Review

Novel roles for genetically modified plants in environmental protection

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Transgenic plants of environmental benefit typically consist of plants that either reduce the input of agrochemicals into the environment or make the biological remediation of contaminated areas more efficient. Examples include the construction of species that result in reduced pesticide use and of species that contain genes for either the degradation of organics or the increased accumulation of inorganics. Cutting-edge approaches, illustrated by our own work, focus on the applicability of genetically modified (GM) plants that produce insect pheromones or that are specifically tailored to the phytoremediation of cadmium or PCBs. This paper discusses the role that the next generation of GM plants might play in preventing and reducing chemical contamination and in converting contaminated sites into safe agricultural or recreational land.

Introduction: the need for (new) GM plants

With the rapid growth in the global population making it increasingly difficult to provide sufficient amounts of food [1], one potential solution is the use of genetically modified (GM) organisms, which might support starving populations through increased crop yield. However, the launch of GM foodstuffs has been impeded, in particular, by the reluctance of different regional jurisdictions to permit the application of GM plants [2].

Another solution, therefore, might be to use remediation techniques to convert contaminated areas* into suitable agricultural land and thereby increase the sites available for food production. Phytoremediation using conventional plants (grasses, sunflower, corn, hemp, flax, alfalfa, tobacco, willow, Indian mustard, poplar, etc.) (Figure 1) shows good potential, especially for the removal of pollutants from large areas with relatively low concentrations of unwanted compounds: areas for which it is not cost-effective to use traditional physical or chemical methods.

Thus far, with traditional dig-and-dump methods being much faster, widespread use of phytoremediation has been limited by the relatively long period of time plants require to reduce contaminant levels [3,4]; several harvest periods generally prove insufficient.

However, gene transfer has already led to the production of GM crop varieties on hundreds of millions of hectares [5]. This irreversible fact, together with recently improved attitudes towards GM plants (even within the EU [6,7], where GM food has traditionally been viewed with distrust), has resulted in calls for the large scale implementation of transgenic plants that can prevent or remove contamination more effectively.

Using GM plants in environmental protection

The generation of transgenic plants for environmental protection involves the two quite separate fields of pollution prevention and pollution removal, with specifically tailored plants already existing for both purposes. Pollution-preventing GM plants can significantly reduce the

Glossary

Allelochemicals: compounds formed and released by one species with the aim of influencing its surroundings (e.g. other, sensitive plant species and their rhizospheres).
Desaturase: enzyme introducing a carbon–carbon double bond, in this case into a fatty acid in a specific position.
GMO (genetically modified organism): an organism with some specific gene(s) introduced or removed artificially.
Herbicides: compounds that are toxic for some plants and used to protect crops against weeds.
Insecticides: compounds toxic to some insects.
Pesticides: compounds toxic to some pests.
Phytoremediation: use of plants to accumulate, remove or render harmless toxic compounds contaminating the environment.
Phytostabilisation: process in which plants are exploited to prevent migration of environmental contaminants to sites where they may pose a danger to human health.
Phytovolatilisation: process by which plants volatilise through their leaf surface some environmental contaminants taken up by their roots.
PCBs (polychlorinated biphenyls): a group of recalcitrant organic compounds that differ in their chlorine substitution on the biphenyl ring. Until the late 1960s, when they were banned because of their toxicity, they were widely used in paints, heat transfer media, electric devices, plastics, etc. because of their technological properties (inflammability, chemical stability and their dielectric constant).
Rhizoremediation: exploitation of microorganisms within the root zone of plants to remove contaminants from the environment.
Sexual pheromone: a compound for chemical communication between females and males within one species.
Transgenic plant: GMO, plant with some specific gene(s) introduced or removed artificially.

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* Typically, such areas have been contaminated by: the spillage of fuels and their additives; the results of industrial accidents; the long-term accumulation of contaminants; the leakage of mine tailings; the long-term treatment of agricultural land with low quality fertilisers; sewage sludge; excess pesticide use; the use of land as army training areas; and pollutant deposits accrued from many years of industrial activity.

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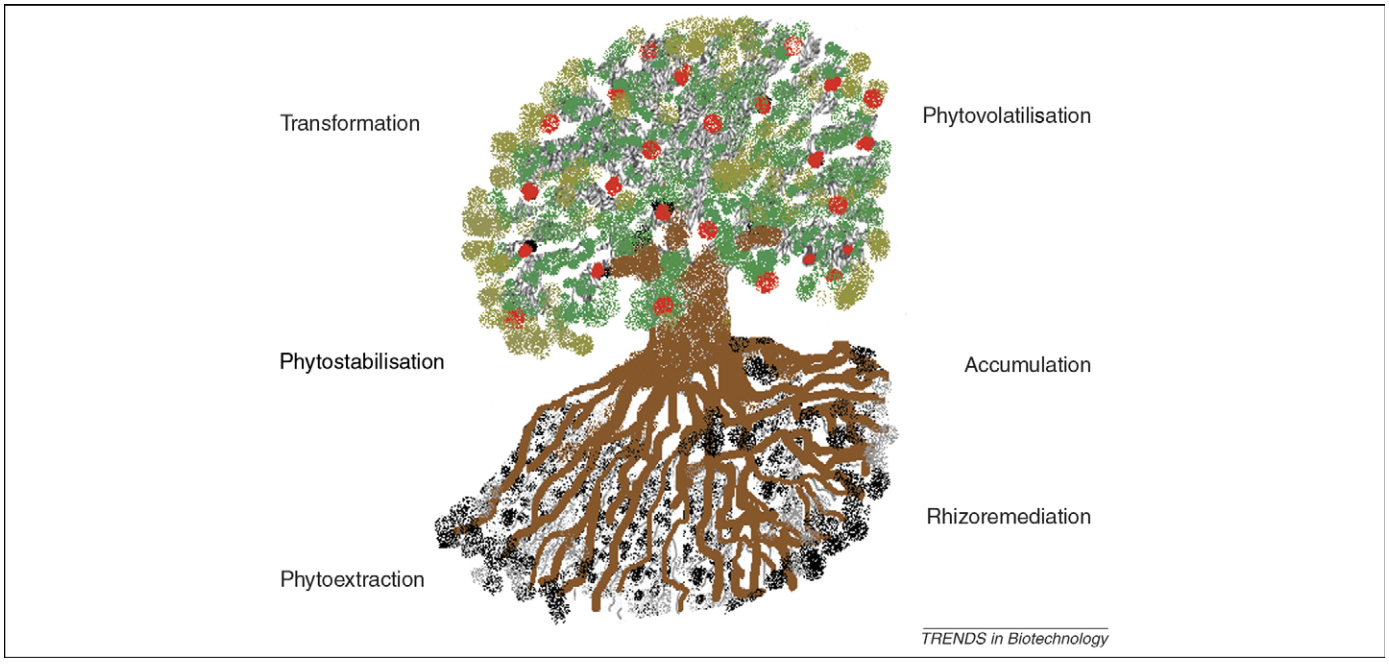

Figure 1. Growing plant roots explore the soil particles and take up water, nutrients, trace elements and other compounds, thus playing an important role in biological remediation. Plant roots can extract contaminants from soil and accumulate, transform and transport them into the parts of the plant that are above-ground. In leaves, fruits or stems, many compounds are stored, transformed or volatilised. Such processes are known as phytoremediation. Via exudate and root turnover, many plant products enter the root zone. Some of these compounds supply soil microorganisms with energy, some act as a carbon source and some can even serve as inducers of degradative pathways. Growing roots also help to spread microorganisms within the soil, thereby supporting rhizoremediation.

amount of agrochemicals needed for crops, thus reducing environmental pollution. Examples include Roundup Ready soya, which enables the use of more environmentally-friendly herbicides, as well as *Bacillus thuringiensis* (Bt)-corn and Bt-cotton, which minimise pesticide use[†]. Recently, however, a new approach to pest management has been developed, based on the construction of plants that produce and emit insect pheromones [8,9]. Pollution-removing GM plants, which deal with contaminations caused by explosives, chlorinated solvents, mercury, selenium, phenolics, etc. [3,4,10,11], have been extensively reviewed in the literature [12–17]. These plants have been developed to contain either transgenes responsible for the metabolisation of organic compounds (thereby leading to the accumulation of less toxic or less recalcitrant compounds) or transgenes that result in the increased accumulation of inorganic compounds. Once optimised, this approach should lead to the accumulation of pollutants in harvestable parts [18,19] and thus either enable their removal or prevent their migration to sites where they may pose a danger to human health [3,17].

Pollution prevention

The first generation of commercially available transgenic plants (e.g. plants expressing the Bt toxin) were able to reduce the loss of crop yield caused by insect damage at the same time as reducing the amount of pesticide required. As both these and herbicide-resistant plants have been the subject of numerous reviews, and their advantages or disadvantages discussed extensively [2,5,20], we will focus on plants that produce and emit insect pheromones (Figure 2). Grown close to or around, for example, a field of food crops requiring protection, this type of GM plant emits a pheromone that attracts male moth pests, thereby reducing their ability to mate effectively. In such cases, the protected crop does not itself need to be transgenic (see Box 1). We tested this novel approach by constructing tobacco plants that produce an insect sexual pheromone from their own fatty acid pool. This was achieved by inserting the gene encoding acyl-CoA-delta^{11}-(*Z*)-desaturase (from the cabbage looper moth), which is responsible for the production of the sexual pheromone in female moths [21,22]. The transformed plants were examined for fatty acid content showing substantial presence of the precursor,

Box 1. Transgenic plants emitting insect sexual pheromones

Transgenic plants capable of synthesising and releasing pheromones into the environment are not intended to destroy entire pest populations, but rather to limit their ability to mate effectively in a field containing a protected crop and thereby to reduce the pest population. In this scenario, GM plants would be planted in the vicinity of the protected crop to disrupt the ability of damaging pests to communicate chemically or to concentrate them in another, desired, location as part of an integrated pest management (IPM) system. Via this method, neither the transgene itself nor its products would be able to enter the human food chain, thus eliminating the possible health risks associated with genetically modified plant (GMP) consumption. Moreover, such an approach means that non-targeted insect life remains unaffected, and insect-resistant strains, such as those been reported with the use of the *Bacillus thuringiensis* (Bt) toxin in transgenic crops, do not develop. Moreover, the synthesis of pheromones, or their intermediates, in GMPs could also be used as an alternative to traditional chemical methods for pheromone production.

[†] It is hard to find good scientific reasons why GM technology has not been universally embraced. All of the widely publicized objections, whether they be the supposed threat to Monarch butterflies or the 'risk' of the inadvertent introduction of allergens into the food chain, have been soundly rebutted and relegated to the status of 'urban myths' [2].

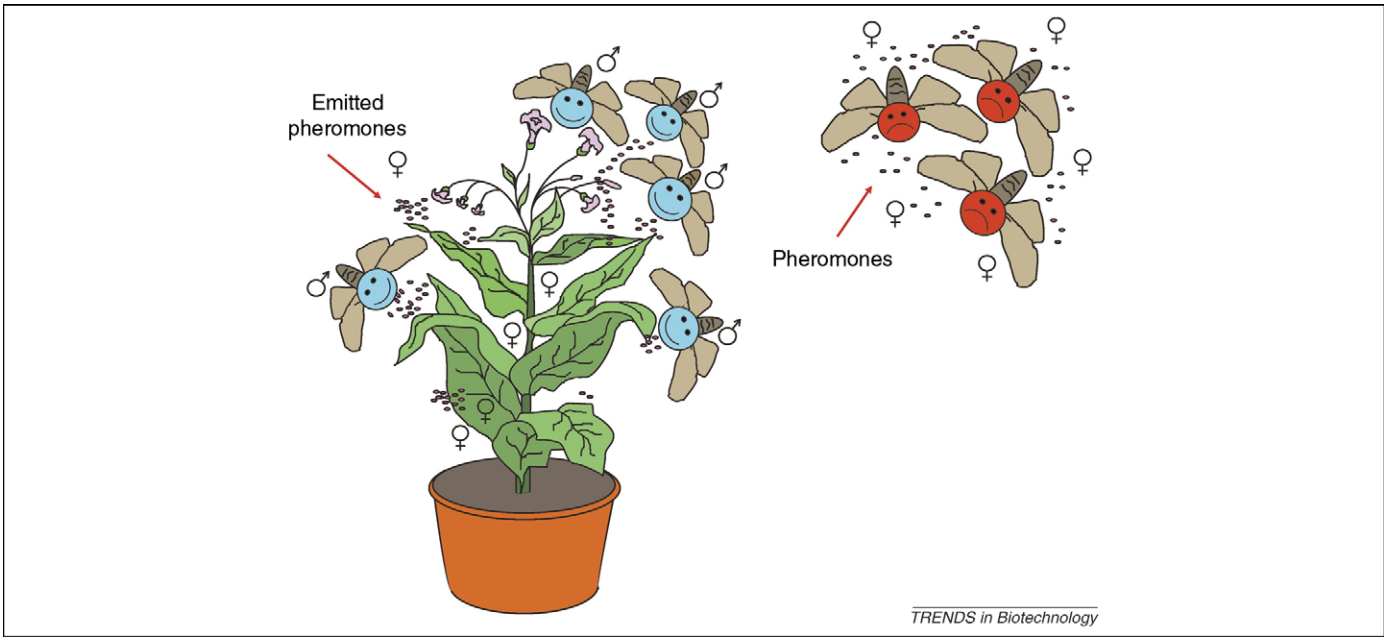

Figure 2. Plants emitting specific insect sexual pheromones compete with female moths in attracting males of the same species, therefore lowering the effectivity of their mating and resulting in a decrease in moth population. This approach cannot eradicate the pest totally, but it will lower the losses of plants that are to be protected.

which was further converted to alcohol by the enzyme normally present in tobacco plants. Our experiments constitute the first example of a GM plant producing measurable amounts of a moth sex pheromone, thereby potentially adding a new method to the existing battery of integrated pest management approaches. Furthermore, we confirmed that these GM plants emit compounds identical to the natural sex pheromone of the African rice borer moth, *Chilo zacconius*, making this moth an ideal candidate for field-testing the application of this system.

Leaves of the same GM tobacco plants also served as a 'cell factory' for the production of monoenic lipids that were chemically converted into the pheromone mixture of another insect, the cabbage moth (*Mamestra brassicae*). This moth exploits a mixture of acetates, rather than the alcohols used by *C. zacconius*. In this case, the GM plants supply the starting material, thereby removing the need for certain synthetic steps and enabling the correct pheromone mixture to be prepared by means of a simple one-pot reaction [8]. The effectiveness of our semi-synthetically prepared mixture has since been successfully trialled in field tests in northern Bohemia.

Pollution removal: phytoremediation and rhizoremediation

Phytoremediation [3,10,11] is not solely a function of plants (Figure 3) but must always be considered in combination with the effect of rhizospheric microorganisms [4,23]. Although they have an inherent ability to detoxify some xenobiotics (i.e. to make them non-phytotoxic), plants, compared with microorganisms [12], generally lack the mechanisms necessary for the complete degradation/mineralisation of toxic compounds.

The potential of genetic engineering to enhance the biodegradation of xenobiotics has been recognised since the early 1980s, with initial attempts being focused on microorganisms. However, there are two main problems with the introduction of GM microorganisms: the legislative barriers blocking their release into the environment and the poor survival rate of those engineered strains that have been introduced into real contaminated soil. The latter problem reflects the inadequate level of knowledge that currently exists about the consortia of microorganisms present in real soil and the ways in which they interact. The survival rate of introduced bacterial species might, however, be improved by the use of strains that have a selective advantage over others, such as strains supported by plants: for example, root colonisers [24].

The use of plants, rather than microorganisms, as genetically engineered environmental cleanup biosystems might also help to overcome the legislative barriers. However, some species, for as yet unknown reasons, are simply more sensitive to contamination than others, so not all plants are equally well suited to metabolise or accumulate pollutants. For remediation purposes, besides their ability to take up, accumulate or metabolise the xenobiotics, one of the most important criteria is the ability of the plant to selectively support the metabolism and survival of degrading bacteria in the rhizosphere [25–27]. Only recently developed methods of detection, such as stable isotope probing, have enabled us to obtain a deeper insight into the effect of pollutants and plants on microorganisms [4,26,28]. Metagenomics, for example, has brought new insights into the presence and activity of degrading microorganisms within rhizosphere consortia, enabling the tracking of responses to compounds released by plants [4,29].

The genetic modification of microorganisms to improve their performance in the rhizosphere represents a challenging possibility that should not be abandoned simply because their release into the environment is currently restricted. The ability of degrading bacteria to colonise roots may be manipulated by improving symbiotic microorganisms. One such example is the rhizoremediation of

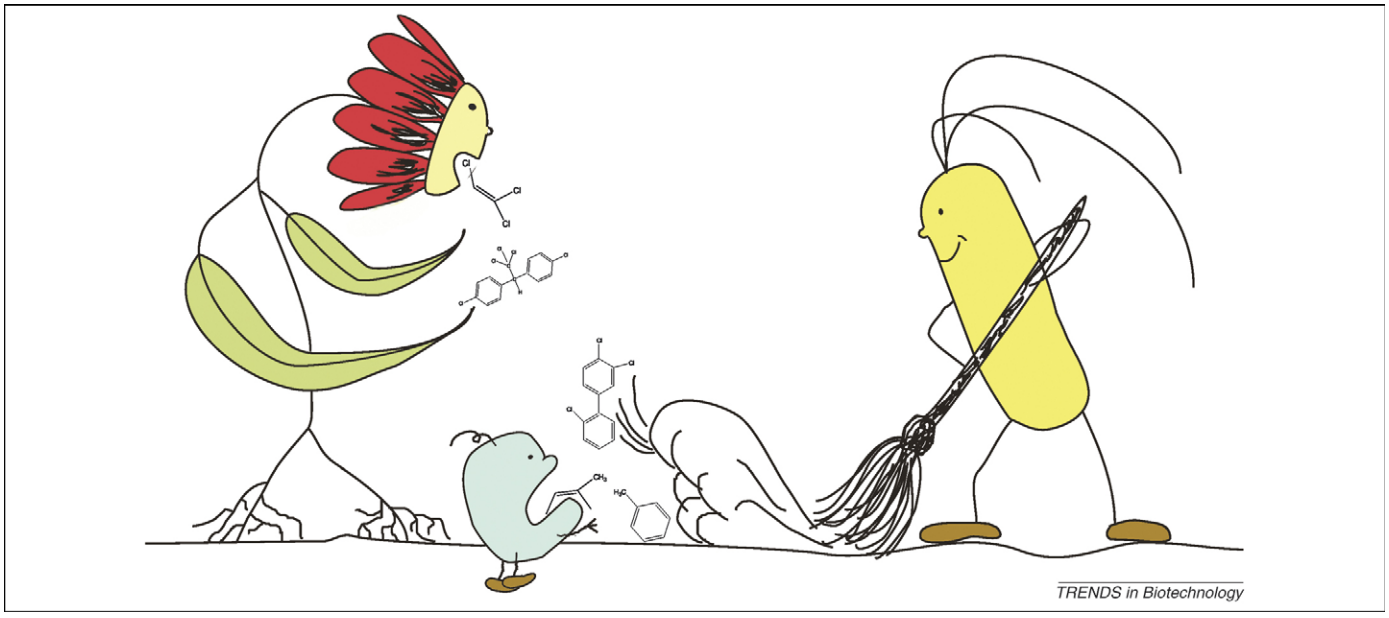

Figure 3. In bioremediation, the roles of the different organisms that are present in the soil cannot always be clearly separated. Some bacterial species (shown in blue and yellow) might degrade selected compounds only to some extent and their end-product can then be further metabolised by other species. Plants can take up the pollutants and the pollutant metabolites formed by other organisms and convert them further or detoxify them. Products of plant metabolism become available to other parts of the complex bioremediation system via leaf fall or root turnover. Therefore, to develop an effective remediation system, all participating components need to be considered.

PCBs by *Pseudomonas fluorescens*, in which biphenyl degradation is regulated using a system that responds to signals from alfalfa roots [24]. The introduction of such GM microorganisms ensures that any changes are limited to the consortia of native bacteria in the rhizosphere and not introduced into the surrounding soil [30].

Another rather promising approach appears to be the development of engineered endophytic bacteria that improve the phytoremediation of water-soluble, volatile organic compounds [31]. Trichloroethylene (TCE)-degrading bacteria have been proven to protect host plants against the phytotoxicity of TCE and to contribute to a significant decrease in TCE evapotranspiration.

Plants with an enhanced ability to accumulate heavy metals

Plants exploit their natural metabolic mechanisms to take up essential trace metals. Cations or oxyanions must either be accumulated in harvestable parts or transformed into less-toxic forms. Although hyperaccumulators, such as *Thlaspi caerulescens*, can uptake sufficient levels of metals to make harvesting and metal recovery economic, they are often limited by their small biomass [10,32]; the amount of pollutant they can remove from soil is a function of their tissue concentration multiplied by the quantity of biomass formed. Despite this, and despite the fact that no universal phytoremediation plant exists, plants that are selective and only capable of accumulating certain elements, are already being used in the cleanup of a broad spectrum of hazardous elements.

In terms of the development of GM plants with improved metal detoxification abilities, we will only discuss the most promising approaches, some of which have been described in more detail elsewhere [13,33–36]. For example, the introduction of bacterial genes is one strategy, which, in US trials in contaminated fields, has already been shown to result in the reduction of toxic organomercurials, as well as in the storage of mercury in its non-toxic form [10,37]. Another promising approach to enhancing metal uptake employed the nicotianamine synthase gene [38] involved in the formation of phytosiderophore, the metal-binding amino acid [39] that increases the bioavailability of metals to plants.

However, the most common strategy involves targeting the proteins involved in metal homeostasis (metallothioneins, phytochelatins, glutathione) for genetic manipulations [40,41]. Although such approaches typically involve the manipulation of plant enzymes responsible for the formation of phytochelatins and related compounds (e.g. overexpression of glutathione synthetase [42], gamma-glutamylcysteine synthetase [43], phytochelatin synthase [44]), manipulations with other enzymes have also been successful. For example, field trials have shown that the overexpression of ATP sulfurylase facilitates increased selenium reduction and its storage in less toxic form [45,46]: a bonus being that such plants also accumulate the potent anticancerogenic compound, methylselenocysteine.

Many papers deal with the expression of metallothioneins in plants [47–49], but our work has focused on improving a plant's ability to accumulate metals by introducing (into the implemented protein) additional metal-binding domains with a high affinity to heavy metals [19]. Such a fusion product with a histidine anchor [18,50] was tested in real contaminated soil, and transfer factors were estimated for cadmium, zinc and nickel [51,14]. In our trials, transgenic tobacco accumulated twice the amount of cadmium in above-ground biomass than did the controls.

A possible enhancement to this approach, currently being tested, involves the cloning of short (cysteine-rich) metal-binding sequences [52] into plants to improve their metal-binding properties. This approach was followed up

by quantum chemical studies of the interactions of metal ions with biologically relevant functional groups: studies that suggested further possible developments in the metal-binding capacities of fusion proteins. Subsequently, theoretical combinatorial chemistry was applied to the complexation and selectivity of metal ions in model sites [53,54], resulting in the design of highly selective combinations of metal-binding sites that might be merged into one polypeptide chain.

Plants with an enhanced ability to detoxify persistent organic compounds

To cope with organic xenobiotics, plants use a mechanism developed to fight allelochemicals, which are toxic compounds produced by other species competing for their resources [10,55,56]. Because the use of plants in the removal of organic compounds has been widely discussed in both reviews [10,12,16,57] and books [3,4], we again will concentrate on only the newest approaches.

To increase their natural abilities, different P450 cytochromes have been introduced into plants. These enzymes are considered to be responsible for the first phase in plant detoxification, the activation reaction of recalcitrant compounds in plants [56]. One illustrative example of this is the enhanced metabolism of halogenated hydrocarbons in transgenic plants containing cytochrome P450 2E1 [58]. Intriguingly, however, overexpression of a basic peroxidase in tomato [59] resulted in increased phenol phytoremediation, thereby supporting the hypothesis that, apart from P450 cytochromes, peroxidases are also involved in this first phase [60,61].

Chloroacetanilide herbicides and explosive compounds [62] have been the focus of other studies. The development of GM tobacco, which overexpresses glutathione-S-transferase for the phytoremediation of chloroacetanilide herbicides [63], addresses the second phase in plant detoxification: namely, the conjugation of the activated compound.

The biodegradation of explosives by transgenic plants expressing pentaerythritol tetranitrate reductase [64] is the classic example of the exploitation of a bacterial gene for phytoremediation. More recently, plants have been constructed that express bacterial enzymes capable of TNT transformation and RDX (hexahydro-1,3,5-trinitro-1,3,5 triazine, an explosive nitroamine widely used in military and industrial applications) degradation [65].

To achieve the aerobic degradation of ubiquitous persistent PCBs, they must first be activated by hydroxylation. The vital missing step in the efficient degradation of PCBs by plant cells is the opening of the biphenyl ring by the bacterial enzyme *bphC*, which is responsible for the cleavage of hydroxylated PCB derivatives, even those formed by plants [66]. Therefore, we have thoroughly studied the cooperation of plants and bacteria in PCB degradation [24–26,28,30]. In particular, we have described the generation of tobacco plants carrying the *bphC* gene [67], subsequently testing the seeds for their ability to germinate in high concentrations of PCB [14]. Improved substrate specificity has since been achieved by the expression of bacterial biphenyl-chlorophenyl dioxygenase genes in tobacco [68].

In addition to these techniques, phytoremediation can even proceed *ex planta*, as shown in the case of trichlorophenol and phenol allelochemicals phytoremediated via an engineered secretory laccase [69].

Conclusion

With most of the GM plants prepared in the EU in the last decade never having reached a real contaminated site, it is results from other regions, primarily the US, that show us the potential for cost-effective commercial applications of the GM approach [34]. Hopefully, therefore, in the near future GM plants will be widely used, not only to significantly reduce pesticide use in agriculture (in particular, reducing the organophosphates and organocarbonates that possess substantial mammalian toxicity), but also to actively remove the residues of agrochemical, industrial and accidental contaminations of the environment. In the great environmental cleanup required, the future lies in tailored phytoremediation-specific plants able to support microbial activities in the rhizosphere. However, to exploit these possibilities on a large scale, it will first be necessary to achieve changes in the existing legislation, overcome regulatory barriers and educate the public into improving their opinion of GM plants.

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Keeping the genie in the bottle: transgene biocontainment by excision in pollen

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Gene flow from transgenic plants is an environmental and regulatory concern. While biocontainment might be achieved using male sterility or transgenic mitigation tools, we believe that perhaps the optimal solution might be simply to remove transgenes from pollen. Male sterility might not be ideal for many pollinators, and might not be implementable using standardized genes. Transgenic mitigation might not be useful to control conspecific gene flow (e.g. crop to crop), and relies on competition and not biocontainment *per se*. Site-specific recombination systems could allow highly efficient excision of transgenes in pollen to eliminate, or at least minimize, unwanted transgene movement via pollen dispersal. There are other potential biotechnologies, such as zinc finger nucleases, that could be also used for transgene excision.

Introduction

Transgenic plants have played important roles in solving current agricultural problems, and hold even greater prospects of alleviating poverty and malnutrition in developing countries. For example, Golden rice contains high levels of β-carotene and could be a great help for people with vitamin A deficiency [1]. Currently, over 3 billion people have micronutrient malnourishment [2]. Micronutrient deficiencies negatively affect human health and cause subsequent societal problems; nearly two-thirds of childhood deaths worldwide are caused directly by malnutrition [2]. Transgenic plants have demonstrated benefits including higher yields, enhanced nutrients, and easier pest control [3]. The Green Revolution has boosted crop yield in many parts of the world but Africa has not realized its benefits. In Africa, impediments such as insufficient water for irrigation and nutrient-depleted soils have resulted in low yields and often crop failure with conventional plant varieties [4]. Biotechnology has the potential to trigger drastically improved agriculture in Africa by adding traits, such as those conferring drought-, salt- or heat-tolerance.

Regardless of its potential benefit, biotechnology has not been exploited fully in very many crops, even in developed countries, because of regulatory and environmental concerns about gene flow. Following the example above, the large-scale deployment of Golden rice has been hampered mainly because of concerns about gene flow to neighboring farms that do not currently contain transgenic plants, and interference with existing vitamin A supplementation [5]. The concern that Golden rice could be grown in a country that lacks sufficient biosafety regulations and monitoring capabilities, coupled with potential gene flow from transgenic rice to weedy rice has stymied its cultivation [6]. Ingo Potrykus, a principal developer and advocate of Golden rice, considers this long delay in its cultivation to be a serious moral downfall [7]. In late 2008, the Rockefeller Foundation promised its financial support for the deregulation process of Golden rice cultivation in several developing countries [8]. It seems, however, that gene flow remains to be a significant regulatory hurdle.

In theory, gene flow could be prevented or rendered a negligible risk if strategies were realized that could contain transgenic traits within cultivated transgenic fields. Uncontrolled transgene escape to non-transgenic crop fields or sexually compatible wild relatives is a particularly important issue if transgene introgression is probable, or even possible, within a crop-wild system [9]. One especially problematic class of transgenes are those used for plant-made pharmaceuticals (PMPs) that are expressed in transgenic food crops or species that are prone to gene flow; that is, those that are outcrossers or have sexually compatible wild relatives [9]. Trace adventitious presence of PMP transgenes in food processed from other non-PMP transgenic crops is not acceptable by either regulators or food companies [10]. In 2002, highly stringent regulatory standard was applied to the biotechnology company ProdiGene for the presence of noncompliant PMP genes in their experimental field trials [11]. Therefore, PMPs probably will be the subject of even higher regulatory scrutiny with regards to gene flow, compared with non-PMP transgenic crops with input traits. These and other biotechnological applications beg for effective methods for biocontainment.

Removal of transgenes from pollen and/or seeds could minimize gene flow problems. Transgene movement from transgenic to non-transgenic plants typically occurs most frequently via pollen dispersal [12]. Therefore, for most plants, the first line of containment is pollen, the long-distance vector for hybridization and introgression. To prevent pollen dispersal, formation of sexual reproductive organisms can be suppressed simply under field conditions for some transgenic crops by harvesting leaves prior to flowering [13]. However, this is not a practically useful

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method for most crop species because the lack of seed production would be significantly disadvantageous. There are a number of biological transgene biocontainment tools that could eliminate, or at least minimize, unwanted transgene escape from transgenic to non-transgenic plant populations, including wild-relatives, or potential negative consequences of transgene flow [14]. These include male sterility and transgenic mitigation, whereas, perhaps the most effective method would be the removal of transgenes from pollen using site-specific DNA recombinases.

While pollen is considered to be the primary agronomy-based vehicle for long-range gene dispersal, it is not the only one. Transgenic seeds can be dispersed as volunteers in the next season, during harvest, transport, and sometimes also mediated by animals. Compared with pollen dispersal, seed dispersal is more predictable because it is most probably caused by human-mediated dissemination, which can be decreased with improved shipment and handling procedures [15]. However, transgene movement via pollen dispersal mediated by insects or wind is almost inevitable without an appropriate pollen biocontainment procedure in place.

Male sterility and chloroplast transformation as potential biocontainment tools

Male sterility is one of the most commonly used transgene biocontainment systems in commercial fields. Mariani *et al*. [16] have generated male-sterile tobacco and canola using a mechanism that prevents pollen formation through the expression of chimeric ribonuclease genes (Barnases). Male sterile plants are able to act as maternal parents and are fertilized with pollen from outside the field. Hybrid seeds from crossing between male-sterile plants and wild relatives can acquire fertility restoration in successive generations via the Barstar gene [17]. Male sterility, however, might have a negative effect on many pollinators that acquire food and nutrients from pollen. For example, the survival of pollen beetles to adulthood has been shown to be reduced in the absence of pollen compared to wild-type flowers [18]. Cytotoxicity of Barnase gene expression results in ablation of tapetal cells and embryos of plants [16,19]. Furthermore, because Barnase toxins have been shown to be cytotoxic in animal and human cell line models, their cell-specific expression to plants parts that are not consumed is required [20]. However, even low amounts of cytotoxic genes, such as Barnases in non-targeted plant parts, caused by leaky expression, might affect plant growth negatively [21]. The cytotoxicity and potentially unregulated expression of Barnase could result in cell death [22], therefore, male sterility might not be the best choice for transgene biocontainment.

Cytoplasmic male sterility (CMS) is another method to contain genes effectively though maternal inheritance, which has been demonstrated in proof-of-principle experiments in transgenic tobacco [23]. However, gene transfer from the cytoplasm to the nucleus occurs at high frequency [24,25]. A high transfer rate of integrated DNA from the cytoplasm to the nucleus, which results in termination of maternal inheritance, might not be an appropriate characteristic for reliable biocontainment systems [26]. Fertility of CMS in *Petunia* could be restored by nuclear gene expression [27]. Also, abnormal morphology of flower parts and poor nectar production in hybrid plants have been reported as unexpected consequences of some CMS systems [28]. CMS systems need to surmount these drawbacks to play effective roles as transgene biocontainment systems.

Plant plastids and their genomes are exclusively maternally inherited in many crop species, thus transplastomic approaches could be effective in biocontainment of male gametophyte-mediated transgene flow. However, maternal inheritance is not universal, which limits the use of plastid transformation for pollen-targeted biocontainment [29]. More than one-third of the species in angiosperm do not have a trait of strict maternal inheritance [30]. Also, efficient tissue culture and selection protocols that are required in order to obtain homoplastomic plants have not been established for most monocotyledonous species plants [13,30]. It has been suggested that additional methods should be paired with plastid transformation to achieve complete transgene containment [31,32].

Genetic engineering for transgene removal from pollen

With regards to pollen biocontainment, transgene removal is an alternative to male sterility, which as mentioned above, can be leaky, and to chloroplast transformation, for which maternal inheritance is typical (no transgenes in pollen), but nevertheless is rather difficult to accomplish in many plant species. One possibility for transgene removal is using site-specific recombination because this simply cuts the transgenes from pollen [33]. With this approach, the entire transgenic construct could be flanked with recognition sites for a site-specific recombinase gene, introduced under the control of a tightly regulated pollen-specific promoter (Figure 1). Upon expression of the recombinase in pollen, the entire transgenic cassette is excised, leaving only a short recognition site in the mature pollen (Figure 1). Similarly, zinc-finger nucleases (ZFNs), which can be designed specifically to bind and cleave target DNA sequences, could also be used to excise transgenes from pollen [34–36] (Figure 2).

In contrast to male sterility, a transgene removal strategy allows for normal production of pollen and fertilization, thus not adversely affecting the many flower-feeding herbivores. Concerns about reintegration of the excised transgene with reversible recombination systems including Cre-*lox* and FLP-*FRT* into pollen genome could be resolved with newly discovered, non-reversible recombination systems, such as ParA-*MRS* or CinH-*RS2* [37]. The potential of this approach has been demonstrated already with the successful removal of integrated transgenes in plants, in particular selectable marker genes, using site-specific recombination systems [38–40]. Furthermore, Luo *et al*. [41] have achieved dramatically increased efficiency of transgene removal in tobacco when they have combined *loxP-FRT* recognition sites with pollen-specific expression of either Cre or FLP recombinases, compared to non-fused recognition sites of Cre-*loxP* or the FLP-*FRT* recombination system. Based on screening of over 25 000 progeny per transgenic event, several transgenic tobacco events have shown complete transgene excision from their pollen [41]. Here, coexpression of both Cre

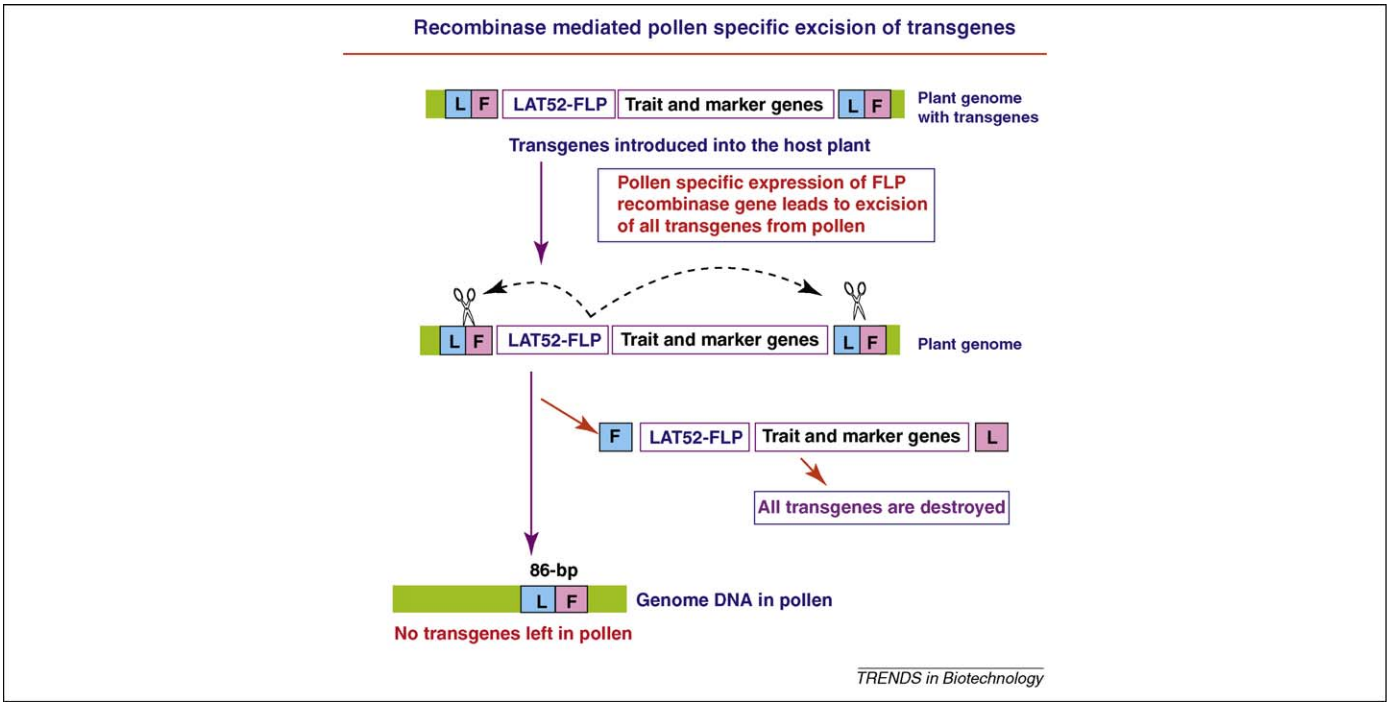

Figure 1. Principle of transgene excision from pollen. Here, L represents the *loxP* recognition sequence from the phage Cre/*loxP* system and F represents the *FRT* recognition sequence of the yeast FLP/*FRT* system. LAT52 is a pollen-specific gene promoter from tomato [42,53]. FLP is a DNA recombinase from the FLP/*FRT* system. Expression of FLP under the control of the LAT52 promoter leads to deletion of all transgenes between the two LF (*loxP-FRT* fusion) sites, including the recombinase gene in pollen specifically. The excised gene sequences will be destroyed by non-specific nucleases present in the cell. Reproduced from Ref. [52] with permission.

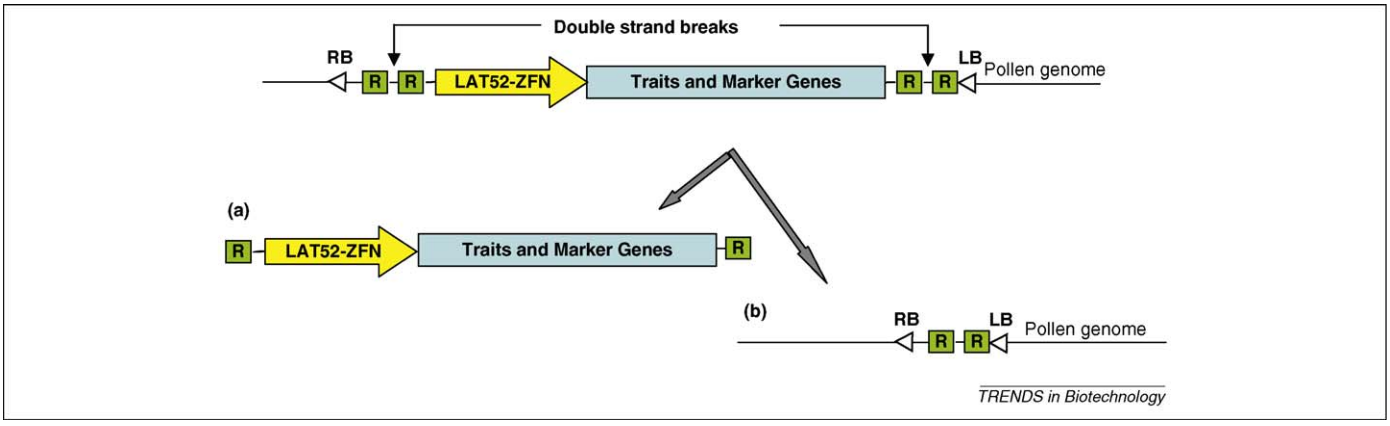

Figure 2. ZFN-mediated transgene excision from pollen. ZFN expression under the control of a pollen-specific promoter LAT52 creates a double-strand break in the spacer region between two adjacent ZFN recognition sites (R) that form one set of ZFN sites. This results in: **(a)** one DNA fragment that contains the functional transgenes, including trait and marker genes that have been excised from pollen genome, and which are destroyed in the cell; and **(b)** the pollen genome with only one set of two adjacent ZFN recognition sites, which by itself, is non-functional.

and FLP recombinases actually decreases the efficiency of transgene removal, which might originate from competition of the two recombinases to bind to adjacent recognition sites [41].

Transgene removal within a particular organ or tissue is made possible by judicious selection of tissue-specific promoters. Several pollen-specific promoters, such as LAT59 and LAT52 promoters from tomato [42,43], ZM13 promoter in maize [44], and DEFH125 promoter in *Antirrhinum* [45] have been characterized as being only activated in pollen cells, with non-detectable activity in other tissues or developmental stages. A site-specific recombinase or ZFN driven by a pollen- or microspore-specific promoter might also be useful for transgene removal from pollen [46]. Availability of several pollen-specific promoters from various sources might provide more chances to use the transgene removal system in many other crop species.

Regulatory and economic considerations

Recently, there has been a trend to decrease the amount of transgenic DNA in plants to the extent that is absolutely necessary to deliver a trait; a development that has been embraced by companies and regulators. The introduction of additional transgenes as means for biocontainment would thus run counter to this trend, except that biocontainment itself might be considered a valuable trait. From an economic perspective, sufficient benefits with regard to significant biosafety gains or sustainability would be

required to outweigh the additional costs for discovering and licensing of the promoters and genes required for transgene removal. Such a transgene removal system that requires initial investment probably would be deployed first in those crops that are the greatest risks with regard to introgression to weedy wild relatives, such as sorghum [9] and switchgrass [47]. However, once the system is established, transgene removal systems in other marketable crops would be significantly cost effective compared to the cost for extensive monitoring and clean-up of accidental transgene contaminants. From a regulatory perspective, it is uncertain at present what degree of decrease in transgene flow constitutes an acceptable risk. In addition, the components required for site-specific recombination would need to undergo a risk assessment analysis for various ecological and food safety parameters. In pollen, very little foreign DNA would remain after excision events because, for example, transgene removal using the Cre-*loxP* recombination system would leave just a single 34 bp *loxP* site in the pollen genome.

There are several choices of well- or partially characterized transgene removal systems. The well-characterized Cre-*loxP* that is derived from phage P1 and the yeast-derived FLP-*FRT* systems are both reversible, which potentially allows the transgene to reenter into the genome, although reintegration of the excised products has not been reported, probably because transgene excision is the preferable reaction in this system [48].

Non-reversible site-specific recombination systems are also available, such as ParA-*MRS* and CinH-*RS2*, which are both derived from the serine resolvase family of recombinases [37]. Transgene removal in plants by ParA recombinase, which was derived from bacterial plasmids RK2 and RP4, has been shown to be precisely site-specific for

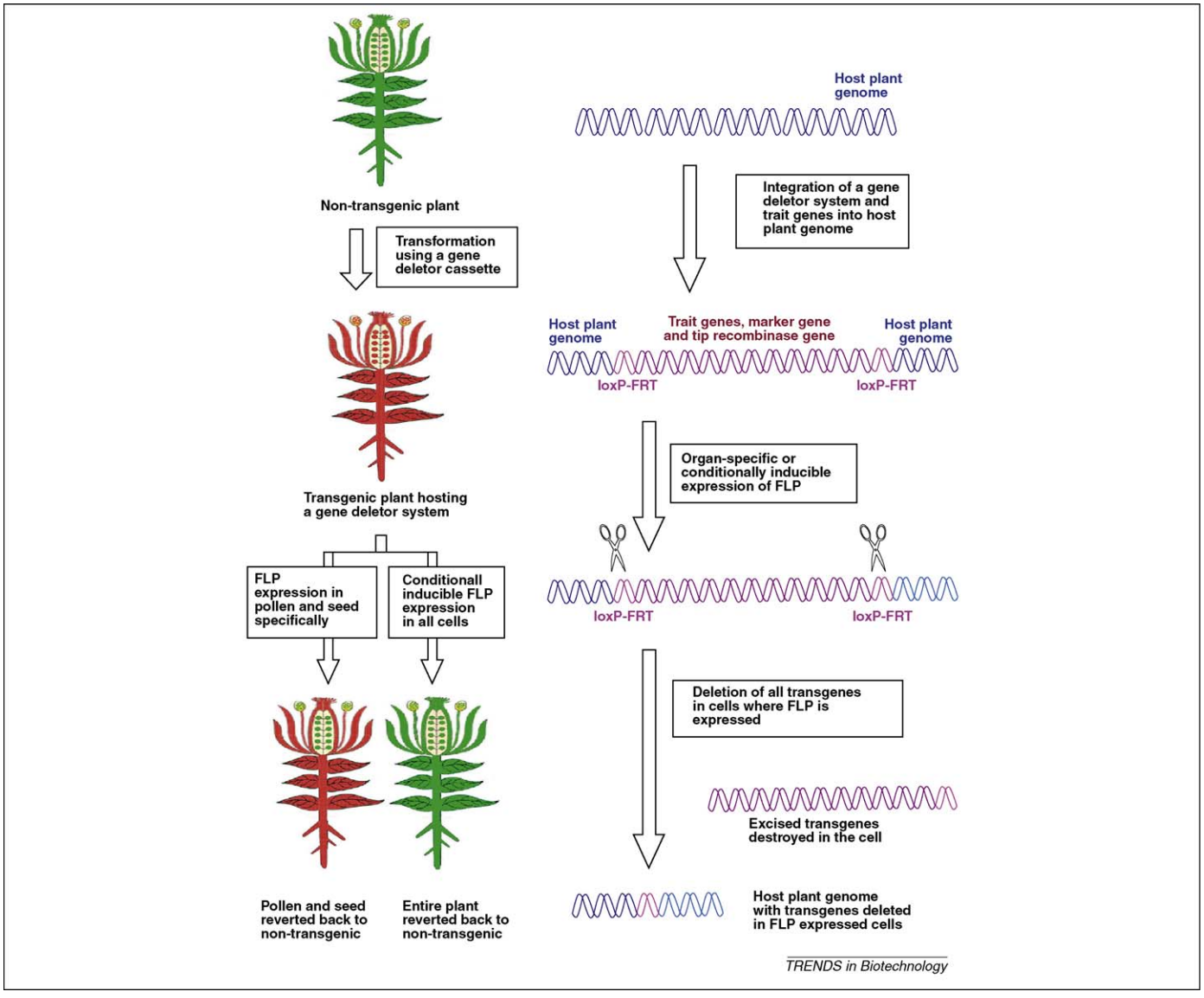

Figure 3. Schematic illustration of biocontainment using a gene deletor system. On the left, the use of a gene deletor system to produce non-transgenic pollen, seed or plants from a transgenic plant is demonstrated. The schematics shown on the right illustrate transgene removal. Any transgenes, such as trait genes, marker gene and FLP or Cre recombinase gene, that have been inserted into the two *loxP-FRT* sites (86 bp in length), will be deleted from any cell, in which the recombinase is expressed. When a pollen- and seed-specific gene promoter is used to control recombinase expression, all functional transgenes are deleted from these specific organs. On the other hand, using a conditionally inducible gene promoter, such as chemically or high-temperature inducible, to control recombinase expression results in the deletion of all functional transgenes throughout the plant upon induction. Adapted from Ref. [41] with permission.

excision of an embedded sequence between the recognition sites [49]. CinH recombinase derived from *Acetinetobacter* plasmids pKLH2, pKLH204 and pKLH205 has shown a site-specific gene excision function in yeast, but it has not yet been deployed in plants [50].

Perspectives

Transgene removal from pollen using site-specific recombination system could be an effective tool for transgene biocontainment; however, no system has so far been tested under agronomic conditions, or even in the field. Any transgene biocontainment system for commercial field application would probably be required to be not leaky and have no pleiotropic effects. Luo *et al*. [41] have been able to achieve complete transgene excision from pollen using fusion recognition sites of *loxP-FRT*, therefore, it appears feasible to employ site-specific recombination as a transgene biocontainment strategy. Further experiments, including those in field settings are needed to increase sample sizes and confidence limits, and also to test for reversal in the bidirectional recombination systems Cre-*loxP* and FLP-*FRT*. Non-reversible recombination systems, such as ParA-*MRS* and CinH-*RS2*, with their longer recognition site sequences, might provide more reliable transgene removal, while removing the possibility of potential transgene re-integration.

Homozygous transgenic seeds cannot be produced with transgene removal using a site-specific recombination system. This could be disadvantageous for seed-propagated plants for commercial purposes. If a transgene removal system were completely efficient, transgenic seed production would rely on the presence of transgenes in eggs; that is, the transgenic female parent (Figure 3). Seeds from transgenic plants that contain the transgene-removal trait by site-specific recombination in their pollen are hemizygous for the transgenic trait, or are non-transgenic. Practically speaking, half of the seeds that contain no transgenic traits could be eliminated for commercial purposes by soaking in a selection agent or by post-germination selection [51]. However, homozygous transgenic seeds could be produced if a conditionally expressed recombinase repression gene is incorporated into the transgene removal system [52]. In this case, expression of the recombinase gene is suppressed conditionally in pollen and seeds at generations in which transgenes need to be maintained, for example, in breeding stock (Figure 3) [52].

Our view on future perspectives on commercial use is cautiously optimistic. Transgenic tobacco plants with laboratory-effective, site-specific recombination systems that contain the fused *loxP*/*FRT* recognition sites [41] are being tested currently under agronomic conditions for the efficacy of transgene removal in pollen in the field. We are also testing multiple systems in *Brassica napus* (canola); again to be challenged under field conditions. If one or more systems perform as well in the field as they do under more controlled conditions, they could be good candidates in a commercially vectored system, and applied to transgenic crops that could otherwise be delayed by regulatory issues. Of special interest is the application in the foreseeable future to crops never before considered for transgenic release, such as outcrossing grasses for bioenergy production. Transgene removal from pollen using site-specific recombination might be the best choice as an environmentally friendly biocontainment strategy with high efficiency.

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Scent engineering: toward the goal of controlling how flowers smell

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Floral scent has an important role in the reproductive processes of many plants and a considerable economic value in guaranteeing yield and quality of many crops. It also enhances the aesthetic properties of ornamental plants and cut flowers. Many floral scent volatiles fall into the terpenoid or phenylpropanoid/benzenoid classes of compounds. Although the biochemistry of floral scent is still a relatively new field of investigation, in the past decade investigators have begun to identify 'scent genes'. Several of these genes, most of which, but not all, encode enzymes that directly catalyze the formation of volatile terpenoid or phenylpropanoid/benzenoid compounds, have now been used to manipulate, through genetic engineering techniques, the mix of volatiles emitted from the flowers of several plant species. The outcomes of these experiments, which are discussed here, have indicated that the genetic engineering approach to altering floral scents has potential; however, they have also revealed the limitations that result from our inadequate knowledge of the metabolic pathways responsible for scents and their regulation.

Why is it useful to introduce or change floral scents?

Floral scent is one of the adaptations that plants have evolved to attract pollinators. The volatiles emitted from the flowers – typically a mixture of several or even scores of compounds – provide potential insect and animal pollinators with information about the location and identity of the flowers. Efficient pollen dispersal, which results in maximum pollen transfer to conspecific flowers, increases fertilization rates and minimizes energy expenditure by the plant, thus increasing fitness. In crop plants where some edible parts of the plant (i.e. the fruit or seed) require fertilization to develop, and where complete fertilization of all the ovaries in a flower is sometimes required for fruits to develop the symmetrical shape favored by consumers, the success rate of fertilization influences both yield and quality.

Sub-optimal pollination rates are common in both cultivated and wild species [1]. The problem is often exacerbated in cultivated plant species where large numbers of conspecific individual plants are crowded together, which, in turn, require large numbers of their specific pollinators. In addition, some crop species are introduced from other parts of the world and do not have their co-evolved pollinators in their present locality [2]. Often, the morphology and biochemistry of such plants have been drastically changed during domestication, without concomitant adaptations in the pollinators.

In extreme cases, a lack of natural pollination can prevent plants from being commercially introduced as crop plants into a new territory [1]. Although breeding for self-pollination or for fruit development without pollination (apomixis) as well as achieving apomixis by chemical means could, in some cases, alleviate this problem [3], self-pollination leads to genetic uniformity and thus increases the probability of the spread of diseases.

In the USA and most other countries where large-scale monoculture agriculture is practiced, domesticated honeybees are used to pollinate many different crops. Beekeepers often derive a large portion of their income from their fees for placing beehives in orchards or crop fields. However, these bees (*Apis mellifera*) are, themselves, non-native to the USA and are not always well suited for the task [4]. Although it is known that the bees find flowers by cues from visual (color, shape) and olfactory signals, the relative importance of the various volatile, color and shape components, and their synergistic interactions, are far from understood [5]. It is clear, however, that bees do not visit all flower species with equal frequency or pollinate them with equal success. In some cases, a lack of scent has been implicated in the failure of flowers to be efficiently pollinated [6]. Recent outbreaks of diseases that have greatly reduced the numbers of domestic and wild honeybees in the USA have exacerbated the problem [7,8].

Genetic engineering of floral scent in crop plants could alleviate the problems listed above. It is envisioned that the scent of both local and introduced plant species could be enhanced to better appeal to local pollinators, thus increasing pollination efficiency and reproductive success.

For humans, scented flowers also constitute a commodity with strong aesthetic and emotional values. Unfortunately, floral scent has been a casualty of plant-breeding programs for the cut-flower market and ornamental plants in general. Despite the oft-expressed sentiment by consumers that they like scented flowers, the cut-flower industry operates under the assumption, based on actual market research, that 'the public in general will not pay an extra cent for scented flowers'. Consequently, breeders in this multi-billion dollar industry have concentrated on producing plants

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with improved vase life, shipping characteristics and visual aesthetic values (i.e. color and shape). Owing to the lack of direct selection, or perhaps because of a negative correlation with any of these traits, many cultivated flowers have lost their scent [9]. Genetic engineering could, perhaps, restore scent to these varieties without sacrificing other important commercial traits and might, thus, produce extra value for the niche market of consumers who really do prefer scented flowers and are willing to pay extra to get them.

Here, we describe recent attempts to modify specifically floral scent by genetic engineering techniques rather than by traditional breeding approaches, with the emphasis on two important pathways that contribute a large number of floral volatiles – the terpenoid and phenylpropanoid/benzenoid pathways. Although the terpenoid pathway was the first and is still the most active area for such attempts to modify not only floral scent but also vegetative and fruit volatiles [10], recent progress in elucidating the phenylpropanoid/benzenoid pathways leading to plant volatiles and the isolation of genes involved in these pathways have opened up new opportunities for the modification of floral scent.

Candidate pathways, general strategies and possible pitfalls

Most of the volatiles in plants belong to one of three major classes of compounds: terpenes, phenylpropanoids or fatty acid derivatives [11,12]. Other volatiles are derived from various amino acids. In general, plant volatiles are considered part of secondary, or specialized, metabolism because most of them are produced only in specific plant lineages and function in specific ecological roles unique to these lineages. They are not as widespread as primary metabolites, which are, by definition, found in almost all plants. However, primary and specialized metabolic pathways are not completely separate; rather, specialized metabolites are mostly produced in the terminal branches of the network of primary metabolism. Thus, in some cases a single reaction and a single enzyme will convert a primary metabolite into a volatile compound, whereas in other cases multiple steps are required [11,12].

For example, a single enzyme converts phenylalanine to phenylacetaldehyde – a volatile found in the floral scent of rose, petunia and many other species [13] – whereas eugenol, another volatile belonging to the phenylpropanoid class, is synthesized in two steps from coniferyl alcohol – an intermediate in the general lignin biosynthetic pathway of plants [14,15] (Figure 1). Likewise, many volatile monoterpenes and sesquiterpenes can be synthesized in a single reaction from geranyl diphosphate and farnesyl diphosphate, respectively; both of these are intermediates in the pathways leading to primary plant metabolites such as sterols, carotenes, chlorophylls, gibberellins and abscisic acid [12]. Numerous terpene synthase genes from various species have now been isolated and characterized. Several genes have also been identified that encode enzymes that convert non-volatile compounds into volatile ones by modification reactions such as methylation, acetylation, and decarboxylation [12]. Expressing these genes in the flower could potentially result in the production of new volatiles. In some cases, there are competing branches in a biosynthetic pathway that lead to volatile and non-volatile (or different volatile) products in the flower, whereby suppressing one branch could lead to enhanced production of the desired volatile.

The choice of specific genes to engineer floral scent thus depends on the availability of genes, the specific goal (i.e. the desired aroma effect for either human consumption or the targeted pollinator), and the ability to siphon compounds from primary metabolism while avoiding undesirable side effects. For example, expressing a gene encoding an enzyme that uses a common primary metabolite to make a volatile might not lead to a noticeable production of this volatile in the flower if the primary pathway is not highly active in this tissue. In addition, expressing such a gene under a constitutive promoter everywhere in the plant could lead to deleterious effects, either from the toxicity of the accumulated compound in non-flower tissue (or even in flowers if it is not emitted fast enough) or from the diversion of the flux of the primary metabolism pathway, which causes a deficiency in a needed compound.

Successful and not-so-successful attempts to engineer scent

To date, the criteria for success in metabolic engineering of floral scent have been based on sensory evaluations by humans, whose odor threshold perception is much lower than that of most animals or insects [16,17]. Unfortunately, the impact of changes in the scent bouquet on insect and animal attraction has not yet been reported.

Engineering of terpenoid volatiles

First attempts to engineer floral scent were focused on modifications of the terpenoid spectrum. The terpenoid pathway was an inviting target because isoprenoid precursors are ubiquitous molecules in plant tissues and, as described above, they also serve as precursors in the biosynthesis of several essential primary metabolites; therefore, they would be available for the synthesis of terpenoid volatiles using introduced terpene synthases. The most often used gene in these initial attempts was linalool synthase (LIS) from the flowers of *Clarkia breweri*, an annual native to California [18]. LIS converts geranyl diphosphate (GPP) to (3*S*)-linalool, a monoterpene alcohol with a sweet, pleasant fragrance that is found in the flowers of many species. Overexpression of LIS under the control of the constitutive 35S promoter in *Petunia hybrida* (petunia) [19] and *Dianthus caryophyllus* (carnation) [20], both of which do not emit this monoterpene from either their leaves or their flowers, indeed resulted in linalool production in both leaves and flowers. However, the synthesized linalool had no effect on the olfactory properties of the flowers or vegetative parts of the transformants. In petunia, most of the linalool was converted by an endogenous enzyme into non-volatile linalyl β-D-glucoside. In transgenic carnation, most of the synthesized linalool was further metabolized into the volatile *cis*- and *trans*-linalyl oxides. Although these extra terpenes constituted almost 10% of the total volatiles emitted from the transgenic flowers, this increase in scent emission was

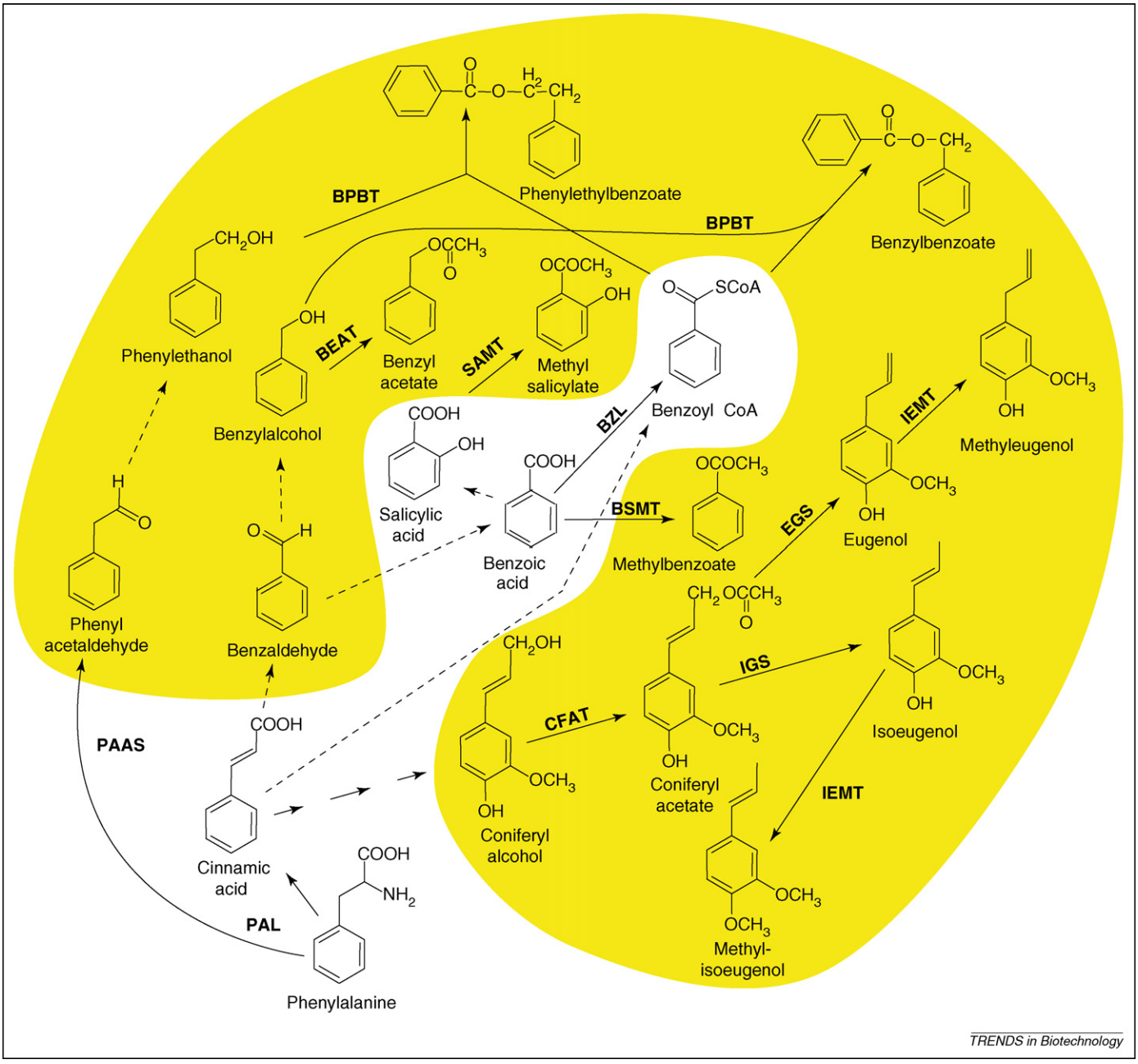

Figure 1. An overview of the biochemical reactions leading to the synthesis of volatile benzenoids/phenylpropanoids found in floral scents of various plants. This chart is a compilation of reactions and enzymes discovered in several plant species that have served as model organisms in the study of floral scent, but all the reactions and volatiles shown here occur in either *Clarkia breweri* or petunia, or both. Solid lines indicate established biochemical reactions, and broken lines indicate possible steps for which enzymes have not yet been characterized. Salicylic acid is shown here as possibly derived from benzoic acid, although it might also be derived from isochorismate [34]. BEAT, acetyl-coenzyme A:benzyl alcohol acetyltransferase; BPBT, benzoyl-CoA:benzyl alcohol/2-phenylethanol benzoyltransferase; BSMT, benzoic acid/salicylic acid carboxyl methyltransferase; IGS, isoeugenol synthase; BZL, benzoate:CoA ligase; CFAT, coniferyl alcohol acyltransferase; EGS, eugenol synthase; IEMT–*S*-adenosyl-L-methionine:(iso)eugenol O-methyltransferase; IGS, (iso)eugenol synthase; PAAS, phenylacetaldehyde synthase, PAL, phenylalanine ammonia-lyase. SAMT, salicylic acid carboxyl methyltransferase. Volatile compounds are shown with a yellow background.

still not enough for most humans to detect a change in floral aroma in smell tests [20]. These pioneering experiments revealed additional problems that have to be considered in the genetic engineering of flower fragrance: the modification of the scent compound into a non-volatile form by endogenous, non-specific enzymes; insufficient levels of emitted volatiles for olfactory detection by humans; or masking of introduced compound(s) by other volatiles [20].

In more recent experiments, successful changes in the terpenoid volatile profile were achieved in *Nicotiana tobacum* (tobacco) plants through the introduction of three lemon monoterpene synthases under the control of the constitutive 35S promoter [21]. These monoterpene synthases use GPP as a substrate but produce multiple products in varying ratios. In addition to the usual terpenoids emitted by the parental line, the transgenic tobacco plants also produced and emitted β-pinene, limonene, γ-terpinene and several other products from their leaves and flowers. Importantly, the emission levels of the new products were sufficient for detection by the human nose [22]. In flowers, the comparably high levels of the introduced monoterpenes did not reduce the emission of linalool,

which was already emitted by the non-transgenic plants. The introduced monoterpene synthases competed for the same GPP substrate, suggesting that the substrate pool is not limiting for monoterpene production [21]. Crosses between transgenic lines harboring different introduced monoterpene synthases resulted in progeny with an even more complex monoterpene emission spectrum [21].

In contrast to LIS-transformed petunia and carnation plants [19,20], no further modification products of the primary monoterpenes were detected in these transgenic tobacco plants. In subsequent experiments, the monoterpenoid profile in these transgenic tobacco plants was further modified by introducing the mint limonene-3-hydroxylase, which catalyzes the hydroxylation of (+)-limonene to form (+)-*trans*-isopiperitenol [23].

Although the terpene synthase genes used in the experiments were introduced under the control of the 35S promoter, the analyzed plants, in general, did not appear to be negatively affected by the expression of the heterologous genes. However, the amounts of the new terpenes synthesized by the transgenic plants was relatively low; and it is probable that plants expressing the introduced genes at a higher level did suffer adverse effects [24] and were, therefore, not selected for further analysis.

Engineering of phenylprpanoid/benzenoid volatiles

Because many of the enzymes for volatile biosynthesis can use multiple substrates, the particular volatiles produced in the flowers of transgenic plants will depend on the substrates available in the floral cells in which the transgene is expressed [11,25]. This is also true for endogenous genes. For example, in petunia flowers the endogenous benzoic acid/salicylic acid carboxyl methyltransferase (PhBSMT) apparently has higher catalytic efficiency with salicylic acid than benzoic acid, but the flowers do not emit methylsalicylate as a result of the small internal pool of free salicylic acid. Thus, the enzyme is responsible for the formation of methylbenzoate using the substantial amount of benzoic acid present within the cells [26,27] (Figure 1). Petunia flowers also emit low levels of benzyl acetate and phenylethyl acetate. When *Rosa hybrida* (rose) alcohol acetyltransferase (RhAAT), which catalyzes the formation of geranyl acetate from geraniol and acetyl-CoA in rose flowers [28], was expressed under the control of the 35S promoter in petunia, it used the endogenous phenylethyl alcohol and benzyl alcohol instead of the unavailable geraniol, and significantly increased the emitted levels of benzyl acetate and phenylethyl acetate in transgenic flowers [29]. Feeding of transgenic flowers with geraniol, the preferred substrate in the *in vitro* assays, or with 1-octanol, an additional potential RhAAT substrate [28], led to the production of their respective acetates, confirming the previous conclusion that the function of the introduced gene *in planta* depends on substrate availability.

To date, the metabolic engineering of floral scent has mainly concentrated on the introduction of the genes responsible for the final steps of the formation of volatile compounds. The redirection of metabolites by restricting the specific fluxes is another approach that has occasionally been tried. For example, antisense suppression of flavanone 3-hydroxylase, an enzyme in the biosynthetic pathway leading to the formation of anthocyanin pigments, resulted in an unpredicted rise in the levels of emitted methylbenzoate in transgenic carnations; the difference could be detected by the human nose [30]. Because both benzoic acid and flavones are ultimately derived from the shikimate pathway, the blockage in the anthocyanin pathway led to an increase in flux in the pathway leading to benzoic acid and, ultimately, methylbenzoate.

Phenylpropanoid/benzenoid floral scent profiles have also been modified by the elimination of some volatile compounds from the scent bouquet. This work has, so far, only been done in petunia. RNAi-mediated silencing of the PhBSMT gene resulted in transgenic petunia plants that lack the major scent component methylbenzoate, with minimal changes in the emission of other volatiles [27]. The change was easily detected by a human sensory panel, which reacted negatively to the decrease in floral scent [27]. More recently, RNAi silencing of the petunia phenylacetaldehyde synthase gene (PhPAAS) not only led to the complete elimination of the emission of phenylacetaldehyde but also of 2-phenylethanol, for which it is a precursor [13] (Figure 1). Silencing the petunia benzylalcohol/phenylethanol benzoyltransferase (PhBPBT) (Figure 1) by RNAi resulted in plants whose flowers did not emit benzylbenzoate or phenylethylbenzoate, although emission of all other volatiles remained unchanged [31]. Interestingly, plants with fully suppressed PhBPBT expression also had clear morphological differences, such as bigger flowers and larger leaves. These differences, which are probably due to an, as yet, unexplained interaction between the benzenoid pathway and auxin [31], demonstrate the unpredictable nature of metabolic engineering in general, but scent engineering in particular. Finally, silencing of coniferyl alcohol acyltransferase (CFAT), the enzyme that catalyzes the formation of coniferyl acetate (the precursor of isoeugenol and eugenol; Figure 1) [14], led to almost complete elimination of isoeugenol emission in petunia flowers, with little effect on the emission of other phenylpropanoid/benzenoid volatiles [15].

Future prospect

The examples described above (and summarized in Table 1) show that metabolic engineering of floral scent is now feasible. However, whether newly introduced 'scent enzymes' will find appropriate substrates, and whether the intended products will be produced and emitted at levels that can be detected by humans and other animals, including insects, will depend on the specific plant–animal pair interactions. These factors cannot be predicted presently because of insufficient understanding of plant metabolic pathways as well as animal olfactory systems. The generation of metabolic flux models of the relevant pathways will provide information for rational metabolic engineering. Useful data for these models are now being obtained from many types of experiments, including those using transgenic technology to increase or change scent production [31], and from the identification and characterization of earlier steps in the scent biosynthetic pathways.

The observation that flowers coordinately synthesize many different scent volatiles that are often derived

Table 1. Approaches used for metabolic engineering of floral scent

Approach	Engineered species	Gene used	Result achieved	Olfactory effect	Refs
Introduction of a single gene	Petunia	CbLIS	Linalyl glucoside	No	[19]
	Carnation	CbLIS	Linalyl oxides	No	[20]
	Petunia	RhAAT	Benzyl acetate and phenylethyl acetate	ND	[29]
Introduction of multiple genes	Tobacco	CITER, CILIM, CIPIN	γ-terpinene, limonene, and β-pinene and side products	Yes	[21,22]
Introduction of multiple steps	Tobacco TERLIMPIN	MsLIM3H	Isopiperitenol and derivatives	ND	[23]
Elimination of some compounds	Petunia	PhBSMT RNAi	Lacks methylbenzoate	Yes	[27]
		PhBPBT RNAi	Lacks benzylbenzoate and phenylethylbenzoate	ND	[31]
		PhPAAS RNAi	Lacks phenylacetaldehyde and phenylethanol	ND	[13]
		PhCFAT RNAi	Lack of isoeugenol	ND	[15]
Blocking of competitive pathways	Carnation	Anti-DcF3′H	Increased methylbenzoate emission	Yes	[30]
Down-regulation of transcription factor	Petunia	PhODO1	Reduced levels of volatile benzenoids	ND	[32]

Abbreviations: CILIM, limonene synthase; CIPIN, β-pinene synthase; CITER, *Citrus limon* γ-terpinene synthase; CbLIS, *Clarkia breweri* linalool synthase; DcF3′H, *Dianthus caryophyllus* flavanoid 3′-hydroxylase; MsLIM3H, *Mentha spicata* limonene-3-hydroxylase; ND, not determined; PhBPBT, benzylalcohol/phenylethanol benzoyltransferase; PhBSMT, petunia benzoic acid/salicylic acid carboxyl methyltransferase; PhCFAT, coniferyl alcohol acyltransferase; PhODO1, ODORANT1 transcription factor; PhPAAS, phenylacetaldehyde synthase; RhAAT, *Rosa hybrida* alcohol acetyltransferase. Tobacco TERLIMPIN is a tobacco transgenic line expressing CITERM, CILIM and CIPIN.

from multiple pathways indicates that the main 'switch' in this process occurs upstream of individual metabolic pathways. Transcription factors that are able to control the multiple pathways leading to the formation of fragrance have not yet been identified. However, the first transcription regulator of the formation of volatile benzenoids in petunia, ODORANT1, was recently discovered [32]. Additional progress in discovering transcription factors will hopefully result in additional tools that can offer an efficient strategy to manipulate the flux through the metabolic pathways to improve scent emission.

Present technology for the genetic engineering of plants in general requires the ability to regenerate plants from callus tissue. To date, successful transformations have been developed for several cut flowers, including commercially important roses, chrysanthemums, carnations and gerbera, although for most varieties it is still an 'art form' [33].

Overall, it is clear that genetic manipulation of floral scent is possible but will require a more rational design based on the correct choice of species, previous knowledge of the pathways involved, including their cellular and subcellular localization, judicious use of promoters, and empirical testing.

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