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Hexenyl Acetate Mediates Indirect Plant Defense Responses

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Oxylipins, lipid-derived signaling molecules have important functions in plant development, reproduction and responses to external stimuli. Jasmonates (JAs), C_6-aldehydes, and their corresponding derivatives, produced by the two main competing branches of the oxylipin pathway, the allene oxide synthase (AOS) and hydroperoxide lyase (HPL) branches, respectively. These two branches share a substantial overlap in their regulatory functions. Majority of experiments to define the role of C_6-aldehydes in plant defense responses were restricted to external application of aldehydes or the use of genetic manipulation of *HPL* expression levels in plant genotypes with intact ability to produce the competing AOS-derived metabolites. To uncouple the roles of the C_6-aldehydes and jasmonates in mediating direct and indirect plant-defense responses, we generated *Arabidopsis* genotypes lacking either one or both of these metabolites. These genotypes were subsequently challenged with a phloem-feeding insect (aphids: *Myzus persicae*) and the volatiles emitted by these plants upon aphid infestation or mechanical wounding were characterized. These experiments led to identification of hexenyl acetate as the predominant compound in these volatile blends. Subsequently, we examined the signaling role of this compound in attracting the parasitoid wasp (*Aphidius colemani*), a natural enemy of aphids.

Keywords: *jasmonates, C_6-aldehydes, hexenyl acetate, volatiles, aphids, plant defense responses*

INTRODUCTION

Plants employ a complex array of physical and chemical defense mechanisms to resist or evade biotic attacks. In addition to the constitutive defense mechanisms such as trichomes, thick secondary wall or toxic compounds, plants are also equipped with inducible defense mechanisms (Paiva, 2000; Walling, 2000). The inducible defenses function either directly via mechanisms such as production of amino acid catabolizing enzymes, antidigestive proteins, and toxic or repelling chemicals (Schoonhoven et al., 1998; Weber et al., 1999; Chen et al., 2005), or indirectly through production and release of volatile organic compounds (VOC) as a signal to the natural enemies of invaders that their prey is in the vicinity (Pare and Tumlinson, 1999; Kessler and Baldwin, 2001; Engelberth et al., 2004; Kessler et al., 2004; van Poecke and Dicke, 2004). Many inducible defense responses are activated by oxylipins, the oxygenated derivatives of fatty acids generated via the oxylipin branch pathways (Creelman and Mullet, 1997; Blee, 2002).

Allene oxide synthase (AOS) and hydroperoxide lyase (HPL) are the two main competing oxylipin-pathway branches producing stress-inducible compounds (Feussner and Wasternack, 2002). The metabolites of the AOS branch are jasmonates (jasmonic acid (JA), methyl jasmonate (MeJA) and their biosynthetic intermediate, 12-oxophytodienoic acid (12-OPDA)). Jasmonates were shown to be involved in many defense responses, including microbial pathogens, herbivores, mechanical and high UV light damages, as well as regulation of carbon partitioning (Devoto and Turner, 2003). The best characterized metabolites of the HPL branch are the green leafy volatiles (GLVs) that predominantly consist of C_6-aldehydes ((*Z*)-3-hexenal, *n*-hexanal) and their respective derivatives such as (*Z*)-3-hexenol, (*Z*)-3-hexen-1-yl acetate, and the corresponding *E*-isomers (Matsui, 2006). The functional role of JAs in mediating plant defense responses has received far more attention than the HPL-derived metabolites (Devoto and Turner, 2003). To examine the defensive function of C_6-aldehydes and their respective derivatives, investigators have altered the levels of GLVs either by the exogenous application of synthetic metabolites (Hildebrand et al., 1993; Bate and Rothstein, 1998; Hamilton-Kemp et al., 1998; Farag et al., 2005; Kishimoto et al., 2005), or by genetic manipulation of the *HPL* expression levels in plant genotypes that are intact in their ability to produce the competing AOS-derived metabolites (Vancanneyt et al., 2001; Halitschke et al., 2004; Kessler et al., 2004; Shiojiri et al., 2006). Collectively, these studies provide strong support for the important role of the HPL-derived metabolites in mediating plant defense responses. However, given the well documented substrate competition between the two branch pathways

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(Feussner and Wasternack, 2002; Matsui, 2006), and the considerable overlap in regulation of gene expression by HPL- and AOS-derived oxylipins (Halitschke et al., 2004), it has not been possible to conclusively determine whether or not each of these metabolites plays a distinct role in mediating direct and/or indirect plant defense responses. To uncouple the signaling roles of the C_6-aldehydes from those of the jasmonates in defense responses, we have generated an ensemble of plant genotypes lacking either one or both metabolites, and subsequently challenged them with various invaders as well as an insect parasitoid. The outcome of the analysis clearly establishes that hexenyl acetate, an acetylated C_6-aldehyde is the predominant wound-inducible volatile that mediates indirect defense responses by attracting the natural enemies of plant invaders to their prey.

MATERIALS AND METHODS

Plant lines and growth conditions

All transgenic and mutant *Arabidopsis thaliana* plants employed in this report were in Columbia-0 background (Col-0) and grown as previously described (Chelab et al., 2006). All the described experiments were performed with 5 week-old plants unless otherwise noted. The *gl-1* seeds (Herman and Marks, 1989) were kindly provided by Dr. Tom Jack (Dartmouth College, Hanover, NH). Seeds for the *aos* plants (CS6149), which were generated in *gl-1* background as a result of a T-DNA insertion in the *AtAOS* (*aos-ko)*, were purchased from the *Arabidopsis* Biological Resource Center (Columbus, OH). We performed PCR analyses and further confirmed the presence of T-DNA insertion within *AtAOS* as previously described (Park et al., 2002). In order to generate *Col-GFP* plants, here designated as Col, the open reading frame of GFP from pEZS-NLGFP vector was cut with *Not*I restriction enzyme and the generated DNA fragment was subsequently subcloned into the binary vector pMLBart, kindly provided by Dr. John Bowman (Monash University, Australia). Upon verification of the DNA insert by sequencing, the construct was used to transform Col-0. In addition, the *Os*HPL3-GFP construct in pMLBart previously described in (Chelab et al., 2006) was used to transform Col-0 and to generate *HPL-OE* plants. Transformation was performed by the floral-dip method (Clough and Bent, 1998), and the *Agrobacterium* strain used was EHA101. T_1 plants were germinated on soil. Selection of transgenics was by treating 10- to 12-d-old seedlings with 1:1.000 Finale (the commercial product that is 5.78% glufosinate ammonium) twice a week. Surviving plants were further screened to select for transgenics containing single inserts which were further propagated to get the homozygous lines used in this report.

To obtain *aos-hpl-GFP*, also designated as *aos-hpl*, and *aos-HPL-OE* plants, pollen from homozygous Col and *HPL-OE* were used to fertilize the male sterile *aos-ko* flowers. Homozygous lines of *aos-hpl* and *aos-HPL-OE* were generated from the segregating F_1 population using kanamycin as well as glufosinate ammonium as selection markers. All transgenes were verified by a number of approaches including PCR analyses using gene-specific primers as described below, in concert with the examination of male sterile phenotype and metabolic profiling of jasmonates and aldehydes, the products of the AOS and HPL branches respectively. All *Arabidopsis* lines containing a T-DNA insertion within the *AtAOS* were confirmed by PCR as previously described (Park et al., 2002). The following primers were further used to verify the presence of the *HPL-OE* transgene (5′-ATGGTGCC GTCGTTCCCGCA-3′ and 5′-TTAGCTGGGAGT GAGCTC-3′) and the *GFP* transgene (5′-ATGGTG AGCAAGGGCGAGGA-3′ and 5′-TACTTGTA CAGCTCGTCCATGCCGAGAGT-3′).

Upon flowering, plants containing the *aos* genotype were sprayed every other day with 2 mM MeJA (Sigma) dissolved in 0.03% Silwett in order to maintain homozygous *aos-ko* and permit an otherwise male sterile plant to produce seeds.

Quantification of AOS- and HPL-derived metabolites

Extraction of JAs (MeJA and JA) as well as 12-OPDA were carried out as previously described (Engelberth et al., 2003; Schmelz et al., 2003) with minor adjustments. In brief, leaf material (~300 mg fresh weight) was collected from intact plants, quickly weighed, and immediately frozen in liquid nitrogen to minimize wound-induced accumulation of oxylipins. Samples were finely ground in mortar while frozen and transferred to a 4 ml screw top Supelco vial containing 1200 µl of 2-propanol/H_2O/HCl (2:1:0.002) and sonicated in a water bath for 10 min. Dichloromethane (2 ml) was added to each sample and re-sonicated for 10 min. The bottom dichloromethane/2-propanol layer was then transferred to a 4 ml glass vial, evaporated under a constant air stream and the resultant pellet was subsequently dissolved in 300 µl of diethyl ether/methanol (9:1, vol/vol) followed by the addition of 9 µl of a 2.0 M solution of trimethylsilyldiazomethane in hexane in order to convert the carboxylic acids into the methyl esters. During this step JA is converted to MeJA. The vials were then capped, vortexed, and incubated at room temperature for 25 min. Then 9 µl of 12% acetic acid in hexane were added to each sample and left at room temperature for another 25 min in order to destroy all excess trime-

thylsilyldiazomethane. The above-mentioned procedure was also used to derivatize carefully calculated amounts of JA (Sigma Inc.) as well as 12-OPDA (Larodan Fine Chemicals Inc., Sweden) in triplicates to generate calibration curves. Methyl ester volatiles were captured on Super-Q (Alltech Inc., State College, PA) columns by vapor-phase extraction as described (Engelberth et al., 2003). The trapped metabolites were then eluted with 150 µl of dichloromethane and analyzed by GC-MS using a Hewlett and Packard 6890 series gas chromatograph coupled to an Agilent Technologies 5973 network mass selective detector operated in electronic ionization (EI) mode. One µl of the sample was injected in splitless mode at 250°C and separated using an HP-5MS column (30 m × 0.25 mm, 0.25 µm film thickness) held at 40°C for 1 min after injection, and then at increasing temperatures programmed to ramp at 15°C/min to 250°C (10 min), with helium as the carrier gas (constant flow rate 0.7 ml/min). Measurements were carried out in selected ion monitoring (SIM) mode with retention times and M^+ *m/z* ions as follows: JA-ME (*trans* 12.66 min, *cis* 12.91 min, 224) and 12-OPDA-ME (*trans* 18.31 min, *cis* 18.75 min, 306).

C_6-aldehydes were measured as previously described (Chelab et al., 2006).

Adsorptive headspace collection and analyses of volatiles emitted from wounded or aphid infested plants

GLVs collections were performed on ~2.2 g of either non wounded or mechanically wounded 5 week-old *Arabidopsis* plants in ~4 L glass desiccators-style containers (Duran Inc., Germany). GLVs were also collected from plants that were either intact or infested with ~500 aphid/plant. These plants were maintained, for the duration of sample collection (72 h), in ~4 L glass desiccators-style containers.

The dynamic headspace collection was performed using an air pump, circulating charcoal purified air in a closed loop at a rate of ~2 L min^{-1}. Emitted volatiles were trapped in a filter containing 50 mg of Porapak Q® (Waters Inc., Milford, MA) at the indicated times and the metabolites were subsequently eluted by applying 200 µl of dichloromethane to the filter. GLVs were analyzed on the same GC-MS instrument described above. One µl of the eluted sample was injected at 250°C in splitless mode and separated on a DB1MS (m×0.25mm×0.25µm). The GC oven temperature was programmed as follows: 5 min at 40°C, ramp to 200°C at 6°C/min with no hold time, but with a post run of 5 min at 250°C. Helium was the carrier gas at 53 ml/min. The mass spectrometer was run in the scan mode. Triplicate measurements from three independent biological samples were carried out for each time point. The identity of (*Z*)-3-hexen-1-yl acetate was determined by comparing the retention time (12.68 min) and mass spectra with that of an authentic standard. The amount of the volatile was computed subsequent to careful preparation of a calibration curve using (*Z*)-3-hexen-1-yl acetate as a standard.

Sources of insects and their maintenance

Green peach aphid (*M. persicae*) colonies were maintained on cabbage seedlings (*Brassica oleracea* var. *capitata*) at laboratory conditions (25±5°C, 50±20% relative humidity, 16 h light). *A. colemani* pupae were obtained from Koppert Inc. (Netherlands). The parasitoids emerged in closed containers at the above described laboratory conditions employed for the development of aphids.

Y-tube olfactometer bioassay using parasitoid wasp

The following bioassay was performed as previously described (Pareja et al., 2007) but with minor adjustments. Briefly, a glass Y-tube (diameter: 2.5 cm; trunk: 26 cm; arm: 12 cm) was used as the bioassay arena. The gas carrying the volatiles was clean in-house air which was filtered through activated charcoal before being split in two. Each stream was passed at 400 ml/min through ~4 L glass container having 2.2 g of mechanically wounded *Arabidopsis* leaves. All connections between the parts described were with Teflon tubing. After every fourth run, the Y-tube and glass vessels washed and rinsed with acetone and placed in an oven at 60°C. All bioassays were carried out at room temperature under artificial lighting in a white cardboard box with the Y-tube vertically placed.

In order to test the response of *A.colemani* to HPL-derived metabolites, volatiles from wounded *aos-hpl* leaves were tested against those of *aos-HPL-OE*. In addition, the parasitoid's response to synthetic hexenyl acetate was tested by allowing it to choose between volatiles from wounded *aos-hpl* leaves placed next to filters spotted with either 100 ng of synthetically pure hexenyl acetate (10 ng μl^{-1} in hexane) or 10 µl of hexane as the control. One-tailed binomial tests were performed to test the significance of the predators' choices for nymph deposition (Zar, 1999).

RESULTS AND DISCUSSION

Hexenyl acetate is the predominant plant volatile synthesized de novo in a transient fashion in response to wounding

We generated an ensemble of plant genotypes lacking either one or both sets of AOS- and HPL-derived metabolites using natural genetic variation and transgenic technologies. The *Arabidopsis* accession Columbia-0, is a natural loss-of-function

mutant in *hpl* and thereby lacks C_6-aldehydes (Duan et al., 2005). The double mutant lacking both C_6-aldehydes and jasmonates (*aos-hpl*) is an engineered T-DNA insertion line in *AOS* resulting in generation of *aos* loss-of-function plants in the trichomeless background (*gl-1*, accession Col-0) (Park et al., 2002). Hence this plant genotype is impaired in its ability to accumulate both JAs and C_6-aldehydes. In addition, this plant is male sterile and can only be maintained as homozygous for the *aos* mutation by spraying the developing flowers with MeJA (Park et al., 2002). We genetically modified these existing single and double mutant lines to produce C_6-aldehydes. To restore the aldehyde-producing capabilities of the wild type Col (WT) background, we had previously generated transgenic plants overexpressing a rice *Os*HPL3-GFP fusion construct (*HPL-OE*), as well as lines expressing GFP alone as the control (for simplicity designated here as Col) (Chelab et al., 2006). The basal and wound induced levels of C_6-aldehydes (hexenals and hexanals) in *HPL-OE* plants were at least 50-fold higher than the negligible levels produced via non enzymatic cleavage of the substrate in the control lines (Figure 1A). Wounding induces

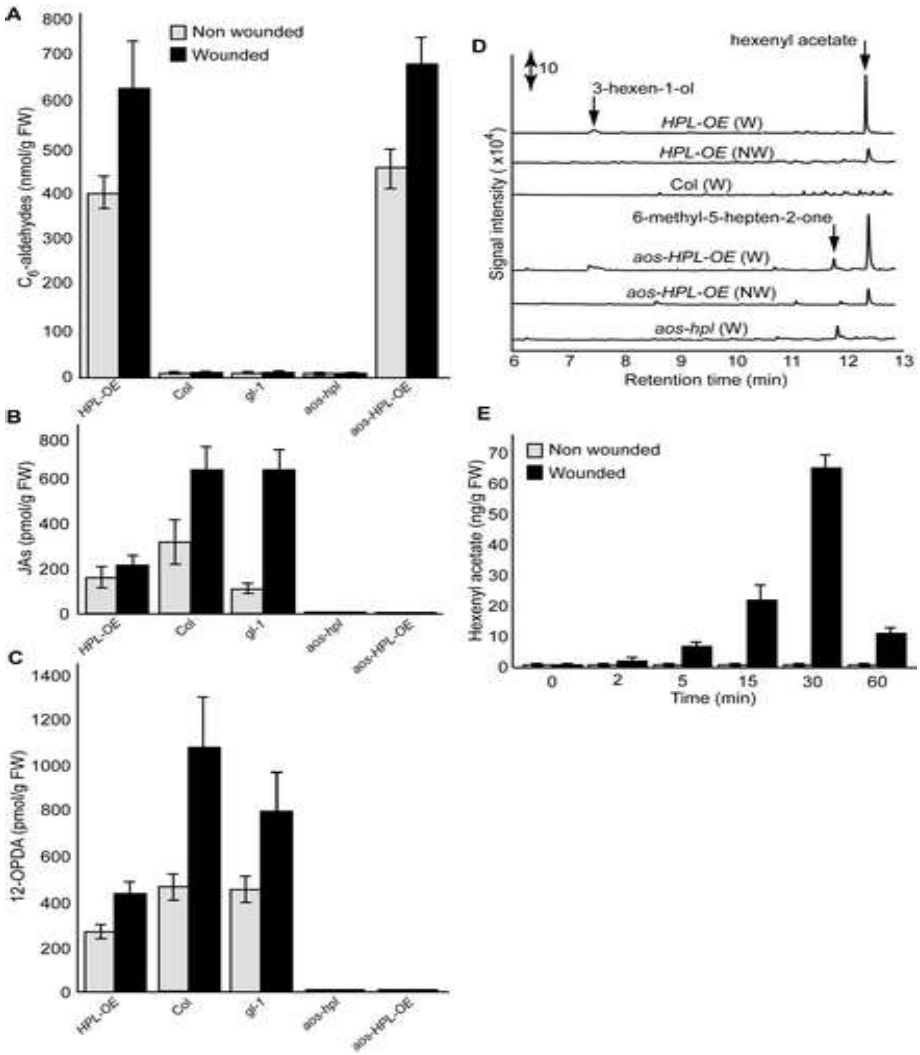

Figure 1. Profiling of the HPL- and AOS-branch pathways metabolites. (A) Levels of C_6-aldehydes, (B) JAs (JA+MeJA), and (C) 12-OPDA determined in non-wounded (grey bar), or wounded leaves 2 hours after mechanical damage (black bar). Each measurement is derived from the mean±standard deviation (SD) of three independent biological replicates. (D) Characterization and quantification of GLVs by adsorptive headspace collection and GC-MS analyses performed on three repeats of three independent biological replicates from wounded and non-wounded *Arabidopsis* genotypes show that hexenyl acetate is the predominant volatile produced in wounded leaves of plants with a functional *HPL*. Double-headed arrow represents a scale for signal intensity. (E) Analyses of the emission rate of hexenyl acetate in non-wounded (grey bar) or mechanically wounded (black bar) *aos-HPL-OE* plants, performed three times on three independent biological replicates show that emission of hexenyl acetate is wound-inducible and transient.

a 33% increase of these C_6-aldehydes in the *HPL-OE* line (Figure 1A). This is in spite of the constitutive expression of *HPL* under the 35S promoter, which may indicate the limited availability of the basal levels of substrates. Concomitant with these increases in the levels of C_6-aldehydes there is a ~60% reduction in the basal and wound-induced levels of JAs (JA and MeJA) and 12-OPDA in the *HPL-OE* as compared to the levels in the control Col, the naturally *hpl* mutant background (Figure 1B and C). These data, consistent with the previous reports (Halitschke et al., 2004), confirm the controlling role of substrate flux in biosynthesis of oxylipins and demonstrate that overexpression of the HPL branch reduces the pool of substrate available for the biosynthesis of jasmonates.

To restore C_6-aldehyde metabolism in the double mutant background (*aos-hpl*), while circumventing any potential influence of the transgene's insertion site or accumulation of second site mutations as the result of the transformation process, we out-crossed the *HPL-OE* to the *aos-hpl* and generated F_1 lines. To select a homozygous *aos-HPL-OE* plant in the subsequent segregating populations, we exploited the male sterile phenotype observed in plants lacking jasmonates (Park, 2002), in combination with the use of the selectable marker employed in generation of *HPL-OE* lines. Profiling of AOS- and HPL-derived metabolites of wounded and non-wounded *aos-HPL-OE* homozygous plants determined that while their jasmonates are below detection levels, their C_6-aldehydes are at levels comparable to those present in the *HPL-OE* line (Figure 1A, B and C). As a control we also out-crossed *aos-hpl* to Col, and generated a homozygous *aos-hpl-GFP* line for simplicity now designated also as *aos-hpl*. These plants, similar to the parental *aos* loss-of-function line in the *gl-1* background, are impaired in the production of both jasmonates and C_6-aldehydes in contrast to the *gl-1* background that is deficient only in C_6-aldehydes (Figure 1A, B and C).

To simultaneously characterize and quantify the wound induced VOCs, we conducted adsorptive headspace collection from all the above described genotypes. This analysis identified 3-hexen-1-yl acetate (hexenyl acetate), the acetylated derivative of (*Z*)-3-hexenol, as the predominant volatile which was released only from the aldehyde-producing plants namely, the *aos-HPL-OE* and *HPL-OE* lines (Figure 1D). Wounding of *aos-HPL-OE* or *HPL-OE* lines led to emission of ~20-fold higher levels of hexenyl acetate than the corresponding non-wounded plants. Additional analyses designed to measure the emission rate of hexenyl acetate established that this plant volatile is synthesized *de novo* and is released rapidly and transiently in response to wounding. Specifically, these data show that a negligible basal level of hexenyl acetate is emitted from the non-wounded plants (Figure 1E). However, 2 minutes after wounding these levels are increased by ~2-fold (3 ng/g FW), reaching the maximum levels (68 ng/g FW) at 30 minutes, and declining by ~6.5-fold (12 ng/g FW) by 60 minutes.

Figure 2. The dynamic headspace collection set.

To specifically examine the role of hexenyl acetate, the predominant wound-induced volatile among the complex blend emitted by the plants, we also performed volatile bioassays with wounded *aos-hpl* in the presence or absence of chemically synthesized hexenyl acetate. These data clearly show that 60% of wasps were attracted to the jar containing *aos-hpl* plants along with the filters spotted with synthetic hexenyl acetate ($P = 0.034$) (Figure 3C).

Hexenyl acetate is the volatile signal from plants to natural enemies of aphids

To characterize VOCs produced by aphid infested plants we conducted adsorptive headspace collection from intact and infested *aos-hpl* and *aos-HPL-OE* plants (Figure 2). Similar to the data obtained from mechanically wounded leaves (Figure 1D), these analyses also identified hexenyl acetate as the prevalent volatile that is predominantly released from the *aos-HPL-OE* plants infested with aphids (Figure 3A). To further examine the role of aldehydes in general and hexenyl acetate in particular in mediating plant indirect responses, we performed volatile bioassays using a glass Y-tube olfactometer

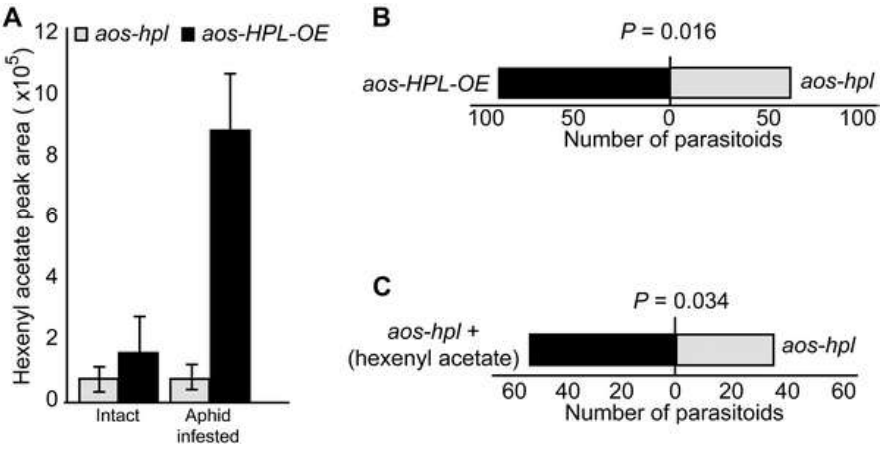

Figure 3. Attraction of parasitoid wasp, *Aphidius colemani,* to the *in vivo* wound-induced or chemically synthesized hexenyl acetate. (A) Characterization and quantification of GLVs by adsorptive headspace collection and GC-MS analyses performed on three repeats of five independent biological replicates from intact and aphid infested *aos-hpl* and *aos-HPL-OE* genotypes show that hexenyl acetate is the predominant volatile produced in aphid infested plants with a functional *HPL*. (B) Volatile bioassays using glass Y-tube olfactometer was employed to determine the response of *A.colemani* to the volatile blend produced from mechanically wounded *aos-hpl* and *aos-HPL-OE* plant genotypes. The bar graph represents the number of parasitoids examined and shows that they are significantly attracted more to the wounded *aos-HPL-OE* than to the *aos-hpl* plants (P = 0.016). (C) Volatile bioassays using glass Y-tube olfactometer was employed to determine the response of *A.colemani* to the presence or absence of synthetic hexenyl acetate in chambers containing wounded *aos-hpl* plant genotype. The bar graph represents the number of parasitoids examined and shows that they are significantly attracted towards the chamber of wounded *aos-hpl* plants with hexenyl acetate-spotted filters as compared to the plant chamber containing the same plant genotype but with hexane-spotted filters as the control (P = 0.034). One-tailed binomial tests were used to determine significance.

and examined attraction of *Aphidius colemani* to wounded *aos-hpl* versus *aos-HPL-OE*. This wasp is parasitic to a range of aphids including green peach aphid. The female wasp finds aphid colonies from a long distance by "alarm signals" produced by an infected plant and lays its egg directly inside the aphid, where the larva feeds and develops into a fully formed wasp killing the aphid in the process. Preference tests using 160 *A.colemani* females released individually show that a statistically significant number of these female parasitoid wasps are attracted to *aos-HPL-OE*, as compared to *aos-hpl* plants (P = 0.016) (Figure 3B).

SUMMARY

This study conclusively establishes the role of C_6-aldehydes in plant defense responses. We exploited the genotypes generated in our laboratory lacking either one or both sets of AOS- and HPL-derived metabolites to demonstrate role of hexenyl acetate, an acetylated C_6-aldehyde, as the predominant wound-inducible volatile signal that mediates indirect defense responses by directing tritrophic (plant-herbivore-natural enemy) interactions.

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