Review

# RNAi-mediated crop protection against insects

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**Downregulation of the expression of specific genes through RNA interference (RNAi), has been widely used for genetic research in insects. The method has relied on the injection of double-stranded RNA (dsRNA), which is not possible for practical applications in crop protection. By contrast, specific suppression of gene expression in nematodes is possible through feeding with dsRNA. This approach was thought to be unfeasible in insects, but recent results have shown that dsRNA fed as a diet component can be effective in downregulating targeted genes. More significantly, expression of dsRNA directed against suitable insect target genes in transgenic plants has been shown to give protection against pests, opening the way for a new generation of insect-resistant crops.**

## Introduction: RNAi in insect genetics and crop protection

A decade has passed since the initial discovery of RNA interference (RNAi) in the nematode *Caenorhabditis elegans* [1], and it is now clear that double-stranded RNA (dsRNA)-mediated gene silencing is a conserved mechanism in many eukaryotes [2,3] (Box 1, Figure 1). Since its initial description the technique has become a valuable tool for functional genomics in insects, particularly in studying gene function in the model insect *Drosophila melanogaster* [4–6]. The preferred delivery methodology in the majority of insect studies has been microinjection of nanogram amounts of long dsRNA, synthesized *in vitro*, into the insect haemoceol [7]. This method of delivery contrasts with the situation in *C. elegans*, where RNAi effects can be produced by feeding bacteria expressing dsRNA [8,9], or even by soaking nematodes in dsRNA solution [10]. Microinjection of dsRNA in insects was considered to be necessary to produce RNAi effects because the complete genome sequence for *D. melanogaster* (and, subsequently, for other insects) has shown that they lack genes encoding RNA-dependent RNA polymerase (RdRP). RdRP is the enzyme necessary for the siRNA amplification step that leads to persistent and systemic RNAi effects [11]. The RdRP function is defined by a characteristic domain, designated PF05183 in the PFAM database (http://pfam.sanger.ac.uk), that has been identified in gene products of eukaryotic microorganisms, fungi, plants, nematodes and a primitive vertebrate (*Branchiostoma floridae* – a cephalochordate) but not in insects, molluscs or other vertebrates. The absence of RdRP in insects predicts that any effects of RNAi will be limited to cells that have taken up dsRNA and will require continuous input of dsRNA to persist. Injection of dsRNA into the body cavity, where it can circulate through the haemolymph, allows short-term effects on gene expression in most cells to be assessed.

The possibility of using RNAi effects to protect plants against insects by downregulating essential gene functions in the herbivore, thus resulting in its death, has been recognized for many years, but the method was considered unfeasible. The absence of dsRNA amplification implies that gene-knockdown effects produced by feeding RNAi to insects would be limited. Effects would only be expected in cells exposed to the nucleic acid; these cells would be those of the midgut and associated structures because these are the only regions of the insect not covered by the chitin exoskeleton (Box 2). Degradation of dsRNA in the gut would require continuous administration of high levels of dsRNA; production of sufficient dsRNA in a transgenic plant and its delivery in a sufficiently undegraded state to the insect would provide another significant technical problem, if a role in defence against insect pests was required. However, recent results have shown that many of these preconceptions were unduly pessimistic and that viable levels of insect resistance can be achieved by producing dsRNAs in plants [12,13].

## RNAi in insects; cellular dsRNA uptake and export

RNAi-mediated gene knockdown in *Drosophila* is localised to the site of dsRNA delivery and effects are temporally limited; indeed, a systemic long-lasting RNAi response has never been observed in *Drosophila*, in contrast to *C. elegans* [1]. The systemic RNAi effect in *C. elegans* is a multistep process that requires the amplification and spread of the silencing signal [11,14]. If a similar system was present in insect pests, it would enable targets to be selected from the whole insect (not just gut-specific targets). In addition, the RNAi amplification step would negate the need for a continuous supply of high levels of dsRNA, and thus could avoid many of the problems associated with the instability of dsRNA in the insect gut.

What lessons can be learned from the use of RNAi in model organisms in relation to a 'real-life' biological problem, such as crop protection against insect pests? Uptake of dsRNA in *C. elegans* has been studied by genetic analysis. A mutant has been identified that is impaired in its ability to mediate a systemic RNAi response when dsRNA is delivered orally [15]. The gene identified, *systemic RNA interference deficient-1* (*sid-1*), is essential and sufficient to mediate systemic RNAi effect in *C.elegans*. When expressed in *Drosophila* S2 cells, *sid-1* enhanced the

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**Box 1. RNA interference – a basic outline**

RNA interference (RNAi) is the specific downregulation of gene expression by double-stranded RNA (dsRNA). The specificity is sequence-based and depends on the sequence of one strand of the dsRNA corresponding to part or all of a specific gene transcript (for recent RNAi reviews see [56–58]). RNAi is a post-transcriptional control mechanism involving degradation of a target mRNA. This degradation is mediated through the production of small interfering RNAs (siRNAs) from the dsRNA, which is cleaved by dsRNA-specific endonucleases referred to as dicers (from the *dicer* gene identified in *Drosophila melanogaster* [59], reviewed in [60,61]). The siRNAs are 21 bp dsRNA fragments carrying two base extensions at the 3′ end of each strand; one strand of the siRNA is assembled into an RNA-induced silencing complex (RISC) in conjunction with the argonaute multi-domain protein, which contains an RNaseH-like domain responsible for target degradation [62,63] (see Figure 1 in main text). The process is closely related to post-transcriptional gene regulation by microRNAs (miRNAs), where the end-result is inhibition of translation initiation, and shares many of the same components. In plants and nematodes, RNAi can have systemic effects on gene expression, so that gene knockout spreads throughout the organism and persists over development. The basis of this effect is thought to lie in the presence of an RNA-dependent RNA polymerase (RdRP) that is able to interact with the RISC complex and generate new dsRNA based on the partially degraded target template by using the hybridised siRNA strands as primers. The synthesized dsRNA is then acted on by the dicer enzymes to generate new siRNAs (secondary siRNAs), thus acting as an amplification step. In this way, once a dsRNA is introduced into a cell, its effect can persist over development; in addition, the dsRNAs can be exported to neighbouring cells and thus spread the gene knockout effect through the organism.

ability of S2 cells to uptake dsRNA at sub-optimal dsRNA concentrations. The gene is predicted to encode an eleven-helix transmembrane channel protein that is expressed on the cell surface and enables uptake of dsRNAs, thereby mediating a systemic RNAi effect. Further potential mechanisms for RNA transport have been suggested by the recent identification of a further *C. elegans* dsRNA uptake mutant, *sid-2* [16]. *sid-2* mutants are unable to mediate an RNAi response when fed bacteria expressing specific dsRNAs. The *sid-2* gene product has been identified as a gut-specific transmembrane protein with a single transmembrane region. To demonstrate functionality, a related nematode, *Caenorhabditis briggsae*, which is defective in uptake of dsRNA from the gut lumen, was transformed with *C. elegans sid-2,* and a systemic RNAi phenotype was restored [16]. This demonstration of the complexity of RNAi-uptake mechanisms and the systemic spread of an RNAi signal in a single organism needs to be bourne in mind when considering RNAi in insects.

Could the absence of RNA transport mechanisms explain why *Drosophila* cannot manifest a systemic RNAi response? Homologues of the *C. elegans sid-1* gene have been identified in insects such as *Tribolium castaneum*, *Bombyx mori* and *Apis mellifera* but not in the *Drosophila* genome. *sid-2* homologues have only been detected in nematodes closely related to *C. elegans*. A *sid-1* homologue has also recently been identified in aphids [17]. However, recent evidence suggests that dsRNA uptake into cultured *Drosophila* S2 cells does not involve a *sid-1*-based mechanism but takes place by receptor-mediated endocytosis [18,19] because pharmacological inhibition of endocytosis also inhibited RNAi effects. Endocytosis of dsRNA also seems to occur in *C. elegans* because knockdown of components of the endocytotic pathway by RNAi results in worms with a 'loss-of-RNAi-function' phenotype [18]. These results suggest that receptor-mediated endocytosis is a widespread mechanism for dsRNA uptake and might well occur across different insect orders. If this is the case, herbivorous insect pests from different orders can be effectively targeted by oral delivery of dsRNA. Further understanding of the complexities of insect dsRNA-uptake mechanisms might facilitate the targeting of specific insect pests.

## Systemic RNAi in insects

To evaluate the potential for systemic RNAi effects in insects, an experimental approach using species other than *Drosophila* has been pursued. Insect systemic RNAi was first documented in the coleopteran *Tribolium castaneum* (flour beetle) by two independent studies. In the first, a homologue of the *Drosophila* sensory bristle-forming gene *Tc-achaete-scute* (*Tc-ASH*) was identified and targeted. Injection of *Tc-ASH* dsRNA into larvae at a single discrete site resulted in a 'loss-of-bristle' phenotype over the entire epidermis of adult insects [20]. In the second study, a parental RNAi effect transmissible between generations was demonstrated by identifying and targeting developmental genes. Injection of dsRNA specific to (i) *Distalless* (leg development gene), (ii) *maxillopedia* (homeotic gene) and (iii) *proboscipedia* (encoding a homeotic protein required for the formation of labial and maxillary palps) was used to produce an RNAi effect in both mother insects (injected) and developing progeny embryos after egg hatch [21]. Thanks to its well-documented, robust systemic RNAi response and the recent completion of its genome sequence, *Tribolium* is becoming an accepted model for the study of systemic RNAi in insects. Intriguingly, a recent genome comparison of *C. elegans* and *Tribolium* revealed a lack of conservation of a systemic RNAi mechanism [22]. For example, *Tribolium* lacks a *C. elegans*-like RdRP, so the signal amplification observed in *Tribolium* must be based on a different gene with a similar activity, or possibly even a different mechanism. RdRP-like activity has been demonstrated in cell-free extracts from *Drosophila* embryos, even though the RdRP gene is not present in insects [23].

Future research aimed at elucidating the mechanism of systemic RNAi in insects is likely to broaden the range of insects amenable to systemic RNAi and of genes that can be regarded as targets for a knockdown effect on expression. RNAi-mediated gene knockdown has been reported in several insect orders, including Diptera, Coleoptera, Hymenoptera, Orthopetra, Blattodea, Lepidoptera and Hemiptera [6,7,20,21,24–32], although most of these studies have used injected dsRNA.

## dsRNA feeding in insects

Development of a robust dsRNA feeding methodology in insects that mimics the results obtainable with *C. elegans* (where efficient suppression of gene expression by orally delivered dsRNA is routine) is a prerequisite for utilization of RNAi for crop protection against insect pests. Turner *et al.* [31] provided a convincing demonstration of RNAi effects after dsRNA feeding in larvae of the light brown apple moth (*Epiphyas postvittana).* dsRNAs

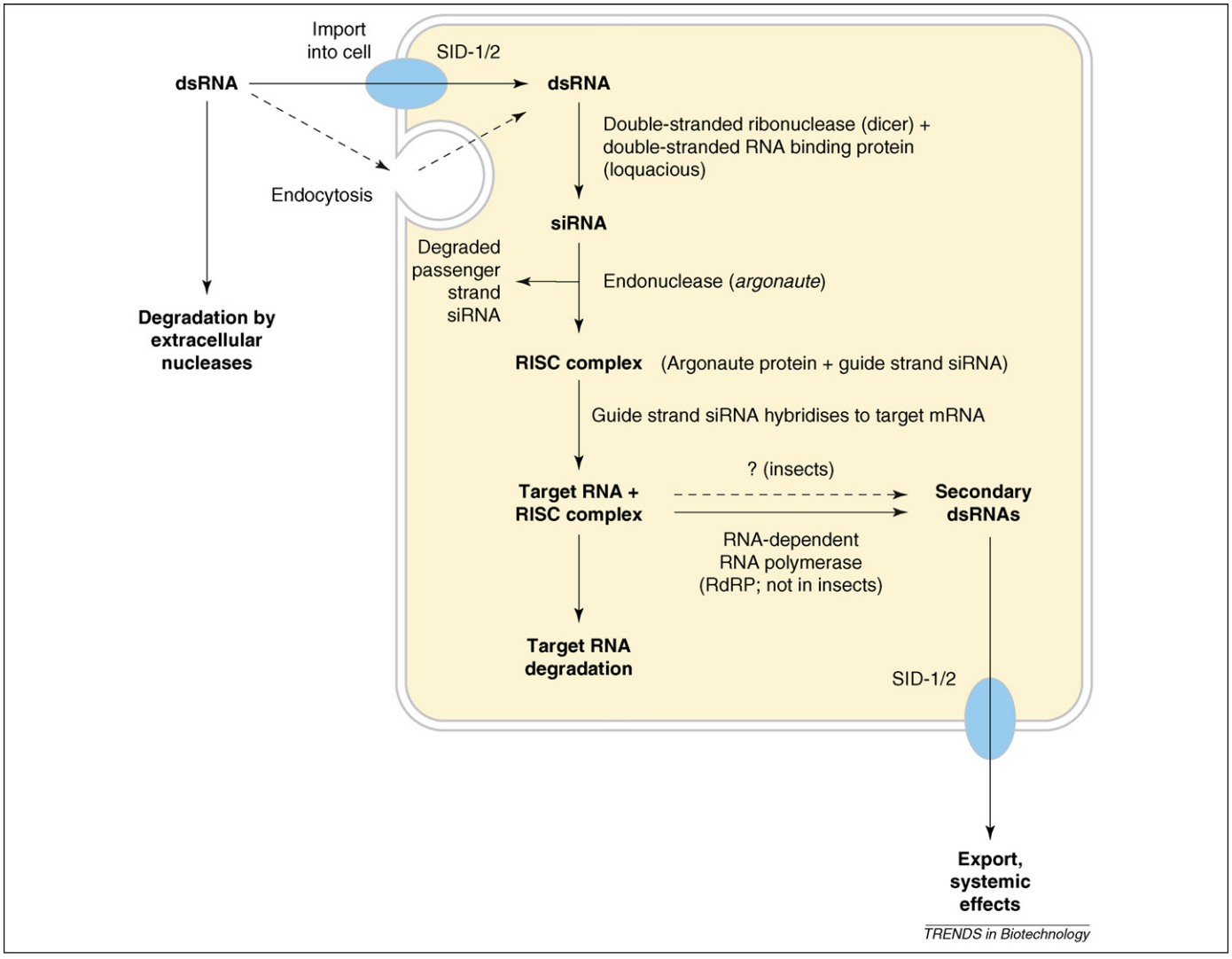


**Figure 1**. Functional stages of gene silencing with double-stranded RNA (dsRNA) in cells of lower animals. The figure shows steps involved in local and systemic gene silencing. Exogenous dsRNA is imported into cells, processed by *dicer* into small interfering RNA (siRNA; 21 bp + 2-base 3′ extensions on each strand) and assembled with the argonaute protein into the RNA-induced silencing complex (RISC). The RISC complex targets and degrades specific mRNAs based on the siRNA sequence. Systemic RNAi effects are mediated through the production of new dsRNAs by RNA-dependent RNA polymerase (RdRP), which uses the target RNA as a template and is primed by siRNA strands. The secondary dsRNAs can be exported from the cell to spread the RNAi effect to other cells. Gene names in italics have been identified in *Drosophila melanogaster*. The transport proteins SID-1 and SID-2 have been identified in *Caenorhabditis elegans*, as has the RdRP enzyme. Transport mechanisms might differ between diffeent organisms.

directed against carboxyesterases were incorporated into an artificial diet. Gene repression was observed after two days of feeding, and maximal repression occurred after seven days. These genes are thought to be gut-expressed, and thus only a local RNAi effect was required for repression. However, in the same investigation, knockdown of a gene expressed in the adult antenna could be achieved through feeding dsRNA to larvae, demonstrating a persistence of the RNAi signal throughout the larval and adult stages and a systemic spread of RNAi signal from the gut to the antennae. In contrast to these positive results, an earlier report showed that midgut aminopeptidase-N gene in larvae of the lepidopteran *Spodoptera litura* was efficiently downregulated by microinjection of dsRNA into the insect haemoceol but stated that attempts to feed dsRNA were unsuccessful in generating an RNAi response [28], although no details of methodology were given. An RNAi response after feeding dsRNA has also been reported in the bug *Rhodnius prolixus* (Hemiptera), where a salivary gland transcript encoding nitroporin 2 (NP2) was targeted both by oral delivery of dsRNA and by microinjection [32]. Both treatments produced downregulation of NP2 expression; however, microinjection was more effective (75% reduction in gene expression) than dsRNA feeding (42% reduction).

Variation in the midgut environment between different species might dictate whether a feeding approach will be successful. However, comparisons based on existing data are difficult because the susceptibilities of different targets to RNAi effects show considerable variation in model species. Some targets have proved to be completely refractory to suppression; for example, most of the neuronally expressed genes of *C. elegans* [33].

## Lessons learned from development of RNAi for plant parasitic nematodes

Plant expression of dsRNAs directed against genes in pathogens has become an established technique, and

**Box 2. The insect gut**

The insect gut is divided into three regions; foregut, midgut and hindgut. Of these the first two are continuations of the 'outside' of the insect and are chitin-lined, so that their surfaces do not present areas of exposed cells (although receptors and transporters are present to allow processes such as taste recognition in the mouth cavity and water transfer in the hindgut to occur). The midgut region is the only part of the gut that contains surfaces of exposed cells, and it is the main site of exchange between the circulatory system (haemolymph) and the gut contents. The midgut itself is responsible for nutrient absorption, whereas excretion and water balance take place primarily in the Malpighian tubules attached to the hind end, which carry out a function similar to that of the kidney in higher animals. RNAi effects occurring in insects as a result of oral delivery of dsRNA are presumably mediated by the midgut surfaces through exposure of cells of the midgut epithelium and the Malpighian tubules to dsRNA in the gut contents.

Conditions in the gut vary considerably between insect orders. Gut pH is an important factor in insect digestion and can vary from predominantly acidic (coleopteran larvae) to strongly alkaline (up to pH 10.5 in some species of Lepidoptera). In addition, within a single insect the pH changes along the gut and with distance from the gut epithelium. The stability of ingested dsRNA in the insect gut could be affected both by chemical hydrolysis (which increases with increasing pH) and by enzymes present in the gut contents.

plants that show increased resistance to a plant virus [34–36] and bacteria [37] through an RNAi effect have been described. The use of dsRNA approaches for the control of plant parasitic nematodes has been recently reviewed in detail [38–40]; however, it is worth highlighting some of the key developments in the application of this technology. Transgenic plants expressing dsRNAs specific to genes encoding a root knot nematode (*Meloidogyne* spp.) splicing factor and integrase (a chromatin remodelling protein) successfully knocked down transcripts in the pest, resulting in almost complete resistance [41]. In another study, a nematode secretory peptide (16D10) that stimulates root growth was successfully downregulated in four closely related species of root knot nematode by transgenic plants expressing dsRNAs, resulting in levels of resistance that varied between 63% and 90% [42]. A further study demonstrated the feasibility of downregulating a root knot nematode transcription factor with plant-expressed dsRNAs; however, in this case loss of function did not result in a deleterious phenotype [43]. To date, there is only one report of the successful downregulation of a cyst nematode transcript via similar approaches [44]; this might reflect the poor uptake of dsRNAs by cyst nematodes, in which the feeding tube has a lower exclusion limit than in root knot nematodes [40].

Although the nematode system clearly differs from insects, it highlights several important points that must be considered in developing an RNAi approach in insect pest species. RNAi effects are species-specific because knockdown experiments and identification of lethal phenotypes in *C. elegans* has not resulted in a universal set of 'nematode target genes' that are useful for protection against plant parasitic nematodes. Therefore, the success of the RNAi approach is dependent on careful target selection (which takes into account differences in specificity between different species) and the ability of the target nematode to mount a systemic RNAi response.

## Using RNAi to produce insect-pest-resistant plants

Despite having been considered for many years, application of RNAi technology to give resistance to herbivorous insects has only just been realised. Two recent papers have described transgenic plants producing dsRNAs directed against insect genes. These plants showed enhanced resistance to the economically important agricultural pests cotton bollworm (*Helicoverpa armigera*; Lepidoptera) and Western corn rootworm (WCR; *Diabrotica virgifera virgifera* LeConte; Coleoptera). The key to the success of this approach is: (i) identification of a suitable insect target and (ii) dsRNA delivery, which includes *in planta* expression of dsRNA and delivery of sufficient amounts of intact dsRNA for uptake by the insect. Although different approaches were used for the generation of insect-resistant plants, careful target selection was common to both (see Figure 2).

Baum *et al.* [12] utilised a screening approach where genes from WCR were identified in cDNA libraries, and genes encoding polypeptides predicted to provide an essential biological function were classified as 'targets'. A total of 290 potential targets were identified, and corresponding dsRNAs were synthesized *in vitro*; their effects on larval performance were determined by delivery in artificial diet feeding trials. Using this approach a total of 14 genes from the initial list demonstrated specific downregulation of target sequences at low dsRNA concentrations and resulted in insect stunting and mortality. The most effective dsRNA, directed against a gene encoding V-type ATPase A, demonstrated rapid knockdown of endogenous mRNA within 24 h of ingestion and triggered a specific RNAi response with low concentrations of dsRNA. The orally delivered dsRNA could produce systemic silencing of genes (encoding both V-type ATPase subunits and β-tubulin) throughout the insect.

The specificity of RNAi-mediated insecticidal effects is an important consideration for the use of this technology in a practical application; effects on non-target insects should be minimised. dsRNAs directed against three target genes (β-tubulin, V-ATPase A and V-ATPase E) demonstrated an effective RNAi response in WCR that resulted in high larval mortality. These dsRNAs were also delivered to three other coleopteran plant pests: Southern corn rootworm (SCR; *Diabrotica undecimpunctata howardii*), Colorado potato beetle (CPB; *Leptinotarsa decemlineata)* and cotton boll weevil (*Anthonomus grandis* Boheman). The dsRNAs demonstrated significant larval mortality in SCR and CPB, although only at higher concentrations than those used for WCR. The sequence identities between WCR and CPB were only 83% and 79% for V-ATPase A and V-ATPase E, respectively. As expected, synthesis of gene-specific dsRNAs for CPB V-ATPase A and V-ATPase E showed increased effectiveness in feeding trials compared with the WCR orthologues. Cotton boll weevil was not only completely insensitive to the three WCR-directed dsRNAs, but was also insensitive to dsRNAs directed against orthologous boll weevil genes, emphasising the differences between insect species in susceptibility to orally delivered RNAi strategies.

To demonstrate the practical application of this technology, transgenic corn was engineered to express dsRNA directed against WCR V-ATPase A. The plants were sub-

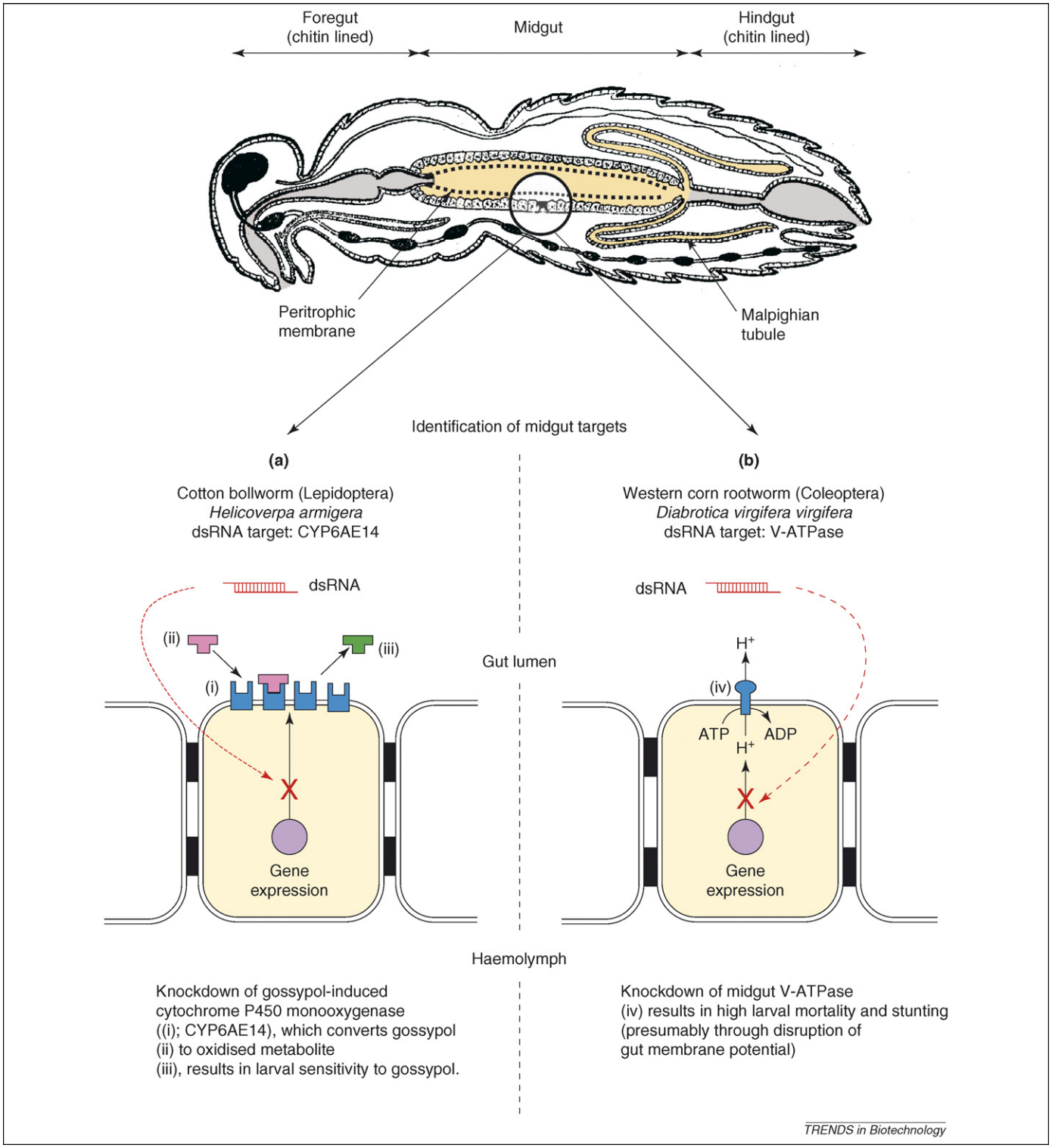


**Figure 2**. Overview of RNAi approaches for insect-resistant transgenic plants. Double-stranded RNA (dsRNA) produced *in planta* can lead to targeted gene silencing in Lepidoptera and Coleoptera pest species [12,13]. dsRNAs corresponding to specific insect targets are expressed *in planta* and are cleaved by endogenous plant Dicer enzymes to produce short interfering RNAs (siRNAs) of around 21 nucleotides. Large dsRNA and siRNA cleavage products are expressed throughout plant tissues and are orally delivered to insect herbivores feeding on transgenic plant material. For gene-silencing to initiate in targeted insect pests, large dsRNAs and siRNAs must persist in the insect gut, and sufficient quantities must be present for uptake into cells in contact with RNAs (the exact uptake mechanism in target insects remains unknown). Approach **(a)**: a gut-specific cytochrome monooxygenase, CYP6AE14, has been identified (i) whose expression correlates with larval growth on diets containing gossypol (ii), a cotton secondary metabolite. CYP6AE14 is presumably involved in detoxification of gossypol (iii) because specific knockdown of this gene product by dsRNAs delivered in artificial diet and by transgenic plant material increases larval sensitivity to gossypol [14]. Approach **(b)**: a related study [13] used a screening approach to identify a lethal phenotype in *Diabrotica virgifera virgifera* when midgut V-type ATPase A (V-ATPase) (iv) was downregulated by dsRNAs delivered in artificial diet feeding trials and transgenic corn. Although no direct evidence was presented for the deleterious effects observed in larvae, it is tempting to speculate that knockdown of V-type ATPase A results in disruption of electrochemical gradient across the gut epithelia, which results in high larval mortality.

jected to WCR infestation and demonstrated a significant level of protection compared to controls; that is, they showed reduced damage from WCR feeding.

A different approach was used by Mao *et al.* [13]. By studying the interaction between cotton bollworm and cotton, they identified a cytochrome P450 gene, *CYP6AE14*, which is highly expressed in the insect midgut and whose expression is correlated with larval growth when gossypol, a cotton secondary metabolite, is added to artificial diets. The authors concluded that expression of *CYP6AE14* is causally related to gossypol tolerance, presumably via detoxification of this compound, and that suppression of the expression of this gene could increase the sensitivity of the insect larvae to the plant's endogenous defence. Tobacco and *Arabidopsis* plants were engineered to produce dsRNAs directed against the bollworm *CYP6AE14* gene. When plant material of both species was fed to larvae, effective repression of the endogenous *CYP6AE14* transcript was observed, and the insects showed increased sensitivity to gossypol when transferred to artificial diets. Interestingly, expression of *CYP6AE14*-directed dsRNA in an *Arabidopsis dicer* mutant (knockout of *Arabidopsis dicer* genes *DCL2*, *DCL3* and *DCL4*) resulted in the production of longer dsRNAs in the plant that were more effective in gene repression of *CYP6AE14*. This result shows that optimal efficiency of repression of targeted genes in pests might require stabilization of dsRNAs. The group of Mao *et al.* [14] has recently reported that they have engineered cotton to express the cotton bollworm *CYP6AE14* dsRNA and that the plants show partial resistance to *Helicoverpa armigera*, as expected[*].

## Future prospects for RNAi-based control of insect pests

The feasibility of using RNAi in the protection of crops against insect herbivores has been demonstrated. This approach holds great promise for the future because it allows a wide range of potential targets for suppression of gene expression in the insect to be exploited. However, at the moment the method compares unfavourably with existing transgenic technologies giving resistance to coleopteran and lepidopteran herbivores. From the limited data currently available for whole-plant bioassays in laboratory trials, protection of maize against corn rootworm, even in the best-performing RNAi-expressing plants, is not as effective as in transgenic maize engineered to produce a modified Cry3Bb *Bacillus thuringiensis* (Bt) toxin [45]. Although it is unfair to compare the resistance of non-optimised research material with a commercial product, RNAi-expressing maize is unlikely to replace Bt-maize in the short term, especially as the effectiveness of the new crop-protection strategy at the field level remains to be determined. However, recent reports of resistance to Bt toxins being observed in field populations of insects exposed to transgenic plants [46,47] will provide an additional impetus for the development of alternative crop-protection strategies.

Which insect genes should be targeted? The screening approach used by Baum *et al.* has already identified a series of potential targets in corn rootworm, of which a gene encoding the β-subunit of a COPI coatomer complex was the most effective in terms of $LC_{50}$ for RNAi in artificial diet. The COPI complex is involved in translocation of proteins from endosomes to the cytoplasm, as well as other potential roles in protein trafficking in the cell, but it is not obvious why interference with this function should be lethal. The screening approach can thus identify targets that would not necessarily be predicted from functional considerations but has the drawback of being very labour-intensive if large numbers of insect bioassays are required. However, the demonstrated efficacy of targeting V-type ATPase A could easily be extended to other insect species. The approach of Mao *et al.*, in which insect detoxification mechanisms towards plant secondary metabolites are targeted, has the advantage of being predictable and specific to pests that feed on a crop producing a defined defensive chemical [48,49]. It can be readily extended to detoxification mechanisms in other plant–insect interactions. Further development of RNAi biotechnology could also seek to complement existing crop protection strategies; for example it might be possible to use technologies in combination to counter broad-range, protein-degradation-based resistance to Bt toxins (observed in highly polyphagous insect pests such as *Heliothis virescens*, which gain resistance through the upregulation of specific proteinase genes [50]).

Further increases in the effectiveness of RNAi strategies might be achieved by utilizing multiple targets. The feasibility of pyramiding multiple targets by RNAi has been demonstrated in *Drosophila* [51] but has yet to be applied to crop-protection strategies. The development of an understanding of the specificity of RNAi gene knockdown in insects should allow crops to be produced that express a cocktail of dsRNAs that are highly effective against target insect pest species. The sequence specificity of dsRNAs can be maximised by a careful bioinformatic approach, although multiple gene knockdown events might be achieved with a single dsRNA by targeting genes belonging to large families with high sequence similarity. However, care must be taken to avoid the possibility that loss of function is compensated for by another untargeted gene.

Although RNAi is unlikely to have an immediate effect on crop protection against lepidopteran and coleopteran plant pests, for which Bt-based strategies offer a high degree of protection, the technology is likely to be taken up for applications where Bt-based approaches have proved difficult, for example protection against flies (dipterans), or where no effective Bt toxins are known, for example protection against sap-sucking homopteran pests such as aphids, leafhoppers and whitefly. Targeting these phloem-feeding insect pests would require *in planta* expression of dsRNAs and transport of dsRNAs in phloem sieve elements. The transport of RNA in plant phloem is well documented; viral RNA genomes, endogenous cellular mRNAs and small noncoding RNAs are known to be transported in plant phloem elements [52–55]. However, there is no evidence for phloem transport of dsRNA; even though systemic RNAi-based gene silencing occurs in plants, recent evidence suggests that siRNAs are transported as single stranded sense and antisense molecules

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[54] and that all RNA in phloem is single stranded. It is possible that dsRNA expressed in phloem cells could be converted to single-stranded RNA (ssRNA) for transport in the phloem by the plant endogenously, but the stability and uptake of ssRNA into insect cells after feeding might then prove a problem. Further experimentation will be required to determine whether dsRNAs can be introduced into plant phloem sap to make targeting specialist phloem feeders by RNAi feasible with current technology.

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Review

# Plants disarm soil: engineering plants for the phytoremediation of explosives

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**Explosives are toxic, recalcitrant to degradation and contaminate large areas of land and ground water. Remediation of these synthetic compounds is difficult and an enormous logistical task. Phytoremediation is a technique that offers an environmentally friendly, low-cost alternative to current remediation techniques; however, this approach is hindered by the low inherent metabolic abilities of plants towards these xenobiotic compounds and the phytotoxicity of these compounds. As a result of recent advances in our knowledge of the biochemistry underlying endogenous plant detoxification systems and the use of genetic engineering to combine bacterial explosives-detoxifying genes with the phytoremediatory benefits of plants, this technology is now poised for testing in the field and in a wider range of plants, such as poplar and perennial grasses.**

## The scale of environmental pollution

Environmental contamination has arisen through the manufacture, use and decommissioning of explosives – the extent of contamination now poses a serious and significant threat to the environment. Explosives-contaminated ground water has the potential to contaminate drinking supplies; a concern for human health. Although global contamination levels are difficult to quantify, the magnitude of the problem in the USA alone is clear: in 2003, the US Department of Defense estimated that the clean up of unexploded ordnance (artillery and military supplies), discarded military munitions and munition constituents on its active ranges, a total of 24.6 million acres (10 million hectares), would cost between US$16 billion and US$165 billion [1]. In addition to active ranges, the US Defense Science Board reported that there could be more than 15 million acres (6 million hectares) of closed sites containing explosives contamination in the form of unexploded ordnance, as well as vast areas offshore [2].

On training ranges, explosives contamination levels are heterogeneous, localized to target areas and regions where former arsenals of corroded, unexploded munitions have leached into the soil and ground water, resulting in hotspots of contamination. In 1997, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX – variously abbreviated from Royal Demolition Explosive, Research Department Explosive or Research Department Unknown Explosive X) was discovered in ground water beneath a training range at the Massachusetts Military Reservation on Cape Cod (USA). This area contains a sole source aquifer that supplies drinking water to half a million people and has led the US Environment Protection Agency (EPA) to halt all training using live munitions at this site (http://www.epa.gov/region1/pr/1997/pr041497a.html). Despite numerous remediation projects, recent reports show that RDX and other pollutants are still detectable in the reservation soil and ground water [3].

Current remediation strategies include removing soil to land fill, composting, incineration or capping of the soil. These methods are themselves environmentally damaging, insufficient for the scale of the problem and expensive. Phytoremediation, the use of plants to clean-up the environment, could present a cost-effective, environmentally friendly and aesthetically pleasing alternative to clean up explosives on contaminated land. With extensive root networks, plants can efficiently penetrate contaminated soil and take up compounds from the ground water. Furthermore, plants, as mostly sessile organisms, have evolved complex detoxification systems to deal with a diverse range of toxic chemicals. The plasticity of this system also enables plants to detoxify relatively recently produced, synthetic pollutants such as explosives. There have been several recent reviews on the phytoremediation of explosives [4–6], and in this article, we look at current studies that are beginning to uncover the detailed biochemistry behind endogenous detoxification systems in plants. We assess the progress of research focused on engineering bacterial genes that confer the ability to detoxify explosives into plants. We also discuss the potential use of these plants in the field to clean up soil in firing ranges and remediate explosives manufacturing sites.

## Characteristics of explosive groups

There are three main groups of explosives: nitrate esters, nitroaromatics and nitramines. Nitrate ester explosives are esters of nitric acid, which commonly contain *O*-nitro groups. The main nitrate esters are glyceroltrinitrate (nitroglycerine, GTN) and pentaerythritoltetranitrate (PETN). At low levels, both GTN and PETN are used clinically as vasodilators, and although higher doses cause symptoms such as headaches and convulsions, these compounds seem to be relatively non-toxic.

Nitroaromatic explosives contain an aromatic ring with multiple nitro groups. The most widely used nitroaromatic explosive, 2,4,6-trinitrotoluene (TNT), contains three nitro groups (Figure 1); these withdraw electrons from the aromatic ring, making electrophilic attack of the ring difficult, and thus TNT is particularly recalcitrant to degradation by microbial oxygenases. In addition, TNT becomes tightly bound to the humic fraction of soil, making it biologically

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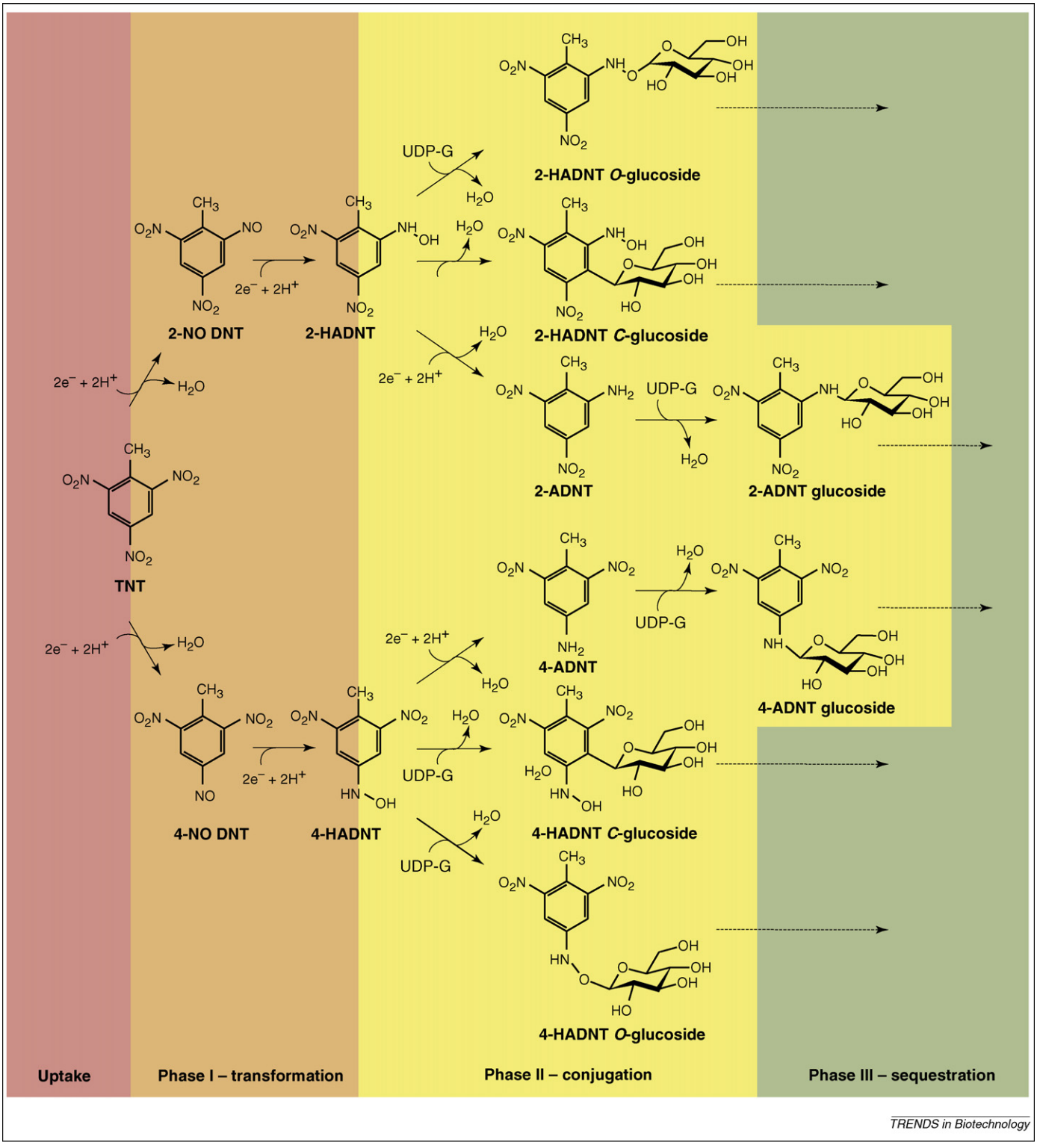


**Figure 1**. Proposed detoxification pathway of the explosive TNT in plants. After uptake, Phase I of TNT detoxification is the transformation via nitroso-dinitrotoluene (NO-DNT) to 2- and 4-hydroxydinitrotoluene (HADNT) isomers. Phase II involves the conjugation of the transformed intermediates to endogenous plant compounds, including sugars. During Phase III, the conjugates are sequestered into the plant biomass, possibly by incorporation into plant cell walls or compartmentalization into vacuoles. Modified from Ref. [28].

unavailable [7,8]. TNT is a component in a wide range of ordnance and demolition explosives. Additional nitroaromatic compounds found as contaminants in military training ranges include dinitrotoluenes (DNTs), which are used in propellants and are also byproducts associated with the manufacture and transformation of TNT. Aminodinitrotoluenes (ADNTs), diaminonitrotoluene and nitrobenzenes can also be present. Soil hotspots of TNT can contain up to 87 000 mg $kg^{-1}$ [9], whereas studies on hand-grenade ranges reveal mean concentrations of between <0.01–36.00 mg $kg^{-1}$ surface soil [10]. TNT and the 2- and 4-DNT isomers are toxic to all organisms tested so far,

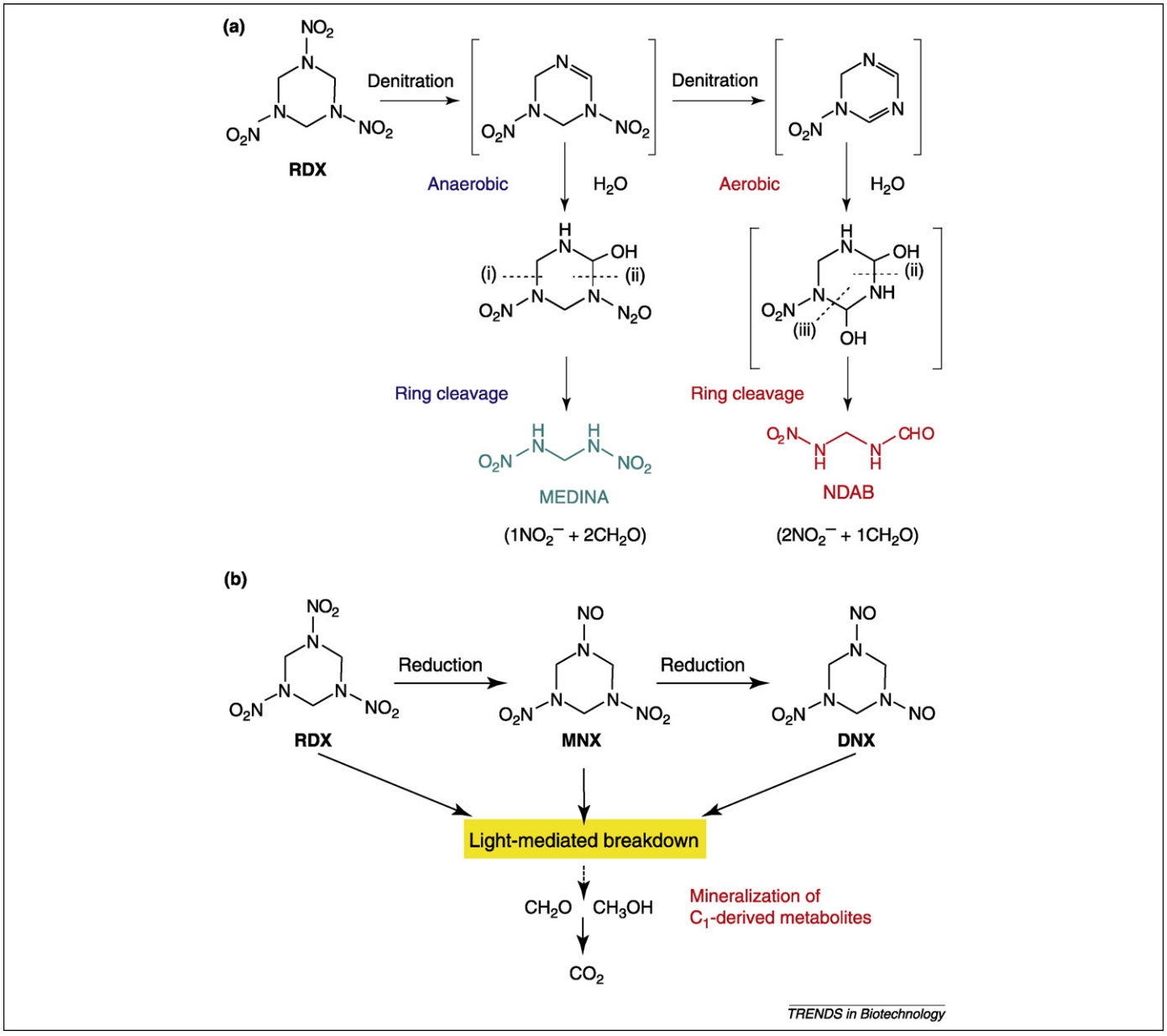


**Figure 2**. Proposed degradation pathways of the explosive RDX. **(a)** Degradation of RDX by the bacterial enzyme XplA under both aerobic and anaerobic conditions, as proposed by Jackson *et al.*, 2007 [63]. The ring cleavage occurs at (i) and (ii) under anaerobic conditions and at (ii) and (iii) under aerobic conditions. Compounds in square brackets are hypothetical, and the pathway is based on product detection and analogy with previous work [68,69]. **(b)** Degradation of RDX in poplar, as proposed by Van Aken *et al.* [48]. Abbreviations: DNX, hexahydro-1,3-nitroso-5-nitro-1,3,5-triazine; MEDINA, methylenedinitramine; MNX, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine; NDAB, 4-nitro-2,4-diazabutanal; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine.

causing anaemia and liver damage in mammals [11] and chlorosis and stunting in plants [12–14]. TNT is listed by the EPA as a possible human carcinogen.

Nitroamines contain *N*-nitro groups. The most important military high explosive currently used is RDX (Figure 2), which is often found, together with TNT, in ordnance, land mines and in the plastic explosive Composition 4. The distribution of RDX is, as for TNT, heterogeneous, with soil hotspots of up to 74 000 mg $kg^{-1}$ at military training ranges [9] and <0.01–51.00 mg $kg^{-1}$ at hand-grenade ranges [10]. RDX, unlike TNT, is highly mobile and can readily leach into ground water, thus potentially polluting subsequent waterways. The nitramine octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX – abbreviated from either Her Majesty's Explosive or High Melting Point Explosive) is used in many anti-tank weapons with military-grade RDX containing ~10% HMX impurity. Levels of HMX reported near firing points at anti-tank rocket ranges are between 100 s and 1000 s mg $kg^{-1}$ within 10 m of the firing point [10]. RDX causes convulsions in humans and other mammals [15,16] and targets the central nervous system [17]. Although no mutagenic effects have been observed in bacterial and mammalian cell cultures treated with RDX or HMX [18], the EPA classifies RDX as a possible human carcinogen.

## Metabolism of explosives by plants

Plants are able to metabolize and detoxify a vast array of chemical compounds. The process of detoxification has been classed into three phases. Xenobiotics lacking a

reactive group enter at Phase I, where a functional group, commonly hydroxyl, amino or sulfhydryl is introduced to the molecule. This transformation enables Phase II to occur, whereby one or more hydrophilic molecules are conjugated to the transformed xenobiotic. In Phase III, the conjugated xenobiotic is sequestered, commonly to vacuolar or cell wall compartments [19–21]. The detoxification phases and pathway for TNT is outlined in Figure 1 and discussed below. Recent work, summarized here, has broadened our knowledge of the biochemistry underlying the endogenous detoxification pathways of explosives in plants.

### *Metabolism of nitrate esters in plants*

The biochemistry of nitrate ester metabolism has not been well studied, but it has been shown that tobacco (*Nicotiana tabacum*) germination is severely compromised by 1 mM GTN [22], whereas cultures of beet (*Beta vulgaris*) are able to take up both GTN and PETN and are able to tolerate and transform 2 mM GTN to glycerol di- and mono-nitrate [23].

### *Metabolism of nitroaromatics in plants*

The toxicity of TNT is species dependent, and the majority of species tested are able to tolerate TNT levels of between 50 and 100 mg $kg^{-1}$ soil (reviewed in Ref. [4]). The type of soil also has a significant effect: soils with high humic content bind more TNT, removing it from the biologically available pool and effectively lowering toxicity [7,13].

Phase I of TNT detoxification in plants commonly constitutes the reductive transformation of one or more nitro groups via a nitroso intermediate to produce hydroxylaminodinitrotoluene (HADNT) (see Figure 1) and then ADNT. This reaction is favoured because the electron-withdrawing properties of the nitro groups of TNT make the aromatic ring of TNT electron-deficient and therefore more easily able to be reduced. Although the ADNT metabolites are relatively stable chemicals, the HADNT intermediates are not stable at room temperature. Two recent studies suggest that previously reported levels of HADNT therefore could have been underestimated [24,25], and it is possible that predominantly HADNT is produced.

Microarray and SAGE gene expression experiments [26–28] have identified a small gene family (five to six genes) of old yellow enzyme (OYE) homologues, the oxophytodienoate reductases (OPRs) in *Arabidopsis thaliana* (*Arabidopsis*) that are upregulated in response to TNT treatment. More-detailed reverse transcription–PCR analysis has confirmed that *OPR2* and, to a lesser extent, *OPR1* are upregulated in response to TNT [29]. Several members of the OYE family have been shown to transform TNT: OPR1, OPR2 and OPR3 possess the conserved, active-site amino acids crucial for this [30–32].

Plants also contain a range of nitroreductases that are likely to be involved in TNT detoxification. In the aquatic plant *Myriophyllum aquaticum*, there is evidence of oxidative transformation of the TNT methyl group and also aromatic hydroxylation [33]. When $^{14}$C-labelled HADNT or ADNT was supplied to *M. aquaticum*, oxidative transformation products of these substrates were not found, suggesting that the oxidative step occurs directly to TNT [33].

After transformation in Phase I, there is now significant evidence that Phase II involves the conjugation of transformed intermediates to sugars and glutathione. Hydrolysable TNT conjugates were observed initially in *Phaseolus vulgaris* [34], then later ADNTs conjugated to one or more six carbon units were identified in Madagascar Periwinkle (*Catharanthus roseus*) root extracts [35]. Further studies in *C. roseus* and *M. aquaticum* demonstrated that additional, conjugated products were formed with TNT that were not present when ADNT substrates were supplied [36]. Mono- and diglycoside conjugates of the less stable 2- and 4-HADNT intermediates, which had not previously been detected owing to their low chemical stability, were subsequently identified [24,36,37]. Recently, microarray analysis [28] has identified the potential involvement of UDP-glycosyltransferases (UGTs) from *Arabidopsis*. Subsequent characterization revealed six purified UGTs that conjugated 2- or 4- HADNT and, to a lesser extent, ADNTs. They also exhibited bias for either the 2- or 4-HADNT isomer, forming both *O*- and *C*-glucosidic bonds. These conjugates were also isolated *in planta*. Overexpression in *Arabidopsis* resulted in increased conjugate production and enhanced seedling root growth, showing that UGTs have an important role in the detoxification process [28]. Gene-expression studies on *Arabidopsis* and *Chlamydomonas reinhardtii* [26–29] have shown that specific glutathione-*S*-transferases (GSTs) are also upregulated, highlighting the involvement of these enzymes in TNT detoxification. The expression of two poplar (*Populus trichocarpa*) GSTs, identified by homology with upregulated *Arabidopsis* genes, is also increased in response to TNT treatment [38]. The identity of the glutathione-conjugated substrate (TNT, HADNT or ADNT) has yet to be elucidated.

Although the subsequent fate of TNT-transformed glucosyl and glutathione conjugates has not yet been determined, studies on other glucosyl- and glutathione-conjugated compounds show that the hydrolysed compound or conjugate can be deposited in the vacuole or cell walls or be excreted [39]. This Phase III step of detoxification supports some of the previous findings. Experiments using radiolabelled TNT in *P. vulgaris* and wheat (*Triticum aestivum*) showed that the majority (~95%) of TNT-derived intermediates and conjugates were found predominantly in the root, where label was distributed evenly between cytosolic and cell wall fractions, with the lignin fraction totalling 27% of the wheat cell wall fraction [40,41]. Similar results were also seen for hybrid aspen (*Populus tremula* x *tremuloides*) [42]. Studies in tobacco cell-suspension cultures identified mono- and diglycoside conjugates of 2- and 4-HADNT, whereas ADNT conjugates were not detected [38]. The presence of HADNT conjugates rather than ADNTs might be because cell cultures lack appreciable levels of lignin as a sink for amine residues. Several genes known to be associated with lignin biosynthesis [43] were upregulated in serial analysis of gene expression (SAGE) data from TNT-treated *Arabidopsis* root, including phenyl ammonium lyase, cinnamate 4-hydroxlase, 4-coumarate coenzyme A (CoA) ligase, hydroxycinnamoyltransferase, cinnamoyl-CoA reductase, caffeic acid *O*-methyltransferase and cinnamyl alcohol

dehydrogenase [26]. Other amino-conjugated xenobiotics have also been located in the lignin fraction [44]. This evidence suggests that a significant proportion of TNT conjugates are biologically incorporated into cell wall lignin complexes. Extremely little is known about the metabolism of the 2- and 4-DNT isomers in plants. The presence of only two electron-withdrawing nitro groups makes electrophilic attack more feasible, and plant oxygenases could theoretically have a role in DNT transformation.

### *Metabolism of RDX in plants*

Plant roots readily take up RDX [45–48], which, in contrast to TNT, is translocated to the aerial organs [45,49]. Experiments using poplar (*Populus deltoides* x *nigra* DN-34) tissue culture and crude leaf extracts fed with $^{14}$C-labelled RDX demonstrated that RDX was reduced to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and hexahydro-1,3-nitroso-5-nitro-1,3,5-triazine (DNX) in intact plant cells. Subsequent mineralization of the heterocyclic ring required both intact cells and light, yielding formaldehyde and methanol. These were further transformed via a light-independent step into carbon dioxide [48] (Figure 2b). Despite the high uptake rates, plants have inherently low endogenous abilities to degrade RDX [49,50], and accumulated RDX therefore becomes biologically available through the food chain via herbivory or is returned to the soil when the plant dies. SAGE analysis on RDX-treated *Arabidopsis* revealed that many of the genes upregulated in response to RDX treatment are also switched on in response to a range of general stresses [51]. Expression analysis on poplar sequences identified from five corresponding *Arabidopsis* TNT-inducible genes (a GST, cytochrome P450, two reductases and a peroxidase) showed that these genes were also upregulated by RDX [52]. Comparisons of SAGE analyses on *Arabidopsis* treated with either RDX or TNT revealed distinct differences in the transcriptome profiles. This suggests that there is little overlap between the detoxification pathways of RDX and TNT and presents a challenge for phytoremediation, because both compounds are often found on contaminated sites together [26,51].

## Engineering plants for the phytoremediation of explosives

Despite the complex detoxification pathways present in plants, their slow generation time compared with that of microorganisms means that plants have had less time to evolve efficient methods for detoxifying these synthetic compounds. Aromatic explosives are particularly phytotoxic, and detoxification rates for all three classes of explosives (nitrate esters, nitroaromatics and nitramines) are inherently low in plants when compared with those of bacterial cultures. Although bacteria isolated from contaminated soil can rapidly detoxify explosives in laboratory cultures, the fact that these explosives persist in the environment suggests that bacteria do not possess enough biomass or metabolic activity to decontaminate these areas significantly. To overcome this limitation, genetic engineering could be used to transfer these bacterial genes into plants, thereby enhancing the ability of plants to detoxify explosives. Data outlined below provide evidence that this clean-up technology can work under laboratory conditions.

### *Metabolism of nitrate esters*

Many bacterial strains that can denitrate the nitrate ester explosives GTN and PETN have been isolated from contaminated land, including *Enterobacter cloacae* PB2 [53]. The *E. cloacae* strain PB2 was isolated from soil enrichments under aerobic and nitrogen-limiting conditions and found to be able to use GTN or PETN as a sole nitrogen source. The gene underlying this ability, designated *onr* (for 'organic nitrate reductase') was subsequently identified and the gene product, termed PETN reductase (PETNr), a monomeric flavin mononucleotide (FMN)-containing protein, was characterized [54]. PETNr sequentially reduces two of the four nitro groups of PETN to yield pentaerythritol dinitrate, which is subsequently oxidized to the dialdehyde [53]. PETNr shares sequence similarities with other related enzymes that have been isolated from bacteria [32,55,56]; these enzymes are members of the OYE family and are homologues of the *Arabidopsis* OPRs. PETNr also possesses activity towards nitroaromatics (described below).

Tobacco plants expressing PETNr were able to germinate and grow normally on solid media containing 1 mM GTN, a concentration that would be lethal to untransformed tobacco. These plants were also able to remediate GTN from liquid culture significantly faster than untransformed seedlings [22].

### *Metabolism of nitroaromatics*

Reductive transformation of TNT via a nitroso intermediate to form 2- or 4-HADNT is the most common pathway for TNT transformation observed in bacteria under aerobic conditions. This step was shown to be performed by a 24.5 kDa, FMN-containing, nitroreductase enzyme (NR). The NR enzyme is encoded by the *nfsI* gene, which, like *onr*, was also cloned from *E. cloacae* [57]. PETNr has been shown to catalyse reductive transformation of TNT. It also has a second activity, the reductive attack of the aromatic ring, releasing nitrite and hydride ($H^-$-TNT) and dihydride ($2H^-$-TNT) Meisenheimer TNT adducts [56]. Characterization of OYE homologues with similar activities to PETNr suggests that the observed liberation of nitrite is likely to be the result of the rearomatization of Meisenheimer dihydride complexes through condensation with HADNTs in a non-enzyme-catalysed chemical reaction to form diarylamines [58].

The NR enzyme can transform TNT significantly faster than PETNr and, when expressed in transgenic plants, NR also confers greater tolerance to TNT than PETNr [22,30,59]. Expression of NR in tobacco conferred the ability to tolerate and detoxify 0.5 mM of TNT in liquid culture, a level close to the solubility limit for TNT at 25 °C and known to be phytotoxic to untransformed tobacco plants.

In soil studies, NR-expressing tobacco plants were able to tolerate levels of TNT contamination that would be toxic to untransformed plants [59]. Measurement of the TNT transformation intermediates isolated from the growth medium of tobacco cultures revealed that NR-expressing plants produced predominantly 4-HADNT and 4-ADNT isomers, indicating that NR favours reduction of the nitro group from the 4 position of the aromatic ring [60]. Recent

studies using aspen transformed with a nitroreductase, *pseudomonas nitroreductase A* (*pnrA*), isolated from the bacterium *Pseudomonas putida* have demonstrated that, compared with untransformed plants, the transgenic trees were able to take up higher levels of TNT from liquid culture and soil. The phytotoxicological limit towards TNT was also significantly higher than for untransformed plants [42], and these properties should now be tested in field trials.

### *Metabolism of nitramines*

Several bacteria, including *Rhodococcus rhodochrous* 11Y, have been isolated that can utilize RDX as a sole nitrogen source for growth. The gene responsible for this metabolism, *xplA*, has been cloned and its protein product identified as a novel, fused flavodoxin-cytochrome P450 enzyme (CYP177) [61,62]. Recent characterization shows that under aerobic conditions, one mole of RDX is degraded by XplA, with the production of one mole of 4-nitro-2,4-diazabutanal (NDAB), two moles of nitrite and one mole of formaldehyde, whereas under anaerobic conditions, one mole of methylenedinitramine (MEDINA), one mole of nitrite and two moles of formaldehyde are produced (Figure 2a) [63].

*Arabidopsis* plants expressing XplA have been shown to remove all of the RDX from a 180 μM solution [62]. This

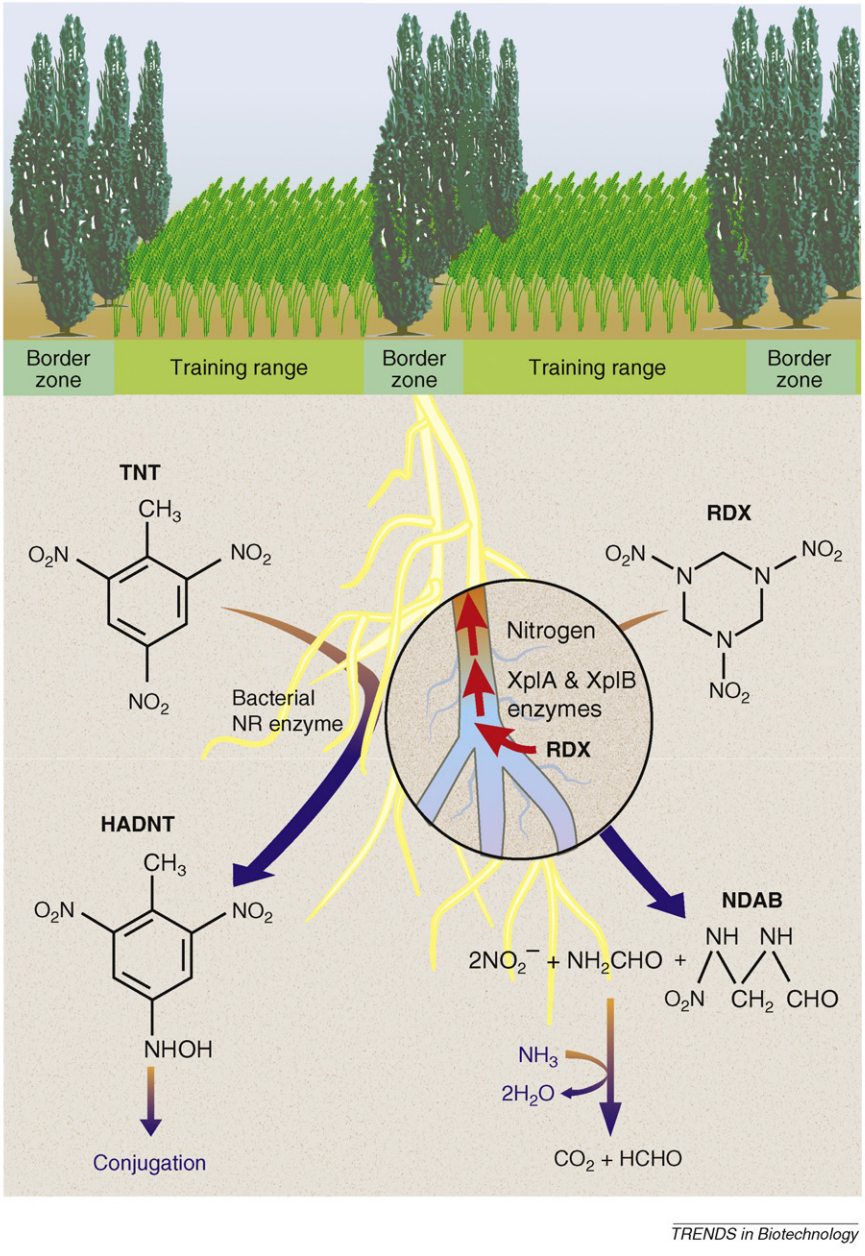


**Figure 3**. Schematic illustration of a proposed method for phytoremediating TNT and RDX from military training ranges using genetically modified plants. Plants are transformed with genes expressing TNT-detoxifying nitroreductase (NR) enzymes and RDX-degrading XplA and XplB enzymes. NR transforms TNT to less toxic compounds (e.g. aminodinitrotoluenes), which are conjugated to endogenous plant compounds before incorporation into the root biomass. RDX is taken up to the aerial parts of the plant, where XplA, together with its reductase partner XplB, catabolize RDX to non-toxic compounds. TNT, which is relatively immobile in soil, and RDX, which is highly mobile in ground water, are removed from training ranges by transformed perennial grasses. Transformed grasses and tree species are planted along range borders to remove RDX from soil leachate as it leaves the training range.

concentration is more than three times that measured in waste water from manufacturing sites [64] and is close to the aqueous solubility limit of RDX [65]. Soil studies have demonstrated that XplA-expressing plants can grow, with no adverse effects, in soil contaminated with RDX at levels of up to 2000 mg $kg^{-1}$, the highest concentration tested, whereas untransformed plants exhibited phytotoxic effects at RDX concentrations of 250 mg $kg^{-1}$ and above. Furthermore, whereas root biomass was significantly reduced in untransformed plants when grown in soil containing RDX concentrations of 2000 mg $kg^{-1}$, XplA-expressing plants had greater root biomasses than untransformed plants or transgenic plants that had been grown in uncontaminated soil. This suggests that XplA-expressing plants utilized RDX as a nitrogen source for further growth [64]. The RDX degradation could be further enhanced by introducing XplB, the reductase partner for XplA, into XplA-epxressing *Arabidopsis*. These plants showed a further 30-fold improvement in the rate of RDX removal from liquid culture and were also able to remove RDX from soil leachate [63], addressing one of the biggest concerns of RDX pollution – its migration through ground water and subsequent contamination of drinking-water supplies.

HMX has a low aqueous solubility limit of 22 μM at 25 °C and has only limited uptake and metabolism in poplar [66]. No activity of XplA towards HMX could be observed under aerobic conditions, and transgenic XplA-expressing lines did not take up HMX from liquid culture significantly more than untransformed plants [63]. It might be possible to increase the activity of XplA towards RDX or to confer activity towards HMX, either by random mutagenesis or, with advances in our knowledge of the structure of XplA, by targeted mutagenesis approaches.

Many explosives are found together on training ranges, particularly RDX and TNT, and the next logical step would be to transform plants with two or more bacterial genes with the aim to detoxify a range of explosives with one plant species. Suitable plant species for phytoremediation would need to be low-growing, fire-resistant and capable of withstanding, and recovering rapidly from, disruption by heavy equipment. The perennial grass species western (*Agropyron smithii*), Siberian (*A. fragile*) and slender (*A. trachycaulum*) wheatgrasses are native to the training ranges of the temperate regions of the USA and are promising candidates for this application. Tree species such as poplar could be trialled in border zones to remove explosives from soil leachate leaving training ranges (Figure 3).

### *The impact of transgenic plants below ground*

The toxicity of high levels of explosives contamination can drastically reduce both the quantity and variety of microorganisms populating the soil rhizosphere [67]. These microorganisms are an essential part of soil fertility and are involved in decomposition and nutrient cycling, and thus the biological health of the soil. So far, only one study has investigated the impact of transgenic plants on the rhizosphere of explosives-contaminated soil. Promisingly for phytoremediation as an environmentally restorative technology, this study demonstrates that detoxification of contaminated soil by NR-expressing tobacco restores the genetic diversity of the microbial community biomass and metabolic activities of the soil [67].

## Conclusions and future directions

The studies reviewed here have enhanced our knowledge of the endogenous, biochemical mechanism of TNT detoxification in plants and demonstrated the potential applicability of genetic-modification technology. Two major breakthroughs have been the isolation of bacterial genes capable of detoxifying, or degrading, the two most widely used explosives, TNT and RDX, as well as the laboratory demonstration that expression of these genes in plants confers the ability to remediate these compounds from soil and ground water. Additional, significant advances have been made in elucidating the endogenous genes and metabolic detoxification pathways for these explosives in plants. Genomic analysis has now also identified gene targets for future characterization, including OPRs and GSTs. It is anticipated that these breakthroughs will enable the development of a range of phytoremediation approaches for tackling the extensive explosives pollution that exists. This could be through selection of native, non-genetically modified species with high endogenous detoxification systems or species such as wheatgrass, a native to military firing ranges in temperate regions, transformed with plant and or bacterial genes. In addition, the molecular structure of enzymes such as XplA could be altered by mutagenesis to give activity towards HMX or increased activity towards RDX. Further screening of explosives-contaminated soils and ground water could also lead to the identification of additional microbial genes encoding activity towards explosives. Although studies involving tobacco and *Arabidopsis* demonstrate the potential for phytoremediation, these species would be unsuitable for field trials because, compared with other species such as grasses and trees, they are not resilient to range training activities and have low biomass and poor root penetration. Recent laboratory studies using NR-transformed aspen to remediate TNT are encouraging [42]. Perhaps the approach with the most potential to work is the creation of plant buffer zones around the perimeter of training ranges to remove RDX from soil leachates. Unlike TNT, which binds tightly to soil organic matter, laboratory experiments have shown that the highly water-mobile RDX is rapidly removed from soil by plants expressing XplA and XplB [63]. Once in the plant, RDX is degraded, unlike TNT, which is transformed to less toxic intermediates that remain stored in the plant biomass. The use of tree species, with their relatively deep, penetrating root networks, could further enhance this approach (Figure 3). To evaluate the potential of this technology for the phytoremediation of contaminated soil and ground water, glasshouse and field trials must be conducted.

However, this new technology is not without drawbacks. Protocols for genetically transforming native grass species such as wheatgrass need to be developed and strategies for gene containment will need to be evaluated. The fate of conjugated TNT intermediates introduced into food chains by herbivory and biodegradation of genetically modified plant biomass needs to be monitored. Furthermore, public acceptance of genetically transformed plants in Europe is

low and this approach might be more feasible in the US, where genetically modified crop species have now been grown for over a decade.

Considering the wider picture, the pool of xenobiotic-degrading genes that could be harvested from microorganisms growing in contaminated environments is only limited by the range of organic pollutants. The studies presented here demonstrate that phytoremediation could potentially be applied to many different types of organic pollutants, such as chlorinated solvents, polycyclic aromatic hydrocarbons, polychlorinated biphenols and pesticides.

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Review

# Evolution of a regulatory framework for pharmaceuticals derived from genetically modified plants

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**The use of genetically modified (GM) plants to synthesize proteins that are subsequently processed, regulated and sold as pharmaceuticals challenges two very different established regulatory frameworks, one concerning GM plants and the other covering the development of biotechnology-derived drugs. Within these regulatory systems, specific regulations and guidelines for plant-made pharmaceuticals (PMPs) – also referred to as plant-derived pharmaceuticals (PDPs) – are still evolving. The products nearing commercial viability will ultimately help to road test and fine-tune these regulations, and might help to reduce regulatory uncertainties. In this review, we summarize the current state of regulations in different countries, discuss recent changes and highlight the need for further regulatory development in this burgeoning, new industry. We also make the case for the harmonization of international regulations.**

## Introduction

The production of pharmaceutical proteins in plants has several potential advantages over current systems such as mammalian and bacterial cell cultures, including the lower costs and scalability of agricultural production, and the absence of human pathogens [1,2]. A large number of plant host systems has been tested, including plant cell cultures, unicellular plants, aquatic plants grown in containment, and, most notably, food and non-food crops, which can be grown in greenhouses, underground growth facilities, or the open field [3].

Research and development in the area of plant-made pharmaceuticals (PMPs) over the past 10 years has focused on agricultural crops, with tobacco, maize, potato, rice and safflower being the most frequently used. However, regulatory uncertainty and technical challenges in downstream processing [4] have prompted the development of PMPs produced in contained systems, such as plant suspension cells [5] (e.g. a carrot cell system developed by Protalix) and the *Lemna* system, as championed by Biolex Therapeutics. Products in these systems have reached phase III and phase II clinical trials, respectively [6]. In 2006, the United States Department of Agriculture (USDA) licensed a poultry vaccine produced in cultured tobacco cells [7]. Since then, several products derived from crop plants have also reached late development stages, including human insulin and carp growth hormone produced in safflower. These are expected to reach the market between 2008 and 2010 (see Table 1).

PMPs present two major challenges for the regulatory bodies. Regulators of agricultural biotechnology are confronted with a novel type of crop use, and drug regulators must deal with a novel drug-production concept. Particular challenges arise in the case of open-field production, in which more than 350 field trials have been approved for crops producing either pharmaceutical or other industrial proteins in the USA, Canada and the European Union (EU) over the past two decades [8]. The USA and Canada have published several discussion papers and drafted PMP-specific guidelines [9–15], yet these guidelines have not been finalized and will probably evolve further with technological developments.

Here, we provide an overview of the regulations governing the cultivation of pharmaceutical plants and the approval of PMP products. We focus on PMPs produced by agricultural cultivation, because these pose a greater regulatory challenge than contained production systems. Non-pharmaceutical products (i.e. plant-made industrials [PMIs]) are outside of the scope of this review. We first set out the requirement for specific regulations and guidance, and then describe the most recent regulatory developments for pharmaceutical plants and the licensing of PMPs at both the national and international levels. We conclude with a discussion of remaining regulatory challenges. A list of relevant websites is provided in Box 1.

## Why do we need specific regulations for PMPs?

### *Regulatory oversight of genetically modified plants*

Several differences have been drawn among first-, second- and third-generation genetically modified (GM) crops. First-generation crops have traits such as herbicide tolerance and insect resistance, second-generation crops have improved food and/or feed (hereafter food/feed) quality, and

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**Table 1. Plant-made pharmaceuticals in advanced stages of development[a]**

| Product | Application | Plant host(s) | Status [b] | Company or academic group |
|---|---|---|---|---|
| Plant-made pharmaceuticals and vaccines – human use | | | | |
| AB | AB cancer vaccine | Tobacco | Phase II clinical trials | Large Scale Biology, USA [c] |
| B subunit of heat labile Escherichia coli toxin LT-B | Oral vaccine against traveller's diarrhoea | Potato, maize | Phase I completed (potato: 1998) [d] | [89] |
| Capsid protein Norwalk virus | Vaccine | Potato | Phase I completed (2000) [d] | [90] |
| CaroRx ™ [e] | AB carries prophylaxis | Tobacco | Phase II clinical trials, approved as medical device in the EU in 2003 | Planet Biotechnology, USA |
| DoxoRx ™ antibody | Side-effects of cancer therapy | Tobacco | Phase I completed | Planet Biotechnology, USA |
| Fusion protein, including epitopes from rabies | Vaccine against rabies | Spinach (virus-infected) | Phase I completed [d] | [88] |
| Gastric lipase | Cystic fibrosis | Maize | Phase II clinical trials, commercialisation expected for 2009/2010 | Meristem Therapeutics, France |
| Hepatitis antigen | Oral vaccine against hepatitis B | Potato | Phase II clinical trials | Azbio, Arizona State University, USA |
| Human glucocerebrosidase (prGCD), | Treatment of Gaucher's disease | Carrot suspension cells | Received FDA approval for Phase II clinical trial of prGCD; marketing expected in early 2008 | Protalix Biotherapeutics, Israel |
| Insulin | Diabetes | Safflower | Path for clinical trials accepted by FDA, commercialisation expected for 2010 | SemBioSys, Canada |
| Lactoferon ™ (α interferon) | Hepatitis C | Lemna | Phase II | Biolex, USA |
| RhinoRx ™ [e] | Common cold caused by rhinoviruses | Tobacco | Phase I/II planned for 2005 [d] | Planet Biotechnology, USA |
| Plant-made pharmaceuticals and vaccines – animal use | | | | |
| Antigen | Vaccine against feline parvovirus | Tobacco | Advanced | Large Scale Biology, USA [c] |
| Antigen | Vaccines against papilloma virus | Tobacco | Early | Large Scale Biology, USA [c] |
| HN protein of Newcastle disease virus | Poultry vaccine | Tobacco suspension cells | Approved by USDA | Dow Agro Sciences, USA |
| Plant-made pharmaceuticals applied as nutraceuticals | | | | |
| Human intrinsic factor | Food supplement; vitamin B12 deficiency | Arabidopsis | Approval from Danish authorities for commercial production in greenhouse; market authorisation in Poland | Cobento Biotech AS, Denmark |
| Human lactoferrin | Developed as food supplement: anti-infection, anti-inflammatory and iron-binding properties | Rice | Advanced [f] | Ventria, USA |
| Human lysozyme | Developed as food supplement: anti-infection, anti-inflammatory and iron-binding properties | Rice | Advanced [f] | Ventria, USA |
| Immunosphere ™ [g] | Carp somatotropin to be used as feed additive for shrimps | Safflower | Only import permits required for USA, Canada or the EU; commercialisation expected for 2008 | SemBioSys, Canada |

Information from references [16,89–91], updated and extended from company websites and literature. Colour code: orange, open field production; green, greenhouse; blue, entirely contained (cell culture, bioreactor-type) production; no colour, production environment unknown. Abbreviations: AB, antibody; EU, Europe; FDA, Food and Drug Administration; HN, hemagglutinin/neuraminidase; LT-B, labile toxin B-subunit; prGCD, plant-cell recombinant glucocerebrosidase; USDA, US Department of Agriculture.
[a]This table cannot be considered a comprehensive list and does also not include PMPs and PMVs that are still in very early phase of development.
[b]For human biopharmaceuticals: phase of clinical trials.
[c]Large Scale Biology filed bankruptcy in 2006.
[d]No updated information available.
[e]Produced from both open fields and greenhouses. Clinical materials have been derived from greenhouses (E. Fineman, personal communication).
[f]Already commercially available as fine chemical.
[g]According to company officials, the carp growth hormone will be used in major shrimp producing countries only (e.g. South America, China, Thailand) and has to seek market authorisation as a food additive in these countries only.

third-generation crops produce added-value products, and thus include PMP crops. First- and second-generation GM crops are mainly intended for food/feed purposes whereas third-generation crops are envisaged as production vehicles for high-value molecules and are not intended for consumption as food/feed. PMP crops are designed to maximize the yield of the target protein, which consequently can accumulate up to 5000 times the level typically found for transgene products in first- and second-generation crops [16]. PMP crops can also undergo

**Box 1. Useful websites concerning the regulation of pharmaceutical plants and their products**

**USA**
- USDA-APHIS: www.aphis.usda.gov/
- US permits for pharmaceutical plants: http://www.aphis.usda.gov/brs/ph_permits.html
- FDA CFSAN: http://www.cfsan.fda.gov/
- EPA: http://www.epa.gov/
- US Excellence through Stewardship Initiative: http://www.excellencethroughstewardship.org/
- Biotechnology Industry Association BIO: http://bio.org/healthcare/pmp/

**Canada**
- CFIA Plant Biosafety Office: http://www.inspection.gc.ca/english/plaveg/bio/pbobbve.shtml; http://www.inspection.gc.ca/english/plaveg/bio/mf/fracad/commere.shtml#3; http://www.inspection.gc.ca/english/plaveg/bio/mf/sumpnte.shtml
- CFIA Feed Section: http://www.inspection.gc.ca/english/anima/feebet/feebete.shtml
- HC: http://www.hc-sc.gc.ca

**Europe**
- EFSA: http://www.efsa.europa.eu
- EMEA: http://www.emea.europa.eu

**International**
- Cartagena Biosafety Protocol: http://www.cbd.int/biosafety/

multiple genetic modifications (i.e. stacking) to co-introduce pest resistance, molecular confinement, changes in glycosylation, and identity preservation traits [17–20]. These multiple modifications can also increase the likelihood of unintended effects on the plant [16]. Furthermore, the pharmaceutically active products are designed to elicit a physiological response in humans, and so inadvertently exposing humans or animals to such plant material is generally perceived as a greater concern than the corresponding risk associated with first- and second-generation crops. Pharmaceutical plants are therefore considered to pose additional environmental and health risks, although the actual risk could differ greatly, depending on the properties and expression level of the protein, the nature of the host plant, and the particular exposure scenarios [16,21–26].

The main concerns raised in stakeholder consultations and crucial reports from consumer and environmental organizations are the risk of contaminating the food/feed chain, and broader environmental impacts, including effects on wildlife [27–34]. Even if the actual risks are negligible, farmers and the food industry are concerned about the economic risks should PMP crop residues appear in food products [35–43]. These concerns are also reflected by the USDA policy of zero tolerance, the history of which is discussed in Box 2. However, the adventitious presence of PMPs in food is probably much less likely than contamination with first-generation GM crops, partly because PMP crops will be restricted to relatively small plots of land. For example, ~15 000 acres of PMP safflower could deliver the entire predicted global demand for insulin in 2012 [44]. The absence of a trade in seeds and viable plants, along with maintaining strictly separated processing streams, should further reduce the risk of food chain contamination.

**Box 2. The ProdiGene case – a trigger for the USDA's zero tolerance policy**

In 2002, the biotechnology company ProdiGene Inc. was fined US$250 000 by the USDA and compelled to carry out a US$3 million clean-up operation after volunteer* maize plants containing the gene for a veterinary vaccine were found among a soybean crop planted in the same field in the following season. Part of the clean-up process included the purchase and destruction of more than half a million bushels of adulterated soybeans, and ProdiGene was also ordered to post a US$1 million bond to fund the development of a compliance programme for future PMP crops.

The ProdiGene case, along with similar incidents involving first-generation GM crops in food products, prompted a robust response by the regulatory agencies; the penalty issued against ProdiGene was the maximum possible under the 2000 Plant Protection Act. This reflected the perceived risk associated with accidental consumption of a pharmaceutical product, and it resulted in a 'zero tolerance' approach to enforcement in which no attempt was made to make penalties proportional to the risk involved. However, it was never shown that the volunteer maize plants were transgenic, or that they produced viable seed containing the vaccine. Nor was there evidence of actual risk. Partly as a result of the controversy over this decision, APHIS envisages moving towards a tiered approach based on the actual risks posed [83].

* A cultivated plant growing from self-sown or accidentally dropped seed.

North American regulators and the biotechnology industry therefore consider pharmaceuticals as a distinct category of GM crops with handling requirements that differ from those required for crops producing food/feed [45]. Existing regulations and guidance documents are considered to be inadequate to govern the commercialization of PMPs and have therefore created regulatory uncertainties for developers. Key elements of proposed regulations and guidance include dedicated machineries and facilities, contract farming, standard operating procedures for many steps of on-farm work, and training programmes for workers (see Box 3). The higher value and lower acreages associated with pharmaceutical crops could make extensive and redundant confinement measures economically feasible. Emerging regulations focus on extensive physical and organizational confinement measures to avoid outcrossing, spillage of seeds or biomass, and co-mingling with food/feed crops [12–14].

PMPs produced in greenhouses and fully contained facilities, such as cell culture systems, fall under different regulations to those governing field-grown crops, and regulations need to be much less stringent as long as containment is maintained. One issue that remains to be dealt with is the level of containment needed. Even within the EU, implementation of GM organism (GMO) legislation at the national level has led to differences in interpretation. For example, GM crops grown in net houses – greenhouses comprising fine-meshed nets instead of glass – are considered as being 'contained' in some EU Member States and as an environmental release in others, with the latter requiring a much more comprehensive dataset for authorization [46].

### *Regulation of pharmaceuticals*

The drug regulators have repeatedly stated that existing guidelines, in principle, also apply to PMPs [13,47–49].

**Box 3. Permit conditions for growing pharma plants in the USA**[†]

- Separation from sexually compatible crops (e.g. one mile for open pollinating maize)
- 50 feet fallow zone surrounding the plot
- No planting of food/feed crops on the test site in the following year
- Dedicated equipment (not for use with food/feed crops)
- Submission of Standard Operating Procedures (SOPs) required, depending on the assigned risk category for the following:
  - Harvesters and planters
  - Storage facilities for seed and equipment used to handle regulated articles
  - Seed cleaning, processing and drying
  - Equipment to off-load, haul or move seed or harvested materials
  - Tractors including attachments
- Monitoring of volunteers during and after completion of field trials
- Growers under contract with the manufacturers only. Annual APHIS training; approval of training programmes for personnel
- Audit of field trial records by APHIS
- On-site inspections by APHIS at least seven times a year before, during and after production

Sourced from the following references [14,45,88].

[†] This box includes examples of specific requirements for confinement measures of pharmaceutical and industrial plants. For full details see the following references [13,14]. Measures depend on host plant, type of protein, location of production and plant handling practices.

However, it is difficult to follow such guidelines to the letter, because they have been developed for cell-based systems, which are sterile and contained processes in which the media and environment can be controlled precisely. By contrast, whole plants are not sterile and are not necessarily contained, and their environment can be variable owing to the weather, soil heterogeneity, and interactions with other organisms, including pests. Cell-based and fermenter-specific terminologies are also difficult to apply to whole plants; for example, the concept of master and working cell banks. For pharmaceuticals produced in mammalian cells, a master cell bank is an archived frozen stock of cells that can be used to replenish a working cell bank, from which the production cells are derived. Given that plants cannot be frozen like cells, it is impossible to apply the same principles to plant-based systems. Another process that is more relevant to pharmaceuticals derived from mammalian cell lines is virus clearance and inactivation, because mammalian cells can support the replication of human pathogens. This is another potential advantage of PMPs, in that such contamination is of little or no relevance to plants, especially in the case of greenhouse-based production. For field-produced PMPs, the only conceivable – albeit still disputed – source of such contamination would be from rodents, birds and workers. Plant viruses, by contrast, are more likely to be present but are not known to present health risks to humans. Nevertheless, regulators have yet to express their views on this.

## Regulations governing the cultivation of PMP crops

Specific regulations and guidance documents for the cultivation of PMP crops have been drafted in jurisdictions with significant commercial research and development (R&D) activity, but not in other areas. This is indicated by the number of field trials that have been approved: 240 in the USA, 90 in Canada, and ~30 in the EU [8]. The development of specific regulations in the USA was largely triggered by a series of compliance failures concerning food/feed GM crops, and – in one case – a PMP crop, which increased public pressure (see also Box 2).

R&D activities have also been tracked in South Africa and Australia, but this has not yet resulted in visible regulatory activities. Some PMP-related commercial R&D is also being conducted in other countries (e.g. South Korea, Japan, China, Chile and Cuba), but little regulatory information is available in the public domain. On an international level, PMP crops have, to date, been taken up only in the context of the Cartagena Protocol on Biosafety (CPB).

### *USA*

The USDA and the Food and Drug Administration (FDA) share responsibility for the cultivation of PMP crops in the USA (Table 2). Within the USDA, the Animal and Plant Health Inspection Service (APHIS) oversees and regulates the release of GM plants into the environment, and also

**Table 2. Statutory authorities, regulations and guidance relevant to growing pharmaceutical crops in the US, Canada and Europe**

| Country | Authority | Scope of regulation | Laws and regulations | Specific regulations and guidance for pharmaceutical crops |
|---|---|---|---|---|
|  | USDA–APHIS Biotechnology Regulatory Services (BRS) www.aphis.usda.gov/ | Development and field production from seed through to grain. Including transport and environmental release | Plant Protection Act (PPA). National Environmental Protection Act (NEPA) | Field Testing of Plants Engineered To Produce Pharmaceutical and Industrial Compounds [88]. Introductions of Plants Genetically Engineered to Produce Industrial Compounds (Interim rule) [50] Draft Guidance for Industry: Drugs, Biologics, and Medical Devices derived from Bioengineered Plants for Use in Humans and Animals [13][a]. Draft Guidance for APHIS Permits for Field Testing or Movement of Organisms with Pharmaceutical or Industrial Intent [14][b]. |
| | FDA CFSAN and CVM http://www.cfsan.fda.gov/ | Additional oversight for food/feed safety | Federal Food Drug and Cosmetic Act (FFDCA) | See above [13] |
| | EPA http://www.epa.gov/ | Reviewing APHIS Environmental Assessments and APHIS regulations; [c] | Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). National Environmental Protection Act (NEPA) Toxic Substances Control Act (TSCA) | Not available |

**Table 2 (*Continued*)**

| Country | Authority | Scope of regulation | Laws and regulations | Specific regulations and guidance for pharmaceutical crops |
|---|---|---|---|---|
|  | CFIA<br>Plant Biosafety Office (PBO)<br>http://www.inspection.gc.ca/english/plaveg/bio/pbobbve.shtml | Environmental release | Canadian Food Inspection Agency Seeds Act and Seeds Regulations | Directive 2007 (Conducting Confined Research Field Trials of Plants with Novel Traits in Canada) and its interim amendment for plant molecular farming field [9]<br>Assessment Criteria for the Evaluation of Environmental Safety of Plants with Novel Traits Intended for Commercial Plant Molecular Farming [92]<br>The PBO is currently developing a regulatory framework for the environmental release of plants which would require closed-loop confinement for commercial production due to potential food/feed, or environmental safety issues, a release termed commercial confined environmental release (CCER).<br>The environmental release of plants intended for plant molecular farming is expected to be regulated under this new framework. |
| | CFIA Feed Section | Use of by-products as feed | Feeds Act and Feeds Regulations | n.i. |
| | CFIA Seed Section | Sale, advertising, import into and export from Canada of seed of pharmaceutical crops | Seeds Act and Seeds Regulations | Although there is no specific guidance pertaining to pharmaceutical crops, for most agricultural crops in Canada, variety registration is required before sale (Seeds Regulations, Part III) |
| | HC | Additional oversight for food safety | | As part of the PBO-CFIA's regulatory framework for CCERs, proponents might be required to submit exposure and hazard data so that impacts on human and animal health resulting from exposure to the plant under review can be assessed. In addition, the potential hazards resulting from the unintentional introduction of plant material into the food and livestock feed chains will be assessed. It is anticipated that HC will review this exposure and the hazard data on behalf of the PBO. |
|  | Member States National Competent Authorities | Field trials (Part B) | Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms [61][d] | Existing guidance is currently being reviewed to assess applicability to non-food crop usage.<br><br>Specific guidance in preparation (announced by EFSA for 2008). |
| | European Commission<br>EFSA<br>Member States National Competent Authorities | Import, cultivation, processing, marketing for commercial purposes (Part C) | | |
| | | Applies to PMPs if (remainders) would be used as/in food/feed; might also apply if a food/feed crop would be used even if used for non-food/feed purposes only. | Regulation (EC) 1829/2003 on genetically modified food/feed [62] | |
| | European Commission<br>Member States National Competent Authorities | Unintentional movements of GMOs between Member States and exports of GMOs to third countries | Regulation (EC) 1946/2003 on transboundary movements of genetically modified organisms [65] | Not available |

Source: Adapted from references [93–95], http://www.inspection.gc.ca/english/plaveg/bio/mf/fracad/ovesure.shtml, http://www.bio.org/healthcare/pmp/factsheet4.asp.
Abbreviations: APHIS, Animal and Plant Health Inspection Service; CFIA, Canadian Food Inspection Agency; CFSAN, Center for Food Safety and Applied Nutrition; CVM, Center for Veterinary Medicines; EFSA, European Food Safety Authority; EPA, Environmental Protection Agency; n.i., not investigated; USDA, United States Department of Agriculture.
[a]Does not cover PMPs used for industrial purposes (e.g. the SemBioSys carp growth hormone).
[b]Does not cover PMPs used for food/feed purposes (e.g. the SemBioSys carp growth hormone).
[c]If manufacture, processing, distribution, use and/or disposal of a PMP produces a chemical substance that represents a risk to health or the environment.
[d]Transposed into national law of each Member State, and thus slight differences in the legislation might occur, especially for contained use and Part B field trials.

monitors GM plant imports, interstate movement (e.g. environmental safety issues and site inspections), the use of by-products, and the disposal of by-products and waste. The FDA also provides additional oversight to ensure safety of the food/feed chain.

In response to the concerns from the food industry and civil society organizations that PMPs might contaminate the food/feed chain, the USDA removed the notification track option, which is a simplified and fast-track procedure designed for agricultural GM crops intended for

food/feed. Furthermore, the USDA increased the criteria required for permission to cultivate PMP crops [45,50]. Draft guidance on the information required by applicants has been provided [13,14] but not yet finalized. However, these guidance documents are non-binding. According to APHIS, the planting of PMP crops requires continuous regulatory oversight, such that the producer must apply for a new permit every year and will not be eligible for deregulation (i.e. effectively releasing GM crops from regulatory oversight), which is currently the case for commodity GM crops following commercialization. More stringent confinement measures than those applied to conventional GM crops must be implemented. Such measures include increased isolation distances, fallow zones, increased inspection, and oversight (Box 3). In cases that require an Environmental Assessment (EA), which is associated with all environmental release permits, the applicant must provide additional information, including details of the potential for gene transfer to and persistence of the transgene in the environment, and the impact on plant and animal communities, agricultural practices and human health [14]. If human health is considered as being potentially affected, an EA also allows for a period of public comment.

The APHIS Compliance and Inspection Branch (CIB) was established in response to several violations of permit conditions [51]. US industry also launched a stewardship policy to 'enhance regulatory compliance and produce quality for consumers', which was recently broadened to become the 'Excellence Through Stewardship Initiative'. The latter initiative brings together the various stewardship measures on confinement of PMP crops, field trial compliance and insect resistance management, and introduces a third-party audit [52–57]. To accommodate the continuing criticism and litigation resulting from APHIS enforcement [58,59], the USDA established a Biotechnology Quality Management System (BQMS) in 2007, to complement the existing APHIS regulatory compliance and inspection process and to address compliance issues proactively together with applicants [60]. Both the Biotechnology Industry Organization (BIO) initiative and the BQMS aim to regain public trust and to prevent further trade disruption from non-compliance and adventitious presence.

### *Europe*

Cultivation of all GM plants in the field constitutes an 'Environmental Release' and as such would require prior notification under Directive 2001/18/EC [61] to the National Competent Authority in the Member States. This Directive covers the deliberate release of food and non-food GM crops into the environment for both R&D purposes (Part B of the Directive) and commercial purposes (Part C), and it thus also covers any PMP crops grown in the field. To date, PMP crops have only been grown under Part B permits, ruling out their commercialization. Pathways for commercialization have yet to be addressed by the European Commission (EC), and it is therefore not entirely clear if applications can only be submitted under Directive 2001/18/EC. In this case, a national Competent Authority (CA) would evaluate the applications, and other national CAs would be asked to comment, with the European Food Safety Authority (EFSA) conducting its own evaluation in case of disagreements only. Alternatively, it is possible that application could be submitted under the centralized procedure set out in Regulation 1829/2003 on GM food/feed. In this case, EFSA would evaluate the application, and national CAs could make comments. With regards to PMPs, only applications for the commercial release of PMP non-food or 'food' crops that are not intended for food/feed purposes are likely to be evaluated under Directive 2001/18/EC rather than Regulation 1829/2003 [62], although this has yet to be clarified (Table 2). EFSA is currently addressing whether any of the existing risk assessment concepts and guidance for food/feed crops can be extended to cover PMP crops, including non-food crops. In 2006, EFSA initiated a self-tasking exercise to address such questions, and their results are scheduled for publication as a draft guidance document in 2008 [http://www.efsa.europa.eu/EFSA/Event_Meeting/GMO_Minutes_37th_plenmeet,3.pdf].

By contrast, the cultivation of PMP crops grown in containment would be regulated by the 'Contained Use' Directive, as amended by Directive 98/81/EC [63,64]. These regulations, overseen at the national level, are far less stringent than Directive 2001/18/EC, because containment does not necessitate a fully fledged environmental risk assessment. The export of live plants, including seeds, would fall under Regulation 1946/2003 [65] on the transboundary movements of GMOs, which would be especially relevant if seeds from PMP crops were exported to other countries (e.g. for field trials or commercial production).

### *Canada*

Currently, the Canadian Food Inspection Agency (CFIA) regulates PMPs in the same way as other plants with novel traits (PNTs), using regulations set out under Canada's Seeds Act and Seeds Regulations (Part V). Canada is also developing these current regulations to cover the environmental release of PNTs specifically intended for commercial plant molecular farming (PMF) (Table 2). The CFIA is developing an approach that focuses on plants that constitute a potential risk to food/feed and/or environmental safety under this new proposed framework [66]. This new framework is likely to enforce a closed-loop production system that aims to keep PMP crops segregated from food/feed chains and, where appropriate, to minimize their environmental exposure. Developers of PMPs would be required to submit environmental, food/feed safety data, as well as to develop a release management strategy (RMS) as part of their application for 'commercial confined environmental release' (CCER) authorization. The applicant's RMS would outline how the developer plans to ensure that these crops would remain segregated from the food/feed chains and how dispersal into the environment would be minimized.

Plants authorized under CCER would then be subject to ongoing regulatory oversight, which would include on-site inspections during seed production, planting, growing, harvest and any post-harvest restriction periods (i.e. ensuring commodity crops are not grown in these locations in rotation). Off-site audits could also be carried out to

examine the developer's records on planting, seeding, monitoring, harvesting, corrective actions (where appropriate), and disposal and storage.

### *Australia*

In Australia, PMP crops are subject to the same regulatory control as commodity GM crops, which are overseen by the Gene Technology Regulator, and both PMP and GM crops are assessed for risks to human health and environmental safety on the same case-by-case basis. The Gene Technology Regulator also has the authority to issue a license that contains specific conditions for managing risks. PMP crops and their products can also be subject to regulation by the Therapeutic Goods Administration, Food Standards Australia New Zealand, the Australian Pesticides and Veterinary Medicines Authority, or the National Industrial Chemicals Notification and Assessment Scheme, depending on the trait, plant species and intended use [67].

### *South Africa*

The regulatory framework governing GMOs in South Africa requires permits for import and export, development, production, use, release and distribution of such organisms within the country. Since the GMO Act came into effect in 1997 [68], thousands of permits have been granted for conventional GM crops as well as for GM-derived pharmaceuticals from non-plant sources, but none have been approved for PMPs, to date. Among the public and private laboratories in South Africa registered to work on GMOs, only two are directly involved in PMP research – the Council for Scientific and Industrial Research (CSIR) and the University of Cape Town.

South Africa's biosafety system has been criticized for its weaknesses in terms of liability, public participation and access to information [69,70], and concerns have been raised that it might not be able to cater adequately for PMP crops [71]. One of the major concerns, as with most countries, is the issue surrounding contamination of the food chain. Therefore, the African Centre for Biosafety recommended that PMPs should not be produced in food crops.

## The Cartagena Protocol on Biosafety

The CPB was established in an attempt to harmonize biosafety issues globally. As part of its remit, the CPB regulates the exchange of information among its 103 signature states as a prerequisite for permission for transboundary movements of GMOs. However, the CBP requirements are not mandatory in the main countries (e.g. USA, Canada and Chile) presently growing PMP crops in open fields, because these have not signed up to or ratified the Protocol, and as such are therefore not parties to the CPB. By contrast, the EU and several countries with recent interest in the technology, such as South Africa, South Korea and Japan, are parties to the CPB. The main CPB mechanism, the Advanced Information Agreement (AIA) procedure, establishes requirements and standards for risk assessment and the mutual exchange of information in case of imports and exports of GMOs, and this procedure is likely to be applicable to PMP crops only if viable plants or seeds intended for commercial exploitation are cultivated in open fields. The import of seeds from PMP crops that originate from field trials or commercial scale production outside the EU and which are intended for processing and extraction would trigger less extensive documentation requirements than the AIA would. Transboundary movements of processed plant material from PMP crops would fall outside the scope of the CBD [46,72].

Whether and how the CBD requirements will be tailored for PMP crops remains to be decided by the Conference/Members of the Parties of the Protocol (COP/MOP). A panel of risk assessment experts from academia, regulatory bodies and stakeholder groups gathered to advise the COP/MOP4 in 2008 and agreed that the general principles and methodologies for risk assessment laid out in the Annex of the CBP should be applied to PMP crops. Based on the experiences with PMP crops in some countries, the panel also identified extra requirements and knowledge gaps in risk assessment (e.g. on the pleiotropic effects of high-level expression, the environmental impact of PMP crop disposal, and occupational hazards [73]).

## Regulations governing the licensing of pharmaceuticals derived from plants

Regulatory activities were triggered by PMPs entering clinical development, primarily within the FDA but also within its EU equivalent, the European Medicines Agency (EMEA). Although both authorities point out that the principles of guidance documents for other biopharmaceuticals apply, specific guidance has already been drafted in both jurisdictions to accommodate unique characteristics associated with PMPs. The respective policies of the different jurisdictions on orphan drugs and biosimilars (known as follow-on biologics in North America) also have a role, because some developers have such products (e.g. insulin, glucocerebrosidase) in their pipelines. However, these cases lie beyond the scope of this review.

### *USA*

In the USA, the FDA oversees the licensing of most drugs and diagnostics, whereas veterinary vaccines are separately regulated by the USDA Center for Veterinary Biologics (CVB) (Table 3). In draft guidelines jointly developed by the USDA and the FDA, specific information is required for the market authorization of PMPs [13]. The FDA guidelines cover PMPs from all conceivable expression platforms, including transient expression using plant viruses, and stable expression in aquatic plants, moss and algae. These guidelines are therefore broader in scope than the corresponding draft from EMEA, which only covers stably transformed higher plants. When applied to PMPs, good manufacturing practice (GMP) guidelines appear to be more flexible at the FDA than at the EMEA (K. Webber [FDA], personal communication). With regards to plant characterization, the manufacturing process and pre-clinical testing, the information required for the commercial regulation of PMPs is similar – regardless of the chosen expression platform.

### *Europe*

Pharmaceutical products derived from GM plants must adhere to the same regulation that covers all biotechnolo-

**Table 3. Statutory authorities, regulations and guidance relevant for clinical trials and market authorisation of PMP products in the US, Canada and Europe[a]**

| Country | Authority | Scope of regulation | Laws and regulations | Specific regulations and guidance for pharmaceutical crops |
|---|---|---|---|---|
|  | FDA<br>www.fda.gov<br>CDER, CBER, CVM | Biopharmaceuticals and vaccines for human use; biopharmaceuticals for veterinary use | Public Health Service Act (PHSA)<br>Federal Food Drug and Cosmetic Act (FFDCA) | Draft Guidance for Industry: Drugs, Biologics, and Medical Devices derived from Bioengineered Plants for Use in Humans and Animals [13] |
| | | Environmental effects from end products | National Environmental Policy Act (NEPA) | |
| | USDA-APHIS<br>www.aphis.usda.gov<br>CVB | Vaccines for veterinary use | Virus, Serum, and Toxins Act | |
|  | HC<br>www.hc-sc.gc.ca<br>Health Products and Food Branch | Biopharmaceuticals and vaccines for human use | Food and Drugs Act and Regulations | No specific guidance yet; drugs derived from pharmaceutical plants are subject to the same oversight as normal drugs. |
| | HC<br>Environment Canada | Environmental and indirect human health effects of new substances – either organisms or chemicals and polymers derived from organisms – before import into or manufacture in Canada that are not covered by other regulations scheduled under the CEPA | Canadian Environmental Protection Act, 1999 (CEPA).<br>New Substance Notification Regulations (Chemicals and Polymers).<br>New Substance Notification Regulations (Organisms) | Although there is no specific guidance pertaining to products derived from pharmaceutical crops, many of these products can be subject to the following guidance documents: Guidelines for the Notification and Testing of New Substances: Chemicals and Polymers [96]; Organisms [97]. |
| | CFIA<br>www.inspection.gc.ca<br>Veterinary Biologics Section | Biopharmaceuticals and vaccines for veterinary use | Health of Animals Act and Regulations | No specific guidance yet; veterinary biologics derived from pharmaceutical plants are subject to the same oversight as normal veterinary biologics. |
|  | EMEA<br>www.emea.europa.eu<br>CHMP, CVMP | Biopharmaceuticals and vaccines for human and veterinary use (assessment only) | Council Regulation (EEC) 2309/93 [74] | Guideline on the quality of biological active substances produced by stable transgene expression in higher plants [49]. |
| | European Commission, National Competent Authorities | Market authorisation | | |

Source: Adapted from references [13,86,94,95,98].

[a]Regulations on medical devices are not included in this table. The plant-made antibody CaroRxTM is authorised in the EU as a medical device.Abbreviations: APHIS, Animal and Plant Health Inspection Service; BREC, Biologic and Radiopharmaceuticals Evaluation Centre; CBER, Center for Biologics Evaluation and Research; CDER, Center for Drug Evaluation and Research; CFIA, Canadian Food Inspection Agency; CHMP, Committee for Medicinal Products for Human Use; CVB, Center for Veterinary Biologics; CVM, Center for Veterinary Medicines; CVMP, Committee for Medicinal Products for Veterinary Use; EMEA, European Medicines Agency.

gically derived drugs, Regulation 2309/93 (Table 3) [74]. The relevant national authorities oversee these drugs during their research and early clinical development phases, and EMEA oversees them during commercial development and application. In 2002, EMEA published draft guidance notes on 'the quality of biological active substances produced by stable transgene expression in higher plants' [48], accompanying the similar document produced by the FDA (see above). Although the FDA guidelines have yet to be finalized, the EMEA guidelines were revised in 2006 and are still under development [49].

In 2004, a five-year EU-funded research programme called Pharma-Planta was launched. This programme had the specific aim to develop efficient and safe strategies for the production of clinical-grade PMPs and to work with the regulators to define appropriate guidelines [75–77]. Throughout 2007 and 2008, Pharma-Planta has been road-testing these latest guidelines by applying them to their own products and trying to help facilitate a better understanding of the specific characteristics of PMPs among regulators. The publication of successful case studies should reduce regulatory uncertainty, encouraging the industry to push their products towards the market; however, as stated above, several regulatory concepts originally developed for cell lines still need to be modified and redefined to be more specific for plants, especially those concepts surrounding master and working cell banks, compliance with GMP and, particularly with regards to batch-to-batch consistency, standard operating procedures for different production systems and downstream processing requirements. There is no 'natural' home or regulatory body for the entire start-to-finish responsibility surrounding the regulation of PMP crops and their products. As such, there is currently an overlap between authorities and a duplication of the information required by the different regulatory bodies, namely EFSA

and EMEA [78]. The precise stage at which each regulatory authority becomes involved, and the ways to deal with potential overlaps in their authority, is currently being investigated.

### *Canada*

Health Canada (HC) is the federal authority that regulates the licensing of drugs in Canada. Before receiving market authorization, a manufacturer must present substantive scientific evidence about the safety, efficacy and quality of the product. The department is currently examining how these regulations apply to PMPs, and a common strategy still needs to be developed (S. Roussel [HC], personal communication).

### *WHO*

The Third Global Vaccine Research Forum of the World Health Organization (WHO) mentioned plant-made vaccines (PMVs) as a potentially important issue [79]. In 2005, a 'WHO Informal Consultation on the scientific basis for regulatory evaluation of candidate human vaccines from plants' reiterated that existing guidance for the development, evaluation and use of conventional vaccines should be applied to PMVs. Other WHO guidelines on Good Agricultural and Collection Practices (GACP) for medicinal plants and for quality aspects of biopharmaceuticals can also be used for harvesting and for developmental genetics, respectively [80,81]. Specific issues that were flagged as being important include seed banking, dose control – in the case of orally delivered vaccines – and the risk of allergenicity. The existing principles of GMP for drugs and/or biologics were generally considered to be applicable, but would need to be modified and supplemented (e.g. by including GMP for early parts of manufacturing, such as agricultural and collection activities). Process validation under GMP was considered to be especially difficult in the case of open-field cultivation, and the Consultation therefore recommended greenhouse cultivation [82].

## Outlook

The current PMP pipeline shown in Table 1 indicates that products from contained systems are on a faster track towards commercialization than PMPs from open-field sites. This partly reflects the perception that contained PMP production attracts a lower regulatory burden, but it might also in some cases reflect the choice of product. For example, high-margin, low-volume products will benefit from contained production, but there will be greater pressure for open-field production in the case of PMPs that give rise to lower-margin, high-volume products, such as nutraceuticals. For this reason, the pressure on regulators to develop a policy framework and appropriate regulatory pathways for the agricultural production of PMPs remains. However, given the concerns of the food industry, farmer groups and civil society groups, and the characteristics of the regulatory challenges, it seems likely that progress towards the regulation of open-field production, at least in the case of food/feed crops, will continue to be slow.

In the USA, the proposed revisions of the APHIS regulations – laid out in their Environmental Impact Statement (EIS) [83] – are likely to pave the way towards a more efficient framework for the cultivation of PMP crops. Instead of the currently applied zero-tolerance policy towards all PMP and PMI crops, a case-specific and risk-based policy formed around adventitious presence is envisaged. APHIS foresees a multi-tiered permit system that would differentiate between PMP and PMI crops, depending on the associated potential health and environmental risks and familiarity (i.e. knowledge of and experience with the crop), as opposed to the present situation in which all cases are considered to be equivalent. The degree of confinement and oversight would also be risk-proportionate and would vary per tier. An additional regulatory track would allow for the commercial production of PMPs and PMIs in open fields while still maintaining regulatory oversight. Multi-year permits are envisaged, although APHIS permit applications would be reviewed every year, even when locations and protocols have not changed [83]. A multi-tiered system is also supported by recent risk assessment case studies on PMP crop risk [25,26].

In Canada, work has been undertaken within individual departments of HC and the CFIA and also in a broader, inter-departmental working group that further includes Agriculture and Agrifood Canada, Canadian Grain Commission, Environment Canada and Industry Canada. This work aims to more clearly define the role that each department should have in the life cycle stages of plant molecular farming. It also aims to decide on the approaches that will be used to further develop regulatory frameworks. The specific strategy of HC is to develop an internal 'roadmap'. This will enable the involved parties to define regulatory pathways that different PMPs could take. By contrast, the CFIA is developing a regulatory framework for the environmental release of plants. This framework would require closed-loop confinement for commercial production, owing to potential food/feed or environmental safety issues.

In Europe, given the institutional separation of scientific risk assessment (undertaken by EFSA) and risk management (carried out at the EC level), the EC will be in charge of exploring and adopting its biotechnology framework. Some consider that confinement measures are part of risk management; as such, the scope of EFSA, which is normally limited to risk assessment, might need to be reconsidered. EFSA's Panel on Genetically Modified Organisms (GMO Panel) has launched an open consultation on the draft Opinion concerning 'the risk assessment of genetically modified plants used for non-food or non-feed purposes' (http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178716609288.htm). These guidelines have the potential to intensify the debate at the level of the EC and within Member States. This might also stimulate discussions to determine how the present regulatory framework for GM crops could be adapted for PMP crops. Currently, the regulations only consider either small-scale non-commercial releases with regulatory oversight or larger-scale releases for unlimited commercial cultivation, processing and trade. The latter category, once approved, is excluded from regulatory oversight. With PMP crops, however, one can expect very small acreages, the absence of free trade in seeds and plant material, contract farming and strict confinement measures. Moreover, not only con-

cerned stakeholders but also the PMP industry will want to keep these crops under strict regulatory oversight [16]. In Europe, it might be difficult to reach agreement across all the Member States, because there is still a divergence of views, even with regards to risk assessment and risk management requirements for first-generation GM crops. Some Member States, including Austria, Hungary, Greece and recently France, are still pushing for stricter requirements. An appropriate regulatory pathway for PMP crops might therefore emerge only after a complex and lengthy negotiation process.

The present situation suggests that the overall approach to regulating PMP crops differs between jurisdictions, with the USA developing a tiered system, and Canada and the EU continuing their case-by-case approach. Regardless of the system, it is necessary to determine how the differences between PMP crops and GM food/feed crops will translate into risk assessment, confinement and monitoring requirements. Will extensive confinement measures and small plots result in less extensive risk assessment or is it anticipated that confinement failures justify fully fledged risk assessment and monitoring requirements [21]? EMEA already oversees pharmaceutical products derived from GM microbes and mammalian cells, and their draft guidance notes are continually revised to accommodate PMP-specific characteristics, such as defining master and working bank cells, cGMP compliance and batch-to-batch consistency. Although contained and controlled plant cell-based systems are likely to fit better into the current guidelines, other potentially important production platforms such as moss, *Lemna* and algae (not discussed in this article) are not yet included within the scope of EMEA's guidance, which focuses only on higher plants [49]. Additional regulations would be needed for alternative platforms such as transiently transformed plants [84] and GM plant viruses [85].

A general challenge facing emerging regulatory frameworks in the USA, Canada and the EU is the need to clarify the various and complex overlaps of regulatory oversight between different regulatory bodies, in particular between the USDA and the FDA in the USA, between CFIA and HC in Canada, and between EFSA and EMEA in the EU. A roadmap for applicants, clearly setting out the remits of these bodies and their responsibilities, could be helpful here.

Considering the issue on a more global level, industrialized countries are more likely to succeed in establishing a tight regulatory framework for PMP crops with rigorous confinement conditions that would be enforced by continuous oversight. Whether developing countries producing their own PMPs could establish and enforce such regulations remains questionable [73,86]. Countries that have weaker biosafety infrastructures could pose a risk to this emerging technology if 'contamination of the food chain' became an issue. This is of particular importance if developers conduct field trials and production in these countries. Strategies to avoid such problems will therefore have to be developed at the international level [87], especially in the contexts of the CPB, the Organisation for Economic Co-operation and Development (OECD), and the Codex Alimentarius.

The development of regulatory frameworks for commercial PMPs and the crops that produce them seems to be evolving by responding to real-world challenges rather than by anticipating them, because such frameworks are only slowly taking shape. Continuing regulatory uncertainty, by contrast, is discouraging PMP developers. To break this circle and to facilitate innovation in PMP development, regulators should adopt a more proactive stance. Nevertheless, a strong pipeline of PMP products would definitely facilitate regulatory development. Research and innovation policy might need to explore possible ways to support possible 'ice-breaker' products.

It is equally important that the regulatory frameworks are developed in an open and transparent manner, by including a broad range of stakeholders. This is particularly the case in Europe, and it might help to avoid or diminish the mutual suspicion and mistrust that has, for a long time, clouded discussions about first-generation GM crops.

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# Potential of *Arabidopsis* systems biology to advance the biofuel field

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**Plant biomass is a renewable and potentially sustainable resource for the production of liquid biofuels and a multitude of bio-based materials. To tailor plants for biofuel production, a powerful gene discovery program targeted to cell wall recalcitrance genes is needed. In parallel, a system is required that reveals the pleiotropic effects of gene modifications and that delivers the fundamental knowledge necessary for successful gene stacking. In our opinion, these objectives can be pioneered through a systems biology approach in *Arabidopsis*. We develop our ideas with a focus on the lignin biosynthetic pathway, because lignin is among the most important factors determining cell wall recalcitrance.**

## Introduction

Global warming and increasing oil prices have catalyzed a worldwide trend to use plant biomass as a source for biofuels and bio-based materials. It has been estimated that only 3.2% of the surface of the land can produce sufficient amounts of plant biomass to provide the current world energy needs [1]. Plant biomass can be processed into liquid biofuels via the conversion of plant cell wall polysaccharides, comprising mainly cellulose and hemicelluloses, into fermentable sugars. This process, called saccharification, proceeds with the help of cocktails of cellulases and hemicellulases (Figure 1) [2,3]. However, most cell walls, particularly secondary-thickened ones, also contain lignin [4,5], an aromatic polymer that rigidifies the plant tissues, but also limits access to polysaccharides for enzymatic degradation [6]. To overcome this bottleneck, lignocellulosic plant biomass is pretreated chemically and/or mechanically to degrade lignin, thereby loosening the rigid cell wall structure and exposing cellulose and hemicelluloses for saccharification [7]. The pretreatment step is one of the costliest in the biomass-to-biofuel conversion process. In addition to lignin, several other cell wall parameters affect the saccharification potential, for example cellulose crystallinity and hemicellulose structure [8–10]. However, the factors that determine cell wall recalcitrance are still largely unknown [11].

Biotechnology holds great promise to tailor plants for optimized conversion to biofuels. Numerous studies have already shown that altering cell wall composition through genetic engineering can lead to improved biomass processing [4,6,9,10,12–16]. However, studies have also noted that the outcome of genetic modifications is not always in agreement with the existing knowledge on the perturbed process [17–19], and that these modifications often have far-reaching effects on other biochemical pathways and processes than those targeted [20–23]. Particularly when taking gene stacking (Figure 2) into account, these unpredictable effects might be exacerbated [12,13,24]. Clearly, fundamental insight into these wider (pleiotropic) effects of gene modification and how genes and pathways interact in complex genetic networks is essential for rational engineering of plant cell walls. In our opinion, a systems biology approach in which consecutive genes in a pathway affecting cell wall recalcitrance are perturbed and the corresponding mutants phenotyped at multiple levels will provide this fundamental knowledge. At the same time, systems biology will surface as a powerful gene discovery tool to define genes closely involved in the perturbed process. A similar systems approach has already shown its merits in studying glucosinolate biosynthesis [25,26]. We will first qualify why *Arabidopsis* is a relevant model system to study cell wall recalcitrance, and then develop our ideas on the potential of *Arabidopsis* systems biology, using lessons from lignin pathway perturbations.

## *Arabidopsis*: a model for biofuel crops

*Arabidopsis* can be used as a model system to discover the genes and genetic networks that determine the saccharification potential of lignocellulosic biomass [9,10,27]. Numerous cell wall biosynthetic mutants are described in the literature [28–30], and preliminary results in our laboratory have demonstrated that senescent *Arabidopsis* inflorescence stems from low lignin mutants such as *cinnamoyl-Coenzyme A (CoA) reductase1* (*ccr1*) and *4-coumarate:CoA ligase1 (4cl1)* [31,32] are easier to saccharify than stems of wild-type plants (Figure 1). In addition, the woody trunks of transgenic poplars defective in the orthologous *CCR* gene had also improved in saccharification potential (Figure 1) [33,34]. Similar results were obtained in transgenic alfalfa lines with altered lignin content [6,18], indicating the feasibility of translating cell wall research from *Arabidopsis* to commercial crops. With gene-stacking in mind, the use of *Arabidopsis* allows for testing the combined influence of multiple mutations/transgenes through double and triple mutants/transgenics much faster than can be achieved in most commercial crops and without the confounding effects of different genetic backgrounds. Lastly, the existing *Arabidopsis* mutant collections and natural accessions [35] are the best-available genetic bases to reveal, through systems biology, how mutations in cell

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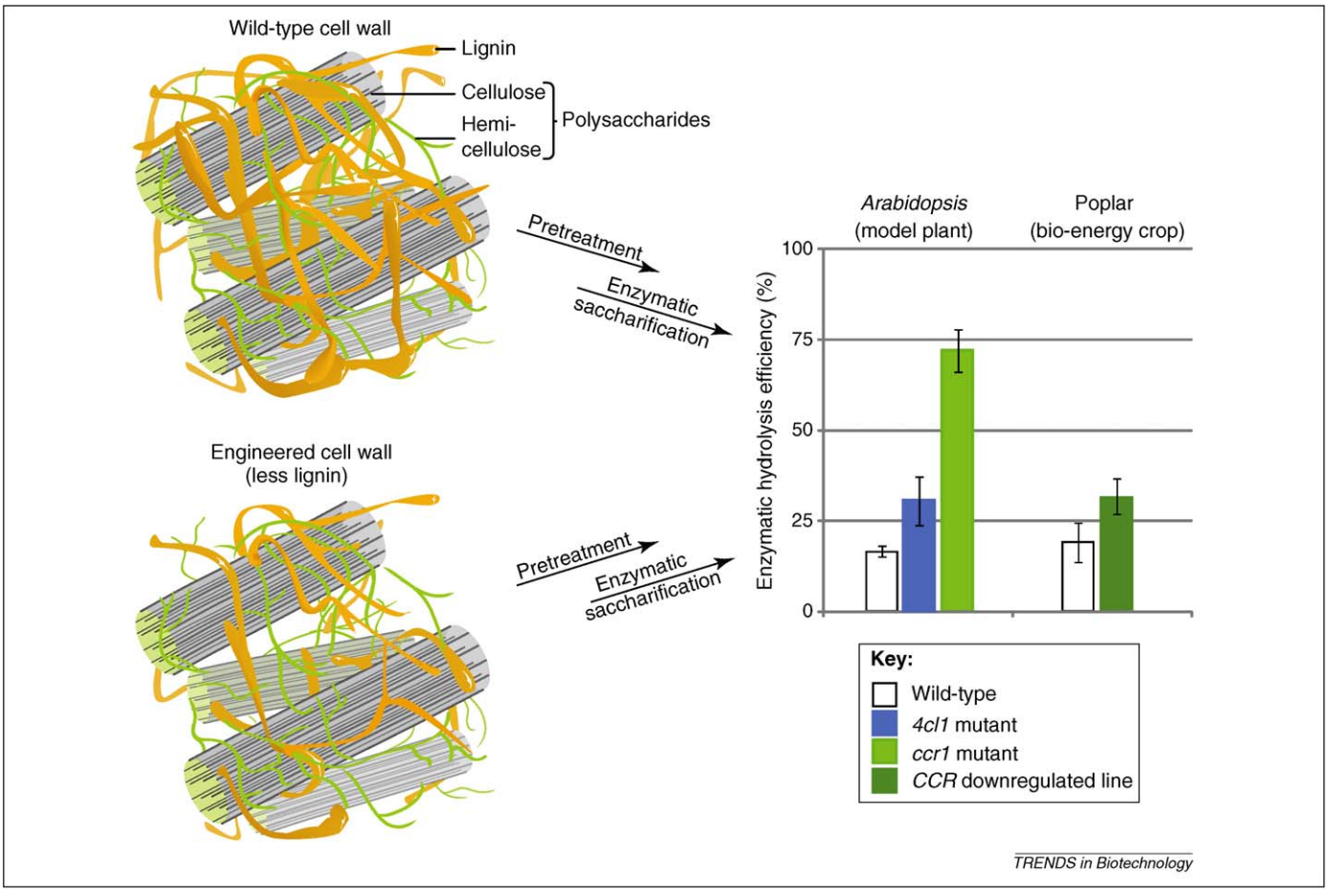


**Figure 1**. Lignin affects saccharification potential. Plant cell walls consist mainly of polysaccharides (cellulose and hemicelluloses) and lignin. The polysaccharides can be enzymatically hydrolyzed into monomeric sugars (a process called saccharification) that can be fermented into biofuels, such as bio-ethanol. However, lignin limits access to the polysaccharides, thereby inhibiting the saccharification process. To overcome this hurdle, a pretreatment (typically a combination of high temperatures and chemicals) is used to disrupt the cell wall structure. Biomass from plants that deposit less lignin, or lignin that is easier to degrade, is more efficiently saccharified. In this example, reduction of the lignin amount increased the saccharification potential (expressed as a percentage of total cellulose hydrolyzed to glucose) approximately twofold and fourfold for *4cl1* and *ccr1* mutants of *Arabidopsis*, respectively (R. Van Acker *et al.*, unpublished data). *4cl1* mutants have a wild-type appearance, whereas *ccr1* mutants are affected in biomass yield. Similarly, the down-regulation of the orthologous *CCR* gene in poplar resulted in less lignin and an approximately 50% increase in saccharification potential. These preliminary results also indicate that cell wall phenotypes in the weedy model species *Arabidopsis* can be translated to commercial crops, such as alfalfa [6] and poplar, as shown here.

wall recalcitrance genes affect biosynthesis in other metabolic and developmental processes – information that will be crucial for the rational design of bio-energy crops.

## Systems biology: lessons from lignin pathway perturbation

The term 'systems biology' has various meanings. Here, we refer to systems biology as the study of the consequences of pathway perturbations, visualized at the transcript, protein, metabolite and phenotype levels, followed by computational analysis of the data and mathematical modeling of the underlying network [36]. Such studies are mostly meaningful in model organisms, such as *Arabidopsis*, because a maximum number of the molecular components of the system (e.g. genes, proteins and metabolites) is known, only in these select species.

### *Gene discovery*

A limited number of studies on the molecular echo of lignin pathway perturbations have already provided a glimpse of the potential behind such a systems biology approach [20–23,33]. For example, transcript profiling demonstrates that a mutation in a single lignin biosynthesis gene can affect the expression of several other genes of the exact same biosynthetic pathway [17,18,20,23]. This implies that within the same differential data set, novel genes (such as biosynthetic genes and transcription factor genes), with hitherto unknown functions, emerge as potential components of lignin biosynthesis by the guilt-by-association principle, a principle based on the observation that genes involved in the same cellular process are often co-expressed ('associated') under different conditions (e.g. tissues, stresses or genotypes) [37–39]. Profiling a single mutant provides a list of genes that are all equally likely to be closely involved in the perturbed process; however, profiling a series of mutants with perturbations in consecutive steps of the same pathway will allow us to select those that are affected by multiple mutations as the prime candidates for a role in lignin biosynthesis. The more perturbations of the very same process – and preferentially in the same tissue – the higher the information content of the data set and the greater the chance that new genes closely associated with the perturbed system will be identified.

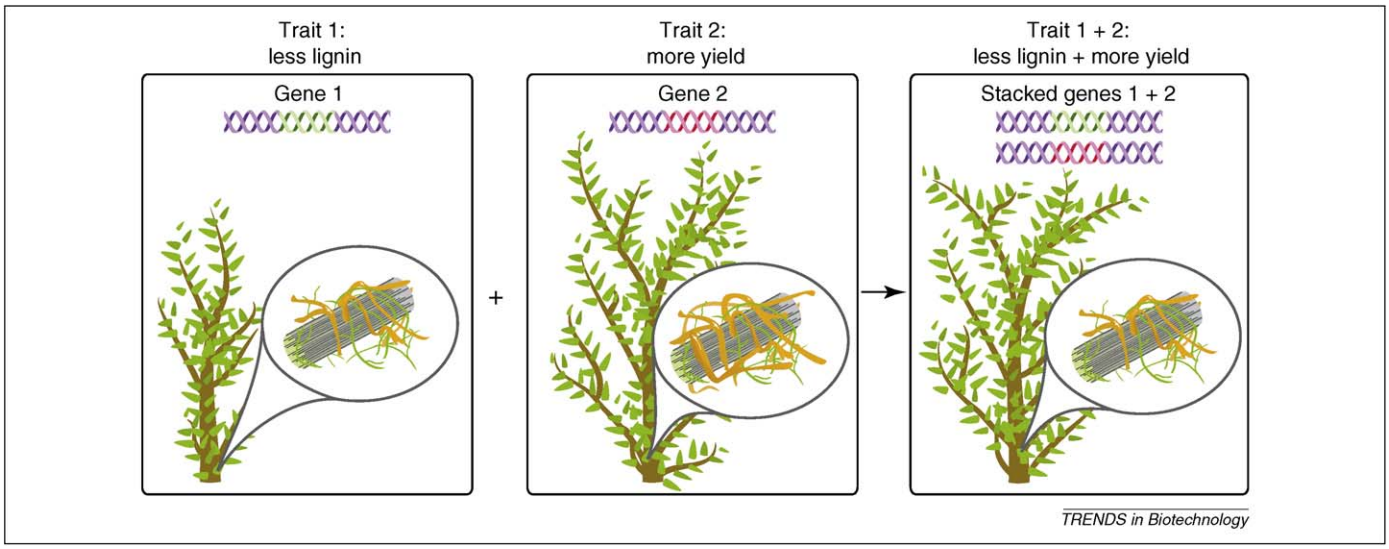


**Figure 2**. With gene stacking, several beneficial traits are combined into a single bioenergy crop. In this example, a gene that leads to reduced lignin content in the cell wall is combined or 'stacked' by crossing, co-transformation, or re-transformation with genes that augment biomass yield [12,13,24]; the end result is a plant with the combined beneficial traits.

Each of these newly identified genes has the potential to affect saccharification efficiency upon altered expression in plants, making systems biology an alternative gene discovery tool to forward genetics, in which mutant collections and natural accessions are systematically screened for saccharification potential. Forward genetics is also becoming an attractive gene identification tool, now that sequence-based gene identification and association mapping have surpassed the tedious map-based cloning procedure [40,41]. However, in contrast to the systems approach that delivers genes closely involved in the perturbed process, forward genetics will identify cell wall recalcitrance genes in an unbiased way and the causal genes will probably be involved in a plethora of processes. Both approaches are thus complementary.

### *System-wide effects*

Although the observation that perturbation of one gene of the lignin pathway affects the expression of other genes of the same pathway is not unexpected, it provides novel insight into the regulation of the lignin biosynthetic pathway [17,20,23]. This regulatory information is often underrepresented in the scientific literature. Indeed, strategies to alter lignin content or composition in plants have hitherto relied on textbook pathways, without taking regulatory feedback loops into account. Understanding these regulatory feedback loops will help explain the unexpected consequences of lignin engineering and allow more informed strategies for cell wall engineering.

Studies on perturbations of the lignin pathway have also highlighted that, in addition to the effects on the lignin pathway itself, the perturbations affect biochemical pathways and biological processes for which a link to lignin biosynthesis is not obvious. For example down-regulation of the phenylalanine ammonia-lyase1 (*PAL1*) or *PAL2* gene in *Arabidopsis* results in far-reaching effects on carbohydrate and amino acid metabolism [20]. Both mutants have reduced lignin content, but no obvious phenotypes at the whole-plant level. These transcriptional and metabolic responses reflect how plants adapt to the genetic defect. In other mutants in which the reduced lignin content is associated with adverse effects on plant development (e.g. *ccr* mutants [23,31–33]), deep phenotyping will help uncover the molecular basis for these pleiotropic effects. Rationalizing further on this topic, the very same systems approach might help in identifying target genes that mitigate these wider and potentially unwanted effects on plant performance. Again, such information is of crucial importance for tailoring bio-energy crops.

### *Pathway discovery*

Systems biology also provides great opportunities for pathway discovery and engineering. Lignin pathway perturbations have been reported to result in the accumulation of aromatic metabolites that are otherwise below the detection limit in wild-type plants [20,23,32,33,42–46], hence revealing fluxes into pathways that remain silent in wild-type plants. Transcript profiling of the same mutants holds promise toward discovering the biosynthetic genes, and altering expression of these genes might have interesting applications. For example, metabolite profiling of CCR-deficient plants (e.g. *Arabidopsis*, tobacco, poplar), which were designed to reduce lignin content in the cell wall, has revealed the unintentional accumulation of ferulic acid-derived metabolites in mutant stem tissues (Figure 3) [31,33]. Interestingly, in these plants, part of the ferulic acid is exported to the cell wall where it is incorporated into the lignin polymer, leading to acetal bonds that are barely detectable in lignin of wild-type plants [47]. These bonds are easily cleaved in mild acid or base solutions, which comprise the basis of many pretreatment processes to degrade lignin. Hence, boosting the biosynthesis of ferulic acid and its export to the cell wall by pathway engineering might be a good strategy to increase the value of bio-energy crops. The principle of shipping alternative lignin monomers to the cell wall to improve lignin degradation has been mimicked by feeding maize cell walls with a monolignol analog, such as coniferyl

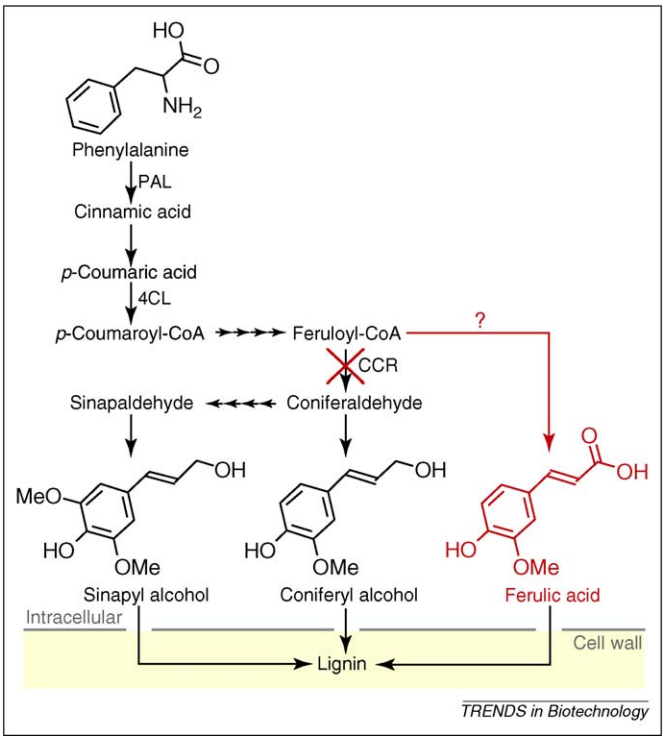


**Figure 3**. The biosynthetic route to lignin. Lignin is an aromatic heteropolymer that provides strength and imperviousness to the cell wall, thereby enabling plants to grow upward and transport water through the vessels. However, these same characteristics also hinder the saccharification process (Figure 1). The biosynthesis of lignin is rather well-established and starts with the amino acid phenylalanine (i.e. the phenylpropanoid and monolignol biosynthetic pathways, shown in black). Each arrow corresponds to one enzymatic step (for more comprehensive descriptions of the pathway, see Refs. [4,5]). In dicots, such as *Arabidopsis* and poplar, the main monomers are coniferyl and sinapyl alcohol. The gray line represents the plasma membrane through which monolignols are transported to the cell wall, where they are polymerized into the lignin polymer. In addition to coniferyl and sinapyl alcohol, several other monomers may co-polymerize into lignin to various extents. It is possible to steer the biosynthesis toward these minor compounds. For example, in *ccr1* mutants, the flux toward the normal monolignols is reduced, but new units derived from ferulic acid appear in the lignin polymer. The red arrow indicates the putative route to ferulic acid in *CCR*-deficient plants. Abbreviations: CCR, cinnamoyl-CoA reductase; 4CL, 4-coumarate:CoA ligase; PAL, phenylalanine ammonia-lyase.

ferulate; this molecule is incorporated into lignin, resulting in a polymer that is degradable at much lower temperatures and lower amounts of chemicals [48,49].

## Limitations and bottlenecks of applying *Arabidopsis* systems biology

The power of systems biology depends on the depth of phenotyping of all components of the system (e.g. metabolites, proteins and transcripts). However, most of these components are still unidentified. In *Arabidopsis*, the molecular function, biological process, or cellular compartment of approximately 30 percent of the genes is known and an additional 40 percent have mere experimental annotations in The Arabidopsis Information Resource (TAIR: www.arabidopsis.org). The situation is worse with respect to metabolites, even in the *Arabidopsis* model. Of the estimated 10 000 metabolites in *Arabidopsis* [50], only approximately 1000 have been structurally resolved [51–53]. There is an urgent need for an international effort in metabolite identification to enrich the information content of systems databases [53–55]. With regard to the most important cell wall recalcitrance factor, namely lignin, this effort should result in a complete catalog of the aromatic metabolites derived from the phenylpropanoid and monolignol biosynthetic pathways and in analytical tools to study how these monomers can potentially co-polymerize in the cell wall [56,57].

In addition to phenotyping at the transcript, metabolite and protein levels, phenotyping at the cellular, subcellular and physiological levels becomes increasingly important. Regarding biofuel applications, multi-level phenotyping of the cell wall composition, structure and quality (via, for example, cell wall proteomics, whole cell wall NMR, UV microspectrometry, and saccharification assays) is crucial to draw correlations and causal connections between the perturbed molecular processes, on the one hand, and the quality of the biomass, on the other [58]. Hence, comprehensive phenotyping platforms will become essential tools to move forward in the design of better bio-energy crops [59].

## Concluding remarks

In the short term, systems biology approaches will undoubtedly provide fundamental insight into the complex interactions within and among individual pathways and will deliver new genes to reduce cell wall recalcitrance. In the long term, the data generated through systems biology should allow modeling of the system and predicting the consequences of genetic engineering more accurately than is possible today. The next step will be to test whether the stacking of genes (Figure 2) will lead to either additive effects or the discovery of more complex interactions than could be anticipated from the individual gene perturbations. Furthermore, as the outcomes from the *Arabidopsis* gene engineering are often transferable to crops, it is reasonable to assume that effective gene stacking in *Arabidopsis* will also be a good predictor for chances of success in a given crop.

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