Isomers of green leaf volatiles in Nicotiana attenuata and their role in plant-insect interactions
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Insects betray themselves in nature to predators by rapid isomerization of green leaf volatiles

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ABSTRACT

Plants emit green leaf volatiles (GLVs) in response to herbivore damage, thereby attracting predators of the herbivores as part of an indirect defense. The GLV component of this indirect defense was thought to be a general wound signal lacking herbivore-specific information. We found that Manduca sexta–infested Nicotiana attenuata attract the generalist hemipteran predator Geocoris spp. as the result of an herbivore-induced decrease in the (Z)/(E) ratio of released GLVs, and that these changes in the volatile bouquet triple the foraging efficiency of predators in nature. These (E)-isomers are produced from plant-derived (Z)-isomers but are converted by a heat-labile constituent of herbivore oral secretions. Hence, attacking herbivores initiate the release of an indirect defense a full day before the attacked plants manufacture their own defensive compounds.

Image description front page

Upper left panel: Close-up of an adult ‘big-eyed bug’ (Geocoris spp.) which is a generalist predator feeding on Manduca eggs and early larval instars. Image courtesy of Matthey Film.
Upper right panel: Egg predation set-up in the field: eggs are glued on the underside of a leaf and scents are smeared onto a cotton swab which is placed close to the leaf. Image courtesy of D. Kessler.
Lower left panel: Second instar M. sexta caterpillar on a N. attenuata leaf. Image courtesy of A.P. van Doorn.
Lower right panel: A first instar caterpillar is attacked by an adult generalist predator who tries to pierce with its stylet into the rear end of the caterpillar. Image courtesy of Matthey Film.
Plants defend themselves against herbivore attack by producing chemical and physical defenses that decrease herbivore performance, but they also release distinct volatile bouquets when attacked. These herbivore induced plant volatiles (HIPVs) can function as indirect defenses by attracting carnivores. Although the attraction of natural enemies to HIPVs has frequently been observed in laboratory studies (1), few studies have demonstrated that HIPVs attract carnivores in nature (1–3).

Elicitors in herbivore oral secretions introduced into plant wounds during feeding (4–6), as well as the rhythm of caterpillar feeding (7), provide the plant with the information required to activate herbivore-specific defenses, including the release of specific volatiles. The composition of the HIPV blend can differ among plant and herbivore species, abiotic conditions, and over time (2, 8–10). HIPVs have several different metabolic origins, of which the isoprene-derived terpenoids and fatty acid–derived green leaf volatiles (GLVs) are the best-studied classes. Terpenoids are released with a delay from the whole plant, not just attacked leaves, after a few hours or with the plant’s next photosynthetic phase (i.e., often a day after the start of herbivore attack) (11–13). Because of their delayed, systemic release, the terpenoids likely function in the long distance attraction of carnivores. GLVs - which consist of six-carbon aldehydes, alcohols, and their esters - are, unlike terpenoids, immediately and likely passively released from wounded leaves (11, 14). Consequently, GLVs likely provide rapid, but nonspecific information about the exact location of a feeding herbivore. GLVs were shown to play a role in host-location of predators and parasitic wasps (15–17). Although changes in constitutive GLV ratios can alter the ability of herbivores to locate their host (18), the degree to which natural enemies use induced GLVs to find plants with prey remains unclear (19, 20).

The GLV blend of mechanically wounded Nicotiana attenuata plants contains large amounts of (Z)-GLVs and proportionally smaller amounts of (E)-GLVs (fig. 3.1). These different isomers result from rearrangements of the double bonds. However, plants that had been attacked for 24 hours by 1, 5, or 10 Manduca sexta neonates, a specialist herbivore of this native tobacco, released (Z)- and (E)-isomers in nearly equal amounts (fig. 3.1A) (21). We compared changes in the (Z)/(E) ratio of GLVs released from mechanically wounded leaves of which the wounds were treated with water (w + w) or M. sexta oral secretions (OS) (w + OS; fig. S3.1) (22). We found that w + w–treated plants emitted high levels of (Z)-GLVs and low levels of (E)-GLVs, whereas treating wounds with M. sexta OS decreased emissions of (Z)-GLVs and increased those of (E)-GLVs, resulting in a distinct change in the (Z)/(E) ratio (fig. 3.1, B and C, and table S3.1). To determine whether the OS-elicited (Z)/(E) shift is
a transient response, we monitored the GLV bouquet for several hours after a single elicitation. Although the GLV burst occurred immediately after a single wounding and vanished after a few hours (fig. S3.2A), the OS-elicited changes in the \((Z)/(E)\) ratio persisted for the duration of the GLV burst (fig. S3.2B).

Fig. 3.1. Herbivory and the application of *Manduca sexta*’s oral secretions to the wounds of wild-type *Nicotiana attenuata* plants lead to a marked change in GLV emissions. (A) Mean \((Z)/(E)\) ratios, with 95% confidence limits, of *N. attenuata* plants attacked by 1, 5, or 10 *M. sexta* neonates (22); CP, caterpillar. (B) Wounded plants release GLVs with a high \((Z)/(E)\) ratio, whereas OS-elicited plants emit a GLV bouquet with a much lower ratio (22). Bars represent the average ratio \((n = 5)\) and their 95% confidence limits. (C) Mean (+ SE) release of GLVs in \(w + w\)-treated and \(w + OS\)-treated plants in the first 20 min after elicitation \((n = 5)\). Values are indicated in ng g FW\(^{-1}\) 20 min\(^{-1}\). LOX2, lipoxygenase 2; HPL, hydroperoxide lyase; ADH, alcohol dehydrogenase; AAT, alcohol acetyltransferase; 13-HPs, 13-hydroperoxides.
Saliva or OS from herbivores can elicit specific responses in a plant, and two main classes of endogenously produced elicitors have been characterized in lepidopteran larvae: fatty acid–amino acid conjugates (FACs) and enzymes such as β-glucosidase and glucose oxidase (6). In addition, OS contain elicitors of plant origin, such as inceptins, which are processed in the oral cavity and reintroduced into the plant during feeding (6). FACs are central elicitors in M. sexta’s OS. In N. attenuata they amplify the wound-induced bursts of the phytohormones, jasmonic acid (JA), and ethylene as well as the release of the sesquiterpene (E)-α-bergamotene (5, 6). We trapped volatiles from plants that had been wounded and treated with the two most abundant FACs in M. sexta’s OS: N-linolenoyl-glutamate (18:3-Glu) or N-linolenoyl-glutamine (18:3-Gln) (5). Neither of the two elicited the OS-induced change in the (Z)/(E) ratio (fig. S3D). Because the alkaline pH of M. sexta OS (pH = 9) elicits the release of methanol during herbivory by activating pectin methyl esterases in leaves (23), we tested the influence of pH on the GLV emissions. In theory, alkaline conditions could convert the relatively unstable (Z)-hex-3-enal into its more stable (E)-isomer, (E)-hex-2-enal. However, an alkaline buffer (0.1MTris, pH 9, in 0.02%Tween-20) equal to the pH of M. sexta OS sufficient to elicit the methanol release did not change the (Z)/(E) ratio relative to w + w–treated plants when applied to puncture wounds (fig. S3.3, A and B). FACs are relatively heat-stable compounds, and heating OS to 90°C for 10 min did not reduce the concentration of FACs in OS (fig. S3.3, E and F). However, heated OS no longer elicited the shift in the (Z)/(E) ratio (fig. S3.3C). Therefore, we conclude that FACs are not involved, but that a heat labile elicitor of M. sexta’s OS directly converts (Z)- into (E)-GLVs or indirectly activates an isomerase in the plant. Additional tests revealed that the M. sexta OS-mediated (Z)/(E) shift is independent of the plant’s JA, salicylic acid, and ethylene-dependent defense signaling pathways (fig. S3.4 and tables S3.2 and S3.3) (22).

To determine whether the OS elicitor responsible for the (Z)/(E) shift functions directly as an isomerase, we added (Z)-hex-3-enal to M. sexta’s OS in an in vitro system and quantified the conversion to (E)-hex-2-enal. More than 50% of the added (Z)-hex-3-enal was converted, which did not occur when water or heated OS were used as the converting solution and only slightly with an alkaline buffer (Fig. 3.2). Additionally, we tested two OS-derived enzymes, glucose oxidase and β-glucosidase, both of which elicit specific responses in plants (6). However, these enzymes did not increase the release of (E)-hex-2-enal. Bovine serum albumin (BSA) has lipophilic properties and increased the isomerization of cis- to trans-JA
when added to plant cell cultures (23). However, BSA had no effect on the (Z) to (E) conversion of leaf aldehydes (fig. S3.5).

![Fig. 3.2. Percentage conversion to (E)-hex-2-enal in vitro (n = 6; light gray bars) and in vivo (n = 6; dark gray bars) (Fig. 3.1) (22). Asterisks indicate significant differences from the control treatment (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001); univariate analysis of variance, F_{6,37} = 99.957, P ≤ 0.001, followed by a Tamhane post hoc test; oral secretions (OS), P ≤ 0.001; OS from artificial diet–fed caterpillars (OS_{AD}), P ≤ 0.001; OS heated to 90°C for 10 min (OS_{boiled}), P = 0.051; 1× buffer (pH 9), P ≤ 0.05; leaf juice (LJ), P = 1.000; LJ + OS, P ≤ 0.01; the control was 0.02% Tween-20 (22).](image)

An (3Z):(2E)-enal isomerase that converts the (Z)-aldehyde to its (E) isomer has been proposed for several plant species; (3Z):(2E)-enal isomerase activity has been found in crude extracts of alfalfa and soybean (25, 26). However, we showed that a plant-derived isomerase was not responsible for the (Z) to (E) conversion of leaf aldehydes, but an unknown OS-derived, heat-unstable compound was necessary and sufficient for the conversion. The addition of crude leaf extract did not increase the conversion rate, and OS from caterpillars never fed on leaf material, but only on an artificial diet (22), converted (Z)-aldehydes to (E)-aldehydes as efficiently as OS from plant-fed caterpillars (Fig. 3.2). The observed (Z) to (E) conversion also appears to be species specific, as the OS from two other generalist lepidopteran species (Spodoptera exigua and S. littoralis) that can feed on N. attenuata were not nearly as active as the OS from M. sexta larvae (fig. S3.6).
Whether this herbivory-specific change is relevant for tritrophic interactions hinges on whether carnivores can use these isomeric changes to augment their prey-hunting abilities. Insects are known to distinguish different isomeric forms of volatiles (27, 28) and two structural isomers elicit responses in distinct antennal olfactory receptor neurons (29). *Geocoris* spp. are generalist predators feeding on eggs and early larval instars of *M. sexta*. They use individual HIPVs, including terpenoids and GLVs, to locate their prey on herbivore-attacked plants (3, 16). We tested whether *Geocoris* could distinguish between (Z)- and (E)-GLVs, and used the changes in the (Z)/(E) ratio to discriminate between an herbivore attacked and a mechanically wounded *N. attenuata* plant in nature.

We created two mixtures (A and B) in lanolin containing either (Z)- or (E)-GLVs (30). We also created GLV mixtures to mimic the GLV emissions of w + w–treated or w + OS–treated plants. To do so, we combined both isomeric alcohols and hexenyl esters (*table S3.5, C and D*) or all eight GLVs used for mix A and B (*table S3.5, E and F*) and added different amounts of each isomer to mimic the 1:1 (Z)/(E) ratio released by OS-elicited plants or the 9:1 ratio to mimic mechanically wounded plants.

We tested the attractiveness of these mixtures to *Geocoris* spp. in a native *N. attenuata* population in the Great Basin desert of southwest Utah by gluing three *M. sexta* eggs per plant to the underside of a lower stem leaf of 21 pairs of plants, as described (3). Plants were of the same size and developmental stage. Developmental stage did not influence the OS-induced (Z)/(E) shift (*fig. S3.7*) (22). On each day, two different mixes were tested by dipping a cotton swab into lanolin paste containing different GLV mixes and placing them immediately adjacent to the leaf with the *M. sexta* eggs.

Predated eggs were counted after 12 and 24 hours. We started with mixes that contained either (Z)- or (E)-GLVs (*table S3.5*, mixes A and B) and *Geocoris* spp. showed a clear preference for those scented with the (E)-GLVs. Whereas 8% of the (Z)-baited eggs were predated, 24% of the (E)-baited eggs were predated (*fig. S3.9*). These results demonstrated that *Geocoris* may distinguish between (Z)- and (E)-GLVs. We tested GLV mixes that consisted of both isomers (*table S3.5C versus S3.5D; table S3.5E versus S3.5F*) in different ratios to determine whether *Geocoris* detected changes in the (Z)/(E) ratio similar to those of OS-elicited plants. In both experiments, predation rates were higher on plants scented with equal amounts of (Z)- and (E)-GLVs [(Z)/(E) = 1:1] relative to those scented with a 9:1 ratio of (Z)/(E)-GLVs (*Fig. 3.3*) (22).
Fig. 3.3. Predation by *Geocoris* spp. in the field. (A) *Geocoris* spp. are generalist predators feeding on eggs and early instar larvae of *Manduca sexta*. [Photo: M. Stitz] (B) Predation assays were performed in a native *Nicotiana attenuata* population in the Great Basin desert of southwest Utah. [Photo: D. Kessler] (C) Average egg predation per plant and day (±SE). Numbers in the plot denote total number of eggs predated per treatment. Treatment pairs with no predated egg were excluded before statistical analysis. Asterisks indicate significant differences between treatments (paired-sample t test, mix E versus F, $t_{17} = 4.600$, ***$P \leq 0.001$; mix C versus D, $t_{12} = 1.594$, $P = 0.137$). The composition of the different mixes tested (mixes C, D, E, and F) are explained in table S3.5. n.s., not significant.

These results show that attack by the specialist herbivore *M. sexta* and the addition of their oral secretions to mechanical wounds elicits a rapid (Z)/(E) isomeric change in the GLV release of *N. attenuata* plants. This change, which lowers the (Z)/(E) ratio of the GLV blend, increases the predation rate of the generalist predator *Geocoris* spp., likely by betraying the location of the feeding caterpillar in a rapid, herbivore-specific, and spatially explicit manner. Why *Manduca* larvae would produce such an apparently maladaptive elicitor in their OS remains to be determined, but the larvae may benefit from the enhanced antimicrobial properties of a GLV blend enhanced in (E)-hex-2-enal (31).
REFERENCES

1. J. D. Allison, J. Daniel Hare, New Phytol. 184, 768 (2009).
21. GLV esters were near or below the detection limit; thus, only the ratios for the aldehyde and the alcohol are shown.
22. See supporting material.
30. We used the four most abundant GLVs of both isomers in N. attenuata: mix A, (Z)-hex-3-enal, (Z)-hex-3-en-1-ol, (Z)-hex-3-enyl acetate, and (Z)-hex-3-enyl butyrate; mix B, (E)-hex-2-enal, (E)-hex-2-en-1-ol, (E)-hex-2-enyl acetate, and (E)-hex-2-enyl butyrate (tables S3.5 and S3.6).
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5) Supporting references
1) Material and methods

a. Plant Material and Growing Conditions

We used seeds of the 22nd and 30th generation of an inbred line of *Nicotiana attenuata* as the wild-type genotype in all experiments (S1). The original seeds were collected in 1988 from a natural population at the DI ranch in southwestern Utah, USA. Seeds of wild-type and genetically transformed plants were germinated as described previously (S1). Briefly, seeds were sterilized and incubated in 1 mM GA3 (Roth, Karlsruhe, Germany; www.carl-roth.de) and 1:50 diluted liquid smoke (v/v) (House of Herbs, Passaic, NJ) before germination on agar plates containing Gamborg’s B5 medium (Duchefa, Haarlem, The Netherlands, www.duchefa.com) at a 26°C/16 h 155 µm s⁻¹ m⁻² light: 24°C/8 h dark cycle (Percival, Perry, IA). Plants were grown in the glasshouse with a day/night cycle of 16 h (26°C–28°C)/8 h (22°C–24°C) under supplemental light from Master Sun-T PIA Agro 400 or Master Son-T PIA Plus 600-W sodium lights (Philips). In the glasshouse plants were grown in 1 L individual pots at 26°C to 28°C under 16 h of light as described by Krügel et al. (S1).

The transformed plants used were *ir-lox3* (A-03-362-2, (S2)), *ir-coil* (A-04-248, (S3)), *as-aco* (A-03-321-10,(S4)), 35s-etr1a (A-03-328-8, (S4)), *ir-npr1* (A-05-213, (S5)) and the cross *ir-sipk* x *ir-wipk* (S6, 7). In *Nicotiana attenuata* lipoxygenase-3 catalyzes the formation of fatty acid hydroperoxides representing the first committed step in the biosynthesis of jasmonic acid (JA). Coronatine insensitive1 (COI1) participates in the perception of JA and regulates gene expression through its interaction with the JASMONATE-ZIM-DOMAIN repressors. *Ir-lox3* (S2) as well as *ir-coil* (S3) *N. attenuata* plants are impaired in JA-mediated direct and indirect defense responses. One of the earliest events initiated by wounding and herbivory described to date is the activation of mitogen-activated protein kinases (MAPK). Two MAPKs in tobacco, the salicylic acid (SA) induced protein kinase (SIPK) and the wound-induced protein kinase (WIPK) are activated in response to multiple biotic and abiotic stresses, including wounding and herbivory. When SIPK and WIPK expression is silenced in *N. attenuata*, the plants are impaired in JA, SA and ethylene biosynthesis (S6, 7).

Another regulatory component that affects JA production in *N. attenuata* is Nonexpressor of PR-1 (NPR1), a negative regulator and an essential component of the SA signal transduction pathway first identified in Arabidopsis (S8). Silencing NaNPR1 reduces the levels of JA and JA–Ile/Leu but increases free SA in herbivore-attacked plants (S5). The two transformants, *as-aco* and 35s-etr1a (S4), are both lines with impaired ethylene emissions. While ACC oxidase (ACO) displays the second committed enzyme in the ethylene
biosynthesis, the ethylene receptor (ETR) is required for perception of ethylene and plants with a mutated (non-functional) ethylene receptor (35s-etr1a) are thus insensitive to ethylene.

b. Plant Treatments

All treatments in the glasshouse were performed with plants in the rosette stage of growth, except when studying the \((Z)/(E)\)-ratio in two different developmental stages (Fig. S3.7). The first fully elongated (+1 position) leaf was used, unless otherwise noted. Plants were wounded with a pattern wheel to punch three rows of holes on each side of the midrib. Wounded leaves were immediately treated with 20 µL of the eliciting solution which was pipetted directly onto the wounded leaf and gently dispersed across the surface with a gloved finger, changing gloves between treatments. The compositions of the different eliciting solutions are summarized in Figure S3.1.

GLV emissions induced by mechanical wounding of the plant were assessed by applying deionized water \((w + w)\) onto the wounds. *Manduca sexta* oral secretions (OS) were collected from third to fifth instar caterpillars reared on *N. attenuata* wild type plants, flushed with argon, stored at -20°C and diluted 1:3 (v/v) with deionized water prior to use.

GLV emissions induced by the alkalinity of *Manduca sexta’s* OS were assessed by applying a 0.1 M Tris, pH 9, buffer solution containing 0.02% (v/v) Tween-20 (1x buffer). This surfactant was added to evaluate its potential eliciting effect, since it was used for the preparation of the FACs solution. The two most abundant FACs in *M. sexta’s* OS, N-linolenoyl-glutamate \((18:3-\text{Glu})\) or N-linolenoyl-glutamine \((18:3-\text{Gln})\) (S9) were used in a concentration equal to 1:3 diluted *M. sexta* OS \((18:3-\text{Glu}, 0.11 \text{mM in 1x buffer and 18:3-\text{Gln; 0.04 mM in 1x buffer}})\). To control for a possible effect of the buffer itself, *M. sexta* OS was diluted 1:3 (v/v) with 3x buffer instead of deionized water in this experiment. Boiled OS \((\text{OS}_{\text{boiled}})\) was heated to 90°C for 10min, flushed with argon, stored at -20°C and diluted 1:3 (v/v) with deionized water prior to use.

To trap volatiles from caterpillar attacked plants, we placed one, 5 or 10 freshly hatched *M. sexta* neonates on the first fully elongated leaf (+1 position) of a wild type plant and let them feed for 24h. After this period we enclosed all caterpillars and the leaf on which they were feeding in two 50 mL food-quality plastic containers and trapped volatiles for 20 minutes as described in section ‘in vivo Volatile Collection and Analysis’.
c. *In vivo* Volatile Collection and Analysis

Single leaves (+1 position for rosette stage and S1 position for early flowering plants) were enclosed immediately after treatment between two 50 mL food-quality plastic containers (Huhtamaki, Bad Bertricher, Germany; [www.polarcup.de](http://www.polarcup.de)) secured with miniature claw-style hair clips. Ambient air was pulled through the collection chamber and a glass tube (ARS, Inc., Gainsville, FL, USA; [www.ars-fla.com](http://www.ars-fla.com)) packed with glass wool and 20 mg of Super Q (Alltech, Düsseldorf, Germany; [www.alltech.com](http://www.alltech.com)). Airflow was created by a vacuum pump (model DAA-V114-GB, Gast Mfg, Benton Harbour, MI, USA; [www.gastmfg.com](http://www.gastmfg.com)) as described by Halitschke et al. (S10). If not otherwise noted volatiles were trapped for 20 min immediately after elicitation. SuperQ traps were eluted with 500 μL dichloromethane (DCM) into a GC vial after spiking each trap with 320 ng tetralin (Sigma-Aldrich, Germany; [www.sigmaaldrich.com](http://www.sigmaaldrich.com)) as an internal standard.

Samples were analyzed on an Agilent 6890N gas chromatograph equipped with an Agilent 7683 autoinjector (Agilent Technologies, Böblingen, Germany; [www.agilent.com](http://www.agilent.com)) and coupled with a LECO Pegasus III time-of-flight mass spectrometer with a 4D thermal modulator upgrade (LECO, Mönchengladbach, Germany; [www.leco.de](http://www.leco.de)) as described by Gaquerel et al. (S11). GLVs were identified and quantified using standard solutions of (Z)-hex-3-enal, (E)-hex-2-enal, (Z)-hex-3-en-1-ol, (E)-hex-2-en-1-ol, [(Z)-hex-3-ethyl] acetate, [(E)-hex-2-ethyl] acetate, [(Z)-hex-3-ethyl] propanoate, [(Z)-hex-3-ethyl] butanoate, [(E)-hex-2-ethyl] butanoate, (Sigma-Aldrich, Germany; [www.sigmaaldrich.com](http://www.sigmaaldrich.com)) and [(E)-hex-2-ethyl] propanoate (Bedoukian Research Inc, Danbury, USA; [www.bedoukian.com](http://www.bedoukian.com)).

(Z)/(E)-ratios were calculated for each sample by dividing each (Z)-GLV by its corresponding (E)-isomer. Ratios were log transformed and the average ratio was subsequently calculated by back transforming the average log ratio and their 95% confidence limits via retransformation of their confidence limits in the log scale following Sokal & Rohlf (S12).

To determine the longevity of GLVs released after a single elicitation and to determine if the OS-elicited (Z)/(E)-shift is transient, rosette staged WT plants were wounded and treated with either water or *M. sexta* OS (1:3, v/v). Leaves were immediately enclosed between two plastic containers as described previously. Traps were exchanged after 20, 40, 60, 150 and 360 minutes ([Fig. S3.2](#)), measured and analyzed as described above.
**d. In vitro Volatile Collection**

To test whether *M. sexta* OS was sufficient to convert (Z)- into (E)-GLVs, we tested *M. sexta* OS and several other eliciting solutions for their ability to convert (Z)-hex-3-enal into its corresponding (E)-isomer, (E)-hex-2-enal. 300 µL of the eliciting solutions were pipetted into a GC vial with an insert and 1 µL of (Z)-hex-3-enal (4.8 µg/µL DMSO) was added to the solution. A volume of 300 µL was chosen to minimize the headspace and thus also the chance of (Z)-hex-3-enal to volatilize without reacting with the eliciting solutions. The GC vial was closed and gently shaken for 20 minutes. After this period the liquid solution was transferred into a 20 mL scintillation vial and volatiles were trapped at room temperature for 20 min from the stirring solution. A constant nitrogen airflow was blown into the scintillation vial and pushed through the glass tube packed with glass wool and 20 mg of Super Q (Alltech, Düsseldorf, Germany; [www.alltech.com](http://www.alltech.com)). The Super Q traps were spiked with 160 ng tetralin and eluted with 250 µL DCM. Samples were analyzed as described in the previous section. (Z)-hex-3-enal is a rather unstable compound and 14.5 % of the pure standard was already converted to (E)-hex-2-enal when directly injected into the GC. A 14.5 % ‘auto-conversion’ was thus included for all measurements when calculating the percentage conversion to (E)-hex-2-enal with the *in vitro* assay. The composition of the different eliciting solutions is summarized in Figure S3.5. All eliciting solutions contained 0.02 % Tween-20 to guarantee a good solubility of the substrate, (Z)-hex-3-enal.

We diluted boiled and unboiled *M. sexta* OS (OS<sub>boiled</sub> and OS) as well as OS that we collected from caterpillars that had fed on artificial diet (OS<sub>AD</sub>, (S13)) 1:3 (v/v) with 0.03 % Tween-20 solution. The control solution (ctrl) was a 0.02 % Tween-20 solution. The (Z)/(E)-conversion induced by the alkalinity of *M. sexta* OS was assessed by using a 0.1 M Tris, pH9, buffer solution that contained 0.02 % (v/v) Tween-20 (1x buffer). We furthermore tested two OS-derived enzymes, glucose oxidase (1 U/mL, from *Aspergillus niger*, Sigma-Aldrich, Germany; [www.sigmaaldrich.com](http://www.sigmaaldrich.com)) and β-glucosidase (8 U/mL, from almonds, Sigma-Aldrich, Germany; [www.sigmaaldrich.com](http://www.sigmaaldrich.com)), both of which are known to function as elicitors when introduced into plants. Both enzymes were diluted in 1x buffer. Concentrations tested were in the range of physiological amounts found in *M. sexta* OS (S14, Fig. S3.10). BSA was dissolved in 1x buffer (0.5% BSA (w/v)).

We tested the enzymatic properties of OS from *Spodoptera exigua* and *S. littoralis* (3<sup>rd</sup> to 4<sup>th</sup> instar) using the *in vitro* system. Both species were reared on artificial diet but had fed on *N. attenuata* plants for at least 24 h before use. OS from all tested species was diluted 1:20 with 0.02 % Tween-20 solution.
To confirm that the (Z)/(E)-conversion is caused by an OS-derived enzyme and that, except for the supply of (Z)-hex-3-enal, no other components of the plant are involved in this process, we tested whether the addition of crude leaf extract could increase the conversion rate. We therefore used a method which was adapted from Bonaventure et al (S15). A fully elongated leaf of a WT *N. attenuata* plant was mechanically wounded with a fabric pattern wheel and 20 µL of 1:3 (v/v) diluted OS was applied to the wounds and gently dispersed across the leaf surface. After 20 min two leaf discs of 12 mm diameter were punched out from the leaf lamina, each leaf disc was placed in a 1.5 mL Eppendorf tube that contained 500 µL of reaction buffer (40 mM MOPS/KOH, pH 7.0, 10 % (v/v) glycerol, 0.02 % Tween-20) and the mixture was homogenized for 20 sec. with an Ultra-Turrax. After a ten second spin, 300 µL of the supernatant of one sample was pipetted into a GC vial with insert and 1 µL of (Z)-hex-3-enal (4.8 µg/µL DMSO) was added to the mixture (sample 1). The 300 µL of the supernatant of the second sample was pipetted into a 1.5 mL tube and, in addition to the (Z)-hex-3-enal, 300 µL OS (diluted 1:3 (v/v) with 0.03 % tween) was added (sample 2). The resulting 600 µL mixture was aliquoted into two GC vials with inserts and all three vials were closed and gently shaken for 20 min. After this period the 300 µL of sample 1 and the 600 µL of sample 2 were transferred into two separate scintillation vials and volatiles were trapped as described above.

**e. Enzyme assays**

The presence of β-glucosidase activity in *Manduca sexta* OS was determined, following the procedure described in Mattiacci et al., (S16). OS samples were assayed in triplicate. The incubation mixture, which contained 5 mM 4-nitrophenyl β-D-glucopyranoside (Sigma-Aldrich, Germany; [www.sigmaaldrich.com](http://www.sigmaaldrich.com)) in 1 mL 0.1 M Tris buffer and 125 µL of OS solution (diluted 1:1 with H2O), was briefly stirred on a vortex and incubated in a water bath at 30°C for 2 h. The reaction was stopped by immersing the incubation tubes in boiling water for 10 min. All tubes were centrifuged at 10,000 x g for 10 min after incubation and the absorbance of the supernatant was measured in an Ultrospec 3000 (Pharmacia Biotech; [http://www4.amershambiosciences.com](http://www4.amershambiosciences.com)) spectrophotometer. The concentration of *p*-nitrophenol, the reaction product, was determined at 400 nm by using a molar extinction coefficient of 18,130. One unit was defined as the amount of enzyme hydrolyzing 1 µmol of substrate per min at 30°C.

Glucose oxidase (GOX) was determined as described (S14) from three replicate *M. sexta* OS samples.
f. FAC Analysis

The two most abundant FACs in *M. sexta* OS, N-linolenoyl-L-Gln (18:3-Gln) and N-linolenoyl-L-Glu (18:3-Glu) were measured by LC-MS from six samples in which each sample consisted of OS from ten third to fifth instar *M. sexta* larvae that were actively feeding on *N. attenuata* plants. Each sample was divided into two aliquots and in each case one aliquot was heated to 90°C for 10min (boiled) prior to analysis while the second aliquot was left untreated.

FAC measurements were done with a 1200L liquid chromatography–triple quadrupole mass spectrometry system (Varian, Palo Alto, CA, USA; www.varianinc.com). 10 μL of each sample were injected onto a ProntoSIL column (C18; 5 μm, 50 x 2 mm, Bischoff, Leonberg, Germany; www.bischoff-chrom.de) attached to a precolumn (C18, 4 x 2 mm, Phenomenex, USA, www.phenomenex.com). The mobile phase comprised solvent A (0.05 % formic acid) and solvent B (MeOH) used in a gradient mode time/concentration (min/%) for B: 0:00/15; 1:30/15; 1:30/98; 12:30/98; 13:30/15; 15:00/15 with a flow of time/flow (mL/min): 0:00/0.4; 1:00/0.4; 1:30/0.2; 10:00/0.2; 10:30/0.4; 12:30/0.4; 15:00/0.4. 18:3-Glu and 18:3-Gln were detected in the electrospray ionization-negative mode. Molecular ions [M-H](-) at m/z 406 and 405, generated from 18:3-Glu and 18:3-Gln, respectively, were fragmented by collision-induced fragmentation with argon under 18.5 and 21.5 V of collision energy. The ion intensities of their respective daughter ions, m/z 128 and 145 were used for quantifications. The total amounts of the two FACs in boiled and unboiled OS were quantified with external standard curves.

g. Field Bioassays

We tested the attractiveness of different mixtures to *Geocoris* spp. in a native *N. attenuata* population in the Great Basin desert of southwest Utah in 2009. Three *M. sexta* eggs per plant were glued to the underside of a lower stem leaf of 21 pairs of native *N. attenuata* plants as described in Kessler and Baldwin (S17). We used a neutral α-cellulose glue which is known to have no effect on predation rates or on plants’ VOC emissions (S17). Plants were of the same size and developmental stage. On each experimental day two different mixes were tested in a paired design. Each pair of plants was separated by an average distance of 1.1 m. A cotton swab was dipped into lanolin paste containing different GLV-mixes and placed immediately adjacent to the leaf with the *M. sexta* eggs. The numbers of eggs predated were counted after 12 and 24 h. Predated eggs are emptied by *Geocoris* and are thus easy to distinguish (Fig. 3.3A). For all experiments we used *M. sexta* eggs from our
own 1 ½ years old inbred colony in Jena or eggs from a 40 year-old inbred colony from the Binghamton University (Biological Sciences Department, Vestal, NY), kindly provided by Carol Miles.

In the first two weeks (Fig. S3.8) no eggs were predated. However, from the third week on, *Geocoris* spp. fed on *Manduca* eggs. This may be due to low population rates, a saturation-based decreased decisiveness (S18), or perhaps since the insects have a broad host range, *Geocoris* needed to associate herbivore-specific changes in the GLV-bouquets with the location of their prey (S19). Since the dietary change closely followed the only natural *Manduca* oviposition event of the season (Fig. S3.8), we speculate that *Geocoris* learned to associate certain volatile compounds with the presence of *Manduca* eggs and hatching larvae.

The mixes that we tested for their ability to attract *Geocoris* are given in Table S3.5. Mix A and B were used to answer the question whether *Geocoris* was able to distinguish between (Z)-GLVs and (E)-GLVs. Mix A consisted of the four main (Z)-GLVs and MIX B of the four main (E)-GLVs that we detected in our 20 min trappings (Fig. 3.1 and Table S3.1). Since the aldehydes and the alcohols were almost 100x more abundant in these trappings than the esters, we took these differences into account and created mixtures that had 100x larger amounts of the aldehyde and the alcohol than the esters.

Mix C and D as well as Mix E and F were used to test whether *Geocoris* was able to sense subtle changes in the (Z)/(E)-ratio. We decided to work with the minimum and maximum ratio we detected in our first measurements (Fig. 3.1B). To mimic the emission of w+OS treated plants we chose a (Z)/(E)-ratio of 1:1, and to mimic the emission of w+w treated plants, we used a mixture in a 9:1-ratio. (Z)-hex-3-enal, which is a relatively unstable compound is available (from Sigma-Aldrich) stabilized with triacetin. To insure that the increased predation rates we saw with the (E)-mixes (Mix B) were not due to a possible repellent effect of the triacetin itself, we tested mixtures that either did not contain the aldehydes and thus also no triacetin (MIX C and D), or we added triacetin to produce mixes that differed in their (Z) and (E) amounts, but contained the same amounts of triacetin (Table S3.5 C, Mix E and F).

h. Headspace collection of lanolin mixes

To determine the actual amounts released from the lanolin paste and to monitor the changes in the emission in the first 12 h of the experiment we collected the headspace from cotton swabs scented with the 9:1 or the 1:1-ratio mix (Mix E and F, Table S3.5 and S3.6). Headspace collections were done in Utah under field conditions (Fig. S3.13). A cotton swab was dipped into the lanolin paste and placed adjacent to a leaf of a GLV-deficient plant (ir-
loxs2) to avoid plant-derived ‘GLV-contaminations’. VOCs were collected with a portable 12V DC vacuum pump (Gast Mfg., Benton Harbour, MI, USA) which pulled air through a 20mL plastic beaker which largely enclosed the cotton swab on to a coconut-charcoal air-sampling trap (ORBO-32™, SUPELCO, Munich, Germany). Volatiles were trapped (from the 9:1-mix, 1:1-mix and from unscented ir-loxs2 plants as control) in a 12h time period, every two hours, for each 30min. The trapping started at 7am and ended at 7pm. This time window was chosen since predation assays were done at the same time.

The GC analyses were performed on a Varian CP-4000 GC coupled with a Varian Saturn 4000 ion trap MS in electron ionisation (EI; 70 eV) mode (Varian, Palo Alto, CA). 1 µL of the sample was injected splitless on a DB-5 column (30m×0.25mm I.D., 0.25 μm film thickness, Agilent, Boeblingen, Germany) with helium at a constant flow of 1 mL min⁻¹ as the carrier gas. The injector was at 250°C. The oven temperature program was: 40°C for 5 min, 120°C at 5°C/min, 30°C/min ramp to 240°C. EI spectra were recorded on Scan mode from 50 to 150 m/z. Identification was performed using identical standards.

i. Statistical Analysis

All statistical analyses were performed with Excel (Microsoft Corporation, Redmond, Washington, USA) or SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Data were transformed, if necessary, to meet the requirements for homogeneity of variance.

2) Supporting text

Due to the space constraints of the main text, we were not able to address four issues that deserve additional consideration. First, the elicitation of direct and indirect defense responses is mediated by a suite of plant hormones, of which JA, SA and ET play central roles. All three affect the induction of HIPVs (S20). To test whether the observed OS-elicited (Z)/(E)-shift is regulated via these phytohormone-signaling systems, we trapped volatiles from transgenic plants impaired in JA-biosynthesis (ir-lox3; LOX3 = lipoxygenase-3; S2)) or perception (ir-coi1; COI1 = coronatine insensitive-1; S3), ET-biosynthesis (as-aco; ACO = ACC oxidase; S4) or–perception (35s-etr1a; ETR1a = ethylene receptor gene-1a; S4), or SA-signaling (ir-npr1; NPR1 = nonexpressor of PR-1; S5). We also examined plants with reduced expression of the mitogen activated protein kinases SIPK (salicylate-induced protein kinase) and WIPK (wound-induced protein kinase; ir-sipk x ir-wipk) which function upstream of the three phytohormones (S6). Although some transgenic lines were impaired in their absolute GLV emissions (Table S3.2 and S3.3), all exhibited M. sexta OS-dependent changes.
in their (Z)/(E)-ratio (Fig. S3.4). Thus, we conclude that the M. sexta OS mediated (Z)/(E)-shift is independent of the plant’s JA, SA and ET dependent defense signaling pathways.

Second, we would like to draw the attention of readers to a number of excellent studies that have examined the function of herbivore-induce plant volatiles (HIPV) in a field context, particularly, those of Thaler (S21) and Shimoda et al. (S22).

Third, we would like to consider the role of GLVs in the context of the entire HIPV blend, which includes a complex mixture of terpenoids and in some cases volatile phenolics, such as MeSA. Two figures are presented in this SOM (Fig. S3.11 and S3.12) depicting gas chromatograms of the headspace composition of a w+OS elicited N. attenuata plant, early after elicitation (0-20 min) and one day after elicitation (22-26 h). From these chromatograms, it’s clear that GLVs dominate the headspace early after elicitation and that other constituents, particularly terpenoids, such as (E)-α-bergamotene (TAB) become more important later in the emission process. However, it should be mentioned that during herbivory and thus when a plant experiences continuous damage GLV emissions remain a prominent feature also in the later emission process. We have published a number of studies about the separate function and regulation of GLVs and TAB: For example, we know that both are important, and when N. attenuata plants in native populations are perfumed with synthetic versions, predation rates increase (S17); we know that plants silenced in total GLV production are “off the radar” of flea beetles, and hence GLV emissions have a “dark side” by functioning as host-location cues for herbivores (S23). We know that GLV emissions are controlled by a SIPK (S7), in addition to the genes involved in their biosynthesis, and that TAB is regulated by a pair of WRKY transcription factors (S24). TAB is slowly and systemically released and likely functions as a long distance signal, which recruits Geocoris from several meters away to an attacked plant, but because of its systemic release, fails to provide information about the exact location of the feeding herbivore. The change in (Z)/(E)-ratio of the GLV blend which results from the putative isomerase in Manduca OS described in this paper, provides predators with a rapid, herbivore-specific signal replete with spatially-explicit information. It is likely that both GLV and terpenoid components are required for optimal indirect defense function, but this will require additional research to verify.

Last, we consider how the change in the (Z)/(E)-ratio of the GLV blend comes about. In the manuscript we have emphasized the role of the yet-to-be identified insect-derived isomerase in Manduca OS which converts (Z)- to (E)-GLVs, because that is the direction where our data points us. However, at this stage, we cannot fully exclude the possibility that an OS-derived constituent might also decrease the stability of (Z)-GLVs without converting
them into the more stable (E)-GLVs. This is a particularly challenging hypothesis to falsify due to the well-described instability of the (Z)-GLVs. As seen from Table S3.6, (Z)-hex-3-enal is particularly unstable in comparison to (E)-hex-2-enal, which is readily detected, even after 12 h in the hot Utah sun. And this brings up an interesting issue. While trappings of the Q-tip headspace onto activated charcoal, elution from the trap, and re-analysis by GC-MS reveals the well-described instability of (Z)-hex-3-enal, it is not clear at which of these three steps the compound is lost. Only losses during volatilization in the headspace are relevant to the biological question which compounds are relevant for the attraction of Geocoris spp., as losses during trapping, elution and re-analysis are simply experimental artifacts of how scientists quantify GLVs. This is an interesting question which will likely only be resolved by a better understanding of the neurobiology of (E)-hex-2-enal/ (Z)-hex-3-enal perception by Geocoris spp. However, it is worth pointing out again that while it’s unclear how much (Z)-hex-3-enal is in the headspace for Geocoris to perceive, it is clear from the tests performed with Mixes C and D (Fig. 3.3 and Table S3.5), that these unstable aldehydes are not required to attract this predator.

3) Supporting figures

**Fig. S3.1.** Elicitation and volatile collection procedures. (A) The first fully expanded leaf of rosette-stage (+1 position) or the first stem leaf of flowering (S1 position) N. attenuata plants was wounded (w) with a fabric pattern wheel, and 20 µL of one of the different eliciting solutions was directly applied to the leaf surface. (B) After applying the solution onto the wounds, the leaf was immediately enclosed between two 50mL food-quality plastic containers. Ambient air was pulled through the collection chamber and a glass tube packed with glass wool and 20mg of SuperQ. Airflow was created by a vacuum pump as described by Halitschke et al. (S10).
Fig. S3.2. The OS-elicited (Z)/(E)-shift persists until the end of the GLV-burst. (A) Mean (+SE) release of (Z)-hex-3-enol after a single elicitation (w+w = wounding + water). Values are given in ng*g FW⁻¹ * 20min⁻¹. (B) For each trapping interval and each group of isomers the (Z)/(E)-ratio was calculated (e.g. (Z)-hex-3-enal / (E)-hex-2-enal). The bars represent the average ratio and their 95% confidence limits (n=6 for w+OS and n=5 for w+w). n.d. = not detected (if the (Z)- or the (E)-isomer or both could not be detected); * = ratio could only be calculated for one replicate and confidence limits were thus not calculated. Experimental setup is described in 'Material and methods'.
Fig. S3.3. Fatty acid-amino acid conjugates and the alkalinity of Manduca sexta’s OS have no influence on the OS-elicited (Z)/(E)-conversion. (A-D) The bars represent the average ratio and their 95% confidence limits (n=5). (A) Control experiment: The influence of M. sexta OS on the (Z)/(E)-ratio. w+w = wounding + water; w+OS = wounding + OS. (B) The influence of the alkalinity of M. sexta OS on the (Z)/(E)-ratio. buffer = pH9. (C) The influence of heat-unstable compounds in M. sexta OS on the (Z)/(E)-ratio. M. sexta OS were heated to 90°C for 10 min prior to use (OS boiled). n.d. = not detected (if the (Z)- or the (E)-isomer or both could not be detected). (D) The influence of fatty acid-amino acid conjugates (FACs) on the (Z)/(E)-ratio. 18:3-Glu = N-linolenoyl-L-Glu; 18:3-Gln = N-linolenoyl-L-Gln. (E-F) FACs are heat-stable components of Manduca sexta OS. Mean (+SE) concentration of 18:3-Glu and 18:3-Gln in M. sexta OS before and after boiling (n=6). Treatments and methods used are described in Fig. S3.1 and in ‘Material and methods’.
**Fig. S3.4. The (Z)/(E)-shift is not signaling elicited.** Different transgenic lines, impaired in JA-biosynthesis (ir-lox3; LOX3 = lipoygenase-3) or JA–perception (ir-coi1; COI1 = coronatine insensitive-1), ethylene-biosynthesis (as-aco; ACO = ACC oxidase) or ethylene–perception (35s-etr1a; ETR1a = ethylene receptor gene-1a), SA-biosynthesis (ir-npr1; NPR1 = nonexpressor of PR-1), or lines that function upstream of all three pathways (ir-sipk x ir-wipk; SIPK = salicylate-induced protein kinase, WIPK = wound-induced protein kinase) were used to test whether influencing one of these signaling pathways also influences the OS-elicited (Z)/(E)-conversion. The bars represent the average ratio and their 95% confidence limits (n=5). Treatments (w+w = wounding + water; w+OS = wounding + OS) and methods used are describe in 'Material and methods'.
In vitro assay to test different eliciting solutions in their ability to convert (Z)-hex-3-enal into (E)-hex-2-enal. 300µL of the different eliciting solutions were pipetted into a GC vial with an insert and 1 µL of (Z)-hex-3-enal (4.8 µg/µL DMSO) was added to the solution. A volume of 300 µL was chosen to minimize the headspace and thus also the chance of (Z)-hex-3-enal to volatilize without reacting with the eliciting solutions. The GC vial was closed and gently shaken for 20 minutes. The liquid solution was subsequently transferred into a 20 mL scintillation vial and volatiles were trapped at room temperature for 20 min from the stirring solution by a constant nitrogen airflow which was blown into the scintillation vial and pushed through the trap. (B) Percentage conversion to (E)-hex-2-enal was calculated for each eliciting solution (n = 3-6).
Fig. S3.6. Percentage conversion to (E)-hex-2-enal by OS from different lepidopteran species. OS from *M. sexta*, *Spodoptera exigua* and *Spodoptera littoralis* were diluted 1:20 with 0.02% Tween-20 and the percentage (Z)/(E)-conversion rate was calculated (*M. sexta* n=6; *S. exigua*, *S. littoralis* n=3) using the in vitro assay as described in Fig. S3.5 and in ‘Material and methods’. Asterisks indicate significant differences from *M. sexta* OS (univariate ANOVA, $F_{2,9} = 31.177$, $p \leq 0.001$, followed by a Scheffé post-hoc test, $p \leq 0.001$ (***)).

Fig. S3.7. (A and B) The OS-elicited (Z)/(E)-shift is not influenced by the developmental stage of the plant. GLVs were trapped from single leaves of rosette stage (+1 position; n = 6; A) and early flowering plants (S₁ position in flowering plants; n = 9; B) immediately after w+w or w+OS elicitation, and (Z)/(E)-ratios were calculated as described in ‘Material and methods’. The bars represent the average ratio and their 95% confidence limits.
Fig. S3.8. Timeline of 2009 Utah field season. The attractiveness of different GLV-mixes to Geocoris spp. was tested during the field season 2009 with a predation assay as described in Kessler and Baldwin (S17) and in the ‘Material and method’ section. On each experimental day two different mixes were tested in a paired design. Mixes used on different experimental days are plotted below the timeline. The composition of each mixture is described in Table S3.5. Environmental events occurring during this period are plotted above the timeline.
Fig. S3.9. Predation by *Geocoris* spp. in the field. Average egg predation per plant and day (n=15). Enhanced predation was observed with the (E)-mix (B) compared to the (Z)-mix (A). The composition of both mixtures is described in Table S3.5. Treatment pairs with no predated egg were excluded prior to the statistical analysis. Asterisks indicate significant differences between treatments (paired sample *t*-test, 28.May, *t*₀ = 4.583, *p* ≤ 0.001 (**)), 03.June, *t*₇ = 3.742, *p* ≤ 0.01 (**)).

Fig. S3.10. GOX and β-glucosidase activity in *M. sexta* OS (n=3).
Fig. S3.11. GLV-related GCxGC-ToF extracted ion chromatograms of the headspace of an OS-elicited *N. attenuata* plant, immediately and one day after elicitation. Representative chromatograms of an OS-elicited WT plant. Volatiles were trapped immediately after elicitation (0-20 min, grey line) and again on the next morning (22-26 h, black line). The single ions 67 and 69 are typical ions for GLVs and have been used for the chromatograms as well as for the 2D-intensity plots. Normalized intensities and magnified areas of the two rectangles in the two-dimensional chromatograms give an overview of the abundance of GLVs in early and late volatile bouquets. G1 = (Z)-hex-3-enal, G2 = (E)-hex-2-enal, G3 = (Z)-hex-3-enol, G4 = (E)-hex-2-enol, G5 = (Z)-hex-3-enyl acetate, G6 = (E)-hex-2-enyl acetate, G7 = (Z)-hex-3-enyl propionate, G8 = (E)-hex-2-enyl propionate, G9 = (Z)-hex-3-enyl isobutyrate, G10 = hexenyl isobutyrate, G11 = (Z)-hex-3-enyl butyrate, G12 = (E)-hex-2-enyl butyrate, T6 = unknown monoterpene 2 (MT), T7 = unknown MT3.
Fig. S3.12. Terpene-related GCxGC-ToF extracted ion chromatograms of the headspace of an OS-elicited *N. attenuata* plant, immediately and one day after elicitation. Representative chromatograms of an OS-elicited WT plant. Volatiles were trapped immediately after elicitation (0-20 min, grey line) and again on the next morning (22-26 h, black line). The single ions 93 and 119 are typical ions for terpenes and have been used for the chromatograms as well as for the 2D-intensity plots. Normalized intensities and magnified areas of the two rectangles in the two-dimensional chromatograms give an overview of the abundance of terpenes in early and late volatile bouquets. T1= α-pinene, T2= β-pinene, T3= β-myrcene, T4= unknown MT1, T5= limonene, T6= unknown MT2, T7= unknown MT3, T8= unknown MT4, T9= α-cedrene, T10= longifolene, T11= (E)-α-bergamotene.
Fig. S3.13. Setup for collecting volatiles from lanolin mixes in the field. Experimental setup is described in ‘Material and methods’.
## Table S3.1. Immediate GLV emissions in the first 20 min after w+w or w+OS treatment in wild type plants.

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<td></td>
<td></td>
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<td>RT2</td>
<td>w + w</td>
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<tr>
<td></td>
<td>[(E)-hex-2-enyl] propanoate</td>
<td>(E)-2-hexenyl propanoate</td>
<td>774</td>
<td>2.82</td>
<td>2.55±0.57</td>
</tr>
<tr>
<td></td>
<td>[(Z)-hex-3-enyl] butanoate</td>
<td>(Z)-3-hexenyl butyrate</td>
<td>912</td>
<td>2.64</td>
<td>189.75±16.31</td>
</tr>
<tr>
<td></td>
<td>[(E)-hex-2-enyl] butanoate</td>
<td>(E)-2-hexenyl butyrate</td>
<td>930</td>
<td>2.70</td>
<td>22.09±3.24</td>
</tr>
</tbody>
</table>

Mean (± SE; n=5) release of GLVs in wild-type (WT) plants. A single leaf (+1 position) of each rosette stage plant was mechanically wounded and treated with water (w+w) or *M. sexta* OS (w+OS). Plant volatiles were collected as described in Halitschke *et al.* (S10) the first 20 min after elicitation and samples were analyzed by GCxGC-ToF MS (S11). Two-dimensional separations were attained using an RTX-5MS column ($RT_1$) followed by a DB-17 column ($RT_2$). Volatiles are listed by chemical classes with their retention times on the first and second dimension ($RT_1$ and $RT_2$). Differences between w+w and w+OS emissions were determined with a Student’s *t*-test and significant differences were displayed in bold (p<0.05).
Table S3.2. GLV emissions in the first 20min after w+w treatment in transgenic lines.

(A and B) Mean (± SE. n>5) release of GLVs in WT plants and transgenic lines in the first 20min after elicitation. A single leaf (+1 position) of each rosette stage plant was mechanically wounded and treated with water (w+w). Differences from WT emissions were determined with a Student’s t-test and significant differences were displayed in bold (p<0.05). Volatiles from ir-lox3 plants were trapped in a separate experiment. n.d., not detected; *, compound was detected only once and thus no statistics were done.
Table S3.3. GLV emissions in the first 20min after w+OS treatment in transgenic lines.

<table>
<thead>
<tr>
<th>Common names</th>
<th>RT (s)</th>
<th>volatile release in ng / g fresh mass</th>
<th>p-value</th>
<th>p-value</th>
<th>p-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT1</td>
<td>RT2</td>
<td>WT</td>
<td>ir-sipk x ir-wipk</td>
<td>ir-col</td>
<td>ir-npr1</td>
</tr>
<tr>
<td>(Z)-3-hexenal</td>
<td>174.1.80</td>
<td>9746.23 ± 722.62</td>
<td>11451.50 ± 1199.86</td>
<td>0.251</td>
<td>10007.75 ± 1534.31</td>
<td>0.881</td>
</tr>
<tr>
<td>(E)-2-hexenal</td>
<td>174.1.93</td>
<td>4533.93 ± 538.47</td>
<td>5260.12 ± 948.80</td>
<td>0.521</td>
<td>4673.60 ± 415.56</td>
<td>0.841</td>
</tr>
<tr>
<td>(Z)-3-hexenol</td>
<td>246.2.58</td>
<td>2561.49 ± 248.01</td>
<td>3632.59 ± 466.56</td>
<td>0.070</td>
<td>2730.44 ± 400.58</td>
<td>0.727</td>
</tr>
<tr>
<td>(E)-2-hexenol</td>
<td>270.2.73</td>
<td>3028.83 ± 254.29</td>
<td>3687.81 ± 581.30</td>
<td>0.323</td>
<td>2998.17 ± 336.21</td>
<td>0.943</td>
</tr>
<tr>
<td>(Z)-3-hexenyl acetate</td>
<td>570.2.89</td>
<td>58.56 ± 3.38</td>
<td>2561.49 ± 248.01</td>
<td>0.052</td>
<td>2730.44 ± 400.58</td>
<td>0.727</td>
</tr>
<tr>
<td>(E)-2-hexenyl acetate</td>
<td>594.2.95</td>
<td>46.45 ± 5.25</td>
<td>57.32 ± 10.47</td>
<td>0.375</td>
<td>45.06 ± 4.66</td>
<td>0.847</td>
</tr>
<tr>
<td>(Z)-3-hexenyl propionate</td>
<td>756.2.78</td>
<td>10.65 ± 1.75</td>
<td>13.57 ± 2.50</td>
<td>0.361</td>
<td>7.34 ± 1.65</td>
<td>0.199</td>
</tr>
<tr>
<td>(E)-2-hexenyl propionate</td>
<td>774.2.82</td>
<td>8.37 ± 1.41</td>
<td>9.96 ± 2.46</td>
<td>0.589</td>
<td>6.92 ± 0.79</td>
<td>0.391</td>
</tr>
<tr>
<td>(Z)-3-hexenyl butyrate</td>
<td>912.2.64</td>
<td>119.33 ± 15.85</td>
<td>135.88 ± 23.32</td>
<td>0.070</td>
<td>118.61 ± 135.88</td>
<td>0.251</td>
</tr>
<tr>
<td>(E)-2-hexenyl butyrate</td>
<td>930.2.70</td>
<td>32.08 ± 2.97</td>
<td>37.28 ± 6.31</td>
<td>0.473</td>
<td>31.73 ± 4.45</td>
<td>0.950</td>
</tr>
</tbody>
</table>

(A and B) Mean (± SE, n>5) release of GLVs in WT plants and transgenic lines in the first 20min after elicitation. A single leaf (+1 position) of each rosette stage plant was mechanically wounded and treated with M. sexta OS (w+OS). Differences from WT emissions were determined with a Student’s t-test and significant differences were displayed in bold (p<0.05). Volatiles from ir-lox3 plants were trapped in a separate experiment.
Table S3.4. Average GLV emissions in the first 20 min after w+w or w+OS treatment in wild type plants from three independent experiments.

<table>
<thead>
<tr>
<th>class</th>
<th>IUPAC Name</th>
<th>common Name</th>
<th>RT (s)</th>
<th>volatile release in ng / g fresh mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RT1</td>
<td>RT2</td>
</tr>
<tr>
<td>aldehyde</td>
<td>(Z)-hex-3-enal</td>
<td>(Z)-3-hexenal</td>
<td>174</td>
<td>1.80</td>
</tr>
<tr>
<td></td>
<td>(E)-hex-2-enal</td>
<td>(E)-2-hexenal</td>
<td>174</td>
<td>1.93</td>
</tr>
<tr>
<td>alcohol</td>
<td>(Z)-hex-3-en-1-ol</td>
<td>(Z)-3-hexenol</td>
<td>246</td>
<td>2.58</td>
</tr>
<tr>
<td></td>
<td>(E)-hex-2-en-1-ol</td>
<td>(E)-2-hexenol</td>
<td>270</td>
<td>2.73</td>
</tr>
<tr>
<td>hexenylester</td>
<td>[(Z)-hex-3-enyl] acetate</td>
<td>(Z)-3-hexenyl acetate</td>
<td>570</td>
<td>2.69</td>
</tr>
<tr>
<td></td>
<td>[(E)-hex-2-enyl] acetate</td>
<td>(E)-2-hexenyl acetate</td>
<td>594</td>
<td>2.95</td>
</tr>
<tr>
<td></td>
<td>[(Z)-hex-3-enyl] propanoate</td>
<td>(Z)-3-hexenyl propionate</td>
<td>756</td>
<td>2.78</td>
</tr>
<tr>
<td></td>
<td>[(E)-hex-2-enyl] propanoate</td>
<td>(E)-2-hexenyl propionate</td>
<td>774</td>
<td>2.82</td>
</tr>
<tr>
<td></td>
<td>[(Z)-hex-3-enyl] butanoate</td>
<td>(Z)-3-hexenyl butyrate</td>
<td>912</td>
<td>2.64</td>
</tr>
<tr>
<td></td>
<td>[(E)-hex-2-enyl] butanoate</td>
<td>(E)-2-hexenyl butyrate</td>
<td>930</td>
<td>2.70</td>
</tr>
</tbody>
</table>

Mean (± SE; w+OS, n= 21, w+w, n=20) release of GLVs in wild-type (WT) plants from three independent experiments (Experiment Fig. 3.1C or Table S3.1, Fig. S3.7 A (rosette plants) and B (flowering plants)). Differences between w+w and w+OS emissions were determined with a Student’s t-test and significant differences were displayed in bold (p<0.05).
Table S3.5. GLV-mixtures used for the field bioassays.

<table>
<thead>
<tr>
<th>A</th>
<th>Compounds (common names)</th>
<th>(A) Only (Z)-mix (µg/µl lanolin)</th>
<th>(B) Only (E)-mix (µg/µl lanolin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Z)-3-hexenal (50% in triacetin)</td>
<td>10.0</td>
<td>0.0</td>
</tr>
<tr>
<td>(Z)-GLVs</td>
<td>(Z)-3-hexenol</td>
<td>10.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>(Z)-3-hexenyl acetate</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>(Z)-3-hexenyl butyrate</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenal</td>
<td>0.0</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenol</td>
<td>0.0</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl acetate</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl butyrate</td>
<td>0.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>Compounds (common names)</th>
<th>(C) (Z)/(E)-mix 1:1; ≈ w+OS, w/o aldehyde (µg/µl lanolin)</th>
<th>(D) (Z)/(E)-mix 9:1; ≈ w+w, w/o aldehyde (µg/µl lanolin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Z)-3-hexenal (50% in triacetin)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>(Z)-GLVs</td>
<td>(Z)-3-hexenol</td>
<td>10.0</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td>(Z)-3-hexenyl acetate</td>
<td>0.1</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>(Z)-3-hexenyl butyrate</td>
<td>0.1</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenal</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenol</td>
<td>10.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl acetate</td>
<td>0.1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl butyrate</td>
<td>0.1</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C</th>
<th>Compounds (common names)</th>
<th>(E) (Z)/(E)-mix 1:1; = w+OS (µg/µl lanolin)</th>
<th>(F) (Z)/(E)-mix 9:1; = w+w (µg/µl lanolin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Z)-3-hexenal (50% in triacetin)</td>
<td>5.0</td>
<td>9.0</td>
</tr>
<tr>
<td>(Z)-GLVs</td>
<td>(Z)-3-hexenol</td>
<td>5.0</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>(Z)-3-hexenyl acetate</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>(Z)-3-hexenyl butyrate</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenal</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>(E)-GLVs</td>
<td>(E)-2-hexenol</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl acetate</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl butyrate</td>
<td>0.05</td>
<td>0.01</td>
</tr>
</tbody>
</table>

|   | triacetin per 10mL mix (derived from (Z)-3-hexenal) | 51.25 µL | 92.2 µL |
|   | triacetin added per 10mL mix | 40.95 µL | 0 µL |
|   | Total amount of triacetin per 10mL mix | 92.2 µL | 92.2 µL |
Table S3.6 Headspace trapping under field conditions of GLV-mixtures used for the field bioassays.

(A) Headspace collections from 9:1 and 1:1 lanolin mixes under field conditions. Volatiles were trapped every second hour for each 30min. Concentrations are given in \( \mu g / 30 \text{min trapping period} \). (B) \((Z)/(E)\)-ratios of the lanolin mixes for each trapping period. n.d. = not detected, if either the \((Z)\)-, or the \((E)\)- compound could not be detected.

### Table S3.6

<table>
<thead>
<tr>
<th>Common names</th>
<th>9:1 mix</th>
<th>1:1 mix</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0h</td>
<td>2h</td>
</tr>
<tr>
<td>((Z))-3-hexenol</td>
<td>38.42</td>
<td>9.54</td>
</tr>
<tr>
<td>((Z))-3-hexenyl acetate</td>
<td>0.62</td>
<td>0.19</td>
</tr>
<tr>
<td>((Z))-3-hexenyl butyrate</td>
<td>0.30</td>
<td>0.18</td>
</tr>
<tr>
<td>((E))-2-hexenal</td>
<td>6.07</td>
<td>1.01</td>
</tr>
<tr>
<td>((E))-2-hexenol</td>
<td>5.30</td>
<td>1.22</td>
</tr>
<tr>
<td>((E))-2-hexenyl acetate</td>
<td>0.22</td>
<td>0.06</td>
</tr>
<tr>
<td>((E))-2-hexenyl butyrate</td>
<td>0.09</td>
<td>0.09</td>
</tr>
</tbody>
</table>

### Table S3.6 B

<table>
<thead>
<tr>
<th>Common names</th>
<th>0h</th>
<th>2h</th>
<th>4h</th>
<th>6h</th>
<th>8h</th>
<th>10h</th>
<th>12h</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexenol</td>
<td>7.25</td>
<td>0.84</td>
<td>7.84</td>
<td>0.92</td>
<td>8.11</td>
<td>0.92</td>
<td>8.25</td>
</tr>
<tr>
<td>hexenyl acetate</td>
<td>2.77</td>
<td>0.89</td>
<td>2.90</td>
<td>0.92</td>
<td>3.34</td>
<td>0.90</td>
<td>3.42</td>
</tr>
<tr>
<td>hexenyl butyrate</td>
<td>1.34</td>
<td>0.57</td>
<td>1.98</td>
<td>0.52</td>
<td>3.83</td>
<td>0.50</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
5) Supporting references