

Exploring multi-trophic plant-herbivore interactions for new crop protection methods

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ABSTRACT

Biological control of arthropod herbivores in agricultural crops depends on antagonists or enemies of the pest organisms. To minimise damage to a crop, it is crucial that the biological control agents are able to find their prey efficiently. Here we discuss the finding that when herbivores feed, plants produce volatiles that are attractive to the predators. The effects of biotic, abiotic and genetic factors on volatile formation and the biochemical and molecular regulation of this indirect defence mechanism are reviewed. The opportunities to use genetically modified plants to further understanding this complex interaction and the possibilities of using our knowledge to improve biological control in agricultural crops are discussed.

INTRODUCTION

Environmentally benign protection of crops against arthropod herbivores can use either direct host plant resistance or biological control. These two approaches are fundamentally different in that host plant resistance depends on direct – constitutive or feeding-induced - defence traits of the crop plants (such as trichomes, toxic secondary metabolites or proteins, and repellents), whereas biological control depends on the use of antagonists or enemies of the pest organisms. For the latter to be effective, it is crucial that the biological control agents are able to find their prey efficiently enough to minimise damage to the crop.

More than a decade ago it was discovered that when herbivores feed, the plants produce volatiles that are attractive to the natural enemies of the herbivore (Dicke *et al.*, 1990a; Turlings *et al.*, 1990) (Figure 1). Thus, plants indirectly defend themselves by enhancing the effectiveness of the natural enemies of the herbivores. The use of predators and parasitoids for biological control is receiving more and more attention and for many years it has been common practice in a number of crops in glasshouse as well as open fields (Van Lenteren, 2000; Kfir *et al.*, 2002). Nevertheless, breeders and agronomists have so far paid little attention to the optimisation of biological control. This is probably due to the relatively recent discovery of the phenomenon of indirect defence, the more complex relationships involved and the difficulties associated with quantification of the effects.

Here we will discuss the principles of plant-mediated multitrophic interactions involved in biological control and our research into the possibilities of using this principle to design new control methods. We use a combination of scientific disciplines varying from ethological studies of predators to plant molecular biology. We focus our work on the induction of

VOLATILE INDUCTION AND EFFECTS ON PREDATOR BEHAVIOUR

The importance of the third trophic level for plant defence was first suggested by Price *et al.* (Price *et al.*, 1980), and was followed by the discovery of the herbivory-induced volatiles that attract parasitoids and predators (Dicke *et al.*, 1990a; Turlings *et al.*, 1990). Upon infestation with two-spotted spider mites (*Tetranychus urticae*), lima bean plants responded by emitting a mixture of volatiles attracting the predatory mite *Phytoseiulus persimilis* (Dicke *et al.*, 1990b) that effectively eliminated local populations of the spider mites (Dicke *et al.*, 1990a). Similarly, corn plants respond to feeding damage of *Spodoptera exigua* caterpillars with the production of volatiles that attracted the parasitoid *Cotesia marginiventris* (Turlings *et al.*, 1990). Since then, it has been shown that this is a common mechanism employed by many plant species in the interaction with many different herbivores, and that these volatiles are not usually emitted in response to mechanical wounding (Dicke *et al.*, 2003).

Figure 2 shows a typical example of the induction of volatiles in cucumber and Figure 3 the results obtained in Y-tube olfactometer experiments on predator attraction by induced plants for a number of plant species. Although treatment with jasmonic acid mimics fairly well the effect of spider mite infestation, some differences (e.g. peak 5, (*E,E*)- α -farnesene) can be found in the volatile blend (Figure 2). Typical volatiles released from a multitude of species after herbivory are the so-called green leaf volatiles such as C6-alcohols, -aldehydes, and -esters, derivatives of the shikimate pathway such as methyl salicylate, and isoprenoids such as (*E*)- β -ocimene, linalool, (*E*)- β -caryophyllene, (*E,E*)- α -farnesene and the homoterpenes 4,8-dimethyl-1,3(*E*),7-nonatriene and 4,8,12-trimethyl-1,3(*E*),7(*E*),11-tetradecatetraene (Figure 1). The isoprenoids are by far the most important components of the induced volatile blend and hence this is the class we focus on.

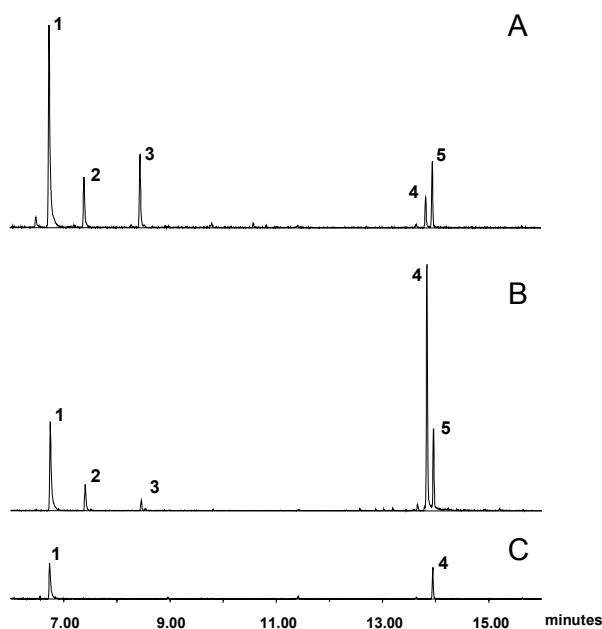


Figure 2. Induction of volatiles in cucumber by spider mite feeding (A) and jasmonic acid spraying (B). Panel C shows the response of control leaves. Peaks are: (*Z*)-3-hexen-1-yl acetate (1), (*E*)- β -ocimene (2), 4,8-dimethyl-1,3(*E*),7-nonatriene (DMNT) (3), (*E,E*)- α -farnesene (4), 4,8,12-trimethyl-1,3(*E*),7(*E*),11-tridecatetraene (5).

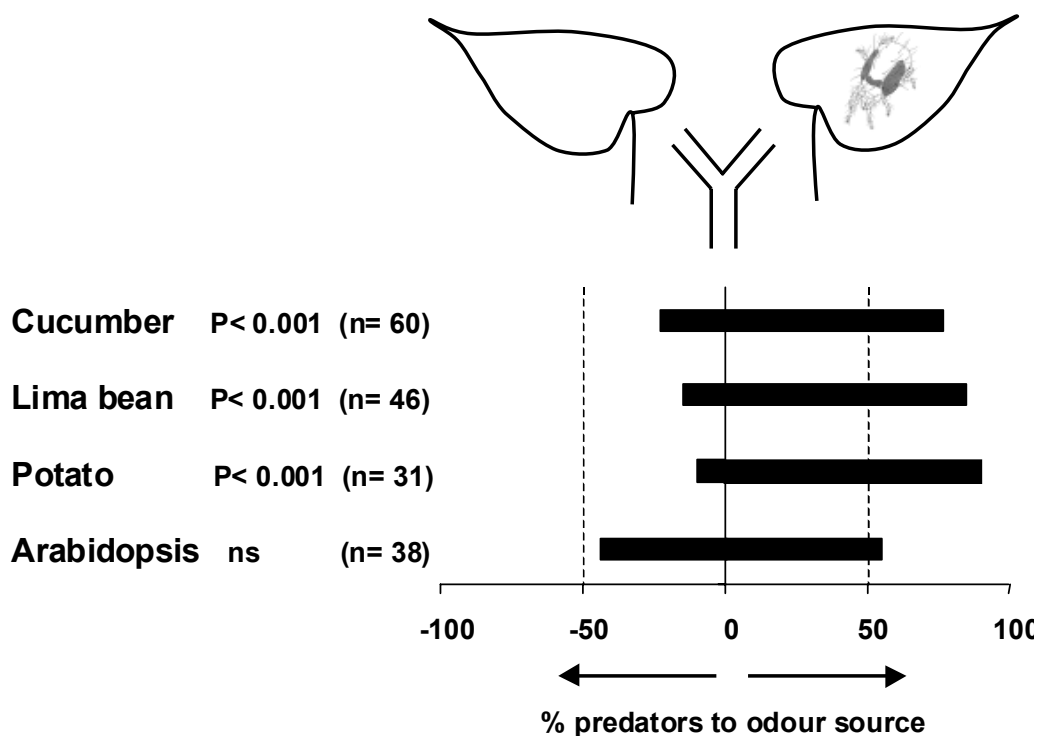


Figure 3. Predator attraction (*Phytoseiulus persimilis*) by spider mite infested cucumber, lima bean, potato and arabidopsis plants in a Y-tube olfactometer.

FACTORS AFFECTING INDUCED VOLATILE FORMATION

Biotic and abiotic factors

Plants have been shown to respond to different herbivore species with quantitatively and qualitatively different volatile blends allowing predators to respond to their specific prey (Sabelis and van der Baan, 1983; Takabayashi *et al.*, 1991; De Moraes *et al.*, 1998; Du *et al.*, 1998). Within a plant species, the quality of the volatile blend may be affected by the developmental stage of the herbivore (Takabayashi *et al.*, 1995), and the volatiles induced by insect egg deposition differ from those induced by feeding (Wegener *et al.*, 2001; Hilker and Meiners, 2002).

There are many reports that the production of non-herbivore induced secondary metabolites by plants is influenced by environmental conditions. It is therefore likely that this also holds for herbivory-induced volatiles and knowledge of these effects may be important for improvement of biological control, e.g. by using optimal conditions for efficient signalling. Some of the factors that have been shown to affect secondary metabolite production such as light, temperature and water availability have also been investigated for their effect on herbivore-induced volatile biosynthesis. High light intensity and water stress are generally reported to increase induced volatile production and/or predator attraction for example in Lima bean, kidney bean, maize and cotton (Loughrin *et al.*, 1994; Takabayashi *et al.*, 1994; Turlings *et al.*, 1995; Gouinguene and Turlings, 2002).

Genetic variation

If conventional plant breeding is to be used to improve biological control through enhanced volatile production there has to be genetic variation in the ability to produce herbivore-induced, predator-attracting volatiles. In gerbera, a number of cultivars differed in composition and amount of volatiles produced after spider mite feeding (Krips *et al.*, 2001) and there were differences between the cultivars in the odour-preference of predatory mites. The composition of the volatile blend seemed to be more important for this difference than the total amount of volatiles produced, and the terpenoids (*E*)- β -ocimene and linalool were possible candidates in determining the difference in attractiveness between cultivars. Also maize cultivars and *Zea* spp. showed large differences in the composition of the volatile blend induced by the application of the oral secretion of *Spodoptera littoralis* to mechanically damaged leaves (Gouinguene *et al.*, 2001).

There are several problems associated with comparing genotypes for their production of induced volatiles, when other differences between the genotypes can not be controlled (Krips *et al.*, 2001). For example, there may be differences in direct defence causing differences in developmental rate of herbivores leading to differences in volatile formation. To circumvent this problem, in addition to spider mite infestation we also used jasmonic acid (JA) treatment in a comparison between seven cucumber genotypes. Earlier research had shown that JA treatment mimics the effect of spider mite infestation in lima bean, gerbera and cucumber plants (Dicke *et al.*, 1999; Gols *et al.*, 1999) (H J Bouwmeester *et al.*, unpublished data). Figure 4 shows that there are large differences in the response of cucumber genotypes to spider mite infestation. In addition, there are similar differences in volatile production among cucumber genotypes after JA treatment. There is a fair correlation between the response to spider mites and JA (e.g. high production in genotypes 1, 6 and 7 for both treatments and low for genotypes 2 and 3). In a preliminary Y-tube olfactometer experiment, the attractiveness of genotypes 1, 4, and 7 to predatory mites upon spider mite infestation was compared, showing a small, but significant preference towards genotype 1 (data not shown).

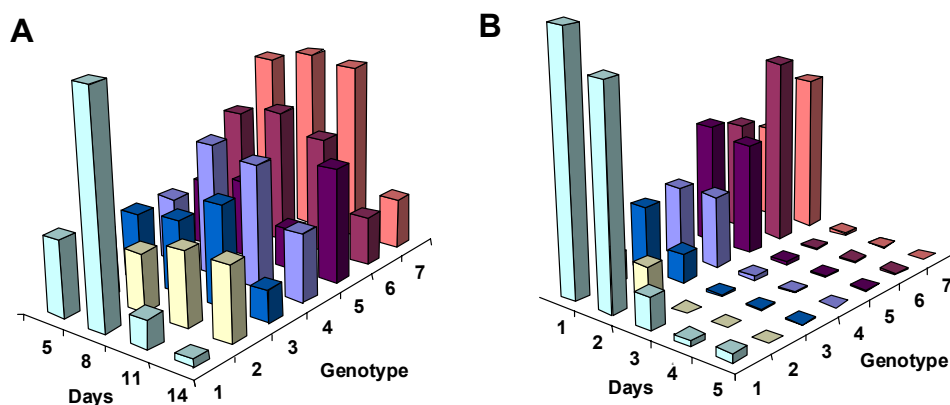


Figure 4. A, Time course of total volatile induction by spider mite feeding and B, jasmonic acid spraying in seven cucumber genotypes. A. 2-Wks old plants were infested with 50 spider mites on day 0. Leaves were sampled after 5, 8, 11 and 14 days for headspace analysis. B. 2-Wks old plants were sprayed with jasmonic acid on day 0. Leaves were sampled after 1, 2, 3, 4 and 5 days for headspace analysis.

HOW IS VOLATILE INDUCTION REGULATED?

Using ^{13}C pulse-labeling experiments it was demonstrated that most of the induced volatiles are biosynthesised *de novo* in response to herbivory (Pare and Tumlinson, 1997). This has led to the search for the induced enzymes involved in the formation of these volatiles. The first herbivory-induced enzyme to be identified was (3*S*)-(*E*)-nerolidol synthase in cucumber and lima bean (Bouwmeester *et al.*, 1999). This enzyme catalyses the conversion of farnesyl diphosphate to (3*S*)-(*E*)-nerolidol, a sesquiterpene alcohol, likely an intermediate in the formation of 4,8-dimethyl-1,3(*E*),7-nonatriene (DMNT) (Donath *et al.*, 1994; Donath and Boland, 1995) (Figure 1). Indeed DMNT is one of the major induced volatiles of cucumber (Figure 2) and all the evidence suggests that (3*S*)-(*E*)-nerolidol synthase is the regulatory step *en route* to this compound. DMNT is also an important constituent of the induced volatiles of other plant species such as lima bean, maize and cotton (Dicke *et al.*, 1990a; Turlings *et al.*, 1990; Takabayashi *et al.*, 1991; Loughrin *et al.*, 1994; Takabayashi *et al.*, 1994) and seems responsible for the attraction of the predatory mite *P. persimilis* to Lima bean and cucumber plants infested with the spider mite *T. urticae* (Dicke *et al.*, 1990b). A synthetic mixture of volatiles, including DMNT, that mimics the blend of volatiles that is emitted by corn plants infested with *S. exigua* caterpillars, attracted the parasitoid *C. marginiventris* which parasitizes the caterpillars (Turlings *et al.*, 1991).

MOLECULAR TOOLS

Elucidation of mechanisms

As mentioned, the elucidation of genotype differences and the role of individual volatiles in herbivory-induced indirect defence, is hindered by the lack of genotypes that differ in one factor only (*e.g.* only differ in the level of volatile production, not in composition and/or direct defence level) (Dicke and Hilker, 2003), but see (Van Poecke and Dicke, 2002) for a first example). With the advent of molecular tools, the use of transgenic plants is now becoming a feasible and exciting way to unravel not only the importance of individual compounds but also the regulation of their induction. Research in our laboratories has focussed on the major components of the blends of induced volatiles, the terpenoids. We have isolated and characterised a multitude of genes involved in terpene biosynthesis such as the monoterpene synthases (+)-limonene synthase, β -pinene synthase and γ -terpinene synthase from lemon (Lücker *et al.*, 2002), α -pinene synthase from strawberry (Aharoni *et al.*, 2003b), the sesquiterpene synthases amorpho-4,11-diene synthase from *Artemisia annua* (Mercke *et al.*, 2000; Wallaart *et al.*, 2001), two isoforms of germacrene A synthase from chicory (Bouwmeester *et al.*, 2002), (+)- and (-)-germacrene D synthase, α -gurjunene synthase and cascarilladiene synthase from *Solidago canadensis* (I G Altug, W A König & H J Bouwmeester, in prep) and a combined mono-/sesquiterpene synthase linalool/nerolidol synthase from strawberry (Aharoni *et al.*, 2003b). Most of these enzymes are involved in the constitutive (or developmentally regulated) biosynthesis of essential oils or flavour compounds in these plant species. They were cloned using PCR with degenerate primers or cDNA microarray analyses from plant tissues that were shown to produce the corresponding products, and hence needed no induction by herbivores.

Some of these enzymes catalyse the formation of compounds that have been shown, in other plant species, to be produced upon induction by herbivores. Examples are limonene and

linalool (Dicke *et al.*, 1990a; De Moraes *et al.*, 1998), germacrene A (Van den Boom *et al.*, 2002) and nerolidol. Nerolidol is found in the induced volatile blend of for example maize but is, more importantly, the first dedicated intermediate *en route* to DMNT (Bouwmeester *et al.*, 1999; Degenhardt and Gershenzon, 2000) (Figure 1). In addition, we have cloned two spider mite induced sesquiterpene synthases from cucumber, (*E,E*)- α -farnesene synthase and (*E*)- β -caryophyllene synthase (P M Mercke, I F Kappers, F W A Verstappen, M Dicke & H J Bouwmeester, in prep), two other major contributors to the induced volatile blend of many plant species (Dicke *et al.*, 1990a; De Moraes *et al.*, 1998) (Figure 1). In addition to the directed cloning of genes encoding enzymes involved in terpenoid biosynthesis, we and others use more generic approaches such as cDNA microarray analysis (Reymond *et al.*, 2000; Schenk *et al.*, 2000; P M Mercke, I F Kappers, F W A Verstappen, M Dicke & H J Bouwmeester, in prep) or DDRT-PCR (Hermsmeier *et al.*, 2001) to explore genes which are regulated by herbivory.

Use of transgenic plants

With the cloning of the genes involved in volatile biosynthesis it is becoming feasible to produce transgenic plants with overexpression or antisense/silencing to produce a new volatile blend. Alternatively, eventually transcription factors may be cloned that could be used to upregulate entire indirect defence pathways. Such plants would be the perfect tools to study the importance of individual compounds, blends, timing and magnitude of the plant's response for predator behaviour. In our laboratories, we have used a number of terpene synthases to transform model plant species. One of the first studies reported the transformation of petunia with the *Clarkia breweri* linalool synthase (Lücker *et al.*, 2001). Instead of the expected emission of linalool, transgenic plants were demonstrated to contain appreciable amounts of linalyl- β -D-glucoside, a non-volatile storage form of linalool, likely formed through the action of an endogenous petunia glycosyltransferase (Lücker *et al.*, 2001). In potato, transformation with a strawberry linalool synthase also led to the formation of glycosides of linalool, and also high amounts of free linalool were emitted, particularly from young plants (A Aharoni, H J Bouwmeester, M A Jongsma *et al.*, in prep) (Figure 5). The difference between the transgenic and wild type plants could easily be detected with the (untrained) human nose.

More straightforward results, i.e. with less side effects caused by endogenous enzymes, were obtained with other monoterpene synthases in tobacco. Tobacco was transformed with three lemon monoterpene synthases that were combined into one plant by crossing (Lücker *et al.*, 2002; Lücker *et al.*, 2003). Transgenic tobacco plants were obtained that produced up to seven new monoterpenes in leaves and flowers (three major products (-)- β -pinene, (+)-limonene and γ -terpinene plus some side products that had also been detected upon heterologous expression in *E. coli*) (Lücker *et al.*, 2003). Finally, the recent successful transformation of arabidopsis with a strawberry linalool/nerolidol synthase (Aharoni *et al.*, 2003a) brings the molecular advantages of arabidopsis into research on the effects of altered volatile blends on multi-trophic interactions. Arabidopsis has shown to be a good model plant for the investigation of induced indirect defence: feeding by the crucifer specialist *Pieris rapae* as well as other herbivorous arthropods results in the emission of volatiles that attract the parasitoid *Cotesia rubecula* (Van Poecke *et al.*, 2001; Van Poecke *et al.*, 2003). Preliminary Y-tube olfactometer experiments with the transgenic potato plants showed that the attractiveness to predators is enhanced by the introduction (or increase) of a single component in the volatile blend (Figure 5).

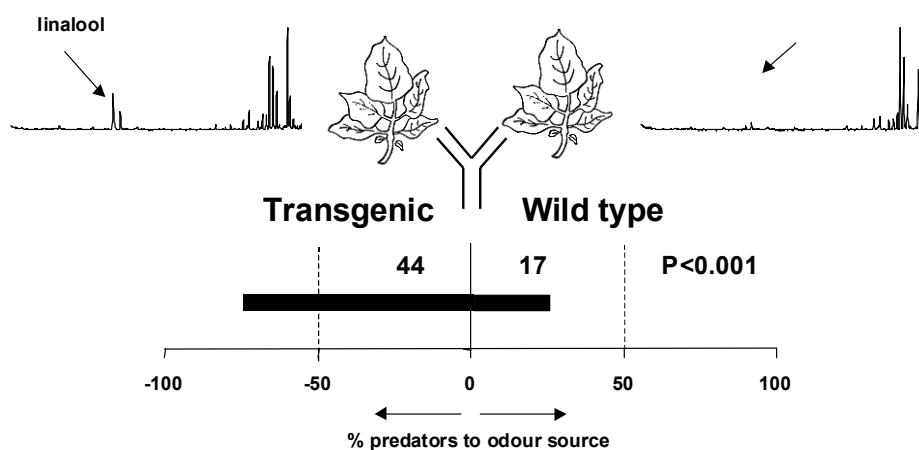


Figure 5. Attractiveness of non-damaged wild type and transgenic, linalool producing potato leaves to predatory mites. Bars represent choices of predatory mites reared on lima bean. Chromatograms show GC-MS analysis of the headspace of the odour sources.

CONCLUSIONS AND OPTIONS FOR NEW CONTROL METHODS

This review shows that our understanding of tritrophic interactions and the chemical signalling molecules and mechanisms involved is increasing rapidly. The use of this knowledge for the design of new control methods will depend on the agricultural system for which the method is intended. Biological control of herbivores in a glasshouse, for example, usually depends on the introduction of the predators, whereas in open systems natural enemies may be present or attracted from elsewhere (although also there it is feasible that introduction of natural enemies will become increasingly important). The introduction of enemies is of course costly and it is important that they survive for some time (which may mean a threshold level of their prey has to be accepted) and are able to find and control their prey quickly once it reaches a threshold level. The advantage of introduction is that the predators are reared under controlled conditions (constant quality) and can even be trained (Dicke *et al.*, 1990c). In open agricultural systems, the farmer in principle depends on the natural presence or invasion of predators, which also have to be kept alive, present and active when necessary. Now that many of the induced volatile blends have been identified, artificial mixtures could be used or alternatively, crops could be sprayed with jasmonic acid to induce volatile production that should lead to the increased presence of natural enemies (Thaler, 1999). However, there are examples where this approach has been unsuccessful (Chiri and Legner, 1983) and several authors have expressed the feeling that this approach will fail because the presence of the volatile cue and the prey are uncoupled (Dicke *et al.*, 1990a; Degenhardt *et al.*, 2003).

If the volatile cues and the presence of the prey are not uncoupled (*i.e.* attractive volatiles are only or mainly produced upon herbivory) then an adequate response of the crop to herbivory is most important. We have reviewed several studies in which the effect of environmental conditions on volatile production and herbivore attraction has been demonstrated and these results should be taken into account when designing future experiments. However, for a practical application, such as the optimisation of biological control, these factors may be

important and it would be of interest to see whether environmental conditions that stimulate induced volatile formation actually improve biological control in a field situation. Another as yet completely ignored factor in the optimisation of biological control is the selection for genotypes with improved (faster, stronger) response. Our results on cucumber (Figure 4), and results with other plant species, demonstrate that genetic variation for this response is available. Further research should demonstrate the effectiveness and the best and easiest way to exploit this variation in breeding.

Finally, it is tempting to speculate about the opportunities that lie within molecular approaches. The knowledge about the molecular regulation of indirect defence volatile formation is rapidly increasing, a multitude of structural genes are already available, and we have shown ample evidence that expression of these structural genes in plants can lead to the production of the expected volatiles. Making this volatile production dependent on herbivore-feeding is only a small step away that will be facilitated by our hunt for herbivore-feeding induced gene expression and hence inducible promoters. There are several indications in the literature that even though the induced volatile blends are sometimes extremely complex mixtures, there are major contributors to the attractive effect of these blends, which increases the chances for success of a molecular approach. Such a molecular approach could include genetic modification or the use of genetic (gene) markers in the selection process. Finally, it is conceivable that changes in the (induced) volatile production in commercial crops could lead to the development of biological control packages in which biological control agents trained specifically for the modified crop are included. It will be exciting to see whether these approaches can lead to plants with altered (improved) predator behaviour and to crops with improved biological control.

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