants, particularly those containing insecticidal Bt toxin, herbicide resistance, or lysozyme, on the microbial community. In general, when observed at all, these effects have been found to be small compared to other major variables such as crop rotation, heat, drought and soil type [8, 82]. Similarly, with regard to phytoremediation, the polluted environment is likely to have a greater effect on the bacterial community than does the transgenic plant.

Discussion

Transgenic bacteria

Thirty years ago, bioremediation by bacteria, and later by recombinant bacteria, seemed a promising, environmentally correct way of depolluting contaminated sites, thus stimulating a great deal of basic research into the genetics and biochemistry of biodegradation. Despite this, little has been achieved in real environmental clean-up situations and, at present, there are few real applications for recombinant bacteria in the field [68, 85]. However, pollution continues, and polluted sites are still costly to clean up by standard means. Thus, as it was 30 years ago, bioremediation remains a feasible alternative. One reason for this paradox lies in the perception of environmental biotechnology by the general public. Public mistrust began with criticism of the ice-minus *P. syringae* [86], and has subsequently extended to recombinant bacteria whether for use in bioremediation or as agricultural supplements. In response to public opinion, the regulatory authorities have responded with strict regulations that discourage research initiatives. Other difficulties involve economic considerations and low profit margins [68]. Thus, only a few priate companies are involved in the field use of naturally occurring microorganisms, while most university research on

transgenic bioremediation bacteria is of a basic nature [85]. The application of the precautionary principle (recently adopted in Europe) may provide an additional way of preventing the use of recombinant bacteria in the environment. Opponents of the precautionary principle have argued that it should be replaced by a risk-risk analysis on a case-by-case basis, since a decision to do nothing is also a decision, and also has its consequences [33, 53, 54].

Transgenic plants

On a more optimistic note, phytoremediation is generally seen as posing fewer biosafety concerns [51, 85]. This is possibly because the general public fears transgenic bacteria, but sees plants as more environmentally friendly (though this may be more true in the United States than in Europe, where there is much antagonism against transgenic plants). In addition, as indicated in this review, genetic mitigation techniques have been suggested, whereby the fate of transgenic plants introduced into the environment may be controlled. It must be made clear, however, that these risk-mitigation methods are preliminary in nature, although they are being continually improved. In particular, none of them have been field tested or applied to plants designed for phytoremediation. Indeed, the state-of-the-art in phytoremediation has not yet progressed to a point where the plants to be used are clearly identified. Thus, this review attempts to foresee ways in which phytoremediation may benefit from genetic risk-mitigation methods designed for agricultural plants. To look still further into the future: is it possible that plants for phytoremediation could be specifically designed for conditional suicide? One could imagine, for example, that plants designed for mercury phytoremediation could be dependent on the continued presence of mercury, and that its depletion, or the escape of the plant to a non-contaminated soil, could activate suicide genes. Similarly, one could imagine designing transgenic plants whereby the suicide genes are under control of a promoter recognizing an external environmental stimulus (e.g., ethanol), so that simply spraying with ethanol would result in plant death, while leaving other plants unscathed.

In conclusion, whereas bioremediation using transgenic bacteria seems presently to be in the doldrums, phytoremediation using transgenic plants could offer some new answers to environmental cleanup of toxic wastes. New genetic method risk-mitigation may help ensure that neither the transgenic plants, nor the transgenes they contain, will escape into the environment.

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