

Herbivore-induced volatiles: the emission of acyclic homoterpenes from leaves of *Phaseolus lunatus* and *Zea mays* can be triggered by a β -glucosidase and jasmonic acid

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Abstract The treatment of healthy, undamaged plants of the Lima bean *Phaseolus lunatus* with solutions of a β -glucosidase from bitter almonds (at $5 \text{ U} \cdot \text{ml}^{-1}$) through the petiole results in an enhanced emission of volatiles to the environment. The compounds are identical with those emitted in response to infestation with the red spotted spider mite *Tetranychus urticae*. Dominant products are the two acyclic homoterpenes 4,8-dimethyl-1,3E,7-dimethylnonatriene (homoterpene I) and 4,8,12-trimethyl-1,3E,7E,11-tridecatetraene (homoterpene II) which are of sesquiterpenoid and diterpenoid origin. Therefore, a β -glucosidase of the herbivore may be considered as the true elicitor for the odor induction. Homoterpene I and most other of the herbivore-induced volatiles can also be triggered by treatment of the plant with solutions of jasmonic acid (JA) at $100 \text{ nmol} \cdot \text{ml}^{-1}$ to $10 \text{ } \mu\text{mol} \cdot \text{ml}^{-1}$. The C_{16} homoterpene II is not significantly induced by JA. The time-course of the enzymatic- and the JA-triggered induction of the volatiles is identical. The dose-response to JA parallels previous reports on alkaloid induction in cell cultures. In corn plants (*Zea mays*) JA triggers the emission of all volatiles which are known to be emitted in response to the damage by the beet army worm *Spodoptora exigua*. In summary, the emission of volatiles after damage by a herbivore resembles the production of phytoalexins in response to an attacking microorganism and uses similar elicitors and internal transduction pathways.

Key words: Volatile (mode of induction); Elicitor; Transducer; β -Glucosidase; Jasmonic acid; *Phaseolus lunatus*; *Zea mays*

1. Introduction

The understanding of chemical communication pathways is of particular interest in the context of mutualistic interactions between plants, within and between insect communities, or in the case of the more complex tritrophic interactions between plants, insects and parasitoids [1]. Recent chemical analyses of such interactions between herbivores, plants and carnivores which feed on the herbivores, have been very successful and revealed that the communication pathways are linked to volatiles which are emitted from the attacked plant [2,3]. Among the many compounds that are released from infested plants, the two acyclic homoterpenes 4,8-dimethyl-1,3E,8-dimethylnonatriene (homoterpene I) and 4,8,12-trimethyl-1,3E,7E,11-tridecatetraene (homoterpene II) were particularly often observed (Fig. 1).

For example, the two tritrophic systems – Lima bean/phytophagous mite/carnivorous mite [2] and corn/beet army worm/parasitic wasp [3] – consist of a di- and monocotyledonous plant and very different herbivores, but homoterpenes I and II are emitted together with other volatiles when damage by the above herbivores occurs. In the Lima bean/spider mite/predatory mite complex, the C_{11} hydrocarbon homoterpene I plays an important role as an attractant for the (useful) carnivorous mites [4]. Biosynthetic studies also support a ubiquitous radiation of homoterpene I and/or II [5,6]. Administration of deuterium-labelled nerolidol to randomly selected higher plants resulted in the emission of homoterpene I and/or II from the leaves of most of these plants within about 24 h of the application of the precursor [7].

In the case of the attacked Lima bean or the corn plant the production of homoterpene I and/or II and other volatiles becomes systemic soon after the plants are damaged by the herbivore [8]. This sequence of leaf damage and induction of volatiles as a systemic response towards the attacking herbivore is strongly reminiscent of the scenario of host/pathogen interactions resulting in the biosynthesis of phytoalexins [9]. In the host/pathogen complex the biosynthetic activities of the attacked plant are triggered by a pathogen-derived elicitor which finally causes gene activation and the de novo synthesis of new compounds which may be toxic to the attacking organism. In the case of the tritrophic interactions, the salivary enzymes of the attacking herbivore may play a similar role to the enzymes [10] or cell wall fragments of the pathogens [11]. This view is strongly supported by our previous finding that β -glucosidases (from bitter almond or potato) induce the biosynthesis of homoterpene I and/or II in Lima beans when solutions of the enzyme are applied to the surface of mechanically damaged leaves [6,7]. A similar induction of volatiles by a β -glucosidase was recently observed with cabbage, and, interestingly, the pattern of the emitted volatiles closely resembled the pattern of volatiles emitted after damage by caterpillars of the large cabbage white butterfly *Pieris brassicae* [12].

While, by now, some knowledge has accumulated about the volatiles emitted after the action of a herbivore, little or nothing is known about the enzymatic activities and the internal signal pathways triggered within the plant in response to the herbivore. In continuation of our work on the biosynthesis and the regulation of the biosynthesis of herbivore-induced volatiles, we now provide the first evidence that, as well as the lytic enzymes of the herbivores, other compounds which are derived from lipid peroxidation (jasmonic acid) play an important role in a selective triggering of the biosynthetic pathways leading to plant volatiles.

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2. Materials and methods

2.1. Chemicals

β -Glucosidase from bitter almonds was purchased from Sigma. Racemic jasmonic acid (JA) was prepared from the racemic methyl ester as described [13].

2.2. Rearing of plant material

Lima beans (*Phaseolus lunatus*, Ferry Morse var. Jackson Wonder Bush, kindly provided by the BASF AG, Ludwigshafen) were grown from seeds in unsterilized garden soil. Individual plants were grown in a pot (diam. = 5.5 cm) at 22–25°C using daylight fluorescent tubes at 800 lux and a photophase of 12 h. Experiments were conducted with 11- to 15-day-old seedlings with two fully developed leaves. Corn plants (*Zea mays*, var. Amador, F1 hybrid) were grown as described above. Ca. 8 week-old plants with 5–6 clearly distinguishable leaves were used for the induction experiments.

2.3. Induction experiments

Freshly cut plantlets of *P. lunatus* or corn seedlings were placed into tap water solutions containing the test substance. β -Glucosidase (from bitter almond) was applied at 5 U·ml⁻¹ and 10 U·ml⁻¹. Induction experiments with jasmonic acid were carried out with solutions containing 100 nmol–10 μ mol·ml⁻¹ of the free acid in tap water. The highest concentration required brief sonication to achieve a homogeneous solution. Control experiments were made by placing freshly cut plants into tap water without additives. The time-course of the induction with JA was followed by a 0, 10 and 20 h preincubation period and a collection period of 20 h resulting in 20, 30 and 40 h of total incubation times. 5 h preincubation was followed by only 5 h of sampling resulting in 10 h total incubation time. The amount of emitted homoterpene I is calculated relative to the sum of signals c, d, f and g from Fig. 2A. Experiments were generally carried out in triplicate.

2.4. Infestation with spider mites

Ca. 1- to 2-weeks-old plants were placed in a plastic chamber covered with a fine mesh. Ca. 50 spider mites (*Tetranychus urticae*; Neudorf, D-31860 Emmerthal, Germany) were introduced, and after 6–7 days the strongly damaged plants (yellow discoloration of the leaves) were withdrawn. To avoid enrichment of the volatiles from the soil, the aerial parts were cut above the surface and placed into tap water. The emitted volatiles were collected over a period of 12–24 h as described.

2.5. Collection and analysis of headspace volatiles

The pretreated plants were placed into a closed system (small desiccator, ca. 750 ml), and the emitted volatiles were continuously collected over a period of 12–24 h on charcoal traps (1 mg charcoal, CLSA-Filter; Winterthur, Switzerland) using air circulation as described [14]. After desorption of the volatiles from the charcoal with dichloromethane (30 μ l), the compounds were analyzed by GC/MS. GC-separation: SE 30 fused-silica capillary column (15 m $\times$ 0.31 mm) under programmed conditions (50°C for 2 min, then at 10°/min to 200°C). MS: Finnigan Ion trap ITD 800; transfer line at 270°C, scan range 35–249 Da/s or Fisons MD 800; GC-interface at 260°C, scan range 35–300 Da/s.

3. Results

Unlike the profile of volatiles from healthy, uninfested leaves of Lima beans (*P. lunatus*), the leaves damaged by the spider mites, *Tetranychus urticae*, emit a number of additional volatiles which have been previously identified and described [2] (Fig. 2B). The induction of the two homoterpenes I and II is particularly noteworthy since the emission of these compounds can not be triggered by simple mechanical damage of the plant. As shown, their biosynthesis requires mechanical damage of the leaf and a simultaneous introduction of a polysaccharide cleaving enzyme, like a β -glucosidase, amylase or a pectinase, into the wound [7]. Such enzymes are assumed to be present in the salivary secretions of many herbivores [15], and a recent analysis of the gut regurgitant from caterpillars of the large cabbage

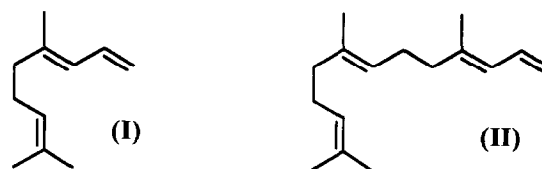


Fig. 1. Formulae of homoterpenes I and II.

white butterfly (*P. brassicae*) confirms this assumption [12]. Fig. 2C demonstrates that the emission of homoterpenes I, II, and several other volatiles can be triggered without a herbivore and without mechanical damage of the leaf surface by simply introducing a solution of the enzyme (β -glucosidase from bitter almonds at 10 U·ml⁻¹) through the petiole of a freshly cut plant. Ca. 18 h preincubation is sufficient to achieve a significant odor induction (Fig. 2C). At lower concentrations (<5 U·ml⁻¹) the induction of homoterpenes I and II or other volatiles is low and not reproducible. In comparing Fig. 2B and C it is obvious that the pattern of volatiles from the infested plant (Fig. 2B) can be largely mimicked by the action of the β -glucosidase from bitter almond (Fig. 2C). (For identification of the compounds refer to the legend of Fig. 2.) Although the sequence of events between the introduction of the β -glucosidase and the emission of the volatiles is unknown, the involvement of low molecular signals and transducers is a reasonable assumption. A highly potent transducer in the plant's defense against herbivores has recently been identified as jasmonic acid (JA) [16–18]. When dilutions of JA were applied through the petiole of freshly cut plantlets of *P. lunatus*, a strong induction of volatiles is observed (cf. Fig. 2D). (3Z)-Hexen-1-yl acetate, (2E)-hex-1-en-3-ol, oct-1-en-3-ol, linalool, the homoterpene I, methylsalicylate and an unknown terpenoid (C₁₀H₁₆O) are significantly enhanced (Fig. 2D). In contrast to the β -glucosidase experiment or the spider mite infection, the emission of the C₁₆ homoterpene II is only moderate after treatment with JA (cf. Fig. 2D). Some experiments showed no induction of homoterpene II. In summary, the products of the lipid peroxidation (hexenylacetates, oct-1-en-3-ol) and terpenoids are the most prominent compound classes which are emitted after treatment with JA.

Interestingly, the plant is able to esterify and to emit the introduced JA, since a significant amount of methyl jasmonate (JA-Me) is found among the collected volatiles. Untreated plants do not release JA-Me.

Using different preincubation times in combination with constant sampling intervals it can be shown that the emission of volatiles starts within 20–30 h of the plants exposure to JA (Fig. 3). The lowest effective concentration of JA is found at about 100 nmol/ml. Rapid and strong induction of homoterpene I is achieved with solutions containing 1–10 μ mol JA/ml. With this high concentration, first effects can already be seen after about 10 h, but the lower limit is difficult to determine and depends strongly on the efficacy of the odor collection device. In contrast to previous experiments with a tomato cultivar (*Lycopersicon esculentum* var. St. Pierre), no significant induction of volatiles is observed if JA-Me is applied to leaves of the Lima bean via the gas phase (ca. 300 μ mol/l air, applied through evaporation from filter paper in a closed vessel) [19].

In order to test JA as a more general transducer en route to homoterpenes I, II, and other volatiles, solutions of JA were

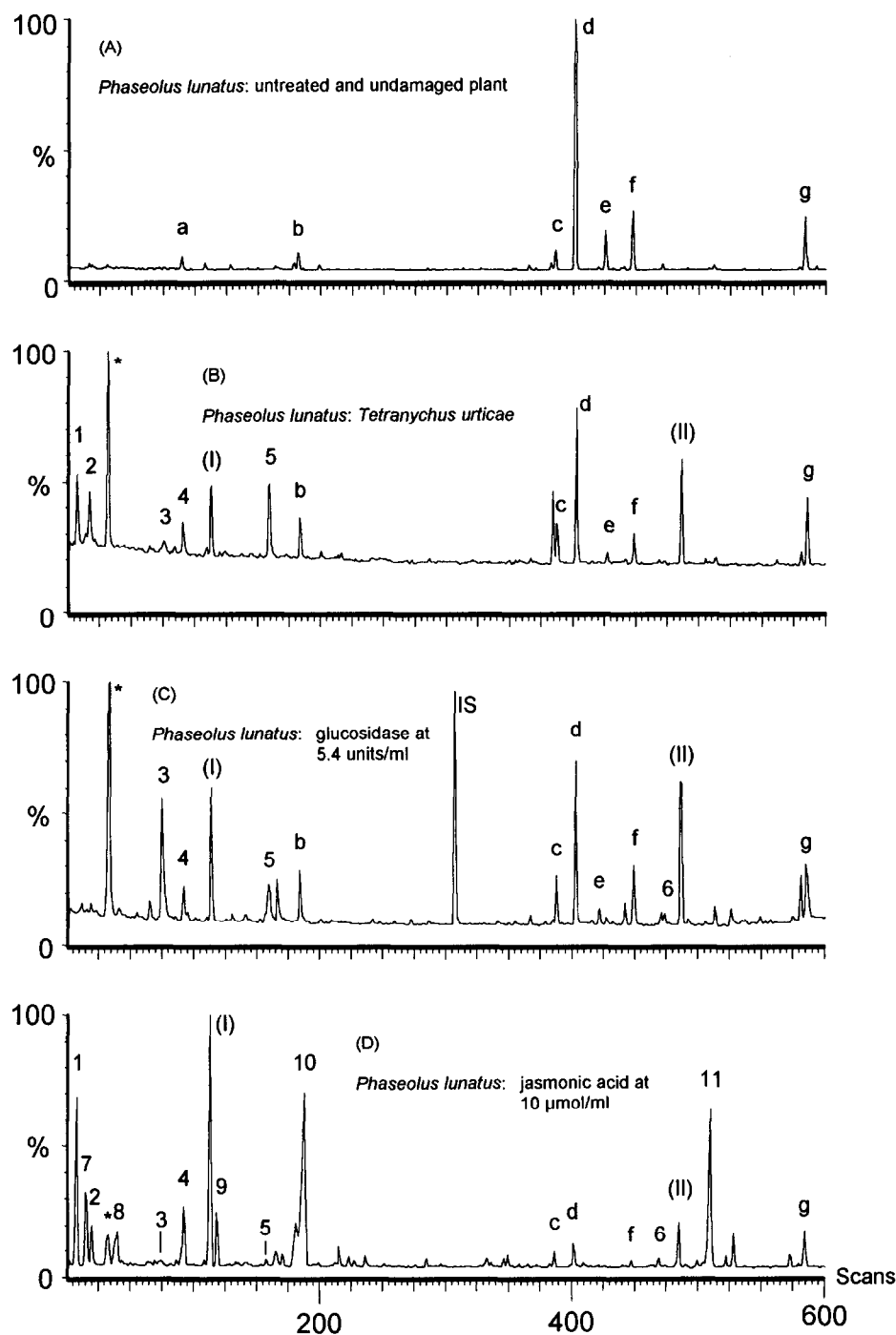


Fig. 2. Gas chromatographic profiles of the volatiles from Lima beans (*Phaseolus lunatus*) after various pretreatments. Compounds were generally separated on a fused-silica column DB1 (15 m $\times$ 0.25 mm) under programmed conditions from 50°C (2 min) at 10 °C min⁻¹ to 200°C, then at 30°C min⁻¹ to 280 °C. Identical numbers or letters denote identical compounds in the figure (A–D). (A) Volatiles from untreated, undamaged plants. Sampling of volatiles, 20 h. Identification of compounds: (a) nonanal, (b) decanal, (c–g) unidentified phenols and oxygenated terpenoids. (B) Volatiles from Lima beans 6 days after infestation with ca. 50 spider mites. Sampling of volatiles, ca. 20 h. Identification of volatiles: (1) (3Z)-hexenylacetate, (2) benzylalcohol, (*) contamination from the circulation pump, (3) methylphenol, (4) linalool, (I) see text, (5) methyl salicylate, (II) see text. (C) Volatiles from Lima beans after pretreatment with a solution of the β -glucosidase from bitter almonds at 10 U $\cdot$ ml⁻¹. Preincubation, 18 h. Sampling of volatiles, ca. 20 h. Identification of compounds: see above, (IS) internal standard (bromodecane), (6) nerolidol. (D) Volatiles from Lima beans after pretreatment with a solution of JA at 10 μ mol $\cdot$ ml⁻¹. Preincubation, 20 h. Sampling of volatiles, ca. 20 h. Identification of compounds: see above, (7) (2E)-hexenylacetate, (8) oct-1-en-3-ol, (9) C₁₀H₁₄, (10) C₁₀H₁₆O, (11) methyl jasmonate (JA-Me).

applied to freshly cut corn plantlets (*Zea mays*). Again, the pretreatment with solutions of JA resulted in a strong emission of novel sesquiterpenoids like bergamotene, β -farnesene, nero-

lidol and a moderate production of the two homoterpenes I and II, while untreated plants released only a series of *n*-alkanes (Fig. 4A,B). The number and the type of the induced com-

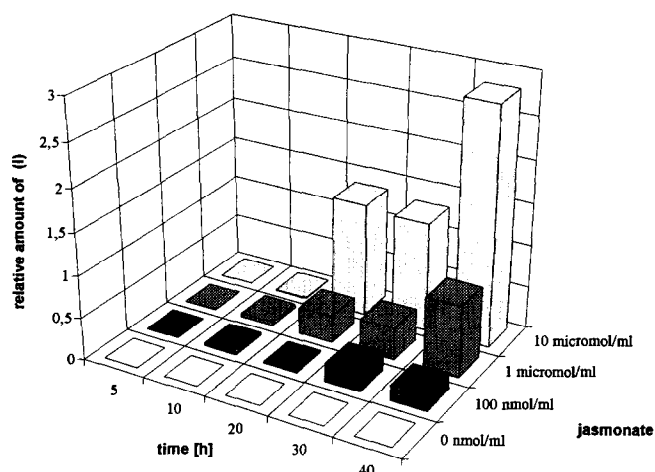


Fig. 3. Time-course and dose-response of Lima beans towards pretreatment with JA. Plotted are the relative amounts of homoterpene I as a function of time and concentration. The time given covers preincubation and sampling; see section 2. The amount of homoterpene I is calculated relative to the sum of the peak areas of c, d, f and g. The figure is representative for the dose-response and time-course of the other induced volatiles.

pounds perfectly match the profile of volatiles released from herbivore-damaged plants (by the beet army worm) [3] and suggest that JA may be the true transducer involved in the corn's defense response to the herbivore.

4. Discussion

In this and previous work it has been shown that the induc-

tion of plant volatiles by herbivores is clearly based on specific interactions of components from the salivary secretions (e.g. a β -glucosidase) of the attacking herbivore with the endogenous signal pathways of the plant's defense response. This explains why the two homoterpenes I and II are not emitted from mechanically damaged leaves until they are additionally treated with an unspecific β -glucosidase [6,7]. Isolation and analysis of extractable, glycosidically bound material from leaves of *P. lunatus* gave, after removal of the sugar moiety, no evidence for bound nerolidol which could serve as the precursor for the biosynthesis of homoterpene I. Furthermore, the simple release of nerolidol from a glycoside through the action of a β -glucosidase from a topically attacking herbivore can not account for the systemic release of homoterpene I and/or II from the whole plant, as is the case in the two systems Lima bean/spider mites and corn plants/beet army worm [3]. Therefore, it appears highly likely that the introduced enzyme(s) hydrolyses cellular structures into oligosaccharides (?) acting as the first signals in a complex network which finally results in gene activation and de novo synthesis of novel metabolites [11]. Since many plants are able to produce homoterpene I immediately after being supplied with nerolidol [7], we conclude that the lytic enzymes (β -glucosidase) will activate, among other things, the biosynthesis of sesquiterpene and diterpene, providing the two terpene alcohols nerolidol and geranylinalool, respectively. The latter are then channelled into the oxidative degradation pathway leading to homoterpenes I and II [20]. In this respect the β -glucosidase of the attacking herbivore is a true elicitor, and the emission of volatiles parallels the production of phytoalexins in response to a pathogen.

Since jasmonic acid (JA, and its biosynthetic precursors [21,22]) has recently been recognized as a potent endogenous

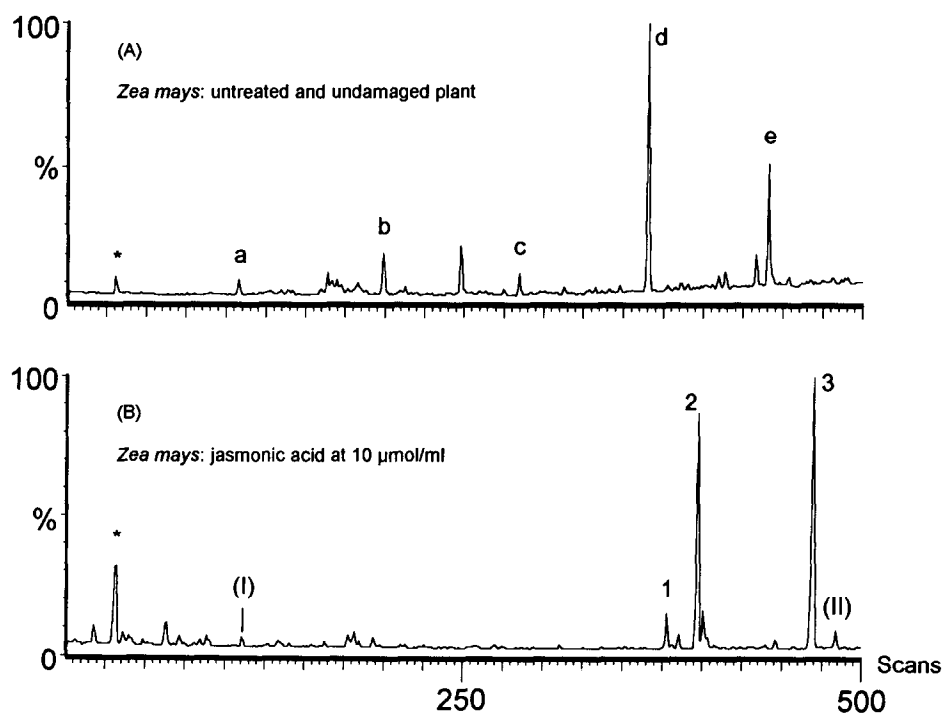


Fig. 4. Gas chromatographic profiles of the volatiles from *Zea mays*. Separation conditions, cf. Fig. 1. (A) Volatiles from undamaged, healthy plants. Sampling of volatiles, ca. 20 h. Identification of compounds: (*) contamination from the circulation pump, (a) *n*-undecan, (b) *n*-dodecan, (c) *n*-tridecan, (d) *n*-tetradecan (e) *n*-pentadecan. (B) Volatiles from undamaged, jasmonate treated plants. JA at 10 $\mu\text{mol}\cdot\text{ml}^{-1}$. Pretreatment, 5 h. Sampling period, 20 h. Identification of compounds [8]: (I) see text, (1) bergamotene, (2) β -farnesene, (3) nerolidol, (II) see text.

signal transducer in elicitor-induced cell cultures, the pronounced effect of this compound on the emission of volatiles fits into this scheme. The dose–response (cf. Fig. 3) is entirely in agreement with the action of JA or JA-Me on the biosynthesis of benzo[c]phenanthridine alkaloids in cell suspension cultures of *Eschscholtzia californica* [17]. The data also fit with the recently reported effect of coronatine on the same system. Coronatine is claimed to be a mimic of jasmonoids and other octadecanoid signalling molecules [23]. In the present case and the coronatine action, the maximum response of the plant is observed within 20–30 h. As shown in Fig. 2C, this is also in agreement with the time–course of the odor induction by the β -glucosidase. Up to now, no attempt has been made to determine the absolute configuration of the JA diastereomer which is active in the odor induction.

The observation that some compounds are triggered by the β -glucosidase and JA (e.g. mono- and sesquiterpenoids) while others are emitted only in response to one or the other of the two externally added compounds, is particularly important. This phenomenon clearly demands different internal signals and transducers, each of which may be responsible for an individual or a group of metabolic pathways leading to (volatile) secondary metabolites. Depending on the substrate specificity of the herbivore-injected β -glucosidase and other lytic enzymes, several low molecular components could be released from cellular structures, and some of them could be recognized by membrane receptors using different signal transducers. Preliminary experiments with the JA precursor 13-hydroperoxyoctadecadienoic acid showed a very pronounced effect on the biosynthesis of the C_{16} homoterpene II, while JA is not very effective in this case (cf. Fig. 2B–D). Owing to the diterpene origin of homoterpene II, and due to sesquiterpene precursor of homoterpene I, this finding strongly supports the selective triggering of different plant defense responses by different elicitors and different transducer molecules.

Besides the few examples of odor induction in Lima beans and corn plants by JA and/or its biosynthetic precursors, the effect of odor induction by octadecanoid signalling molecules might be widely distributed in nature and may even be regarded as a general phenomenon. Results of initial experiments point in this direction and will be reported soon.

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