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ORIGINAL ARTICLE

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(*Z*)-3-Hexenol induces defense genes and downstream metabolites in maize

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Abstract In response to insect feeding, corn plants (*Zea mays* cv. Delprim) release elevated levels of volatile organic compounds (VOCs), including the C_6-volatile (*Z*)-3-hexenol. The level of mRNA accumulation for a series of defense genes was monitored in response to application of (*Z*)-3-hexenol (50 nmol) to undamaged plants. The induction of transcripts for *hpl* (hydroperoxide lyase), *fps* (farnesyl pyrophosphate synthase), *pal* (phenylalanine ammonia-lyase), *lox* (lipoxygenase), *igl* (indole-3-glycerol phosphate lyase) and *mpi* (maize proteinase inhibitor) were compared with metabolites generated from the respective pathways. While headspace VOC analysis showed an increase in (*Z*)-3-hexenyl acetate and methyl salicylate with *lox* and *pal* induction, respectively, MPI accumulation was not observed with an increase in *mpi* transcripts. Moreover, (Z)-3-hexenol treatment did not elevate sesquiterpene emissions or activate *fps* transcription. Chemical labeling and bioassay experiments established that exogenous (*Z*)-3-hexenol can be taken up and converted to a less active acetylated form. These data indicate that (*Z*)-3-hexenol can serve as a signaling molecule that triggers defense responses in maize and can rapidly be turned over *in planta*.

Keywords C_6-volatiles · (*Z*)-3-Hexenol · Methyl jasmonate · Terpenes · Volatile organic compounds · *Zea*

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Abbreviations BAW: Beet armyworm · FPS: Farnesyl pyrophosphate synthase · GAP C: Glycerol phosphate dehydrogenase, cytosolic form · HMGR: Hydroxymethylglutaryl-coenzyme A reductase · HPL: Hydroperoxide lyase · IGL: Indole-3-glycerol phosphate lyase · LOX: Lipoxygenase · MeJA: Methyl jasmonate · MeSA: Methyl salicylate · MPI: Maize proteinase inhibitor · PAL: Phenylalanine ammonia lyase · VOC: Volatile organic compound

Introduction

Plants can initiate a series of inducible defense responses following herbivore damage, including the accumulation of polyphenol oxidases (PPOs) and proteinase inhibitors (PIs), as well as the release of volatile organic compounds (VOCs) that can serve as a signal for beneficial insects. VOCs can include monoterpenes, sesquiterpenes, shikimic acid pathway derivatives such as methyl salicylate (MeSA), as well as the lipoxygenase (LOX) derivatives methyl jasmonate (MeJA) and C_6-volatiles (Paré and Tumlinson 1999). While there are many reports on the role of the oxylipin derivative MeJA in intra- and inter-plant signaling, much less is known about possible functions of C_6 oxygenated components as elicitors of plant defense responses (Farmer 2001).

The role of MeJA was originally identified with respect to the wound-induced expression of PIs that protect plants against digestive serine proteinases of herbivorous insects (Farmer and Ryan 1990; Codero et al. 1994). MeJA has also been found to induce phytoalexin accumulation in bean and barley (Weidhase et al. 1987; Croft et al. 1993). Recent studies have indicated that the octadecanoid pathway with jasmonic acid (JA) and/or its intermediate precursor phytodienoic acid serve as components involved in herbivore-induced VOC emissions (Koch et al. 1999; Thaler et al. 2002). In cotton and tobacco plants, exposure to MeJA results in the production of volatiles that mimic those emitted with

insect damage (Halitschke et al. 2000; Saona et al. 2001). Other plant volatiles that can regulate defense-related genes include MeSA in tobacco (Shulaev et al. 1997), terpenes in lima bean (Arimura et al. 2000) and ethylene in soybean (Hoffman et al. 1999).

C_6-volatiles are released from all green plant tissues with insect or mechanical damage and include unsaturated C_6-alcohol and aldehydes that are produced from a branch of the oxylipin pathway catalyzed by the hydroperoxide lyase (HPL) enzyme (Hatanaka et al. 1987). There is some evidence that plant exposure to these compounds can reduce herbivore feeding (Hildebrand et al. 1993) and seed germination frequency in soybean (Gardener et al. 1990), as well as phytoalexin induction in cotton balls (Zeringue 1992). C_6-volatiles have also been proposed to prime neighboring plants against impending herbivory by enhancing inducible chemical defense responses triggered during insect feeding (Engelberth et al. 2004). Molecular data have demonstrated that aerial treatment of *Arabidopsis* and lima bean with a synthetic C_6-volatile, (*E*)-2-hexenal, induces the transcription of defense-related genes, including *lox* and *pal* (Bate and Rothstein 1998; Arimura et al. 2001). Treatment with C_6-volatiles also triggers VOC emissions in tomato (Farag and Paré 2002), with (*Z*)-3-hexenol the most potent of the volatiles tested.

Induction of VOC emissions is an active process that triggers de novo biosynthesis, and a lag period exists between treatment and subsequent release (Paré and Tumlinson 1997). In contrast, constitutive volatile release occurs at the time of physical damage to leaf tissue. To probe the nature of VOC induction, sites of regulation must initially be identified along the linear path from a gene to a functional metabolite. Most genes are thought to be regulated at the transcript level, although certain chloroplastic genes have been shown to be primarily regulated by posttranscriptional events, including transcript stability, translation, protein turnover, and protein activity (Danon 1997). In addition, increases in mRNA levels do not always correlate with increases in protein levels or enzymatic activity (Gygi et al. 1999).

The aim of this research is to combine phytochemical and molecular approaches to provide insight into whether C_6-volatiles can play a role in initiating plant defense responses at genomic and metabolic levels. Owing to the wealth of knowledge of plant–insect interactions in maize, most notably the identification of the first herbivore elicitor 17-hydroxy linolenoyl glutamine (volicitin), that trigger defense responses (Alborn et al. 1997; Frey et al. 2000), this system is likely to provide a good model for assessing the role of C_6-volatiles as signal molecules in plant defense. Indeed, maize was the first species selected to examine endogenous hormone levels in response to exposure to insect feeding damage (Schmelz et al. 2003a), the roles of JA and ethylene inhibitors having already been probed (Schmelz et al. 2003b).

In this study, plant volatile emissions were measured in conjunction with levels of mRNA accumulation to examine if transcriptional induction triggered by oxygenated C_6 hydrocarbons is linked to downstream products. Specifically, we compared differences in phytochemical release and mRNA levels for several previously characterized enzymatic steps in defense pathways for insect damage, MeJA or C_6-alcohol treatment. Based on results from these experiments, we postulated that applied (*Z*)-3-hexenol can be taken up by maize plants and metabolized into a less active acetylated form. To clarify the role of (*Z*)-3-hexenyl acetate as a chemical elicitor, synthetic (*Z*)-3-hexenyl acetate was applied to maize and its efficacy as a signaling molecule was compared to that of other synthetic C_6-volatiles.

Materials and methods

Plants, insects and reagents

Maize (*Zea mays* L. cv. Delprim) plants were maintained in an insect-free facility in which temperature was maintained at 29 ± 4°C with a relative humidity of 40 ± 10%. Plants were grown under metal-halide and high-pressure sodium lamps in a 16-h/8-h light/dark photoperiod with a total light intensity of 700 μmol m^{-2} s^{-1}. Plants were grown in 16-cm-diameter pots using Pro-gro potting soil containing Osmocote fertilizer (Scotts-Sierra Horticulture, Marysville, OH, USA). Five-week-old plants that were 60–70 cm tall and had four distinguishable leaves were used.

Eggs of beet armyworm (BAW; *Spodoptera exigua*) obtained from USDA–ARS (Stoneville, MS, USA) were placed in an incubator in which temperature (26°C), relative humidity (ca. 90%) and the light/dark cycle (16 h/8 h) were held constant. Larvae were provided with an artificial pinto bean diet, following the method of King and Leppla (1984). Two-week-old larvae (late third- or early fourth-instar caterpillars) were starved for 8 h and then placed on plants to generate insect damage.

n-Hexanol, (*Z*)-3-heptenol, (*E*)-2-hexenal, (*Z*)-3-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, (*E*)-2-pentenal and MeJA [20% (*Z*)-epi-form content] were obtained from Bedoukian Research (Danbury, CT, USA) and were of ca. 99% purity, as determined by capillary GC–FID analysis. Solvents used were of GC or HPLC grade.

Treatment and harvest of plants

For insect damage, 15 BAW larvae were allowed to feed continuously on maize plants for 2 h. For chemical treatments, a single 50-nmol dose of a C_6-volatile or MeJA was suspended (by vigorous shaking) in 12 ml water and uniformly sprayed on the aerial portion of the plant at an estimated amount of 0.15 nmol cm^{-2} of leaf surface area, similar to the procedure of Thaler (1999) and Keinänen et al. (2001). The same volume of pure water was sprayed on untreated control plants, and

plants were harvested at 0 and 24 h. For analysis by reverse transcription–polymerase chain reaction (RT–PCR), plants were harvested at different time points (0, 1.5, 3, 6, 12, 24 h) after the 2-h insect feeding or a single chemical treatment. For structure–activity relationships, samples were harvested at 0, 3 and 12 h after C_6-volatile treatments. All plant materials were harvested during the light period to avoid light/dark effects on gene expression. Individual plants were used for each time-point harvest, except in the case of non-destructive volatile analysis in which data were collected from the same plant at different time points. Test solutions were administered in the open air to prevent the build-up of volatilized materials either applied to the plant or emitted from the plant in response to the chemical treatment.

Collection and analysis of volatiles

To analyze for volatiles emitted from intact treated maize, plants were placed in collection chambers 1 h after treatment. Volatiles were collected from treated plants for a 2-h interval at different time points (2, 4, 8, 24, 48 h) as previously described (Farag and Paré 2002). All treatments were run at least in triplicate.

Volatiles collected on Super-Q adsorbent traps were eluted with 150 µl of dichloromethane, and nonyl acetate was added as an internal standard. Extracts were analyzed by capillary GC on a fused silica column (15 m long, 0.25 mm i.d.) with a 0.25-µm-thick layer of bonded methyl siloxane (Quadrex, New Haven, CT, USA). Injections were made in the splitless mode for 30 s, and the gas chromatograph was operated under the following conditions: injector 230°C, detector 250°C, column oven 40°C for 3 min, then programmed at a rate of 12°C per min to 180°C and finally ramped at a rate of 40°C to 220°C°for 2 min; the linear flow velocity of the He carrier gas was 20 cm s^{-1}. For C_6-volatiles, samples were collected and analyzed in the same manner except that a DB5 column (30 m long, 0.25 mm i.d.; J&W Scientific, Folsom, CA) was used under the same GC conditions as listed above. Quantification was based on comparison of area under the GC–FID peak with that of 400 ng of the internal standard. For comparisons of the same compound under different treatments, response factors for individual compounds were assumed to be equal. Selected samples were also analyzed by GC–MS on a (ion trap) mass spectrometer (GCQ plus; Thermoquest, Austin, TX, USA) interfaced to a gas chromatograph (Trace GC2000) and operated in the electron impact mode. Injections were made in the splitless mode for 30 s and samples were analyzed on a DB5 column (30 m long, 0.25 mm i.d.; J&W Scientific) under the same conditions previously mentioned for GC–FID analysis. The transfer line and ion-source were adjusted to 230°C and 180°C, respectively. The components of the plant volatile emission were identified by comparison of GC retention times with those of authentic standards and by comparison of mass spectra with spectra of an EPA/NIH database.

Plant ^{13}C labeling

Maize seeds were germinated for 4 days under a moist paper towel in the dark. To reduce the amount of stored ^{12}C-labeled carbon available for the growing plant, seeds were trimmed by using a razor blade to remove ca. 60–70% of the endosperm without cutting into the germinating embryo. Four trimmed seeds were planted in sterilized soil. The pot was placed in a glass sleeve (45 cm long, 20 cm diameter) closed at the base and with a removable glass lid at the top. A glove port 10 cm in diameter is positioned on the side of the cylinder, flush with the base. Multiple ports for air flow are located around a portion of the base and the lid perimeter. The chamber is sealed by covering the ports with Teflon septa held in place by screw caps, clamping down the glass top and mounting a latex glove over the glove port. The chamber was flushed with nitrogen at a rate of 5 l min^{-1} for 5 min to remove air-borne $^{12}CO_2$. To generate $^{13}CO_2$, a vial of 5 mmol $NaH^{13}CO_3$ (Sigma, St. Louis, MO, USA) was opened into a 100-ml beaker containing 1.2 mol of 70% perchloric acid. Maize plants were grown in the labeled environment for 12 days (ca. 25 cm in height). The chamber was then flushed with charcoal-filtered air (5 l min^{-1}) for 10 min and exhausted into a 1 M KOH bubbler/trap to remove any residual $^{13}CO_2$ before the chamber was opened. Plants were immediately sprayed with a dose of 50 nmol of (Z)-3-hexenol, and volatiles were collected for an 8-h period between 1000 hours and 1800 hours on day 1, as previously described. To determine the amount of ^{13}C incorporated in each compound, samples were analyzed by GC–MS and each selected mass ion was quantified via computer software analysis. The fraction of each compound that incorporated ^{13}C was computed on a per molecule basis (Paré and Tumlinson 1997).

Reverse transcriptase-mediated PCR

Total leaf RNA was isolated with an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Four micrograms of RNA was reverse-transcribed into cDNA using 2.5 µl MuMLV-RT enzyme (Promega, Madison, WI, USA). Each reaction mixture contained 4 µg of RNA, 5 µl oligo dT, 4 µl dNTPs (2.5 M), 1 µl RNasin, 4.4 µl DEPC water and 10 µl of 5× MuMLV buffer, and samples were incubated at 37°C for 1 h. For the determination of transcript quantities, the first-strand cDNA was amplified in a PCR reaction using gene-specific primers:

– *fps*: 5′-CATGGATGACTCTCACACTC-3′ and 5′-GTCATCCTGGACTTGAAAG-3′ corresponding to nucleotides 405–424 and 801–820 in maize *fps* (farnesyl pyrophosphate synthase) cDNA;

- *gap c*: 5′-GCTAGCTGCACCACAAACTGC-3′ and 5′-TAGCCCCACTCGTTGTCGTAC-3′ corresponding to nucleotides 454–474 and 945–965 in maize *gap c* (glycerol phosphate dehydrogenase, cytosolic form) cDNA;
- *hpl*: 5′-TACGAGATGCTGCGGATG-3′ and 5′-CTCGAAGTCGTCGTAGCG-3′ corresponding to nucleotides 1048–1066 and 1387–1404 in barley *hpl* (hydroperoxide lyase) cDNA;
- *igl*: 5′-CTCCGCGATCAAGGCTGCATC-3′ and 5′-GAGTGAGAGCACACGAGTTCC-3′ corresponding to nucleotides 6–24 and 608–628 in maize *igl* (indole-3-glycerol phosphate lyase) cDNA;
- *lox*: 5′-GTTCGGAACATCGGAAAGATC-3′ and 5′-GGAGGCGTGGTTGTTCTTGAC-3′ corresponding to nucleotides 3–24 and 361-378 in maize *lox* (lipoxygenase) cDNA;
- *mpi*: 5′-ATGAGCTCCACGGAGTGC-3′ and 5′-TCAGCCGATGTGGGGCGTC-3′ corresponding to nucleotides 1–18 and 204–222 in maize *mpi* (maize proteinase inhibitor) cDNA;
- *pal*: 5′-CGAGGTCAACTCCGTGAACG-3′ and 5′-GCTCTGCACGTGGTTGGTGA-3′ corresponding to nucleotides 1089–1118 and 1388–1407 in maize *pal* (phenylalanine ammonia-lyase) cDNA.

The PCR volume was 50 µl, containing 100 ng of each primer, 4 µl dNTPs, 5 µl of cDNA, and 0.5 units of Taq DNA polymerase (Takara, Japan). A PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA, USA) was used for the amplification of *fps*, *gap c*, *hpl*, *igl*, *lox*, *mpi* and *pal* cDNAs, using 24, 32, 24, 24, 23, 23, 25, 24 and 23 cycles, respectively, under optimum dynamic ranges before reaching the plateau. Equal amounts of PCR products were separated by electrophoresis in 1.5% agarose gels and detected by staining with ethidium bromide. *gap c*, a gene that is not induced with defense responses in maize (Frey et al. 2000), was used to ensure equal loading of the lanes. RT–PCR analysis was performed at least in triplicate using RNA from two different sets of plants. Signals of the amplified PCR products were quantified using a Kodak Digital Science image station using Kodak ID image analysis software (http://www.Kodak.com/go/1D3). Normalization of signal was based on the housekeeping gene *gap c*.

To confirm the identity of amplified fragments, PCR products were ligated in the pGEMT-easy vector (Promega) according to the manufacturer's protocol and both strands were sequenced using an ABI 310 genetic analyzer. Sequences were searched using Blast and alignments were performed with Vector NTI software.

Western blot analysis

Total protein extracts were prepared from insect-damaged, chemically treated and control plants by using 0.1 M Na_2HPO_4 (pH 7.2) containing 14 mM β-mercaptoethanol as the extraction buffer. Leaf samples were frozen in liquid nitrogen and subsequently ground in a mortar with the extraction buffer at 4°C. The buffer extracts were then centrifuged at 12,000 *g* for 15 min at 4°C. Protein concentrations in the supernatant were determined by the Bradford (1976) assay. Protein extracts were separated on a 15% SDS–PAGE gel (30 µg protein per lane) and electroblotted to polyvinylidenedifluoride (PVDF) membranes according to the procedure of Sambrook and Russell (2001). Blots were incubated for 60 min at room temperature with the anti-MPI antiserum diluted 1:1,000, followed by incubation with 20 µl alkaline-phosphatase-conjugated goat anti-rabbit IgG (Sigma). The serological color reaction was developed using 10 ml of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) liquid substrate system (Sigma). Western blot analysis was performed in triplicate using protein extracts from three different sets of plants with a representative blot presented.

Statistics

Analysis of variance was run using SAS statistical software. Means were separated using Duncan's multiple range test at $P < 0.05$.

Results

Induction of *lox* and emission of products

Undamaged maize plants released relatively small amounts of VOCs into the atmosphere (Fig. 1a). However, when herbivore larvae such as BAWs fed on the leaves, elevated levels of low-molecular-weight compounds were observed (Fig. 1b). The VOC profile of oxylipin derivates included (*Z*)-3-hexenal, (*E*)-2-hexenal and (*Z*)-3-hexenol (Fig. 1c), as well as the hexenyl derivative (*Z*)-3-hexenyl acetate. To identify what role endogenous oxylipins might play in the induction of defense responses, plants were sprayed with a dilute aqueous suspension of individual C_6 components. Selected defense-related transcripts, as well as downstream products including phenyl propanoids, terpenoids, nitrogenous compounds, maize proteinase inhibitors and LOX metabolites, were assayed over a given time period. Based on recent studies showing that the oxylipin product MeJA induces defense responses when sprayed on plants as a dose in the micromolar to millimolar range (Kessler and Baldwin 2001; Martin et al. 2003), MeJA at the low, albeit biologically active, dose of 50 nmol was applied as a positive control. The same dosage of (*Z*)-3-hexenol (50 nmol) was applied to maize plants to allow for equimolar comparisons of the two oxylipin products. A 500-nmol dose of (*Z*)-3-hexenol was also tested as an elicitor of VOC emissions. When VOC

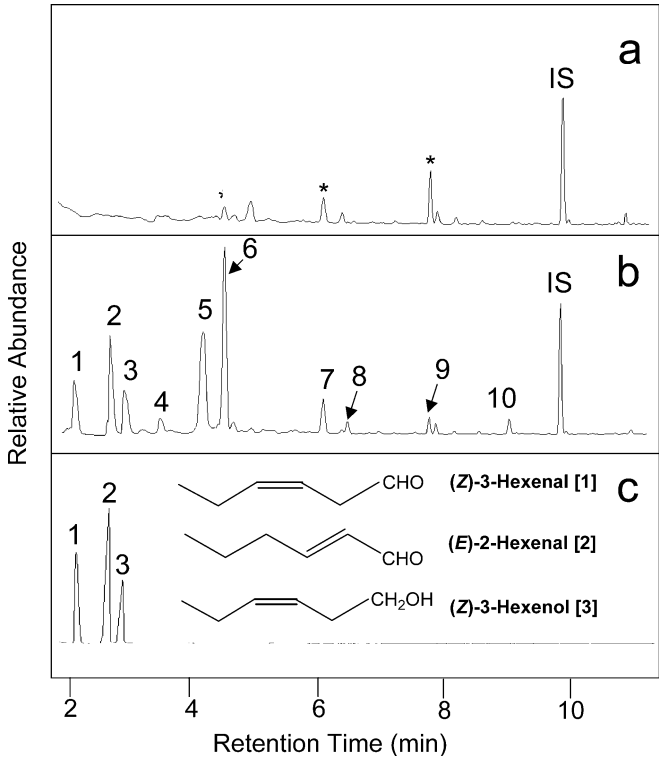

Fig. 1a–c Chromatographic profiles of VOCs emitted at 30-min intervals from undamaged (**a**) and continuously BAW (beet armyworm: *Spodoptera exigua*)-damaged (**b**) maize (*Zea mays*) plants, and comparison with a mixture of synthetic C_6-volatiles (**c**). *Peaks*: *1*, (*Z*)-3-hexenal; *2*, (*E*)-2-hexenal; *3*, (*Z*)-3-hexenol; *4*, α-pinene; *5*, myrcene; *6*, (*Z*)-3-hexenyl acetate; *7*, linalool; *8*, nonatriene; *9*, hexenyl butyrate; *10*, indole. An *asterisk* (*) designates that a compound aligns with the numbered peak in the corresponding chromatogram. *IS*, Internal standard

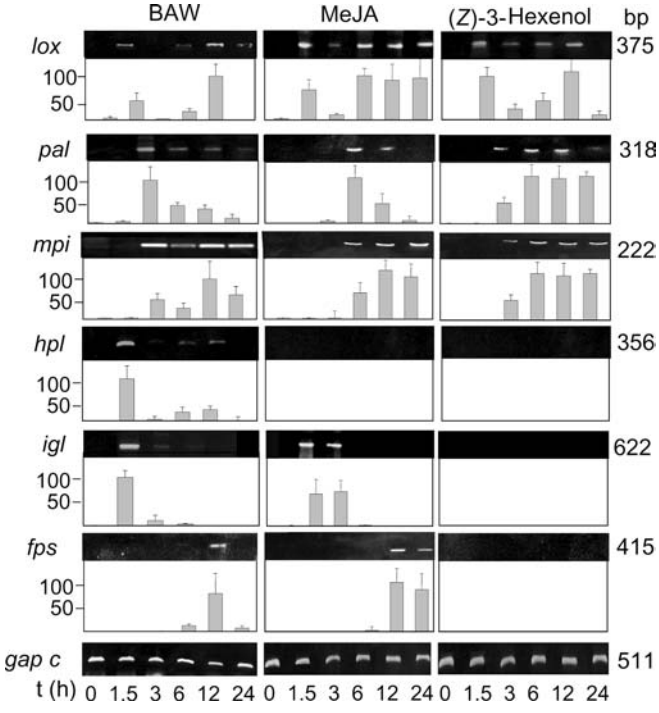

Fig. 2 Expression of defense genes in response to BAW damage, and 50 nmol MeJA and 50 nmol (*Z*)-3-hexenol treatments, in maize plants harvested at the indicated times. Numbers on the right-hand side represent different probe sizes for the RT–PCR analysis. Graphs show quantification for *lox*, *pal*, *mpi*, *hpl*, *igl* and *fps* amplified PCR products as a percent of the maximum band intensity (100%) recorded within each treatment; signals were normalized based on *gap c*. Error bars represent SE ($n = 3$)

emissions were collected for a 6-h interval, 24 h after spraying maize with (*Z*)-3-hexenol, the 500 nmol dose caused less than a 3-fold increase in total emissions compared with the 50 nmol dose (128 ± 7 versus 53 ± 5 µg, respectively); therefore, the 50-nmol dose was deemed closer to levels that might arise in natural plant systems.

In maize, the wound-responsive LOX enzyme catalyzes the first reaction of the octadecanoid pathway, converting linolenic acid into both 9 and 13 hydroperoxy linolenic acid (Hildebrand et al. 1988; Kim et al. 2001, 2002). Treatment of maize plants with (*Z*)-3-hexenol resulted in a transient increase in *lox* transcripts at 1.5 h and a sharp reduction after 12 h; with BAW damage and MeJA treatments, levels of *lox* mRNA increased within 1.5 h and were sustained to 24 h (Fig. 2). The LOX product (*Z*)-3-hexenyl acetate was detected between 2 and 8 h after (*Z*)-3-hexenol treatment (Fig. 3a). BAW damage triggered a similar, albeit attenuated, release of (*Z*)-3-hexenyl acetate and, in addition, a second release at 48 h (Fig. 3a). HPL catalyzes the second step in the oxylipin pathway, using 13-hydroperoxy linolenic acid as a substrate to produce C_6-volatiles (Kim et al. 2002). While the *hpl* gene and the products derived from the HPL enzyme, (*Z*)-3-hexenal, (*E*)-2-hexenal and (*Z*)-3-hexenol, were not induced with (*Z*)-3-hexenol or MeJA treatment at either the transcript (Fig. 2) or metabolite level (Fig. 3b), C_6-volatile induction was observed with BAW damage.

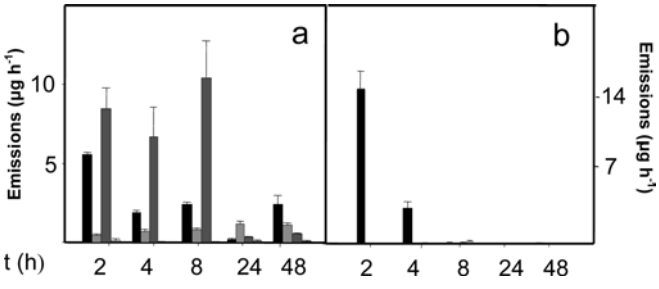

Fig. 3a,b Emissions of (*Z*)-3-hexenyl acetate (**a**) and C_6-volatiles [(*Z*)-3-hexenal, (*E*)-2-hexenal and (*Z*)-3-hexene-1-ol] (**b**) from maize subjected to BAW damage (*black columns*), to 50 nmol MeJA (*light grey columns*) and 50 nmol (*Z*)-3-hexenol (*dark grey columns*) treatments, and from control plants (*white columns*). Collections were made for 2 h. Error bars represent SE ($n = 3$). Means were separated using Duncan's multiple range test at $P < 0.05$

Biogenetic origin of (*Z*)-3-hexenyl acetate

The burst in (*Z*)-3-hexenyl acetate emissions with (*Z*)-3-hexenol treatment raised the possibility that (*Z*)-3-hexenyl acetate is derived at least in part from the (*Z*)-3-hexenol that was applied to the plant. To determine the source of (*Z*)-3-hexenyl acetate released in response to exogenous (*Z*)-3-hexenol treatment, endogenous and exogenous C_6-volatile components were separately labeled with ^{13}C and ^{12}C, respectively. Plants were grown under $^{13}CO_2$ conditions and chemically labeled plants were exposed to unlabeled [$^{12}C_6$]-(*Z*)-3-hexenol. Dilution of ^{13}C label in C_6-volatiles was used as a measure of exogenous [$^{12}C_6$]-(*Z*)-3-hexenol incorporation into hexenyl acetate emissions. In mechanically damaged plants, 58 ± 5% enrichment of [$^{13}C_6$]-(*Z*)-3-hexenyl acetate was observed, while dilution of the ^{13}C label to 7 ± 1.5% was observed with [$^{12}C_6$]-(*Z*)-3-hexenol treatment (Fig. 4a,b). This 88% dilution of the ^{13}C label with the application of unlabeled (*Z*)-3-hexenol indicates plant biochemical conversion of the C_6 alcohol to the acetylated form.

Induction of the *pal* gene and emission of MeSA

MeSA is a product of the phenylpropanoid pathway; PAL is the first committed step in MeSA biosynthesis starting from phenylalanine (Buchanan et al. 2000). *Pal* transcripts were induced by treatment with BAW, MeJA and (*Z*)-3-hexenol, although the timing of induction varied. BAW damage resulted in maximum *pal* levels at 3 h while the MeJA treatment maximum was at 6 h (Fig. 2). In the case of (*Z*)-3-hexenol treatment, induction was more gradual, with higher transcript levels at 3 h, and was sustained to 12 h (Fig. 2). An increase in MeSA emissions was observed 48 h after BAW or (*Z*)-3-hexenol treatment, although MeJA did not activate MeSA emissions (Fig. 5). We speculate that MeSA emission is controlled by SA-methyl transferase activity; however, such a gene has yet to be cloned, so transcripts levels could not be monitored.

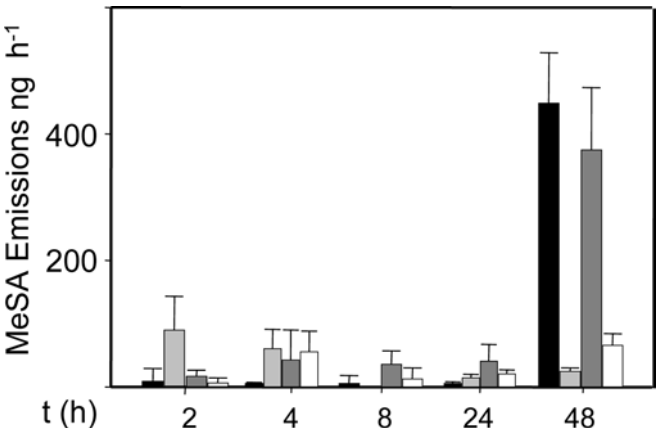

Fig. 5 Emissions of methyl salicylate (MeSA) from maize subjected to BAW damage (*black columns*), to 50 nmol MeJA (*light grey columns*) and 50 nmol (*Z*)-3-hexenol (*dark grey columns*) treatments, and from control plants (*white columns*). Collections were made for 2 h. Error bars represent SE ($n = 3$). Means were separated using Duncan's multiple range test at $P < 0.05$

Induction of maize proteinase inhibitor

Maize proteinase inhibitor, MPI, belongs to a family of serine proteinase inhibitors that inhibit digestive proteases of insects (Codero et al. 1994; Tamayo et al. 2000). The level of *mpi* transcripts increased within 3 h after BAW and MeJA treatments, and the increased levels were sustained through 24 h. An increase in *mpi* transcripts was also observed with (*Z*)-3-hexenol although induction was delayed to 6 h after (*Z*)-3-hexenol exposure (Fig. 2). BAW damage and MeJA treatment caused higher levels of MPI protein accumulation than that detected in control leaves 24 h after treatments (Fig. 6). However, induction of the *mpi* gene by (*Z*)-3-hexenol did not result in an accumulation of

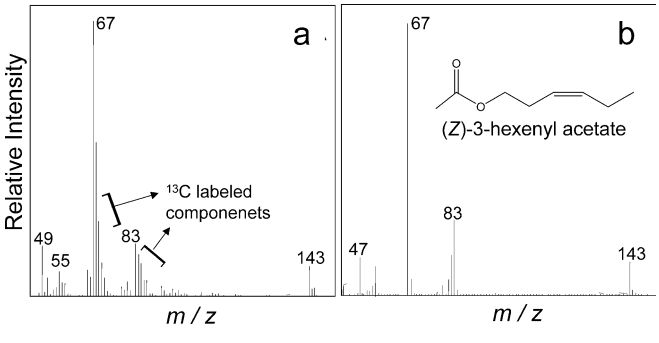

Fig. 4a,b Abundance of ^{13}C label in plant-metabolized (*Z*)-3-hexenyl acetate, as analyzed by electron ionization mass spectroscopy. Maize plants were subjected to mechanical damage (**a**) or exposed to ^{12}C-labeled (*Z*)-3-hexenol (**b**). The ion species at *m*/*z* 67 was used to calculate for ^{13}C enrichment levels

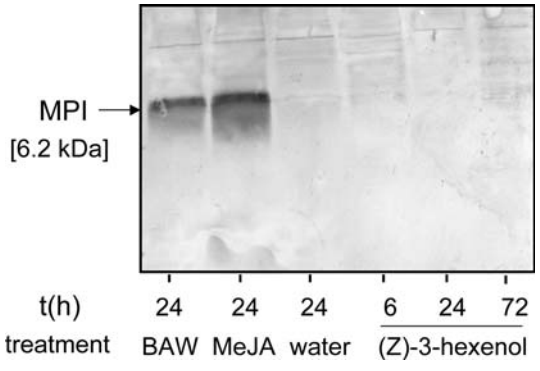

Fig. 6 Accumulation of MPI protein in maize plants subjected to BAW damage, treatment with 50 nmol MeJA or 50 nmol (*Z*)-3-hexen-1-ol, and in untreated control plants at the indicated time points, as measured by Western blot analysis

MPI protein in leaves harvested 6, 24 or 72 h after treatment.

Selective induction of the indole and sesquiterpene pathways

Indole-3-glycerol phosphate is utilized by IGL to yield volatile indole (Frey et al. 2000). Indole release activated by BAW and MeJA was preceded by *igl* gene induction within 1.5 h of treatment and declined within 6 h; induction of the *IGL* gene expression was not observed with (*Z*)-3-hexenol treatment (Fig. 2). Indole release increased by 40-fold and 85-fold at 8 h with BAW and MeJA treatments, respectively, while (*Z*)-3-hexenol did not trigger indole emissions compared to control (data not shown).

Sesquiterpenes represent the major group of volatiles induced by BAW damage. Regulation of sesquiterpene biosynthesis was followed by monitoring transcripts of *fps*, a gene for the enzyme that catalyzes the synthesis of C_{15}-farnesyl pyrophosphate unit from C_5 isoprene units. While *fps* transcript levels were elevated at 12 h with BAW damage, as well as 12 and 24 h with MeJA treatment, no transcript induction was observed with (*Z*)-3-hexenol treatment (Fig. 2). At the metabolite level, both BAW damage and MeJA treatment increased the release of the sesquiterpenes *β*-caryophyllene, α-humulene, *β*-farnesene and (*E*)-nerolidol. The sum of these terpenes was released at 40- and 150-fold greater levels than that of control plants. Sesquiterpene emissions were significantly greater at 8 h for BAW and MeJA treatments than for control plants (Duncan's, $P < 0.05$) and were highest at the 24- and 48-h collection periods (Fig. 7a). Sesquiterpene emissions were not significantly different from those of control plants (Duncan's, $P < 0.05$) with (*Z*)-3-hexenol treatment (Fig. 7a).

No gene specific for monoterpene synthesis was monitored. An increase in the monoterpenes α-pinene, *β*-pinene and limonene was observed at 2 and 4 h after BAW damage, with levels decreasing to those of the control within 8 h. No subsequent increase in monoterpene was observed when monitored over 5 days (data not shown). An exception to this pattern was an increased release of the oxygenated monoterpene linalool with BAW damage that extended from 4 to 24 h (Fig. 7b); significantly higher levels of linalool were also detected with MeJA between 2 and 24 h after chemical treatment (Duncan's, $P < 0.05$). (*Z*)-3-Hexenol treatment resulted in the emission of linalool at a level 4 times greater than that of control plants at 4 h, although this was less than the amounts observed with BAW or MeJA treatment at levels 6 and 9 times higher than for control plants, respectively (Fig. 7b).

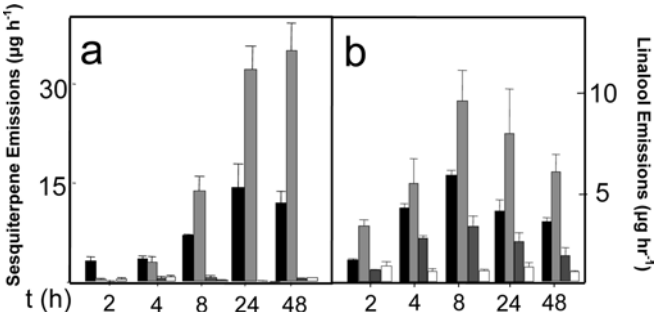

Fig. 7a,b Emissions of total sesquiterpene [*β*-caryophyllene, α-humulene, *β*-farnesene and (*E*)-nerolidol] (**a**) and linalool (**b**) from maize subjected to BAW damage (*black columns*), to 50 nmol MeJA (*light grey columns*) and 50 nmol (*Z*)-3-hexenol (*dark grey columns*) treatments, and from control plants (*white columns*). Collections were made for 2 h. Error bars represent SE ($n = 3$). Means were separated using Duncan's multiple range test at $P < 0.05$

Biological activity of natural and synthetic C_6 analogues

To compare structure–activity relationships, C_6-volatiles released from maize [(*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, (*E*)-2-hexenal, (*Z*)-3-hexenal] as well as other structural analogues (*n*-hexanol, (*Z*)-3-heptenol, (*E*)-2-pentenal) were tested for their efficacy to activate the (*Z*)-3-hexenol-inducible VOCs (*Z*)-3-hexenyl acetate, linalool and MeSA (Table 1). (*Z*)-3-Hexenol and (*E*)-2-hexenal were found to be the most active in triggering a select group of VOCs. A significant increase in linalool emissions was detected with (*Z*)-3-hexenol and (*E*)-2-hexenal treatments, while an increase in MeSA emission levels was only observed with (*Z*)-3-hexenol treatment (Table 1). (*Z*)-3-Hexenyl acetate was emitted from plants treated with (*Z*)-3-hexenol and (*Z*)-3-hexenal at levels ca. 100 and 13 times higher, respectively, than from untreated control plants (Table 1). Plant-emitted (*Z*)-3-hexenyl acetate could not be differentiated from applied (*Z*)-3-hexenyl acetate and thus was not measured with (*Z*)-3-hexenyl acetate applications.

To account for differences in MeSA release with (*Z*)-3-hexenol and (*E*)-2-hexenal treatments, *pal* transcript levels were monitored at 3 and 12 h post elicitation. *Pal*

Table 1 Quantification of maize (*Zea mays*) VOCs [μg (10 h)$^{-1}$] emitted in response to treatment with C_5-, C_6- and C_7-volatiles. (*Z*)-3-Hexenyl acetate and linalool were collected on day 1; MeSA collections were made on day 3. Values represent mean ± SE ($n = 4$). Different letters indicate significant difference between treatments; *NM*, not measured

Treatment	(*Z*)-3-Hexenyl acetate	Linalool	Methyl salicylate
(*Z*)-3-Hexenol	32 ± 6^a	8 ± 2^a	5.5 ± 1.5^a
(*E*)-2-Hexenal	0.1 ± 0.03^b	5 ± 0.2^b	3 ± 0.9^b
(*Z*)-3-Hexenal	4 ± 0.4^c	3 ± 0.6^c	0.6 ± 0.2^b
(*Z*)-3-Hexenyl acetate	NM	2 ± 0.3^c	0.7 ± 0.1^b
n-Hexanol	0.8 ± 0.02^b	1 ± 0.1^c	0.3 ± 0.1^b
(*Z*)-3-Heptenol	0.2 ± 0.05^b	3 ± 0.5^c	0.5 ± 0.9^b
(*E*)-2-Pentenal	0.2 ± 0.03^b	2 ± 0.7^c	0.4 ± 0.1^b
Control (water)	0.3 ± 0.1^b	1.4 ± 0.3^c	0.5 ± 0.3^b

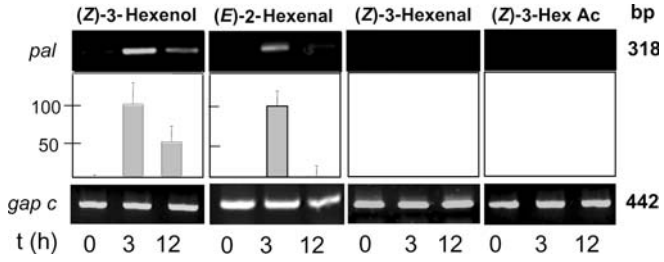

Fig. 8 Time courses of expression of the *pal* gene in maize plants in response to C_6-elicitor treatments. Numbers on the right-hand side represent different probe sizes for the RT–PCR analysis. Graphs show quantification for amplified PCR products as a percent of the maximum band intensity (100%) recorded within each treatment; signals were normalized based on *gap c*. Error bars represent SE ($n = 3$)

was also monitored for treatments with the C_6-volatiles (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenal, which did not trigger MeSA emissions. At the transcript level, *pal* was induced with (*E*)-2-hexenal at 3 h as well as with (*Z*)-3-hexenol treatment at 3 and 12 h; no transcript induction was observed with (*Z*)-3-hexenyl acetate or (*Z*)-3-hexenal (Fig. 8).

Discussion

The role of C_6 components as plant signals normally generated in response to leaf tissue damage and present within plant tissue was examined. Since the scope of this study did not include plant–plant communication via C_6-volatile emissions (Farag and Paré 2002; Engelberth et al. 2004), spray application of aqueous oxylipin components onto the leaves instead of volatilization of C_6 compounds was selected. Indeed, the aqueous spray of (*Z*)-3-hexenol was effective in passing through the waxy coating of the maize leaf and in being metabolized by the plant, as observed by the stable-isotope labeling studies. The application of (*Z*)-3-hexenol to maize plants activated mRNA transcription of *lox*, *pal*, and *mpi*, as well as accumulation of the downstream gene products (*Z*)-3-hexenyl acetate, linalool and MeSA. The proteinase inhibitor MPI showed a slight induction at the transcriptional level and no accumulation at the protein level. These induced responses are a subset of a full array of genes/gene products activated by either BAW or MeJA treatment that also include *igl*, *fps*, *mpi* gene transcripts and the gene products indole, sesquiterpenes and MPI. Induction of *hpl* and downstream C_6-volatile components was triggered with BAW feeding although not by MeJA or (*Z*)-3-hexenol treatment.

For all genes examined, induction was compared with a control treatment at time 0 and 24 h both of which showed no transcript induction. PCR products were not detected when the PCR reaction was run without reverse transcription. DNA size markers confirmed that PCR products were the amplification of the appropriate sequence size of the transcribed gene. Since there exists the possibility of small gene families in maize and the primers might recognize more than one member, the PCR products from PAL, LOX, and GAP C were sequenced to verify the specificity of the primers used (see Electronic Supplementary Material). MPI PCR product was also sequenced since induction of the gene without accumulation of the downstream protein was observed. For each of the genes sequenced, only one maize cDNA from the NCBI Gene Bank exhibited high sequence homology with the amplified PCR product.

An up-regulation of the allene oxide synthase (AOS) branch producing JA involves induction of *lox* and *aos* (Harms et al. 1998), with JA or MeJA acting as a positive feedback regulator of its own biosynthesis, as observed in rice and tomato (Sivasankar et al. 2000; Ha et al. 2002; Xu et al. 2003). Such a positive feedback loop was not observed in maize with C_6-volatile treatment. (*Z*)-3-Hexenol treatment induced *lox* transcripts but not the *hpl* gene that encodes the second enzyme involved in C_6-volatile production (Fig. 2); *lox* induction by C_6-components has also been observed in *Arabidopsis* with (*E*)-2-hexenal treatment (Bate and Rothstein 1998). The LOX enzyme examined in this study has an unusual dual positional specificity, producing both C-9 hydroperoxides and C-13 hydroperoxides, with the latter acting as a substrate for HPL to form C_6-volatiles (Kim et al. 2002). The absence of *hpl* induction (Fig. 2) and release of C_6-volatile emissions upon exposure to (*Z*)-3-hexenol (Fig. 3b) eliminates signal amplification by C_6-volatile components and may explain the attenuated effect of (*Z*)-3-hexenol in triggering defense responses compared to that of MeJA. The ability of MeJA to up-regulate C_6-volatile release is species-specific. In cotton and tomato, C_6-volatiles were not released by MeJA treatment (Saona et al. 2001; Farag and Paré 2002); however, in tobacco and barley, MeJA treatment was found to trigger C_6-volatiles (Kessler and Baldwin 2001; Kohlmann et al. 1999).

While C_6-volatiles are released only from insect-damaged leaves, (*Z*)-3-hexenyl acetate is released distal to the site of insect damage (Turlings and Tumlinson 1992), suggesting that C_6-volatiles cannot be released from undamaged leaves unless converted into an acetate form (Paré and Tumlinson 1998). The observation that exogenously applied (*Z*)-3-hexenol can be derivatized into an acetylated form for export opens up the possibility that endogenously generated C_6-components can follow the same derivatization pathway for export. Indeed, (*Z*)-3-hexenyl acetate was detected as a major C_6-volatile component in the headspace volatiles collected from insect-damaged plants (Figs. 1, 3a). While (*Z*)-3-hexenol activated the MeSA biosynthetic pathway (Fig. 5), (*Z*)-3-hexenyl acetate was found to be inactive at both transcript and metabolite levels (Fig. 8, Table 1). Interestingly, in tomato, both C_6-aldehydes and alcohols reduced aphid fecundity whereas (*Z*)-3-hexenyl acetate was biologically inactive; the observed biological activity is thought to be due to induced changes in the leaves upon which the aphids have fed (Hildebrand et al. 1993).

Hence, acetylation of (*Z*)-3-hexenol appears to have an important role in the inactivation and rapid turnover of this signaling molecule and may account for the attenuated effect of C_6-volatiles in triggering VOC emissions. This deactivation by chemical modifications is analogous to the biochemical conversion of JA to *cis*-jasmone, providing a shunt for the turnover of this stress hormone in jasmine flowers (Koch et al. 1997). We speculate that MeSA emission is controlled by SA-methyl transferase activity; however, the gene for such an enzyme has yet to be cloned, so transcripts levels could not be monitored.

Salicylic acid (SA) is another plant hormone that can exist in either the free acid or methyl ester form. MeSA has been reported to serve as an airborne signal, activating disease resistance and expression of defense-related genes in tobacco (Shulaev et al. 1997). SA is also an endogenous signaling molecule that can modulate JA-dependent and JA-independent defense responses. In lima bean and tobacco, MeSA emission is preceded by an increase in endogenous SA levels (Shulaev et al. 1997; Engelberth et al. 2001). In maize, (*Z*)-3-hexenol treatment mimicked BAW in activating *pal* and MeSA, while MeJA resulted in *pal* induction with no increase in MeSA emissions (Figs. 2, 5). Since the JA and SA defense pathways can respond antagonistically (Doares et al. 1995; Rao et al. 2000), an elevated level of applied MeJA may play some role in the down-regulation of MeSA emissions as well as endogenous SA levels.

(*Z*)-3-Hexenol induction of *lox* and *pal* triggered an increase in (*Z*)-3-hexenyl acetate and MeSA emissions; however, induction of *mpi* by (*Z*)-3-hexenol (Fig. 2) failed to trigger accumulation of MPI protein (Fig. 6). In tomato, (*E*)-2-hexenal was found to induce the systemic peptide signal systemin that activates proteinase inhibitor (*pin*) accumulation (Sivasankar et al. 2000); however, production of systemin by C_6-volatiles failed to result in an induction of *pin* in tomato (Sivasankar et al. 2000).

In maize, (*Z*)-3-hexenol treatment did not activate sesquiterpene biosynthesis at either the genomic (Fig. 2) or metabolic levels (Fig. 7a), whereas an induction was observed with BAW and MeJA treatments. In Arabidopsis, *hmgr-1*, which encodes the enzyme that serves as an entry point into sesquiterpenes biosynthesis, was found to be induced by MeJA and not with (*E*)-2-hexenal treatment (Bate and Rothstein 1998). The fact that a significant increase in sesquiterpene emissions was observed with BAW and MeJA treatments at an earlier time point than *fps* gene induction suggests that induction of *fps* may not be the rate-limiting step in the regulation of sesquiterpene biosynthesis.

Structural specificity of volatile elicitors is evident in the case of the herbivore elicitor volicitin in which the L-isomer of the amino acid is active while the D-isomer is inactive in triggering VOC emissions (Truitt et al. 2004). Structural specificity was also observed among C_6-volatiles, with (*Z*)-3-hexenol and (*E*)-2-hexenal the only active components of those tested in triggering VOC emissions. One structural motif that has been proposed to be particularly reactive in triggering defense responses in plants is the presence of an α,β-unsaturated carbonyl group in an aldehyde form (Farmer 2001). In maize, conjugated (*E*)-2-hexenal triggered linalool emissions and *pal* transcripts, whereas no induction was observed with the non-conjugated (*Z*)-3-hexenal forms. In comparing C_6-alcohols, the presence of a double bond appears to be important in conferring activity, with (Z)-3-hexenol exposure triggering plant VOC emissions at a level ca. 8 times greater than *n*-hexanol (Table 1). Another important structural motif for C_6-volatile elicitation is the six-carbon chain length, as demonstrated by the administered compounds (*Z*)-3-heptenol and (*E*)-2-pentenal. Reduction or extension of the original C_6-unit proved to deactivate the alcohol or aldehyde, respectively, in triggering VOC emissions, even though the tested compounds contained the functional motif that triggered VOC emissions when part of the C_6-unit (Table 1).

The results presented here show that (*Z*)-3-hexenol can initiate a subset of regulatory steps and downstream metabolites to initiate defense responses in maize. This structural specificity reduces the likelihood that non-specific hydrophobic or polarity effects are predominantly responsible for the induction of VOC emissions. With these results in hand, an examination of how C_6-volatiles biochemically interact with plant tissue in triggering defense responses can proceed.

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