Attracting friends to feast on foes: engineering terpene emission to make crop plants more attractive to herbivore enemies

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When attacked by herbivorous insects or mites, some plant species call on other arthropods for help. They emit mixtures of volatile compounds, dominated by terpenes, to attract carnivorous arthropods that prey on or parasitise herbivores and so reduce further damage. This fascinating defence strategy offers a new, environmentally friendly approach to crop protection. Using recent advances in the biochemistry and molecular genetics of terpene biosynthesis, it should now be possible to engineer crop plants that release terpenes for attracting herbivore enemies. By introducing or selectively altering the existing rate of terpene emission and composition, plant breeders could enable attacked plants to attract enemies and reduce additional herbivory, without compromising the effectiveness of other modes of defence.

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Abbreviations

DMAPP	dimethylallyl diphosphate
DXP	1-deoxyxylulose-5-phosphate
FPP	farnesyl diphosphate
GPP	geranyl diphosphate
HMGR	3-hydroxy-3-methylglutaryl-CoA reductase
IPP	isopentenyl diphosphate
MEP	methylerythritol-4-phosphate

Introduction

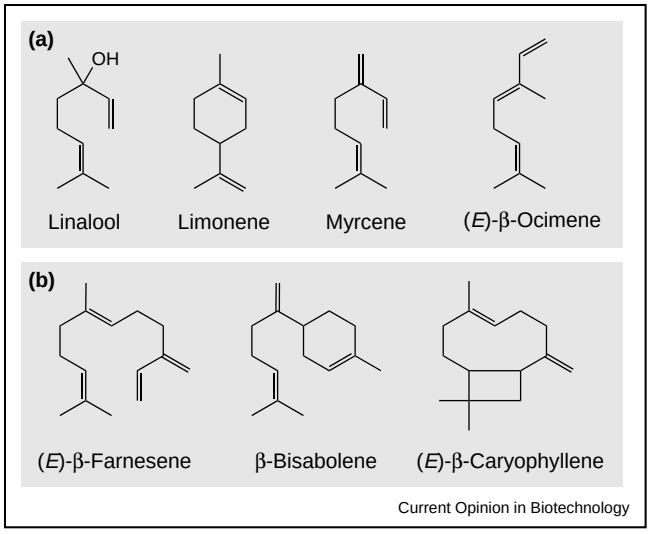
It is now more than ten years since researchers in the Netherlands and the United States first observed that herbivore damage to certain plants induces the emission of volatile organic compounds that attract natural enemies of the herbivores [1,2]. Termed 'indirect defence' this phenomenon has been reported in more than 15 different plant species after feeding by an assortment of arthropod herbivores [3–6]. The herbivore enemies that respond to volatiles from herbivore-damaged plants include various carnivorous arthropods, both predators and parasitoids. Attraction of herbivore enemies has been shown to benefit the plant by reducing subsequent herbivory and increasing reproductive fitness [7,8••,9•], although such advantages are not realised in all cases [10].

In an agricultural setting, the value of indirect defence has been shown by the co-cultivation of maize with an African grass (*Melinis minutiflora*) that releases abundant volatile compounds [11••,12]. The proximity of this grass led to a significant reduction in damage to maize plants by lepidopteran larvae as a result of increased parasitism by braconid wasps. These results suggest that the manipulation of volatile emission in crops may be a valuable strategy to attract herbivore enemies and thus minimise pest problems in an environmentally benign way. Herbivore enemies may be especially useful against herbivores that are tolerant of the direct defences (toxins, feeding deterrents and morphological barriers) of a plant.

The majority of volatile compounds released after herbivore damage are monoterpenes and sesquiterpenes (Figure 1). These C_{10} and C_{15} members of the terpene family of natural products are responsible for many of the characteristic smells of plant oils, resins, fruits and flowers. They are the dominant components of many natural volatile blends that lure herbivore enemies and so are assumed to play a central role in attraction [13]. Moreover, many monoterpenes and sesquiterpenes are emitted specifically in response to herbivore feeding and not after simple mechanical damage [14]. Emission from both wounded and non-wounded organs is often systemic and prolonged, lasting for hours or days [13].

In this review, we outline how monoterpene and sesquiterpene emission might be employed to increase the visitation of herbivore enemies in agricultural systems. Then we discuss how emission could be introduced or enhanced by metabolic engineering. Although there are still many gaps in our knowledge of the indirect defences in plants, the release of volatile signals to attract herbivore enemies upon herbivore attack appears to be a general property of plants that could help provide effective defence against herbivores. Therefore, a consideration

Figure 1



Examples of volatile **(a)** monoterpenes and **(b)** sesquiterpenes that are emitted by plants after herbivore damage and which are components of volatile blends that attract herbivore enemies.

of the potential of this phenomenon for improving crop protection seems warranted.

Befriending the right enemy

One of the first requirements for exploiting indirect defence in an agricultural context is to identify one or more herbivore enemy species that are not only capable of being attracted by herbivore-induced volatiles, but which are also present in abundance in the region where the crop is likely to be grown. Even more critical is the ability of the enemy species to control herbivore populations sufficiently to significantly decrease plant damage. Certain herbivore enemies, including predators and parasitoids, can dramatically reduce herbivory on plants [4,7,8••,9•], but this is not always the case, especially for parasitic wasps that do not immediately kill their hosts. In one experiment, larvae of the large cabbage white butterfly parasitised by the wasp *Cotesia glomerata* consumed leaf material at a higher rate than unparasitised caterpillars and had prolonged larval development [10].

Sniffing out the right volatile blend

The volatile blend to be introduced or enhanced should be chosen to closely match the major attractants known for the enemy species selected. Unfortunately, detailed preferences of herbivore enemies for particular compounds or mixtures of compounds have not been determined. However, duplicating the exact blend of volatiles previously known to be attractive may not be necessary as most enemy species are capable of learning; they orient positively to whatever odours they perceived during previous successful bouts of foraging or oviposition [15,16]. Analyses of the mixtures of terpenes released after herbivore damage show that although many compounds are common to more than one species, others are species-specific [4,17]. In addition to terpenes, C_6 alcohols and aldehydes from the octadecanoid pathway ('green leaf volatiles'), phenolics, such as methyl salicylate, and indole are often also detected [5,18,19]. The composition of herbivore-induced volatile blends is influenced not only by the species damaged, but also by the herbivore and the type of damage inflicted and by the nature of the elicitor molecules in the herbivore's oral secretions [17,20,21]. Whether small qualitative and quantitative differences in the blend are critical in attraction remains to be determined. On the one hand, such differences have been shown to be decisive in leading certain parasitic wasps to their correct host species and even to the appropriate caterpillar instar [22,23]. On the other hand, given the amount of genotypic [24,25], developmental [21] and environmental [21,26] differences reported in terpenoid emission, herbivore enemies must accommodate considerable variation in the signal they respond to. We need to learn much more about indirect defence before we can designate with confidence which specific herbivore-induced terpenes are required for enemy attraction.

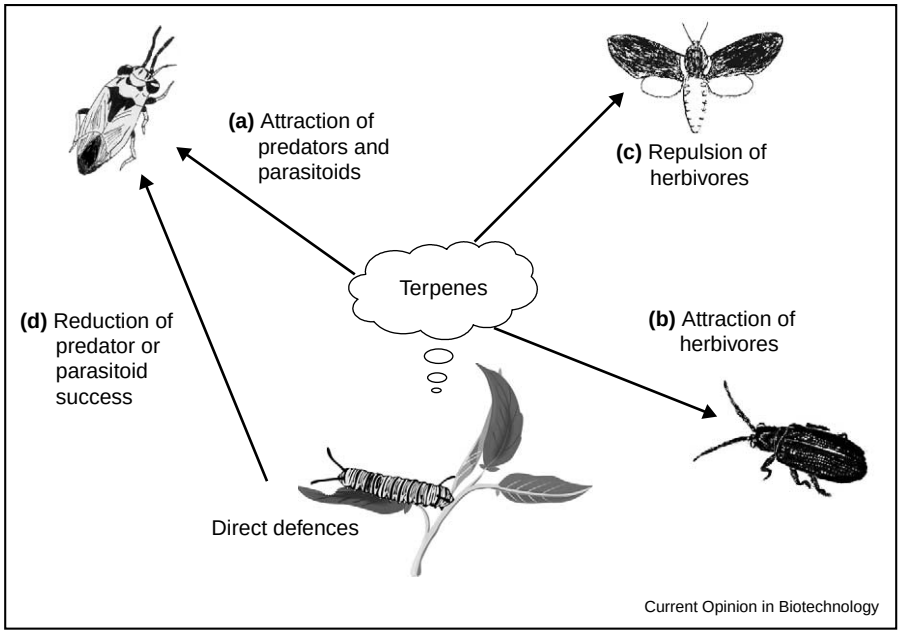
Adjusting the timing of volatile release

It has been suggested that the effectiveness of indirect defences in attracting herbivore enemies would be increased if terpene volatiles were released continuously rather than only following initial herbivore damage. However, this suggestion ignores the fact that most herbivore enemies have a remarkable facility for learning the odours associated with their prey or hosts [15,16,23]. A continuously released signal provides misleading information about the location of feeding herbivores and is likely to be ignored by foraging enemies. In addition, continuous emission of terpenes might have negative consequences for populations of enemies, because individuals would spend time in fruitless searches for prey or hosts for parasitism. The same problem is likely to arise if attractive terpene volatiles were directly applied to unattacked plants or released from artificial dispensers in fields, an approach found unsuccessful in stimulating indirect defence [27]. Therefore, to exploit terpene volatiles in attracting herbivore enemies, release should be synchronised with the presence of herbivores. When modifying existing volatile release in a crop plant, it seems best to leave formation of terpenes under the control of existing herbivory-triggered signalling networks. When engineering volatile release in a crop without any previous vestige of such a system, terpene formation might be placed under the control of a herbivore-responsive promoter, created in a similar manner to the synthetic pathogen-induced promoters recently described [28].

Avoiding uninvited guests

The defensive value of releasing terpenes to attract herbivore enemies could be compromised if additional

Figure 2



Interactions between plants, herbivores and herbivore enemies mediated by volatile terpenes. **(a)** After herbivore damage triggers the release of terpenes, these substances attract predators and parasitoids that prey on the herbivores or use them as hosts for their larvae. Both are examples of indirect defence. The same terpenes can also affect other herbivores, either **(b)** attracting them to feed or **(c)** repelling them from feeding or oviposition. **(d)** In addition, plant traits involved in direct defence against herbivores (e.g. glandular trichomes or toxins) can negatively affect predator or parasitoid success. Attempts to engineer terpene emission in crop plants must balance the advantages (a,c) against the possible disadvantages (b,d).

herbivores were also attracted to this signal (Figure 2). In fact, several insects, particularly beetles, are known to be attracted to terpene-rich volatile blends from damaged leaves [29–31]. However, not all herbivores are attracted to damage-induced volatiles. Certain adult lepidopterans have been shown to avoid damaged plants for oviposition [8••,32]. For crops in which engineering of enhanced volatile emission is contemplated, it will be necessary to investigate whether any of their major herbivores are likely to aggregate in response to volatiles. If so, it might be possible to create a volatile mixture that attracts herbivore enemies, but which does not attract herbivores.

Trading-off between direct and indirect defences

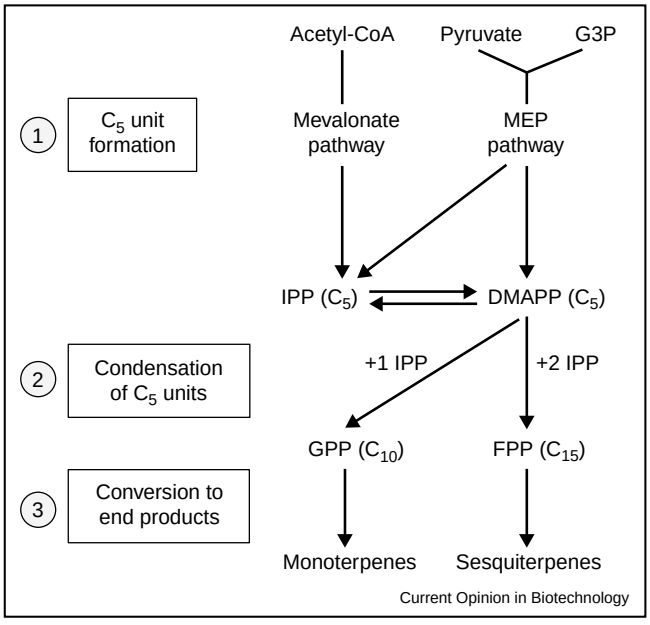
Crop plants, like those growing in the wild, often possess direct chemical defences against herbivores in addition to indirect ones (Figure 2). Unfortunately, these two modes of defence can sometimes work at cross purposes if herbivores that are tolerant of toxins store them to use as a defence mechanism against their own enemies. For example, tobacco hornworm larvae feeding on cultivated tobacco plants (*Nicotiana tabacum*) retain sufficient nicotine in their hemolymph to reduce the growth and survival of endoparasitic wasps [33]. Thus, plants may have to choose between deploying direct and indirect defences. When attacked by tobacco hornworm larvae, a species of wild tobacco (*Nicotiana attenuata*) reduces its induced nicotine production and releases volatile terpenoids to attract herbivore enemies [34,35].

In other instances, direct and indirect defences may be compatible: prolonging herbivore development times with sublethal levels of direct defences could, in theory, increase herbivore exposure to enemies [36,37]. In practice, tobacco budworm (*Heliothis virescens*) larvae on tobacco plants engineered with *Bacillus thuringiensis* (Bt) toxin suffered enhanced parasitism because they were exposed to their enemies for longer times [38]. Hence, it may be possible to engineer both modes of defence simultaneously into agricultural plants. Weakened herbivores also help sustain natural enemy populations, which is crucial for achieving sustainable biological control [37].

Metabolic engineering of volatile terpene biosynthesis

The manipulation of plant terpene metabolism has long been a focus of interest in plant biotechnology, because many terpenes have high economic importance as pharmaceuticals (Taxol), insecticides (pyrethrins), flavours (menthol) and fragrances (linalool). Unfortunately, progress in this area has been slowed by lack of basic tools and knowledge. For many branches of terpenoid biosynthesis, the genes encoding the critical biosynthetic

Figure 3



The three major phases in the biosynthesis of monoterpenes and sesquiterpenes involved in the attraction of herbivore enemies. (1) Construction of the basic C_5 units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), via the mevalonate pathway from acetyl-CoA or the methylerythritol-4-phosphate (MEP) pathway from pyruvate and glyceraldehyde-3-phosphate (G3P). (2) Condensation of two or three C_5 units to form geranyl diphosphate (GPP; C_{10}), the precursor of monoterpenes, or farnesyl diphosphate (FPP; C_{15}), the precursor of sesquiterpenes. These reactions are catalyzed by prenyltransferases. (3) The conversion of GPP and FPP to individual terpene end products, catalysed by terpene synthases.

steps are not yet available and even the reactions themselves are not known with confidence.

The formation of the monoterpenes and sesquiterpenes involved in attracting herbivore enemies can be divided into three phases [39,40] (Figure 3). In the next two sections, we consider how each of these phases might be manipulated in turn to enhance the formation of monoterpenes and sesquiterpenes. As these compounds do not generally accumulate in plants, but are released soon after synthesis [41], manipulation of their biosynthesis can be expected to have direct effects on emission. Either genetic engineering or conventional breeding could be employed to manipulate terpene biosynthesis, because for crops such as cotton [42], beans [24] and maize [25] varieties are known that differ in the amount and composition of volatile terpenes they produce.

Engineering the early steps: increasing flux to terpene volatiles

Manipulation of the steps involved in construction of the C_5 units in either of the basic pathways should have broad consequences for the formation of many terpenoid metabolites. The mevalonate pathway, localised in the cytosol, produces isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) that enter sesquiterpene, sterol and triterpene biosynthesis [39,43]. In theory, overexpression of the gene encoding the presumptive regulatory enzyme in the mevalonate pathway, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), might be expected to elevate the synthesis of sesquiterpenes and sterols, as well as other metabolites. However, in practice, constitutive overexpression of a heterologous HMGR in tobacco had no effect on sesquiterpenes, but did lead to a three- to tenfold increase in total sterols [44,45]. In *Arabidopsis*, overexpression of the endogenous HMGR had no effect on terpene biosynthesis at all [46].

Manipulation of the methylerythritol-4-phosphate pathway (MEP) has been more successful in promoting increased terpenoid formation in plants. This recently discovered pathway, which in higher plants is located in the plastids, supplies IPP and DMAPP for monoterpene, diterpene and tetraterpene (carotenoid) formation [40,47]. Overexpression of the first step, the condensation of pyruvate and glyceraldehyde-3-phosphate to form 1-deoxyxylulose-5-phosphate (DXP), catalysed by DXP synthase, resulted in elevated levels of carotenoids in *Arabidopsis* as well as greater amounts of chlorophylls and tocopherols, which contain terpene moieties [48]. For the second step, conversion of DXP to MEP, overexpression of the corresponding enzyme in peppermint led to an increase of almost 50% in the monoterpenes responsible for the characteristic flavour of this herb [49••]. Thus, increased flux through the basic terpenoid pathway could raise the level of many desired end products, such as the terpenes released upon herbivore damage.

Nevertheless, this approach must be used cautiously, as elevating the levels of many terpenoids indiscriminately could have unforeseen negative consequences for the plant. Some terpenoids have critical regulatory roles in the plant as hormones (e.g. gibberellins, abscisic acid and brassinosteroids) or as components of signal transduction pathways (e.g. prenylated proteins). When the desired terpenes are known to be made in specific cell types, it might be best to elevate flux through the basic terpenoid pathway in those cells only. Monoterpene formation in peppermint, for instance, is localised in the glandular trichomes found on the leaf surface [50]. Thus, the use of trichome-specific promoters for engineering terpene metabolism [51] might be judicious in this species. Unfortunately, the site of synthesis of the terpenoid volatiles that attract herbivore enemies is not yet known.

When rate-controlling steps of the basic pathway are encoded by a gene family, one can attempt to manipulate specific members of the family that control formation of the desired terpenoids. For the MEP pathway, it was recently discovered that plants have two distinct types of

DXP synthase genes (sharing only 70% similarity in their corresponding amino acid sequences). One of these gene types seems to specifically regulate the formation of terpenoid secondary metabolites, such as monoterpenes [52]. Hence, this gene might be a good candidate for future efforts to engineer increased volatile terpene emission.

In the second phase of terpene biosynthesis, IPP and DMAPP condense to form geranyl diphosphate (GPP) and farnesyl diphosphate (FPP), the precursors of monoterpenes and sesquiterpenes, respectively. Modification of either of the prenyltransferase enzymes that catalyse these reactions could allow one to manipulate the levels of monoterpenes and sesquiterpenes independently. Although genes encoding both GPP [53–55] and FPP synthases [56,57] are available, there have so far been few attempts to employ them to alter terpene biosynthesis. However, overexpression of a cotton FPP synthase in *Artemisia annua* resulted in a two- to threefold increase in artemisinin, an antimalarial sesquiterpene in this plant [58•].

Engineering the later steps: changing the composition of volatile compounds

GPP and FPP are converted to specific monoterpenes and sesquiterpenes in the third phase of terpene formation by enzymes known as terpene synthases. These are important enzymes in the formation of terpene attractants to herbivore enemies, because most of the attractants are direct products of terpene synthases. Sometimes additional biosynthetic steps are required for the formation of volatile compounds following the action of terpene synthases. Yet, even here, terpene synthases may catalyse the rate-controlling step in the formation of volatile end products [59]. Therefore, engineering of terpene synthases provides numerous opportunities to alter the specific composition of terpene volatiles and their rate of formation.

The most frequently used terpene synthase in such studies is linalool synthase from *Clarkia breweri* [60], which converts GPP to (3*S*)-linalool, a widespread monoterpene emitted by flowers of many species and by plants after herbivore attack. In one investigation, the gene encoding this enzyme was transformed into tomato under the control of a promoter conferring expression during fruit maturation with the aim of enhancing the aroma and flavour of the fruit [61••]. Fruits from transgenic tomato plants contained significant concentrations of (3*S*)-linalool and 8-hydroxylinalool, a product of allylic hydroxylation of linalool, neither of which were found in untransformed control plants. In investigations with carnation, transformation with the linalool synthase gene under the control of a constitutive promoter was able to trigger linalool emission in a variety of this ornamental plant that previously lacked monoterpenes [62•]. These results demonstrate the feasibility of introducing monoterpene production into plants that do not display this ability, a good precedent for attempting to introduce monoterpenes that attract herbivore enemies into crop plants that do not usually emit these compounds. The transformants made in these studies all had normal morphology, suggesting that sufficient GPP is available for diversion to monoterpenes without unduly affecting the supply of intermediates to the rest of the terpenoid pathway. However, in a past attempt to increase carotenoid formation, diversion of metabolic flux to this branch of the terpenoid pathway led to a decrease in the formation of the gibberellins, resulting in dwarfed plants [63].

Unfortunately, not all efforts to introduce linalool formation have been successful. In petunia, considerable linalool was formed after genetic transformation with linalool synthase, but it was almost completely converted to the non-volatile β-D-glucoside [64•]. Even in carnation, a significant fraction of the linalool formed was further metabolised [62•]. These results demonstrate that certain additional endogenous enzyme activities must be considered in attempting to engineer monoterpene production in plants.

Unlike the *C. breweri* linalool synthase, many other terpene synthases have the unusual ability to produce more than one product [65]. Therefore, introduction of a single terpene synthase activity to a crop plant is likely to lead to the formation of several new volatile products. This is not necessarily a problem as herbivore enemies are often attracted by a mixture of terpenes, some of which may be derived from a single terpene synthase activity [66]. However, if behavioural studies point to one or two individual terpenes as critical in luring herbivore enemies, it may soon be possible to deploy modified terpene synthases capable of producing single products. Analyses of the crystal structures of terpene synthase are beginning to define structural residues responsible for the formation of particular end products [67–69].

Conclusions

Biological control methods are often proposed as alternatives to synthetic insecticides in an effort to reduce the environmental impact of modern agriculture. In this context, natural enemies of pests show great promise to limit crop damage in an environmentally safe manner if they can be summoned in sufficient abundance during pest outbreaks. The recent discovery of volatile plant terpenes that attract enemies may help harness the full potential of biological control in agroecosystems.

To enlist enemies of pests in crop defence, it is now feasible to try to engineer crop plants to emit strong, readily detectable bursts of terpenes to signal the presence of herbivores using our increasing knowledge of plant terpene metabolism and expanding collection of terpene biosynthetic genes. Herbivore-induced terpene emission could be introduced into crops that do not

presently have this trait or existing emission could be augmented by increasing flux through the terpenoid pathway. In both cases, the composition of the volatile blend could be modified to match the preferences of particular enemy species and to avoid simultaneous attraction of more herbivores. To preserve signal reliability, however, it is critical to maintain the temporal synchrony between herbivory and volatile emission. The effectiveness of natural enemies could be enhanced further if volatile terpene emission were coordinated with direct defences (e.g. toxins or feeding deterrents) to prolong the time that herbivores remain vulnerable to attack from foraging enemies. At the same time, direct defences that could be sequestered by herbivores for their own defence or that impair enemy foraging behaviour (e.g. glandular hairs) should be avoided.

In natural environments, plants are under strong selection to optimise the use of natural enemies in their own defence. Further studies of the interactions between plants, their herbivores and the enemies of their herbivores should provide more clues to facilitate the application of indirect defences in agriculture.

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After much work on indirect defences in cultivated plants, this is the first study to show their operation in natural ecosystems. The release of terpenes and other volatiles from a wild tobacco species was found to increase predation on the eggs of herbivore larvae as well as to deter further oviposition.

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