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Priming by airborne signals boosts direct and indirect resistance in maize

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Summary

Plants counteract attack by herbivorous insects using a variety of inducible defence mechanisms. The production of toxic proteins and metabolites that instantly affect the herbivore's development are examples of direct induced defence. In addition, plants may release mixtures of volatile organic compounds (VOCs) that indirectly protect the plant by attracting natural enemies of the herbivore. Recent studies suggest that these VOCs can also prime nearby plants for enhanced induction of defence upon future insect attack. However, evidence that this defence priming causes reduced vulnerability to insects is sparse. Here we present molecular, chemical and behavioural evidence that VOC-induced priming leads to improved direct and indirect resistance in maize. A differential hybridization screen for inducible genes upon attack by *Spodoptera littoralis* caterpillars identified 10 defence-related genes that are responsive to wounding, jasmonic acid (JA), or caterpillar regurgitant. Exposure to VOCs from caterpillar-infested plants did not activate these genes directly, but primed a subset of them for earlier and/or stronger induction upon subsequent defence elicitation. This priming for defence-related gene expression correlated with reduced caterpillar feeding and development. Furthermore, exposure to caterpillar-induced VOCs primed for enhanced emissions of aromatic and terpenoid compounds. At the peak of this VOC emission, primed plants were significantly more attractive to parasitic *Cotesia marginiventris* wasps. This study shows that VOC-induced priming targets a specific subset of JA-inducible genes, and links these responses at the molecular level to enhanced levels of direct and indirect resistance against insect attack.

Keywords: priming, volatile organic compounds, induced resistance, jasmonic acid, *Spodoptera littoralis*, *Cotesia marginiventris*.

Introduction

In response to attack by herbivorous insects, plants activate inducible defence mechanisms. Many of these inducible defences directly target the herbivore's physiology, and may protect the plant against further damage (Karban and Baldwin, 1997; Walling, 2000). In addition to this direct defence, herbivore-attacked plants are also capable of defending themselves in an indirect manner by releasing complex mixtures of volatile organic compounds (VOCs) that can attract predatory or parasitic arthropods, which are natural enemies of the attacking herbivore. This so-called indirect induced defence has been described for several agro-ecosystems, and constitutes an important plant strategy to reduce insect damage (Dicke *et al.*, 2003b; Pare and Tumlinson, 1999; Pichersky and Gershenzon, 2002; Turlings and Wäckers, 2004).

Apart from their role in indirect induced defence, herbivore-induced VOCs are also thought to enhance the level of resistance in neighbouring plants. Since the first experimental indications that herbivore-induced VOCs may be involved in defence activation in nearby plants (Baldwin and Schultz, 1983), various research groups have reported similar observations under both laboratory and field conditions (Baldwin *et al.*, 2006; Dicke *et al.*, 2003a). In an attempt to identify the key volatiles behind VOC-induced resistance,

many experiments have been performed in which plants were exposed to relatively high concentrations of synthetic, purified volatiles. These studies revealed that – in addition to methyl jasmonate – terpenoid volatiles and C6 green-leaf volatiles (GLVs) can activate jasmonic acid (JA)-dependent defence reactions in plants (Arimura *et al*., 2000, 2002; Bate and Rothstein, 1998; Farag and Pare, 2002; Ruther and Kleier, 2005; Yan and Wang, 2006). However, the intensity of these VOC-induced defence reactions remained usually weak in comparison with the defence reaction triggered by JA or elicitors from insect regurgitant. Furthermore, the concentration of the applied volatiles was often unrealistically high, and many of these chemicals induced less than the complete set of JA-inducible defence-related genes. For these reasons, the phenomenon of VOC-induced resistance has remained controversial for many years (Dicke *et al*., 2003a).

Engelberth *et al.* (2004) were the first to provide evidence for an alternative mechanism by which VOCs may enhance resistance in neighbouring plants. They demonstrated that VOCs from *Spodoptera exigua*-infested maize do not induce defence directly, but sensitize the plant for augmented induction of defence responses upon subsequent elicitation of defence by wounding and caterpillar regurgitant. This so-called priming for defence could be mimicked by exogenous application of three green GLVs, (*Z*)-3-hexenal, (*Z*)-3-hexen-1-ol or (*Z*)-3-hexenyl acetate. In contrast, exposure to VOCs from regurgitant-treated plants with only trace amounts of GLVs failed to evoke a reaction in neighbouring plants, suggesting that GLVs are the active compounds in the caterpillar-induced blend of VOCs. In contrast to these findings, Paschold *et al.* (2006) recently demonstrated that the VOC blend of *Manduca sexta*-infested *Nicotiana attenuata* fails to prime neighbouring *N. attenuata* for defence. Surprisingly, this blend contained compounds that had previously been shown to induce priming and/or defence in other plant species (GLVs and terpenoids; Engelberth *et al*., 2004; Ruther and Furstenau, 2005). On the other hand, VOCs from detached leaves of sagebrush (*Artemisia tridentata*) can prime *N. attenuata* for enhanced proteinase activity on attack by *M. sexta* (Kessler *et al*., 2006), which demonstrates that VOCs from clipped sagebrush contain priming-inducing compound(s) that are absent in the blend of *M. sexta*-infested *N. attenuata*. Hence VOC-induced priming entails a common and realistic defence phenomenon in plants, although the priming-inducing compound(s) may differ among different plant species.

Despite the fact that VOC-induced priming has been demonstrated in different plant species, it remains unclear how far this priming actually contributes to enhanced protection against insect attack. This prompted us to assess further the impact of VOC-induced priming on the efficacy with which the plant defends itself against insect attack. In this study we show that VOCs from caterpillar-infested maize prime neighbouring plants for augmented expression of a subset of defence-related, caterpillar-inducible genes. In addition, we provide combined evidence that this VOC-induced priming confers enhanced levels of direct and indirect protection against a herbivorous insect, the lepidopteran pest *Spodoptera littoralis*.

Results

Identification of defence-related, Spodoptera littoralis*-inducible genes*

To identify genes that are transcriptionally induced by *S. littoralis* feeding, a cDNA library was constructed from 2-week-old maize plants that had been infested for 48 h. Amplified cDNA clones were screened for differential hybridization between radiolabelled cDNA from non-infested plants and cDNA caterpillar-infested plants. Of the 376 cDNA clones tested, 19 clones showed enhanced hybridization to cDNA from *S. littoralis*-infested plants (caterpillar-inducible), whereas 14 showed enhanced hybridization to cDNA from non-infested control plants (caterpillar-repressed). Sequence analysis of 13 *S. littoralis*-inducible clones resulted in 10 different gene sequences (Figure 1a; Table 1). BLASTn homology analysis revealed that nine clones are identical to previously identified expressed sequence tags (ESTs). One EST clone (no. 4) did not correspond to any known maize sequence, but showed significant DNA homology with a pathogen-inducible EST from *Saccharum officinarum* (sugarcane). BLASTx analysis of the predicted amino acid sequence revealed that most ESTs represent genes with functions related to plant defence. The predicted amino acid sequence of EST clones 2, 3 and 7 are identical to a B73 lipoxygenase (B73 Lox), a maize proteinase inhibitor (MPI), and a cystatin II proteinase inhibitor (Cystatin II PI), respectively (Table 1). The predicted amino acid sequences of EST clones 5 and 9 show high levels of homology to other cystatin proteinase inhibitor proteins of maize (Table 1). BLASTx analysis of EST clone 10 revealed homology to a pathogen-inducible lectin from wheat (*Triticum aestivum*), whereas clones 1, 6 and 8 displayed homology to a L6E ribosomal protein, a serine protease inhibitor, and a putative esterase/lipase, respectively, from rice (*Oryza sativa*; Table 1). BLASTx analysis of EST clone 4 did not result in any statistically significant hit, probably because this EST represents the 3′-untranslated region of the gene. However, BLASTx analysis of the corresponding *S. officinarum* homologue revealed significant levels of similarity with various glycine-rich proteins from rice and maize (data not shown).

Expression pattern of Spodoptera littoralis*-inducible genes*

The expression profile of the 10 *S. littoralis*-inducible ESTs was studied by Northern blot analysis in 8-day-old

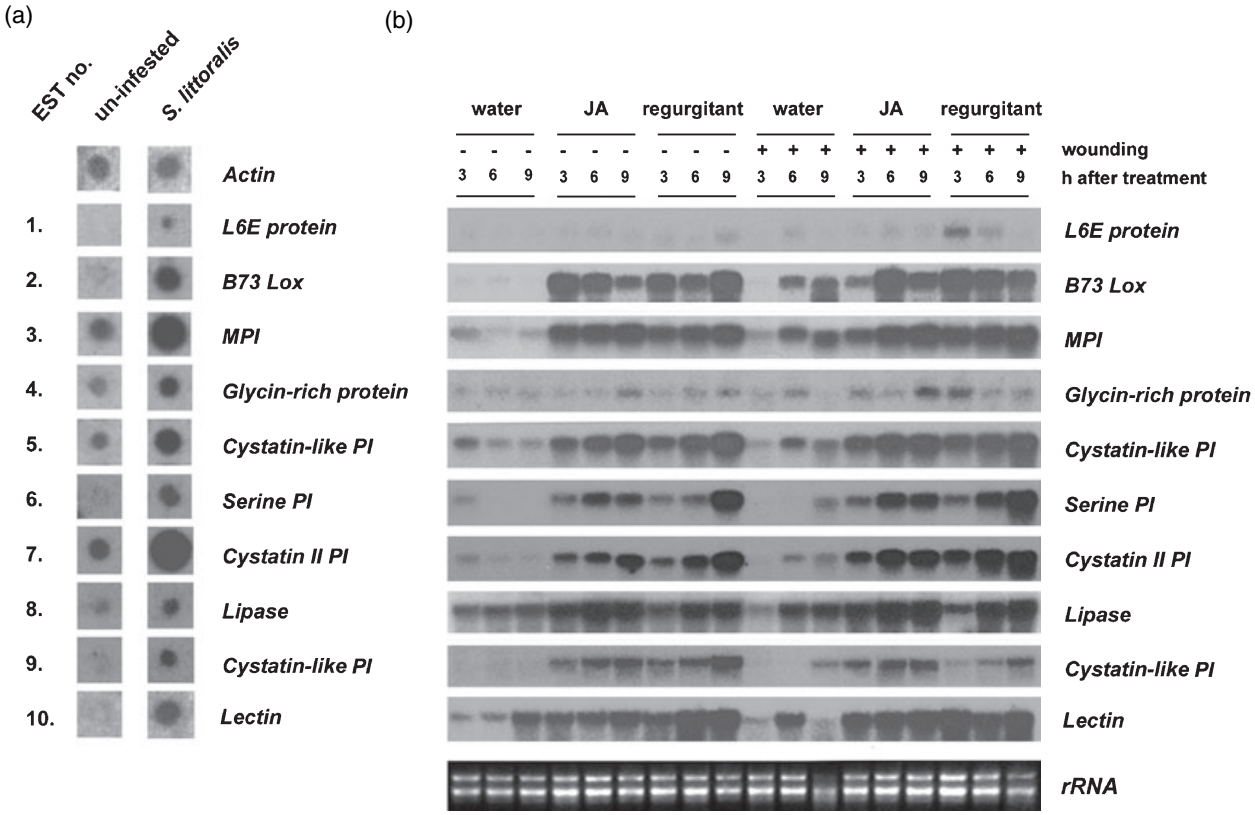

Figure 1. Differential expression of 10 *Spodoptera littoralis*-inducible maize genes.
(a) Differential hybridization between cDNA from uninfested and *S. littoralis*-infested maize to 10 EST clones from *S. littoralis*-infested maize.
(b) Northern blot analysis of 10 *S. littoralis*-inducible ESTs at 3, 6 and 9 h after treatment with water, 200 μM jasmonic acid (JA) or 50% v/v *S. littoralis* regurgitant, with or without additional wounding.

Table 1 BLAST analysis of 10 *S. littoralis*-inducible EST clones

	DNA homology (BLASTN)			Protein homology (BLASTX)			
EST no.	Accession number[a]	Identity (%)	*E*-value	Accession number[a]	Identity (%)	*E*-value	Putative function
1	AY103559	100	0	XP_472843	84	4e-35	L6E ribosomal protein (*Oryza sativa*)
2	AF465643	100	0	AAL73499	100	0	B73 lipoxygenase (*Zea mays*)
3	X78988	100	0	CAA55588	100	0	Proteinase inhibitor MPI (*Z. mays*)
4	CA196921	76	6e-14	–	–	–	Glycine-rich protein (*S. officinarum*)
5	CK827737	100	0	AAY57866	97	1e-45	Cystatin-like proteinase inhibitor (*Z. mays*)
6	BM382058	100	0	NP_910046	48	2e-15	Serine protease inhibitor (*O. sativa*)
7	D38130	100	0	BAA07327	100	0	Cystatin II proteinase inhibitor (*Z. mays*)
8	AY105196	100	0	AAV59435	87	5e-72	Lipase/esterase (*O. sativa*)
9	BM072984	100	0	CAG29028	98	6e-69	Cystatin-like proteinase inhibitor (*Z. mays*)
10	CF032590	100	0	AAW48295	54	1e-08	Lectin (*Triticum aestivum*)

[a]GenBank accession number.

seedlings on treatment with water, 200 μM JA or 50% (v/v) *S. littoralis* regurgitant, with or without additional wounding. Nine genes displayed relatively weak induction by wounding alone, but much stronger induction by treatment with JA or *S. littoralis* regurgitant. This suggests that JA is the primary signal in the activation of these genes. However, EST 1 encoding a putative L6E protein was not responsive to JA treatment, but showed significant induction upon the combined treatment of wounding and regurgitant (Figure 1b). Apparently the activation of this gene requires caterpillar-induced signals additional to JA.

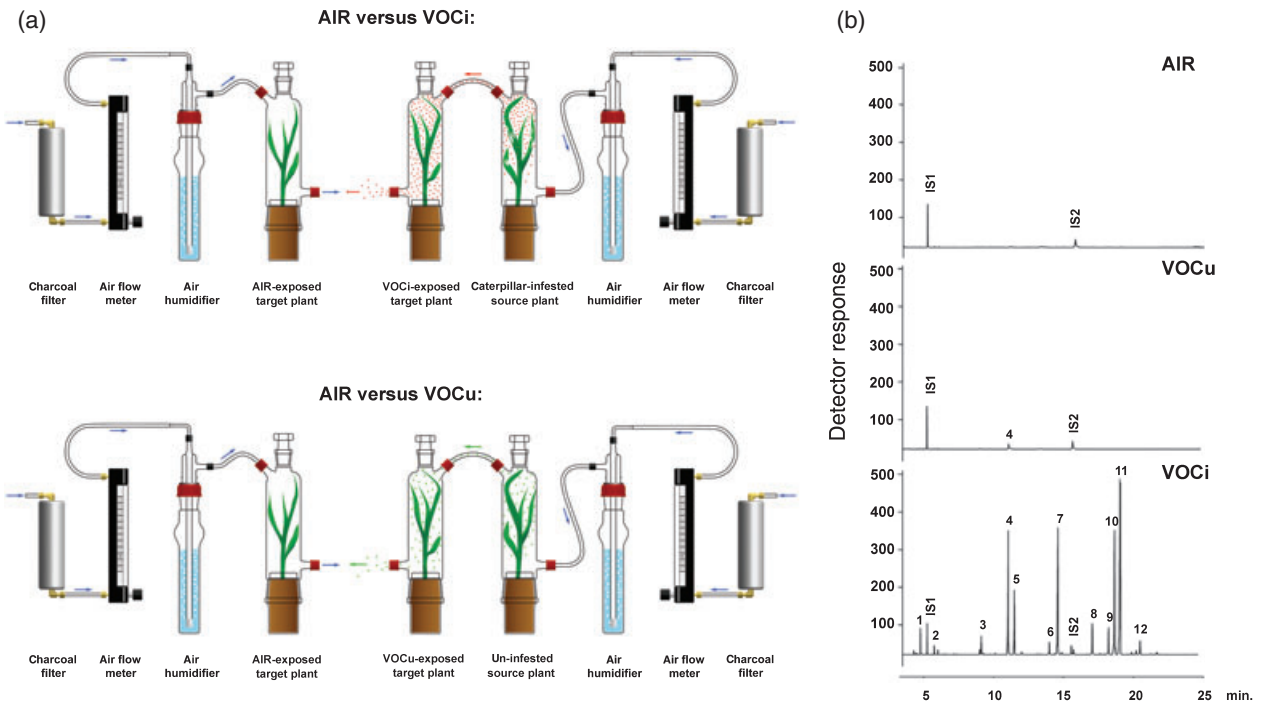

Figure 2. Experimental conditions of the different exposure treatments.
(a) Schematic drawing of the two experimental set-ups used to expose maize to volatile organic compounds (VOCs) from neighbouring plants. A continuous stream of charcoal-filtered, humidified air was pushed through the system at a speed of 0.4 l min^{-1}. Target plants were placed in glass vessels and exposed for 16 h to clean air, or to caterpillar-infested source plants (AIR versus VOCi). As an extra control, air-exposed plants were also compared with plants exposed to VOCs from uninfested plants (AIR versus VOCu).
(b) Gas chromatograms (GC-FID) of the three different odour sources. 1, (*Z*)-3-hexenal; 2, (*E*)-2-hexenal; 3, (*Z*)-3-hexenyl acetate; 4, linalool; 5, 4,8-dimethyl-1,3(*E*),7-nonatriene; 6, phenethyl acetate 7, indole; 8, geranyl acetate; 9, (*E*)-β-caryophyllene; 10, (*E*)-α-bergamotene; 11, (*E*)-β-farnesene; 12, β- sesquiphellandrene. IS, internal standard.

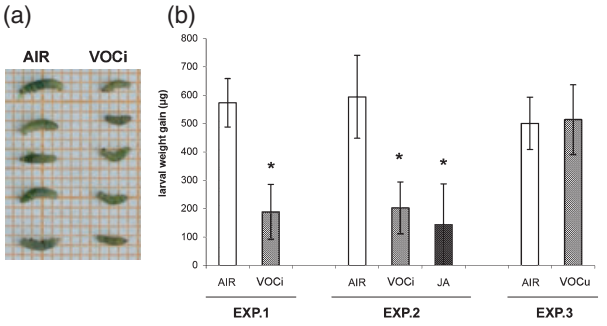

Figure 3. Volatile organic compound (VOC)-induced resistance against *Spodoptera littoralis* caterpillars.
Plants were exposed for 16 h to clean air (AIR), VOC from *S. littoralis*-infested plants (VOCi), or VOC from uninfested plants (VOCu). As a positive control, plants were soil-drenched with 500 µM jasmonic acid (JA) 1 day before infestation.
(a) Representative differences in size between caterpillars from plants that had been exposed to clean air and plants that had been exposed to VOC from infested plants. Pictures were taken at 11 h of infestation.
(b) Larval weight-gain values in different experiments. Plants were exposed to clean air, VOC from infested plants or VOC from uninfested plants, or treated with JA. Values represent means ± SD; $n = 60$. Asterisks indicate statistically significant differences from air-exposed control plants (Student's *t*-test, $\alpha = 0.05$).

VOCs from infested plants reduce Spodoptera littoralis *feeding in neighbouring plants*

To test whether VOCs from caterpillar-infested maize plants induce insect resistance, we used an experimental set-up that allows continuous air flow from *S. littoralis*-infested plants to non-infested target plants (Figure 2a). Control plants were exposed to clean air for a similar period. After exposure, plants were infested with *S. littoralis* caterpillars for 11 h, upon which larval weight gain was assessed. Plants pre-exposed to VOC from caterpillar-infested plants (VOCi) allowed significantly lower levels of caterpillar growth than plants pre-exposed to clean air ($P < 0.05$; Figure 3). This effect on caterpillar growth was less pronounced for longer periods of infestation (data not shown), suggesting that differences in foraging behaviour at the early stages of infestation account for the observed effects. The level of VOC-induced resistance was almost comparable with that of plants that had been soil-drenched with 500 µM JA (Figure 3). On the other hand, plants that had been exposed to VOCs from uninfested plants (VOCu) allowed similar levels of caterpillar growth to air-exposed plants (Figure 3). Hence VOC-induced resistance is caused by compounds that are specific for the blend from infested plants.

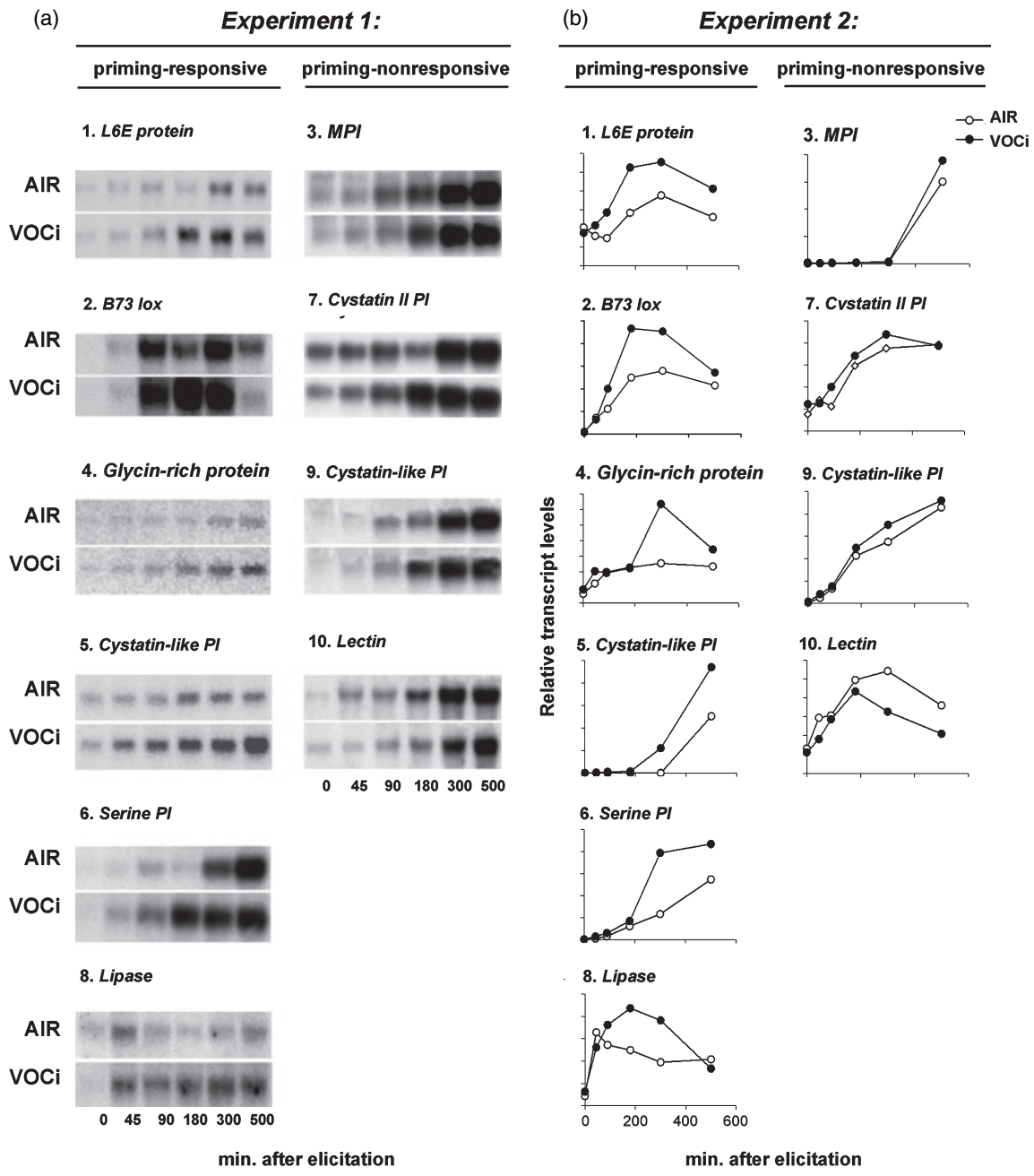

Figure 4. Volatile organic compound (VOC)-induced priming for enhanced expression of defence-related, caterpillar-inducible genes in two independent experiments.
Plants were exposed for 16 h to clean air (AIR) or VOCs from *Spodoptera littoralis*-infested plants (VOCi). Shoot material was collected immediately after exposure treatment or at different time points after defence elicitation by wounding and *S. littoralis* regurgitant. Defence-related gene expression was quantified by Northern blot analysis (a) or quantitative (Q)-RT-PCR analysis (b).

VOC-induced priming targets a subset of Spodoptera littoralis*-inducible genes*

To examine whether the observed induced resistance against *S. littoralis* is based on direct defence activation or priming, we quantified expression of the *S. littoralis*-inducible defence genes in two independent experiments. Plant material was collected directly after exposure treatment and at different time points after subsequent defence elicitation by applying *S. littoralis* regurgitant on wounded leaf areas. The level of gene expression was quantified in two independent experiments by Northern blot analysis (first experiment; Figure 4a) or quantitative (Q)-RT-PCR; second-experiment; Figure 4b). In both experiments, plants

pre-exposed to caterpillar-induced VOCi showed no direct activation of JA-inducible defence genes compared with air-exposed control plants. This suggests that the observed VOC-induced resistance is not based on direct induction of JA-inducible defence mechanisms. Upon subsequent defence elicitation by wounding and regurgitant, all genes showed induced expression. Interestingly, six genes consistently showed an earlier and/or stronger transcriptional induction in the plants that had been exposed to VOCi. The four other genes remained unaltered in the speed and intensity with which they were induced (Figure 4). This demonstrates that only a subset of caterpillar-inducible defence-related genes is responsive to VOC-induced priming.

VOCs from infested plants prime for enhanced production of aromatic and terpenoid volatiles

To examine whether VOC-induced priming also targets the plant's indirect defence response, levels of induced VOC emission were measured at different time points after defence elicitation (Figure 5a,b). Exposure to VOCi alone did not enhance VOC emissions in the target plants (Figure 5b). However, at different time-points after defence elicitation, emissions of aromatic volatiles such as indole, and various terpenoid compounds such as linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*)-β-farnesene, were significantly enhanced in the VOCi-exposed target plants (Figure 5a,b). The release of GLVs was not statistically different between both treatments. Moreover, target plants that had been exposed to VOCu from undamaged plants did not show augmented emissions of aromatic and terpenoid compounds. Hence VOCi from caterpillar-infested maize prime neighbouring plants for enhanced production of aromatic and terpenoid compounds.

VOC-induced priming enhances attractiveness to parasitic Cotesia marginiventris *wasps*

To investigate whether the observed augmentation in the emission of aromatic and terpenoid compounds renders primed plants more attractive to parasitic *C. marginiventris* wasps, air- and VOCi-exposed plants were tested in a six-arm olfactometer (Turlings *et al.*, 2004). As shown in Figure 6, elicited plants attracted considerably more wasps than non-elicited control plants. Within the defence-elicitation treatments, VOCi-exposed plants attracted more wasps than air-exposed plants (Figure 6). This difference in attractiveness was statistically significant ($P < 0.01$) in the

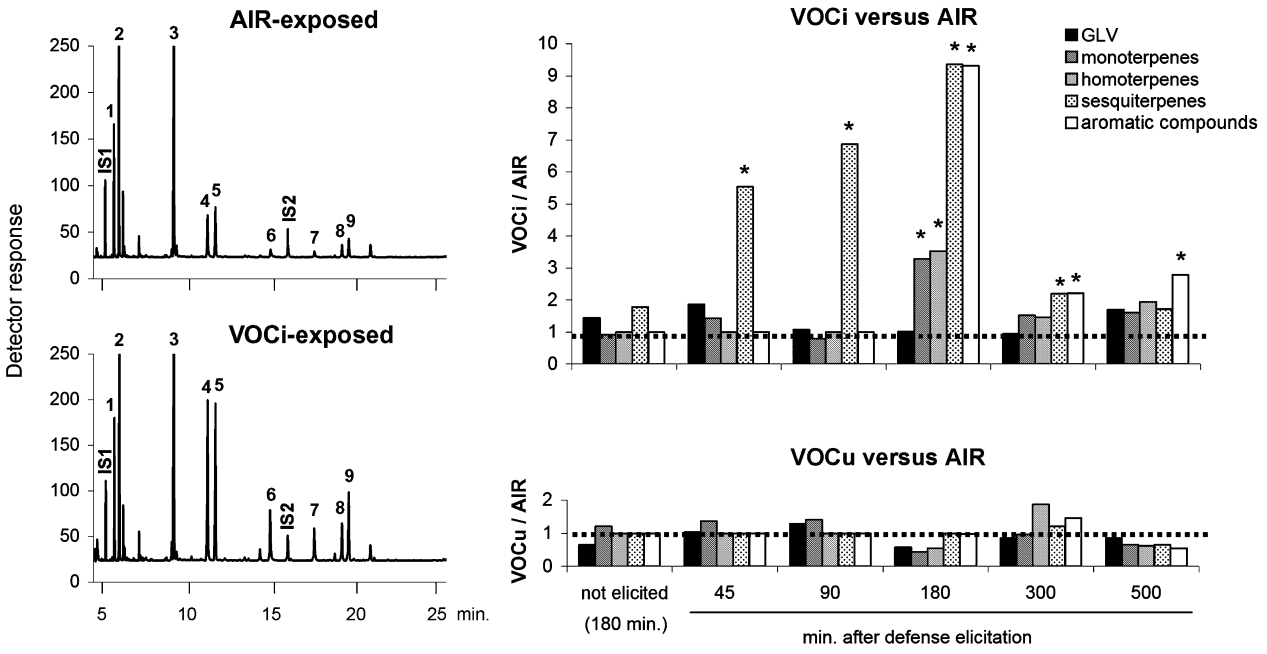

Figure 5. Volatile organic compound (VOC)-induced priming for enhanced emission of various VOCs upon defence elicitation by wounding and *Spodoptera littoralis* regurgitant. VOCi, volatiles from infested plants; VOCu, volatiles from uninfested plants.
(a) Typical gas chromatograms of VOCs from plants at 180 h after elicitation of defence. Prior to elicitation treatment, plants were exposed to air (AIR) or VOC from infested plants (VOCi) for 16 h. Major compounds were: 1, (*E*)-2-hexenal; 2, (*Z*)-3-hexen-1-ol; 3, (*Z*)-3-hexenyl acetate; 4, linalool; 5, 4,8-dimethyl-1,3(*E*),7-nonatriene; 6, indole; 7, geranyl acetate; 8, (*E*)-α-bergamotene; 9, (*E*)-β-farnesene. IS, internal standard.
(b) VOC emission ratios between air- and VOC-exposed plants at different time points after elicitation treatment. Values represent ratios between average amounts of the different classes of VOCs, as specified in Experimental procedures. Ratios were set to one if none of the compounds was detected in either treatment. Asterisks indicate statistical differences between total average amounts from air- and VOC-exposed plants (Student's *t*-test, $\alpha = 0.05$).

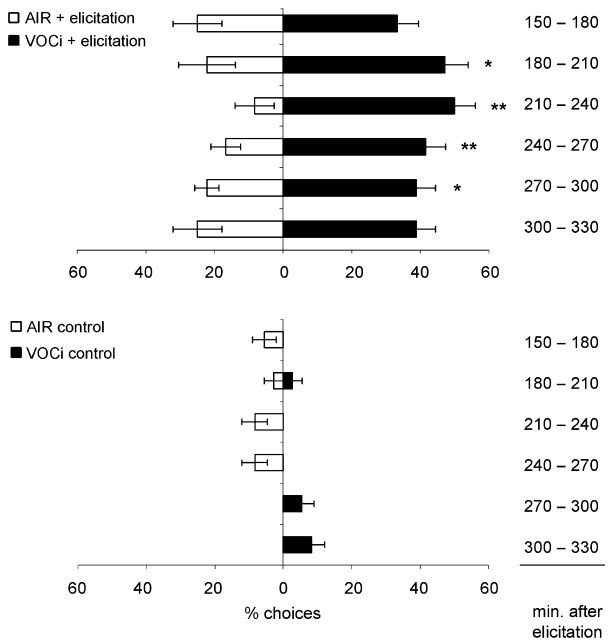

Figure 6. Responses of the parasitic wasp *Cotesia marginiventris* to plants exposed to clean air (AIR) or volatile organic compounds from *Spodoptera littoralis*-infested maize (VOCi). Values shown are average percentages of parasitoids choosing an olfactometer arm containing air- or VOCi-exposed plants at different time points after defence elicitation. Control plants were not subjected to elicitation treatment. Asterisks indicate statistically significant differences in attractiveness between air- and VOCi-exposed plants (*$P < 0.01$; **$P < 0.001$).

period between 180 and 300 min after defence elicitation (Figure 6), which corresponds exactly to the period when the augmented production of aromatic and terpenoid compounds was most pronounced (Figure 5). These findings indicate that VOC-induced priming for enhanced production of aromatic and terpenoid compounds boosts the effectiveness of the plant's indirect induced defence response.

Discussion

In this study we used molecular, chemical and behavioural approaches to determine the effectiveness of VOC-induced priming against herbivory. Exposure to VOCi from infested maize plants reduced the growth of *S. littoralis* caterpillars on neighbouring plants, and concomitantly conferred priming for augmented induction of six caterpillar-inducible defence genes. Although this correlation provides no evidence for a causal relationship between the induced resistance, on the one hand, and the function of the priming-responsive genes, on the other, most of the priming-responsive genes encode proteins with functions related to plant defence (Table 1). This strongly suggests that VOC-induced priming boosts the direct defence response of the plant, resulting in reduced feeding by caterpillars. Interestingly, this induced resistance was most pronounced during the first hours after caterpillar application. Differences in feeding behaviour during the earlier stages of infestation appear to account for the observed effects. The loss of differential plant resistance at later stages of infestation may be explained by the slower defence response of non-primed control plants having 'caught up' with that of primed plants, but could also be the result of an adaptation of the caterpillars to the plant's inducible defence mechanisms. The latter explanation is supported by several reports that *Spodoptera* caterpillars have the ability to activate gut proteases that are resistant to the protease inhibitors of the host plant (De Leo *et al.*, 1998; Jongsma *et al.*, 1995).

The VOCi-induced priming for augmented transcription of defence-related genes was observed in direct comparison with air-exposed control plants (Figure 4). The possibility that VOCu from uninfested plants trigger a similar transcriptional priming is highly unlikely. In our continuously flowing exposure system, amounts of basal VOCu emissions from source plants (linalool) would have been negligible compared with the internal concentrations of these compounds in target plants. Secondly, the VOCu blend from uninfested plants did not contain any detectable amounts of GLVs (Figure 2b), which have been demonstrated to be important for VOC-induced priming in maize (Engelberth *et al.*, 2004). Finally, it is very likely that most plants had already been exposed to VOCu prior to testing, as they had all been cultivated in close proximity to each other. This is supported by our control experiments, which clearly demonstrated that exposure to VOCu had no effect on the level of induced resistance and primed VOC emission in comparison to air-exposed plants (Figures 3 and 5b). It is therefore plausible to assume that the six *S. littoralis*-inducible genes are responsive to priming by VOCi from caterpillar-infested plants. As such, the augmented induction of these genes can be used as a marker for primed defence expression in maize.

Over time, we also found a temporary augmentation of indirect defence. VOC-exposed plants produced enhanced levels of aromatic and terpenoid volatiles between 180 and 300 min after defence elicitation, which coincided with improved attraction of parasitic *C. marginiventris* wasps during this period. Hence exposure to VOCi from caterpillar-infested plants accelerates and intensifies both direct and indirect defence mechanisms, contributing to enhanced resistance at the second and third trophic levels. In accordance with this, Heil and Kost (2006) have recently demonstrated VOC-induced priming for indirect defence in lima bean. It was shown that exposure to an artificial blend of VOCs, which resembled the blend from herbivore-infested plants, conferred enhanced secretions of extrafloral nectar upon wounding. The fact that this treatment had previously been shown to cause enhanced attraction of predatory and parasitoid insects, as well as reduced herbivore damage and increased production of inflorescences and leaves (Kost and

Heil, 2006), suggests an important ecological relevance of VOC-induced priming for indirect defence.

The physiological and molecular mechanisms behind the VOC-induced priming remain largely unknown. Nevertheless, it seems evident that VOC-induced priming targets defences that are controlled by JA (Engelberth *et al.*, 2004). The fact that some JA-inducible genes were not priming-responsive (Figures 1 and 4) suggests that JA is not the only defence regulatory compound that controls the augmented defence response in primed plants. It is therefore conceivable that there are additional layers of regulation that involve as-yet unidentified signalling compounds. In this context, the gaseous hormone ethylene may be an important priming factor in addition to GLVs. Ethylene emission is induced upon insect attack (De Vos *et al.*, 2005; Schmelz *et al.*, 2003), and has been shown to function as an important modulator of JA-inducible defences against insects (Harfouche *et al.*, 2006; Van Loon *et al.*, 2006). Interestingly, ethylene can enhance the induction of some JA-dependent genes (Penninckx *et al.*, 1998), whereas other JA-inducible genes are unaffected, or even repressed, by ethylene (Anderson *et al.*, 2004; Shoji *et al.*, 2000). Furthermore, Ruther and Kleier (2005) recently showed that ethylene synergizes the emission of maize sesquiterpene compounds upon treatment with high doses of the GLV (*Z*)-3-hexen-1-ol. Future experiments with mutant plants that are impaired in the production or perception of ethylene could further specify the regulatory role of ethylene in VOC-induced priming.

Priming for JA-dependent defence has also been reported in response to other resistance-inducing agents. In Arabidopsis, treatment with the root-colonizing *Pseudomonas fluorescens* WCS417r triggers an induced systemic resistance (ISR) effective against pathogens and insects that are sensitive to JA-dependent resistance mechanisms (Ton *et al.*, 2002b). Root colonization by these *P. fluorescens* WCS417r bacteria does not result in direct activation of JA-dependent defence responses, but primes for augmented expression of JA-responsive genes upon subsequent pathogen attack (Van Wees *et al.*, 1999; Verhagen *et al.*, 2004). Hence *P. fluorescens* WCS417r-mediated ISR is based on priming for JA-dependent defences that are specifically engaged during pathogen attack. Large-scale transcription profiling experiments of ISR-expressing Arabidopsis have revealed that a distinct subset of JA-inducible genes are priming-responsive to ISR treatment with rhizobacteria (Pozo *et al.*, 2005; Verhagen *et al.*, 2004). Interestingly, the promoter regions of these priming-responsive genes are enriched in binding sites for specific transcription factors (M.J. Pozo and C.M.J. Pieterse, Utrecht University, The Netherlands, unpublished data), which suggests a sophisticated regulatory mechanism where specific transcription factors regulate the augmented expression of the JA-inducible, priming-responsive defence genes.

Several recent studies have shown that herbivore-inducible VOCs prime for direct and indirect plant defence mechanisms (Engelberth *et al.*, 2004; Heil and Kost, 2006; Kessler *et al.*, 2006). The current study reveals further details about the molecular mechanisms behind VOC-induced priming. In addition, we have demonstrated that VOC-induced priming can target organisms at the second and third trophic levels. Priming for defence against pathogen infection has been shown to offer a significant advantage over induction of direct defence (Van Hulten *et al.*, 2006). In the case of VOC-induced defence priming against insects, Kessler *et al.* (2006) were able to demonstrate priming under field conditions in wild tobacco upon exposure to VOCs from clipped sagebrush. Although both plant species rarely co-occur in nature, their study does demonstrate a potentially ecological relevance of VOC-induced priming for direct plant defence. Here we show this potential for both direct and indirect plant defence. A detailed cost–benefit analysis of priming for defence against insects has yet to be performed. If the advantages of VOC-induced priming against insects are comparable with those of priming against pathogens (Conrath *et al.*, 2002; Van Hulten *et al.*, 2006), the phenomenon offers an extremely useful concept for future strategies in crop protection (Turlings and Ton, 2006). Therefore a complete understanding of the molecular mechanisms and ecological consequences of VOC-induced priming will be an important next step towards exploitation of the phenomenon in sustainable agriculture.

Experimental procedures

Plant material, cultivation and treatments

Seedlings (*Zea mays* var. Delprim) were grown individually in plastic pots (10 cm high, 4 cm diameter) in potting soil (Ricoter Aussaterde, Aarberg, Switzerland) under controlled conditions (23 ± 2°C; 60% RH; 16 h light/8 h dark; 50 000 Lux). Material for the cDNA library was derived from shoots of 14-day-old plants that had been infested for 2 days by 10 second-instar *S. littoralis* larvae. Material for gene-expression analysis in response to wounding, JA or *S. littoralis* regurgitant was derived from detached shoots of 8-day-old plants that had been incubated in 2 ml water, 200 μM (±) JA (Sigma-Aldrich, Buchs, Switzerland), or 50% (v/v) regurgitant for 3, 6 or 9 h. Wounding was inflicted by scratching the undersides of two leaves over an area of approximately 1 cm^2 on both sides of the central vein with a razor blade. In all other experiments, plants were 10–13 days old with three fully developed leaves. Source plants for the VOC-exposure experiments were introduced individually into glass vessels (Turlings *et al.*, 2004) and infested with 15 second-instar *S. littoralis* larvae by applying them into the whorl of the youngest leaves. Infested source plants were maintained for 6 h under laboratory conditions with supplemented light (26 ± 1°C; 40 ± 10% RH; 16 h light/8 h dark; approximately 8000 Lux at plant height). Target plants were similarly introduced into glass vessels and connected to a specifically developed multiple air-delivery (MAD) system via Teflon tubing (Figure 2). This system consisted of a central wooden tray with two rows containing six glass odour

vessels (Turlings *et al.*, 2004), a metal frame with eight neon tubes (four Osram 18W/21-810 alternated with four Sylvania Gro-Lux F18W/GRO-T8), and two manifolds with six flow meters (Aalborg Instruments & Controls; Monsey, NY, USA), each followed by charcoal filters and water bubblers filled with MilliQ-water (Model VCS-HADS-6AF6C6B; ARS Analytical Research System, Gainsville, FL, USA). Plants were exposed to humidified clean air; air from herbivore-infested plants; or air from uninfested plants at a flow-rate of 0.4 l min^{-1} for 16 h (8 h light/8 h dark). In the bioassays for induced resistance against *S. littoralis* infestation, induction treatment with JA was performed by soil drenching to a final concentration of 500 μM, 1 day before challenge infestation. In the bioassays for priming for gene expression and VOC production, defence elicitation was performed by scratching the undersides of two leaves at two different locations over an area of approximately 1 cm^2 on both sides of the central vein with a razor blade, after which aliquots of 10 μl 50% (v/v) *S. littoralis* regurgitant were distributed evenly over the scratched leaf areas. Regurgitant was collected from fourth-and fifth-instar *S. littoralis* that had been feeding on maize leaves for at least 2 days, and stored at −76°C until use.

Insect material

Larvae of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) were reared from eggs on artificial wheatgerm diet as described previously (Turlings *et al.*, 2004). One day before testing in the induced resistance bioassays, second-instar larvae were maintained on 2-week-old maize leaves. Adult *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) parasitoids were reared as described previously (Turlings *et al.*, 2004). Before testing in the behavioural assays, female parasitoids were trained by allowing them to oviposit two to three times into second- or third-instar *S. littoralis* larvae while being exposed to the complete VOC blend of 2-week-old maize plants that had been infested by second-instar *S. littoralis* for 24 h.

RNA extraction, Northern blotting and Q-RT-PCR

Total RNA was extracted from pooled shoot samples ($n = 3$–5). Frozen leaf tissue was homogenized in extraction buffer (0.35 M glycine, 0.048 N NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% (w/v) SDS; 1 ml g^{-1} leaf tissue), extracted with phenol/chloroform, and RNA was precipitated using LiCl as described by Sambrook *et al.* (1989). For Northern blot analysis, 10 μg RNA was denatured using glyoxal and dimethyl sulfoxide (Sambrook *et al.*, 1989). Samples were separated electrophoretically on ethidium bromide-stained 1.5% agarose gels, checked for equal loading, and blotted onto Hybond-N^+ membranes (Amersham, Dübendorf, Switzerland) by capillary transfer as described (Ton *et al.*, 2002a). DNA probes were derived from PCR products (M13/rev) of cDNA clones in pGEM-T easy vectors by random labelling with α-^{32}P-dCTP using the Prime-a-gene labelling kit (Promega, Wallisellen, Switzerland). Quantitative (Q)-RT-PCR was performed essentially as described previously (De Vos *et al.*, 2005). Total RNA (5 μg) was digested with Turbo DNA-free (Ambion, Huntingdon, UK) and checked for genomic DNA contamination by conventional PCR (40 cycles) of the *Actin-1* gene (forward: 5′-TTGCCCTTGATTATGAACAG-3′; reverse: 5′-AGGTGGCGCAACTACTTTA-3′). DNA-free RNA was converted into cDNA using oligo-dT20 primers, 10 mM dNTPs and Superscript (Invitrogen, Breda, the Netherlands). Efficiency of cDNA synthesis was assessed by Q-RT-PCR using primers of the constitutively expressed *Glycerol phosphate dehydrogenase C* gene (GAPC; forward: 5′-GCATCAGGAACCCTGAGGAA-3′; reverse: 5′-CATGGGTGCATCTTTGCTTG-3′). Based on the C_T (threshold cycle) values of GAPC, cDNA samples were diluted to similar quantities. Primers for Q-RT-PCR analysis of the *S. littoralis*-inducible ESTs are shown in Table 2. Q-RT-PCR was performed in optical 96-well plates with a MyIQ Single Color Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands), using SYBR Green to monitor dsDNA synthesis. Each reaction contained 1 μl cDNA, 0.5 μl of each of the gene-specific primers (10 pmol μl^{-1}) and 10 μl 2 × IQ SYBR Green Supermix reagent (Bio-Rad) in a final volume of 20 μl. The following PCR program was used for all Q-RT-PCR reactions: 95°C for 3 min; 40 cycles of 95°C for 30 sec, 59.5°C for 30 sec, and 72°C for 30 sec. C_T values were calculated using OPTICAL SYSTEM software ver. 1.0 for MyIQ (Bio-Rad). C_T values of the *S. littoralis*-inducible genes were normalized for differences in double-stranded DNA synthesis using GAPC C_T values.

Differential hybridization screen

Total RNA was extracted from pooled shoot samples ($n = 5$) of 14-day-old plants that were uninfested (control), or had been infested for 2 days by 10 second-instar *S. littoralis* larvae. Purification of mRNA was performed using the Oligotex mRNA midi-kit (Qiagen AG, Basel, Switzerland). Double-cDNA synthesis and directional cloning into λUni-ZAP XR was done using the ZAP-cDNA synthesis kit (Stratagene, Amsterdam, the Netherlands). Size fragmentation of cDNA fragments >350 bp was performed by gel extraction (Promega) after separation on a 1.0% agarose Tris–acetic acid–EDTA gel. Appropriate dilutions of the λ cDNA library (10^{-3} to 10^{-5}) were plated onto *E. coli* XL-1 Blue agar plates. Gene clones were amplified by PCR (35 cycles) after transferring individual plaques into 96-well plates containing 30 μl standard PCR mix with M13/rev primers. Amplified clones were spotted *in duplo* (10 μl) onto Hybond+ membranes (Promega) using the bio-dot micro-infiltration apparatus (Bio-Rad, Reinach, Switzerland). Membranes were hybridized

Table 2 Primer sequences used for Q-RT-PCR analysis of the different ESTs

EST no.	Forward primer	Reverse primer
1	5′-TCAAGTCTGGCCTGCTCCTT-3′	5′-ACTTGGCGACATCAACACCA-3′
2	5′-GCGACACCATGACCATCAAC-3′	5′-GCTCGGTGAAGTTCCAGCTC-3′
3	5′-ATGAGCTCCACGGAGTGC-3′	5′-TCAGCCGATGTGGGGCGTC-3′
4	5′-GGCGACGATAAATTTGAATGC-3′	5′-TCAAAAGCCAGACACATGCAC-3′
5	5′-AGGGCTTGTTCGGTTAGGTG-3′	5′-TGCAGAATAAGGAGCCATGC-3′
6	5′-GACGGAGGAGGAAGGAGGAG-3′	5′-ACCTGATGCACTGCTTGCAC-3′
7	5′-TGCCCTGCTCATACTGCTTG-3′	5′-GCGAGTTCCTGGAGGTGAAG-3′
8	5′-CCAAGAGCCTCATCATCGTG-3′	5′-CGTGGTAGTGGTCCGTGTTG-3′
9	5′-CAAGGAGCACAACAGGCAGA-3′	5′-GGACATGAGCTGGCGATTTT-3′
10	5′-TCGTCGTCCTTGGAGAGCTT-3′	5′-CATCTGCCAAGTCCCCTTCT-3′

with ^{32}P-CTP-labelled cDNA from either uninfested or *S. littoralis*-infested maize. Labelled cDNA was synthesized from total RNA using the Omniscript reverse transcriptase kit (Qiagen). Differentially expressed sequence tags were re-amplified from the original 96-well plates by PCR with T7/SK primers. PCR products were sequenced directly or sub-cloned in pGEM-T easy (Promega), transformed into *E. coli* XL-1 Blue, re-amplified by PCR (M13/rev), and sequenced. Sequence reactions were performed using an automated capillary sequencer ABI Model 3730 (Mycrosynth, Balgach, Switzerland).

Induced-resistance bioassays

Second-instar *S. littoralis* larvae of approximately equal size were selected, divided into groups of 10 for each plant, weighed, and released into leaf whorls. Infested plants were maintained in glass vessels under laboratory conditions for 11 h. Subsequently, larvae were recollected and weighed. For each individual plant, larval weight gain was calculated as the difference between average larval weight before and after infestation. Differences in larval weight gain between different treatments and their controls were analysed for significance using a *t*-test ($P < 0.05$).

Bioassays for priming for gene expression

Immediately upon exposure treatment, plant shoot material was harvested to detect potentially direct effects on gene expression (time point 0). The remaining plants were used to quantify priming-responsive gene expression. To this end, shoots were collected for RNA extraction at 45, 90, 180, 300 and 500 min after elicitation treatment. RNA was extracted from pooled shoot material of at least three different plants originating from independent experiments.

Bioassays for priming for VOC emission

After exposure treatment, all plants were kept outside the odour vessels for 30 min. To quantify potentially direct effects, undamaged plants were put back in clean odour vessels without further elicitation treatment. To quantify priming-responsive VOC emission, plants were subjected to elicitation treatment and put back into clean odour vessels. VOCs were collected from 12 odour sources simultaneously by extending the MAD system (see above) with a VOC collection system by attaching a Super-Q trap (Alltech Associates Inc., Deerfield, IL, USA; described by Heath and Manukian, 1992) at the upper part of each odour vessel. Purified air from the MAD system entered the source vessels via Teflon tubing at a rate of 1.2 ml min^{-1} and was exhausted through the Super-Q trap at a rate of 0.6 l min^{-1} via Teflon-tubing, a second set of flow meters and a vacuum pump (model ME2; Vacuubrand, Wertheim, Germany). The overflow of incoming air was vented through a second opening of the odour source vessel. Super-Q traps from vessels containing undamaged plants were collected after 180 min, and from vessels containing elicited plants at 45, 90, 180, 300 or 500 min after elicitation treatment. Detection and quantification of VOCs were performed as described previously (D'Alessandro and Turlings, 2005). To obtain an estimate of the different classes of VOC, total amounts of the following compounds were summed: GLV: (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate; monoterpenes: (*Z*)-β-ocimene and linalool; homoterpenes: 4,8-dimethyl-1,3(*E*), 7-nonatriene and 4,8,12-trimethyl-1,3(*E*),7(*E*),11-tridecatetraene; sesquiterpenes: (*E*)-β-caryophellene, (*E*)-α-bergamotene, (*E*)-β-farnesene, β-sesquiphellandrene and (*E*)-nerolidol; aromatic compounds: indole and methyl anthranilate. Differences in VOC emission between air- and VOC-pretreated plants were analysed for significance using a *t*-test ($P < 0.05$).

Bioassays for Cotesia marginiventris *behaviour*

Attraction of *Cotesia marginiventris* was quantified using a six-arm olfactometer. This device allows parasitoids to be exposed to six different air streams that enter a central choice chamber (Turlings *et al.*, 2004) Groups of six wasps were exposed to four different odour sources from (a) elicited, air-pretreated plants; (b) elicited, VOC-pretreated plants; (c) non-elicited, air-pretreated plants; and (d) non-elicited, VOC-pretreated plants. The two residual arms remained empty. To enhance responsiveness of *C. marginiventris* to VOC from caterpillar-infested maize, parasitoids were given oviposition experience on *S. littoralis* larvae as described above. Each bioassay allowed six releases of six parasitoids between 150 and 330 min after defence elicitation. In total, six independent bioassays were performed with alternating placements of the different odour sources. The behavioural responses of the parasitoids to different odour sources were analysed statistically using a log-linear model and fitted by maximum quasi-likelihood estimation in the software package R (ver. 1.9.1; R-project, Vienna, Austria) as described previously (Schnee *et al.*, 2006).

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