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Prospects of genetic engineering for robust insect resistance☆

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Secondary plant metabolites are potentially of great value for providing robust resistance in plants against insect pests. Such metabolites often comprise small lipophilic molecules (SLMs), and can be similar also in terms of activity to currently used insecticides, for example, the pyrethroids, neonicotinoids and butenolides, which provide more effective pest management than the resistance traits exploited by breeding. Crop plants mostly lack the SLMs that provide their wild ancestors with resistance to pests. However, resistance traits based on the biosynthesis of SLMs present promising new opportunities for crop resistance to pests. Advances in genetic engineering of secondary metabolite pathways that produce insecticidal compounds and, more recently, SLMs involved in plant colonisation and development, for example, insect pheromones, offer specific new approaches but which are more demanding than the genetic engineering approaches adopted so far. In addition, nature also offers various opportunities for exploiting induction or priming for resistance metabolite generation. Thus, use of non-constitutively expressed resistance traits delivered *via* the seed is a more sustainable approach than previously achieved, and could underpin development of perennial arable crops protected by sentinel plant technologies.

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Introduction to the concept of creating robust insect resistance by genetic engineering of plant secondary metabolism

Pesticides, and particularly insecticides, serve agriculture well, with the currently registered compounds presenting an extremely low risk to the environment and to human health, when used in crop protection under legally enforced protocols. However, seasonal application of pesticides to the crop is unsustainable in terms of the carbon footprint particularly associated with application. In addition, the readiness by which selection for insecticide resistance occurs *via* target site modification, often involving only one non-synonymous single nucleotide polymorphism (SNP), or *via* upregulation of a particular gene associated with metabolism [1], aggravates the problem of the inherent unsustainability of this type of intervention against pests. Breeding delivers resistance to crop plants *via* the seed and, by definition, is more sustainable, but has not produced sufficiently durable insect control for replacement of insecticides in mainstream food production. This is also true of weed control with regard to herbicides, but is less the case for pathogen control, although without current fungicides there would be unacceptable losses in food production [2]. Delivery of insecticides is extremely inefficient, with a very small percentage applied to the crop reaching the target insect and even less so the target site, but such compounds are robust in terms of stability and, provided delivery ensures sufficient bioavailability for effective toxic effects and does not lead to environmental contamination, selectivity is based mostly in unique aspects of the target insect physiology. Secondary plant metabolites can be small lipophilic molecules (SLMs) with similar physicochemical properties and toxicities to pesticides, and many have provided the lead structure or inspiration for synthetic insecticides, for example, pyrethrum for the pyrethroids [3], nicotine and other nicotinic acetylcholine receptor agonists for the neonicotinoids [4••] and stemofoline for the very recently introduced butenolides [4••]. Some insecticides are natural products, for example, spinosad, which comprises the natural spinosyns A and D. Thus, genes for the biosynthesis of the natural insecticidal SLMs are available in nature for exploitation by genetic engineering. The potential of doing this has been demonstrated by pioneering groups led originally by Gierl [5] and Moller [6], who established, respectively, the enzymology for the secondary plant metabolism that produces insecticidal SLMs, the hydroxamic acids [benzoxazinoids, e.g. DIMBOA (**I**), $R_1 = OCH_3$, $R_2 = H$] (Figure 1), and the precursors, the cyanogenic glucosides (**II**) and glucosinolates (**III**) for the toxicants hydrogen cyanide (HCN) and organic isothiocyanates (RNCS). Compound **I** can also be stored as a glucoside, which then also releases the toxic product upon tissue damage, for example, caused by insect feeding. Together with storage tissue location, *in situ* release can confer selectivity to insects. With the demonstration that non-producing crop plants can be genetically engineered in the laboratory to express these defence

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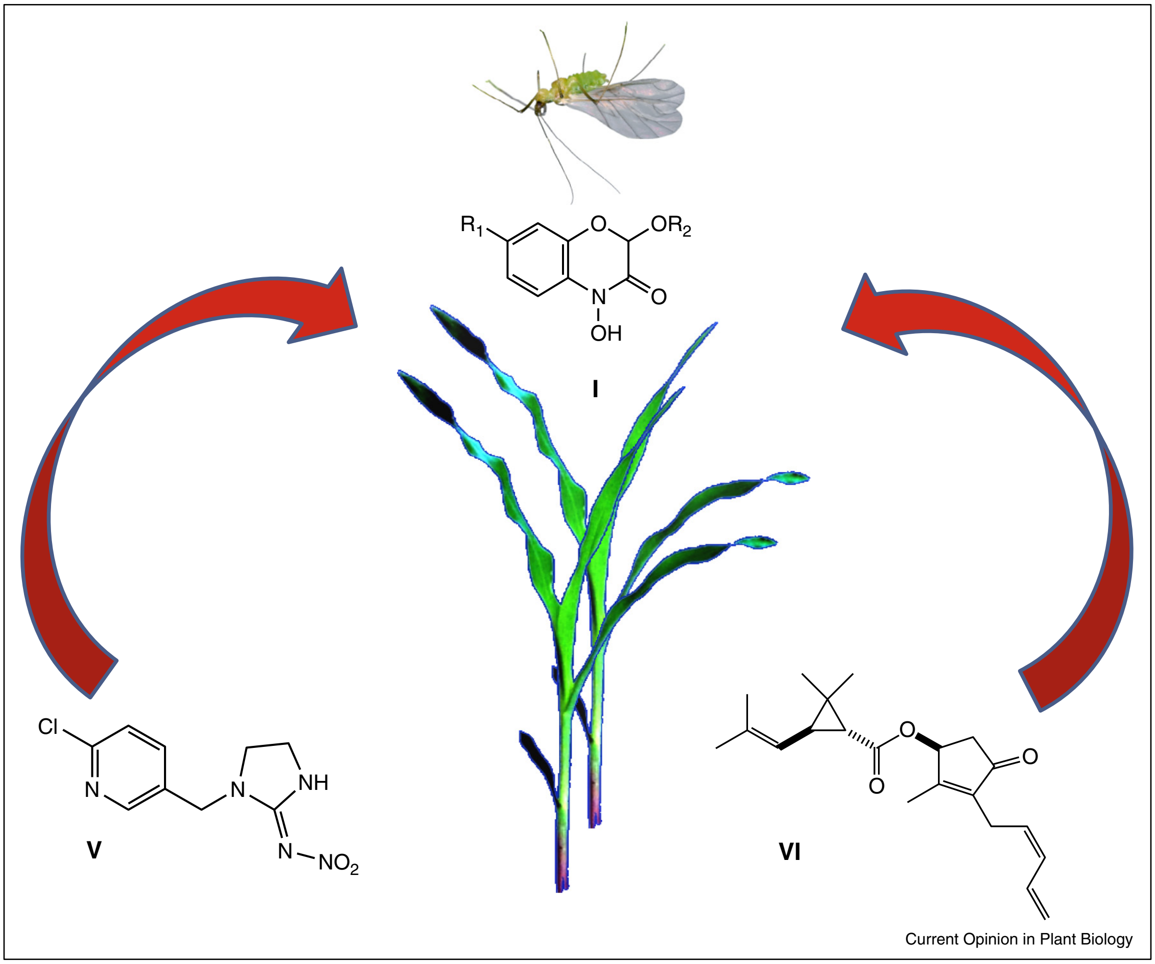
[1] Authors contributed equally to this work.

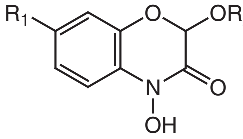
Figure 1



Current Opinion in Plant Biology

Naturally occurring insect toxicants (e.g. **I**) are small lipophilic molecules like commercial insecticides (e.g. **V**) or lead compounds (e.g. **VI**) for these.

pathways [7] comes the prospect for developing this approach to insect control [8••]. There is further evidence from the long term success of engineering plants to produce proteins derived from *Bacillus thuringiensis* (Bt) [9•] against lepidopterous pests. Here, although not SLMs, the protein structures are unique in providing high levels of toxicity against lepidopterous larvae. However, ingestion of the protein is required and certain gut conditions have to be present for activity. No similarly valuable related structures have become available. Although the enzymology for producing insecticidal secondary metabolites can be substantially more complicated than for Bt related proteins, it is evident that the discovery of metabolic gene clusters will provide less demanding routes for this type of genetic engineering [10••]. Non-target toxicity is dealt with by risk analysis before registration, but there are considerable concerns expressed in the media, particularly regarding neurophysiologically active insecticidal components but, also more importantly, the problem of rapid selection for resistance to toxicants where deployment is widespread. Both of these problems can be alleviated by targeting pheromones and other semiochemicals, another group of natural SLMs that affect insect behaviour and development. Here, much more complicated genetics are associated with the response by the pest to these compounds and so selection for resistance is more physiologically demanding for the pest. These SLMs, being highly specific signals that act at extremely low levels, are also intrinsically more benign than insecticidal SLMs. Indeed, the high volatility and chemical instability of many semiochemicals that contribute to their low risk also present problems of deployment, but such problems can be overcome by genetic engineering of plants for production on release, and this can indeed be achieved [11]. An alternative is to use genetically engineered plants as a factory for synthesis of pheromones otherwise only available by expressive chemical synthesis [12••]. Besides genes for pathways for the insect toxicants and semiochemicals being available for engineering insect resistance, there are phytopheromones that can induce or prime production so that the defence is only activated when the pest arrives, or the plant is primed to produce a greater activation when insect colonisation



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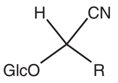

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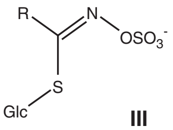

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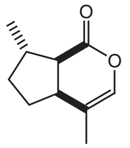

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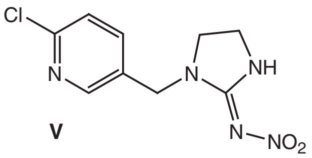

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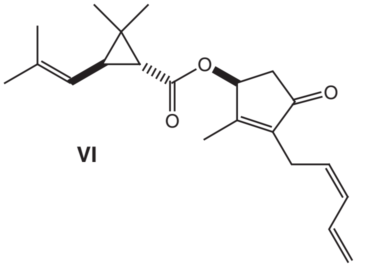

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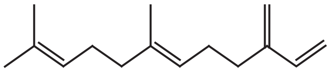

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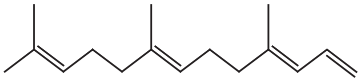

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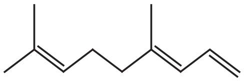

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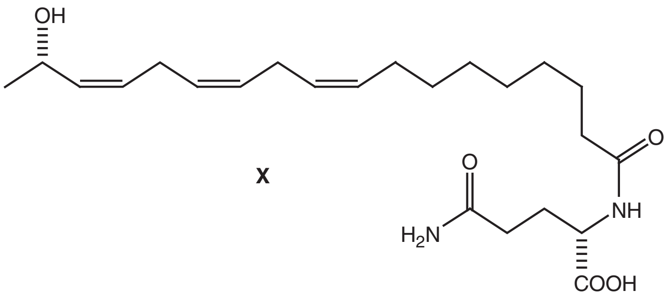

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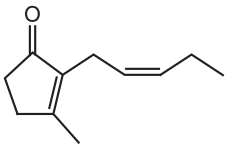

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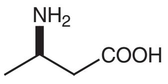


XII

begins for which there are evolutionary trade-offs [13,14••]. Thus, the scene is set for robust non-constitutive insect resistance to be engineered into crop plants, opening opportunities for further advances in sustainable agriculture, including perennial arable crop protection by sentinel plant technology .

Following on from molecular pathology

The relative success of controlling fungal and other plant pathogens by breeding and *via* more conventional genetic engineering, without targeting secondary metabolites such as the phytoalexins, provides lessons for exploiting secondary metabolites. Firstly, there is an advanced understanding of pathogen/plant interactions [15] that underpins these successes. It may also be that the advanced level to which molecular pathology has ascended has not uncovered the acute need for, as with insect control, the role of SLMs. However, there are differences between the pest and disease-causing kingdoms and their respective modes of plant resource exploitation. Insects, as comprising a class of animals, have highly developed sensory systems which, together with mobility, determine interactions with plants, particularly with respect to host plant acceptability. Pathogens, except for the zoospores of oomycetes, are not so mobile, relatively sessile, and have generally a more intimate molecular interaction with the plant. As insects become closer to this model, there appears to be more opportunity to develop molecular recognition-based approaches to management. This is potentially true for the sucking insects (e.g. aphids [Aphididae, Homoptera]) [16], and we await realisation of opportunities here but certainly, in terms of insect/plant recognition processes, there are analogies with pathogens [17]. Nonetheless, fungicides remain in heavy use, even with resistant cultivars [18], and their use can help preserve resistance mechanisms. Therefore, the lessons of value may eventually divert work to genetic engineering of plants in relation to SLMs against pathogens involved as elicitors and in recognition, but will relate to the main thrust of engineering based on secondary metabolite targets.

Constitutively expressed toxicants

Constitutive toxicants, having evolved from the so-called ‘arms race’ between plants and organisms at the second trophic level employing these as a food resource, encompass all secondary biosynthetic pathways. Some are weakly toxic and only effective when in the necessary tissues and at high expression rates. Polyphenolic compounds, biosynthesised from phenylalanine and related to the structural plant components such as lignin, are often weakly active inhibitors of digestive enzymes and are typical of the defence of highly apparent plants such as long lasting trees [19]. Essential oils, usually comprising isoprenoids, although naively popular targets for developing alternative secondary metabolite based pest management, also usually comprise weakly active compounds. However, where components of essential oils act specifically as semiochemicals, for example, the nepetalactones (**IV**) [20••], there may be particular value in their use.

Alkaloids are biosynthesised widely in plants and involve various biosynthetic pathways, with the essential nitrogen arising from amino acid primary metabolism. These compounds can be highly toxic, for example, as neurotoxins acting as agonists of acetylcholine or inhibitors of acetylcholine esterase activity [4••]. As lead compounds for commercial pesticides such as the neonicotinoid imidocloprid (**V**), pest resistance may be aggravated by their natural ecological presence [21••]. Although pyrethrin I (**VI**), the lead compound for the pyrethroid insecticides targeting the insect voltage gated sodium channel, is found in the pyrethrum daisy, *Tanecetum cinerariifolium*, its location within the acenes removes this compound largely from the defence of the parent plant (Figure 1).

Prominent targets for engineering insect resistance include avenacins, which are steroidal lectins [22], and although many biosynthetic enzymes are involved, co-genomic location to an extent simplifies the task [10••]. The pyrethrins are targeted [23••] and also the acylsugars [24••], but studies are ongoing regarding mode of action.

Inducible and primed defence

Compounds that are broadly active and which also cause phytotoxicity are commonly associated with insect defence. However, storage as an inert precursor is also common, for example, as N-oxides for alkaloids, as glycosides, mostly glucosides, for many toxicants such as the hydroxamic acids, for example **I**, and as cyanogenic glycosides (**II**) and the glucosinolates (**III**) that, by chemical rearrangement and catabolism of the aglycones, give HCN or RNCS. All are under consideration as genetic engineering targets and production can be induced by insect attack. This may simply involve the action of glycosidases brought into contact with the glycoside isolated within storage vacuoles or trichomes. Alternatively, induction of precursor production can occur. This may be a direct process *via* elicitors (see later) or a primed effect. Priming is an even more economic evolutionary solution than induction and could involve a primed production with release of the toxicant *via* later damage, or priming of much earlier processes involving epigenetic or other regulatory events [25–27]. A dramatic upregulation of a specific glucosidase gene on local feeding by the bird-cherry-oat aphid, *Rhopalosiphum padi*, can rapidly release **I** from the respective glucoside [28]. This could be exploited in insect control, if the biosynthetic pathway of **I** can be enhanced by genetic engineering of one or more of the genes, mainly cytochrome P450s that are co-located in the genomes of crop plants such as rye, *Secale cereale*, maize, *Zea mays* and wheat, *Triticum aestivum*. Indeed, a mutant lacking the gene for the first committed biosynthetic step in this pathway that involves the dedicated generation of indole from indole glycerol phosphate is highly susceptible to caterpillars and aphids [29].

Plant genetic engineering for insect pheromones

Secondary metabolism was highly evolved before the appearance of the more advanced kingdoms, and parsimony in pathways between plants and animals can be exploited. As for plant toxicants, all pathways are found in insects and so there is a very wide range of options. For isoprenoids, the aphid alarm pheromone, common for most pest aphid species, which naturally causes dispersal of aphids when attacked and recruits foraging by predators and parasitoids, (i.e. parasitic organisms that kill their host), is a simple sesquiterpene hydrocarbon, (*E*)-β-farnesene (**VII**), biosynthesised directly from farnesyl diphosphate (pyrophosphate). A gene causing production of **VII** has been overexpressed constitutively in the model plant, *Arabidopsis thaliana*, to produce **VII** sufficiently pure for alarm activity [30] and which repels aphids and increases foraging by the parasitic wasp *Diaeretiella rapae*, which lays its eggs in aphids that feed on plants in the family Brassicaceae [11]. Together with a plastidial locating peptide sequence this, as a synthetic gene with codon usage optimised for expression in the Poaceae, has been overexpressed in wheat with similarly dramatic results in the laboratory. The engineered wheat, comprising the elite hexaploid cultivar Cadenza with and without increased expression of the synthetic gene for generating the precursor farnesyl diphosphate is currently being tested in the field in comparison with the unengineered commercial cultivar, with full containment, under ACRE (the Advisory Committee on Releases in the Environment), sown as spring and winter crops.

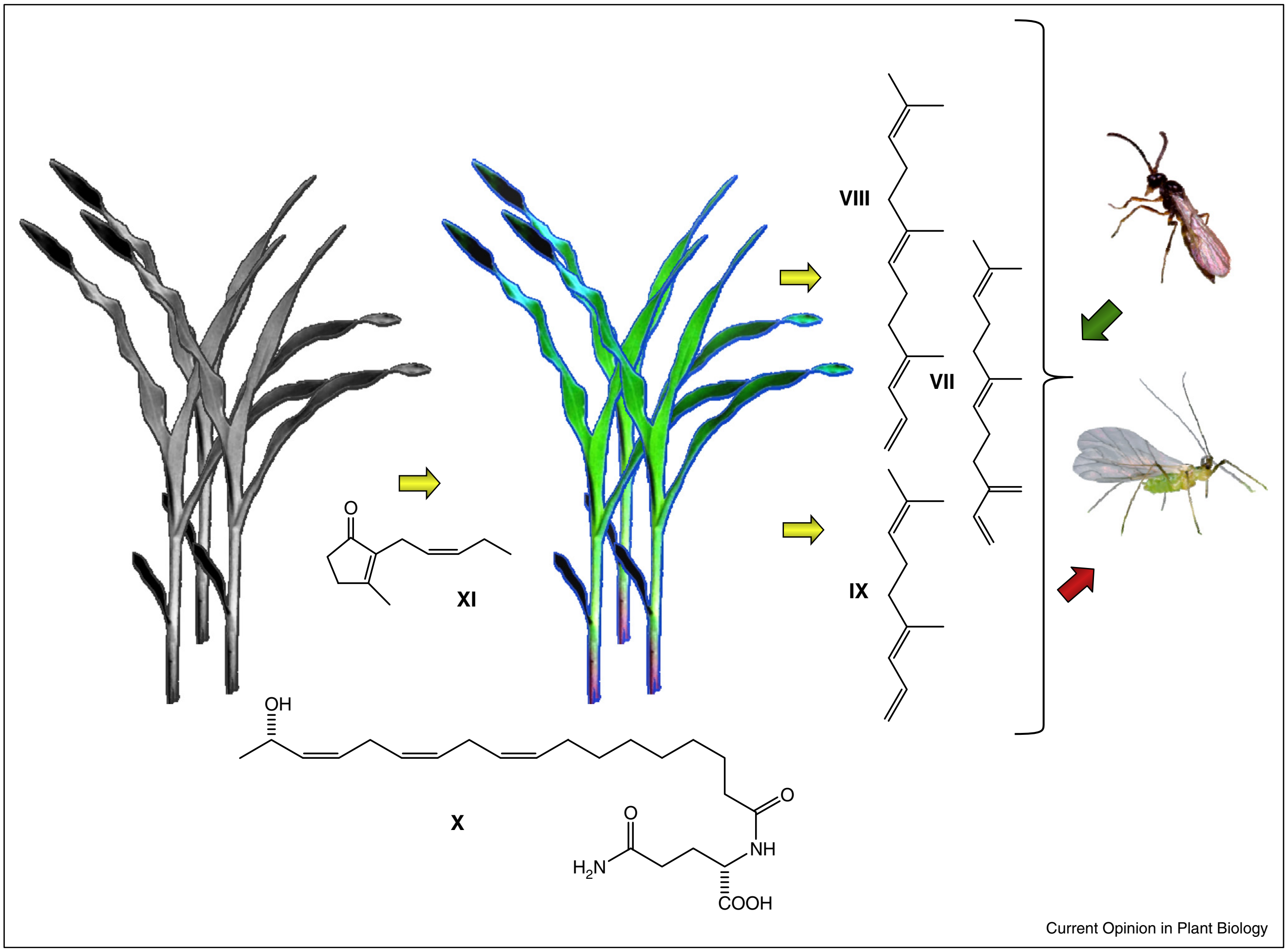
The aphid sex pheromones comprise specific isomers of iridoid nepetalactones, for example **IV**, and their biosynthesis in the Madagascan periwinkle, *Catharanthus roseus* (Apocynaceae), has recently been elucidated [31••]. Although some aspects remain under investigation, the discovery of a novel reductive cyclisation in nepetalactone biosynthesis provides a bioinformatics-based route to identification of the corresponding synthetic genes in the full genomic sequence now published for the pea aphid, *Acyrthosiphon pisum* [32]. This is being investigated to provide new genes for plant genetic engineering to avoid co-production of other compounds apparently associated with the biosynthetic pathway in plants.

The sex pheromones of moths (Lepidoptera) have attracted considerable biosynthetic and genetic investigations. Although also involving simple SLMs mostly derived from fatty acids by desaturation, chain shortening and altered functional groups, these can be expensive to produce by chemical synthesis particularly with sufficient purity. Therefore production in plants in chemical factories is under development [12••] with the products converted, by cheap green technologies, into the final pheromones for deployment in crops after appropriate formulation.

Plant genetic engineering for other insect semiochemicals

Plants produce a diverse array of semiochemicals that affect insect behaviour and development. Repellents and attractants can be deployed in a push–pull system and the prospect for genetic engineering of plants for these functions has been reviewed [33]. Numerous attractants (Pull), already identified as simple polyunsaturated fatty acids oxidation products such as (*E*)-2-hexenal [34,35••,36•], could easily be targeted. For repellency (Push), either specific SLMs or mixtures characteristic of plants unsuitable as hosts could be utilised. These could comprise SLMs specific to non-host taxa, for example, the monoterpenoid (1*R*,5*S*)-myrtenal which is typical of plants in the Lamiaceae that are non-hosts for the black bean aphid, *Aphis fabae*, and which is repellent even in the presence of the host [37]. However, by modifying the characteristic mixtures of SLMs from the host plant, the common bean *Vicia faba*, this plant can be rendered unattractive [34]. The latter could present a highly economic approach simply by altering upwards or downwards expression of the synthetic gene for just one SLM [20••,38]. Perhaps the most promising approach currently investigated is the engineering of the biosynthesis of homoterpenes, or more correctly,

Figure 2



Elicitors associated with plant damage by insects (e.g. **X** and **XI**) can induce or prime, naturally or in GM plants, the production of insect pheromones (e.g. **VII**) or semiochemical based plant defence compounds (e.g. **VIII** and **IX**).

tetranorterpenes, for example, **VIII** and **IX**, which are synthesised by oxidative cleavage of isoprenoid secondary alcohols (*E*)-nerolidol and (*E*,*E*)-geranyllinalool, respectively [39,40••]. These compounds are both volatile and highly unstable; thus biosynthesis in plants would solve the practical problems of their deployment which would provide both repellency of herbivorous pests and, at the same time, attraction of their enemies [41].

Switching on engineered genes by phytopheromones and other elicitors (Figure 2)

When insects feed, the plant can respond to a range of compounds generated by this process, including proteins derived from the insect, as well as catabolites derived from the plants themselves. The elicitor volicitin (**X**), isolated from caterpillar regurgitate, induces indirect defence involving the homoterpenes [42], but requires insect feeding in order to enter a plant vascular system [43]. However, on damage, plants can release volatile SLMs that cause neighbouring plants to undergo induction of defence involving toxicants as well as semiochemical based indirect defence [44••]. Initially, the volatile, and thereby externally acting, component methyl jasmonate, of the jasmonate hormone pathway, showed promise, but effects were erratic [45•] and could be phytotoxic. However, *cis*-jasmone (**XI**), which signals differentially from methyl jasmonate and is also volatile, by having lost the carboxylic acid function, elicits upregulation of defence associated genes, for example, *At Cyp81D11* [46•,47•], and others, without the negative effects associated with jasmonate induction [48]. There are now several examples of *cis*-jasmone inducing defence by repelling pests and attracting beneficial insects, particularly as a consequence of upregulation of homoterpenes [49,50•]. *cis*-Jasmone also induces production of **I** [51] and has a positive effect on barley, which must involve other inducible defence chemistry as barley is without the hydroxamic acid pathway. Aphids also cause elicitation of effects during feeding [16] and the aphid *Lipaphis erysimi*, specialising on Brassicaceae, suppresses floral volatile production in the mustard plant, *Sinapis alba*, as opposed to generalist aphid feeding [52••]. Since the discovery of **X**, the same group has identified other novel insect associated elicitors [53]. Commercial GM programmes have sought elicitors but not apparently exploiting the natural elicitors, even though IP can be secured as was done for *cis*-jasmone. Use of such tools could provide a route to securing non-constitutive expression of currently commercialised insect control genes, for example, those related to Bt endotoxin expression.

Modifications of plant secondary metabolism place only a relatively low burden on diverted photosynthate [14••]. Nonetheless, priming, because of an even lower burden, could be more valuable and yet has produced few routes to exploitation. The main SLM studied as a priming agent is β-aminobutyric acid (BABA) (**XII**), but associated phytotoxicity is a problem and suitable analogues have not yet been identified. Recently, *cis*-jasmone has been shown to prime maize for a stronger response when later attacked by the leafhopper *Cicadulina storeyi* [54] and shows promise for this approach for the future.

Induction *via* the rhizosphere also shows promise, and in the ecosystem, novel semiochemical based processes are being developed, for example, attraction of insectivorous nematodes to maize roots damaged by the rootworm, *Diabrotica virgifera virgifera* [55••]. In the same way as through the air, signals from stressed plants can pass to intact plants and induce defence [56]. In addition, a powerful 'highway' for signalling is established *via* arbuscular mycorrhizal hyphae, which causes dramatic induction in the indirect defence chemistry of undamaged plants [57••].

Conclusions and further ways forward

The few practical examples, and the many proven opportunities, demonstrate value for genetic engineering of plant secondary metabolite based insect resistance. Where this involves volatile SLMs acting as pheromones and other semiochemicals, resistance to these processes would be encountered when widely adopted in agriculture. Nonetheless, by using highly sophisticated techniques, such as insect peripheral neuroelectrophysiology, developed originally to identify the semiochemical targets, we can quickly identify new semiochemicals arising as alternative signals through resistance. Already, there is evidence for this evolutionary strategy [58•,59•] being associated with development of resistance to pheromones and other semiochemicals, and arising where, if the insect ceases to use semiochemical cues, it will as a consequence become evolutionarily disadvantaged. Signalling from one plant to another, whether through the air or the rhizosphere, facilitates the development of sentinel plants that by causing upregulation of visual marker genes, could warn of attack and other problems. Such sentinel plants could also be developed to switch on defence in the main crop after initial attack on the sentinel plants.

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The iridoids comprise a large family of distinctive bicyclic monoterpenes that possess a wide range of biological activities. The discovery of iridoid synthase, a plant-derived enzyme that generates the iridoid ring scaffold, as evidenced by biochemical assays, gene silencing, co-expression analysis and localisation studies, is reported.

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A plastidial terpene synthase gene, PlTPS2, from lima bean (*Phaseolus lunatus*) was characterised. The recombinant PlTPS2 protein was multifunctional, producing linalool, (*E*)-nerolidol and (*E,E*)-geranyllinalool, precursors of (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [TMTT]. The manipulation of TMTT is an ideal platform for pest control via the attraction of generalist and specialist predators in different manners.

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Biotechnological manipulation of plant defence pathways can increase crop resistance to herbivores and pathogens. The use of multi-gene cassettes which mimic natural gene clusters can facilitate coordinated regulation of a pathway and speed transformation efforts. The targeting of defence pathway genes to organs and tissues in which the defensive products are typically made and stored can also increase yield as well as defensive potential.

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Jasmonic acid (JA) signalling can influence plant defence and the production of plant volatiles that mediate interactions with insects. Results indicate that JA seed treatment can make tomato plants more attractive to predatory mites, but that direct effects on herbivorous mites are variable and cultivar dependent.

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cis-Jasmone treatment leads to an increase in CYP81D11 (At3g28740) gene expression. This gene was chosen for a more thorough analysis of the potential biological relevance of the *cis*-jasmone induced defense response. Additional experiments which are carried out give further support to a role of CYP81D11 in the direct or indirect defense response of *A. thaliana*.

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Changes in the defence volatile profile of cotton, *Gossiypium hirsutum* induced by the naturally occurring plant elicitor *cis*-jasmone are investigated and whether these changes modify the behaviour of cotton aphids, *A. gossypii*. The results in this study show that *cis*-jasmone can induce the production of *A. gossypii*-induced homoterpenes with potential for deployment in novel crop protection strategies.

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Extensive knowledge exists on the effects of insect herbivory on volatile emission from vegetative tissue, but little is known about its impact on floral volatiles. In this study, it is shown that herbivory by phloem-feeding aphids inhibits floral volatile emission in white mustard *Sinapis alba*. The findings provide new insights into interactions between insect herbivores and plant chemistry.

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Plant strengthener is a generic term for several commercially available compounds of mixtures of compounds that can be applied to cultivated plants in order to 'boost their vigour, resiliance and performance'. Studies into the consequences of boosting plant resistance against pests and diseases on plant volatiles have found a surprising and dramatic increase in plants attractiveness to parasitic wasps.

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Common mycorrhizal mycelial networks can determine the outcome of multitropic interactions by communicating information on herbivore attack between plants, thereby influencing the behaviour of both herbivores and their natural enemies.

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Sex pheromones play a pivotal role in the communication of many sexually reproducing organisms, and speciation is often accompanied by pheromone diversification enabling proper mate finding and recognition. The genetics of a newly evolved pheromone phenotype in wasps is elucidated with results from behavioural experiments indicating how the evolution of a new pheromone component occurred in an established sender–receiver system.

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The role of strain-specific sexual communication as a prezygotic mating barrier between two strains of *Spodoptera frugiperda* is investigated by analysing strain-specific variation in female pheromone composition of laboratory and field strains, and also male attraction in wind tunnel and field experiments. Although corn-strain individuals are more restricted in their production of and response to pheromones than rice-strain individuals, differences in sexual communication between corn-strain and rice-strain individuals are not strong enough to cause assortative mating.