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Differential Induction of Plant Volatile Biosynthesis in the Lima Bean by Early and Late Intermediates of the Octadecanoid-Signaling Pathway[1]

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Plants are able to respond to herbivore damage with de novo biosynthesis of an herbivore-characteristic blend of volatiles. The signal transduction initiating volatile biosynthesis may involve the activation of the octadecanoid pathway, as exemplified by the transient increase of endogenous jasmonic acid (JA) in leaves of lima bean (*Phaseolus lunatus*) after treatment with the macromolecular elicitor cellulysin. Within this pathway lima bean possesses at least two different biologically active signals that trigger different biosynthetic activities. Early intermediates of the pathway, especially 12-oxo-phytodienoic acid (PDA), are able to induce the biosynthesis of the diterpenoid-derived 4,8,12-trimethyltrideca-1,3,7,11-tetraene. High concentrations of PDA result in more complex patterns of additional volatiles. JA, the last compound in the sequence, lacks the ability to induce diterpenoid-derived compounds, but is highly effective at triggering the biosynthesis of other volatiles. The phytotoxin coronatine and amino acid conjugates of linolenic acid (e.g. linolenoyl-L-glutamine) mimic the action of PDA, but coronatine does not increase the level of endogenous JA. The structural analog of coronatine, the isoleucine conjugate of 1-oxo-indanoyl-4-carboxylic acid, effectively mimics the action of JA, but does not increase the level of endogenous JA. The differential induction of volatiles resembles previous findings on signal transduction in mechanically stimulated tendrils of *Bryonia dioica*.

During evolution, plants have developed a multitude of defense mechanisms against adverse biotic and abiotic impacts. Plants are sessile organisms and this property in particular may have forced evolution of the plethora of adaptive mechanisms to environmental stresses that we see today. To overcome the peculiar constraints that result from their stationary way of life, plants utilize volatile signals for long-distance interactions. For example, volatiles serve as attractants for pollinators (Knudsen et al., 1993; Langenheim, 1994) and seed-dispersing animals (Howe and Westley, 1986), and they attract prey to carnivorous plants (Kite, 1995). Herbivore-induced plant volatiles can even serve as cues to direct predators into the vicinity of their prey (Dicke et al., 1990; Turlings et al., 1990; Takabayashi and Dicke, 1996; De Moraes et al., 1998). This signaling by the plant to higher trophic levels has been interpreted as the plant's "cry for help" (Dicke and Sabelis, 1992).

Airborne volatiles, especially methyl jasmonate and methyl salicylate, volatiles corresponding to nonvolatile endogenous hormones such as jasmonic acid (JA) and salicylic acid, may function as signals for neighboring uninfested plants by activating defense-related genes (Farmer and Ryan, 1990; Bruin et al., 1995; Miksch and Boland, 1996; Shulaev et al., 1997). A comparable function may be attributed to ethylene, which is emitted from infested or herbivore-damaged plants (Chaudhry et al., 1998; Lund et al., 1998). The significance of such volatile-induced prophylactic defenses, however, remains to be established in the field. The volatile profiles released from a specific plant after damage by different herbivores or microorganisms may differ in their quantitative and qualitative composition (Takabayashi et al., 1991; De Moraes et al., 1998).

The existence of such herbivore- or microorganism-specific responses requires a distinct recognition of the attacking organism by the plant. Different feeding or infection mechanisms may account for some of the differences, but different elicitors may also be involved. β-Glucosidase (Hopke et al., 1994; Mattiaci et al., 1995) and cellulysin from the plant parasitic fungus *Trichoderma viride* (Piel et al., 1997) are examples of high-M_r elicitors of volatile biosynthesis. Among the low-M_r elicitors, JA, the bacterial phytotoxin coronatine (Ichihara et al., 1977; Boland et al., 1995), the structural analog indanoyl-L-Ile (Krumm et al., 1995), and the recently identified herbivore-specific volicitin (Alborn et al., 1997; Paré et al., 1998) are particularly noteworthy (see Scheme 1).

The first events following leaf damage and introduction of the elicitors are not well understood, but in several cases the activation of the octadecanoid-signaling cascade has been demonstrated (Blechert et al., 1995; McCloud and Baldwin, 1997; Piel et al., 1997). The cascade starts with the formation of (13*S*)-hydroperoxylinolenic acid from free and/or bound linolenic acid (Vick and Zimmermann, 1984; Feussner et al., 1995). Next, the hydroperoxide is converted to an unstable allene oxide and then into 12-oxo-phytodienoic acid (PDA) (Hamberg, 1988), recently recognized as the most important signal for plant mechanoreceptors (Weiler, 1997; Blechert et al., 1999). PDA is probably exported to the cytosol, reduced to 10,11-dihydro-PDA (Schaller et al., 1998), and degraded, by three consecutive β-oxidation cycles, to epi-JA (for review, see

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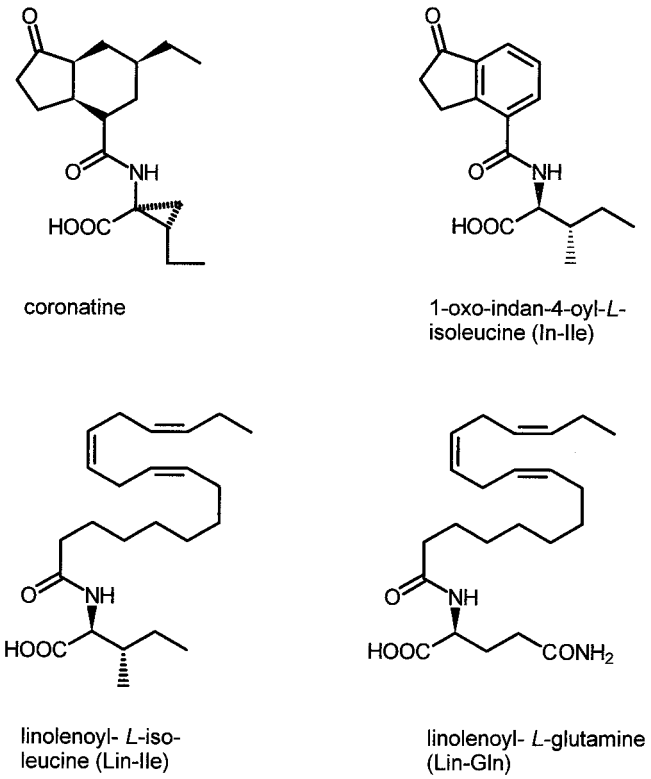

Scheme 1. The phytotoxin coronatine, isolated from *P. syringae* or *Xanthomonas campestris*, the accordingly designed synthetic analog 1-oxoindanoyl-Ile, and the amino acid conjugates of linolenic acid Lin-Ile and Lin-Gln, represent potent elicitors of plant volatile biosynthesis.

Beale and Ward, 1998; Müller, 1998). The mode of subsequent signal processing that precedes gene activation is unknown. Figure 1 shows that the lipid-based signaling pathway is composed of at least four structurally different types of compounds, probably endowed with signaling qualities: (a) acyclic fatty acids and functionalized derivatives; (b) cyclopentanoid C_{18} fatty acids; (c) cyclopentanoid C_{12} fatty acids such as epi-JA and JA; and (d) amino acid conjugates of the intermediates of the cascade, in particular that of epi-JA (Krumm et al., 1995; Tamogami et al., 1997; Wasternack et al., 1998).

We provide quantitative data on cellulysin-dependent endogenous JA production. Furthermore, we demonstrate that early and late intermediates of the octadecanoid pathway, along with certain amino acid conjugates, induce the biosynthesis of different volatile patterns in leaves of the lima bean (*Phaseolus lunatus*). In particular, the induction of certain terpenoids of cytosolic and plastidic origin is dependent on the application of early and late intermediates of the signaling pathway. Moreover, the amino acid conjugates coronatine, 1-oxo-indan-4-oyl-L-iso-Leu (In-Ile), and a conjugate of linolenic acid with Gln [Lin-Gln], a deoxa analog of volicitin) mimic volatile patterns typical of early intermediates of the cascade, but these elicitors do not induce the de novo biosynthesis of epi-JA.

MATERIALS AND METHODS

Plant Material

Induction experiments were carried out using lima bean (*Phaseolus lunatus* Ferry Morse cv Jackson Wonder Bush). Individual plants were grown from seed in 5.5-cm-diameter plastic pots at 23°C and 80% RH using daylight fluorescent tubes at approximately 270 μE m^{-2} s^{-1} with a photoperiod of 14 h. Experiments were conducted with 12- to 16-d-old seedlings with two fully developed leaves (Hopke et al., 1994).

Induction Experiments

Plantlets of lima bean were cut with razor blades and immediately transferred into vials containing a solution of the test substance in tap water. In order to achieve a high concentration of emitted volatiles in the headspace, the vials with the cut plantlets were enclosed in small (750 mL) desiccators. The experimental setup was maintained at 25°C and continuously illuminated during incubation. Solutions of JA and In-Ile were applied at 1 mM; PDA was

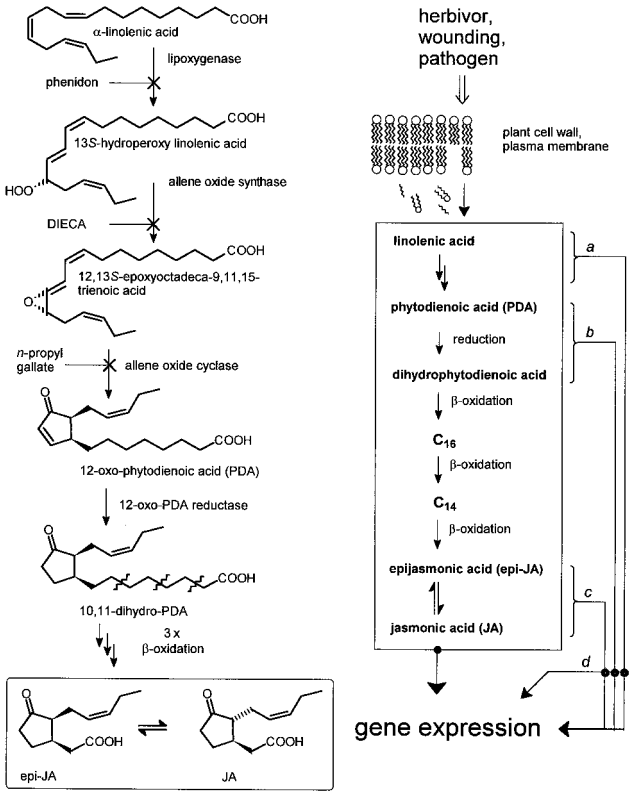

Figure 1. The Vick-Zimmerman pathway of JA biosynthesis from α-linolenic acid. Inhibitors and their putative targets are indicated. Phenidone interferes with lipoxygenase activity, DIECA reduces hydroperoxides to alcohols, and *n*-propyl gallate inhibits both lipoxygenase and allene oxide cyclase activity. The signaling sequence may be activated by pathogen infestation or herbivore feeding. The intermediates may have distinct and different biological activities: *a*, acyclic octadecanoids; *b*, cyclopentanoid C_{18} fatty acids; *c*, epi-JA and JA; *d*, amino acid conjugates of *a*, *b*, and *c*.

used in a range from 0.05 to 1 mM. The highly active coronatine was applied as a 0.1 mM aqueous solution. Commercially available cellulysin (a cocktail of cellulases and endoglucanases) was used at a concentration of 50 μg/mL. Linolenic acid and the two conjugates of linolenic acid, linolenoyl-L-iso-Leu (Lin-Ile) and Lin-Gln, were applied at 2.0 mM. To overcome problems with the low solubility of the linolenic acid conjugates, small amounts (10 mg/100 mL) of Triton X-100 had to be added.

The inhibitors phenidone and *n*-propyl gallate (Hamberg, 1988) were used at a 1 mM concentration. Freshly cut plantlets were pre-incubated with the inhibitor solutions for 48 h prior to the induction experiments. Control experiments were run by placing freshly cut plantlets into tap water. In assays using linolenic acid and linolenic acid conjugates, the control also contained the detergent Triton X-100. The total uptake of the test solution during a typical incubation experiment was about 1 mL per plantlet (approximately 3 g fresh weight) corresponding to 300 nmol/g fresh weight, for example, in JA. If not stated otherwise, experiments were carried out in triplicate.

Collection and Analysis of Headspace Volatiles

The volatiles emitted by the (pre-)treated plants were continuously collected over a period of 24 h on small charcoal traps (1.5 mg of charcoal, CLSA-Filter, Le Ruisseau de Montbrun, Daumazan sur Arize, France) using air circulation as described previously (Donath and Boland, 1995). After desorption of the volatiles from the carbon trap with dichloromethane (2 $\times$ 15 μL), and the addition of an internal standard (1-bromodecane, 5 μL of a 7.2 mM solution in dichloromethane), the total volume was adjusted to 40 μL. The extracts were directly analyzed by GC-MS using fused-silica capillary tubes (15 m $\times$ 0.25 mm; Alltech, Unterhaching, Germany) coated with DB 1 (0.1 μm). Helium at 60 kPa served as the carrier gas. Separation of the compounds was achieved under programmed conditions (40°C for 2 min, then at 10°C min^{-1} to 200°C). MS analysis was performed (model MD800, Fisons, Bellevue, WA) with the GC interface at 260°C and the scan range at 35 to 300 D. Individual compounds (peak areas) were quantified with respect to the peak area of the internal standard.

Quantification of Endogenous JA

The quantification of endogenous JA followed the protocol of Baldwin et al. (1997). Treated leaves (1.0 g of tissue) were frozen and ground under liquid nitrogen. The resulting powder was suspended in a solution of acetone and 50 mM citric acid (70:30, v/v). [9,10-$^2H_2$]Dihydro-JA (146 ng) was added as an internal standard. The organic solvent was allowed to evaporate overnight at room temperature to avoid losses of volatile fatty acid compounds. The resulting aqueous solutions were filtered and extracted with 3 $\times$ 10 mL of diethyl ether. The pooled extracts were then loaded onto a solid-phase extraction cartridge (Varian, Darmstadt, Germany) containing 500 mg of the sorbent aminopropyl. After loading, the cartridges were washed with 7 mL of a solvent mixture of trichloromethane:2-propanol (2:1, v/v). Bound JA and the standard were eluted with 10 mL diethyl ether:acetic acid (98:2, v/v).

After evaporation of solvents and esterification of the residue with excess diazomethane, the sample was adjusted to 50 μL with dichloromethane. The solutions were analyzed by GC-MS without further purification. The methyl esters of JA and the standard [9,10-$^2H_2$]JA eluted separately, allowing quantification of the former. To enhance the sensitivity of the method, spectra were recorded in the selective ion mode, monitoring only the fragment ion at m/z = 83, corresponding to the base peak of both JA and [9,10-$^2H_2$]JA. The amount of endogenous JA was calculated from the peak areas of JA and the [9,10-$^2H_2$]JA standard using a previously produced calibration curve.

Chemicals

Cellulysin, phenidone (1-phenyl-3-pyrazolidinone), and salicylic acid were purchased from Sigma-Aldrich. 12-Oxo-PDA was obtained from Campro Scientific (Veenendaal, The Netherlands). Solvents were purified prior to use. Free JA was obtained from the methyl ester (provided by Dr. R. Kaiser, Givaudan-Roure, Dübendorf, Switzerland) by saponification. The methyl ester of In-Ile was prepared as described previously (Krumm et al., 1995; Krumm and Boland, 1996). Coronatine was isolated from cultures of *Pseudomonas syringae* pv *glycinea* according to the procedure described by Nüske and Bublitz (1993).

[9,10-$^2H_2$]Dihydro-JA

A catalytic amount of platinum dioxide was stirred in dry diethyl ether (15 mL) and reduced by ^2H gas until a black suspension resulted. Then a solution of methyl jasmonate (1.0 g, 4.46 mmol) dissolved in dry diethyl ether (10 mL) was added, and stirring was continued under a ^2H atmosphere until GC analysis indicated complete reduction of the double bond. The catalyst was then filtered off and the solvent removed. The product was purified by chromatography on silica gel using pentane:diethyl ether (80:20, v/v) for elution. The yield was 0.72 g (71%). ^{1}H-NMR (400 MHz, $CDCl_3$): 0.75 to 0.81 (t, J = 7 Hz, 3H, 1-H); 1.07 to 1.51 (m, 7H); 1.66 to 1.74 (m, 1H); 1.98 to 2.30 (m, 5H); 2.50 to 2.57 (m, 1H); 3.53 (s, 3H,-OCH_3). ^{13}C-NMR (400 MHz, $CDCl_3$): 14.0 (t, ^3J = 2.8 Hz, 1-C); 22.4 (td, ^2J = 10.4, ^3J = 2.3 Hz, 2-C); 26.0 (m, 4-C); 27.2 (9-C); 27.7 (dt, ^2J = 10.4, ^3J = 2.5 Hz, 5-C); 31.8 (m, 3-C); 37.7 (10-C); 38.0 (8-C); 38.9 (11-C); 51.7 (OCH_3); 54.2 (6-C); 172.7 (12-C); 219.8 (7-C). MS (EI, 70 eV): 228 (4); 197 (3); 156 (36); 96 (10); 83 (100); 55 (10). High-resolution MS (HR-MS) 228.1692 ($C_{13}H_{20}D_2O_3$, calculated value = 228.1694).

Lin-Gln

Linolenic acid (100 mg, 0.36 mmol) and triethylamine (40 mg, 0.40 mmol) were dissolved in tetrahydrofuran (4 mL) and ethyl chloroformate (43 mg, 0.40 mmol) was added with stirring at −10°C. After 5 min, L-Gln (105 mg, 0.72 mmol) dissolved in aqueous sodium hydroxide (2.8 mL, 0.29 M) was added and stirring was continued for 15 min at

room temperature. The reaction mixture was acidified with 2 N hydrochloric acid and extracted with ethyl acetate. The combined organic layers were dried (Na_2SO_4) and, after removal of solvents, the residue was washed with diethyl ether. The yield was 126 mg (86%). ^{1}H-NMR (400 MHz, DMSO-d_6): 0.92 (t, J = 7.7 Hz, 3H); 1.20 to 1.33 (m, 8H); 1.41 to 1.52 (m, 2H); 1.65 to 1.79 (m, 1H); 1.85 to 1.97 (m, 1H); 1.98 to 2.14 (m, 8H); 2.71 to 2.84 (m, 4H); 4.06 to 4.19 (m, 1H); 5.23 to 5.40 (m, 6H); 6.78 (s, 1H); 7.29 (s, 1H); 8.05 (d, J = 6.5 Hz, 1H); 12.47 (s, 1H). ^{13}C-NMR (400 MHz, THF-d_8): 14.7; 21.3; 26.2; 26.3; 26.5; 28.1; 29.0; 30.2; 30.3; 30.4; 30.7; 32.6; 36.5; 52.6; 128.0; 128.5; 129.0 (2x); 130.9; 132.4; 173.3; 174.2; 175.5. MS (EI, 70 eV): 406 (M^+, 4), 388 (10), 359 (3), 170 (21), 147 (46), 130 (100), 121 (27), 108 (58), 95 (63), 79 (85), 67 (45), 55 (27). HR-MS 406.2817 ($C_{23}H_{38}N_2O_4$, calculated value = 406.2832).

Lin-Ile

Lin-Ile was prepared from linolenic acid (100 mg, 0.36 mmol) and L-Ile (94 mg, 0.72 mmol), as described for Lin-Gln. After evaporation of the solvent, the crude product was purified by chromatography on silica gel using diethyl ether:pentane:acetic acid (150:75:4, v/v) for elution, followed by rechromatography on Sephadex LH-20 (trichloromethane:methanol, 2:1, v/v). Yield: 104 mg (74%). ^{1}H-NMR (400 MHz, $CDCl_3$): 0.84–0.94 (m, 9H); 1.07–1.33 (m, 9H); 1.36–1.49 (m, 1H); 1.51–1.63 (m, 2H); 1.82–1.93 (m, 1H); 1.94–2.05 (m, 4H); 2.12–2.25 (m, 2H); 2.67–2.80 (m, 4H); 4.56 (dd, J = 8.5, 4.8 Hz, 1H); 5.20–5.37 (m, 6H); 6.00 (d, J = 8.5 Hz, 1H); 8.74 (s, 1H). ^{13}C-NMR (400 MHz, $CDCl_3$): 11.6; 14.3; 15.4; 20.6; 25.1; 25.5; 25.6; 25.7; 27.2; 29.2 (2x); 29.3; 29.6; 36.7; 37.6; 56.4; 127.1; 127.7; 128.3 (2x); 130.3; 132.0; 173.8; 176.0. MS (EI, 70 eV): 391 (M^+, 5), 362 (2), 246 (2), 260 (5), 132 (93), 86 (100), 79 (20). HR-MS 391.3070 ($C_{24}H_{41}NO_3$, calculated value = 391.3087).

RESULTS

Induction of Volatiles by Early and Late Intermediates of the Octadecanoid-Signaling Pathway

Treatment of freshly detached leaves of lima bean with solutions of JA has been shown previously to induce the de novo biosynthesis of volatiles (for lima bean, Donath, 1994; Piel et al., 1998; for maize, Paré et al., 1998). The pattern of the induced compounds was largely, but not completely, identical to that observed after spider mite damage (Dicke et al., 1990). When similar induction experiments were conducted with early intermediates of the octadecanoid pathway, such as linolenic acid, treatment of the leaf resulted in the release of a volatile pattern clearly different from that obtained after JA treatment (compare Figs. 2 and 3). Linolenic acid was active only at relatively high concentrations (2.0 mM) and, surprisingly, induced only the biosynthesis of two homoterpenes, 4,8-dimethylnona-1,3,7-triene (DMNT) and (at a much more pronounced level) 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT). Both compounds represent degradation products of the tertiary terpenoid alcohols nerolidol and geranlyllinalool (Gäbler et al., 1991; Boland et al., 1998) and can be synthesized by many angiosperms (Boland et al., 1992).

When the leaves were pretreated with inhibitors of the octadecanoid pathway, such as phenidone (Peña-Cortés et al., 1993; Doares et al., 1995; Piel et al., 1997) and *n*-propyl gallate (Hamberg, 1988), prior to treatment with linolenic acid, no induction of de novo synthesis of DMNT and TMTT was observed. Upon treatment of the leaves with low concentrations of PDA (0.1 mM), the first cyclopentanoid member of the pathway, more of these and other volatiles were released. However, the two homoterpenes DMNT and TMTT, already observed after linolenic acid treatment, clearly prevailed (see Fig. 2). Higher concentrations of PDA (1.0 mM) resulted in the emission of a rather complex pattern of compounds that closely resembled those observed after treatment with JA (Boland et al., 1995), the last member of the octadecanoid pathway (Fig. 3). The major and most significant difference between the two volatile patterns after PDA and JA treatment was the lack of the C_{16} homoterpene TMTT in plants treated with JA.

Induction of Volatile Biosynthesis by Coronatine and In-Ile

As reported previously, treatment of leaves of the lima bean with the bacterial phytotoxin coronatine induced a significant de novo biosynthesis of volatiles (Boland et al., 1995). When low concentrations (0.05 mM) of coronatine were applied, a volatile profile closely resembling that after PDA treatment was observed (Fig. 4). Again, the C_{16}-homoterpene TMTT was one of the dominant compounds in the GC profile of the collected volatiles, but the quantitative composition of the PDA profile was not reproduced.

Unlike coronatine, the synthetic analog In-Ile, which was originally designed as a structural analog of coronatine (Krumm et al., 1995; Krumm and Boland, 1996), failed to induce the biosynthesis of TMTT at any concentration but stimulated the emission of a volatile pattern closely resembling that after JA treatment (Fig. 4).

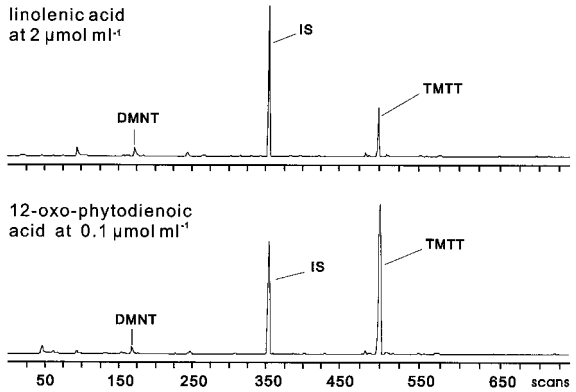

Figure 2. GC profiles of the induced volatiles after treatment of lima bean leaves with linolenic acid (2 μmol mL^{-1}) and PDA (0.1 μmol mL^{-1}). Both compounds induce the biosynthesis of the two homoterpenes DMNT and TMTT, but PDA proved to be significantly more active. IS, Internal standard.

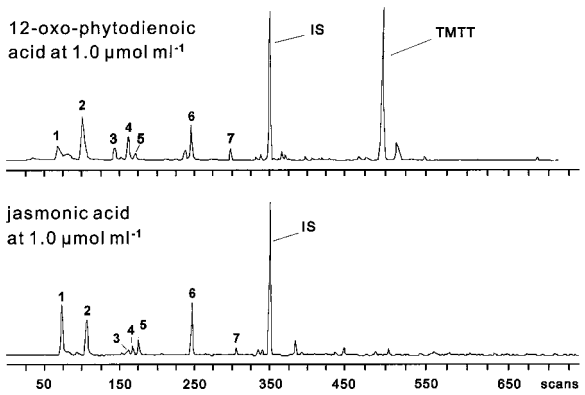

Figure 3. GC profiles of the induced volatiles after treatment of lima bean leaves with PDA (1 μmol/mL) or JA (1 μmol/mL). PDA applied at higher concentrations induced the biosynthesis of a complex blend of volatiles largely resembling the JA-induced pattern of volatiles. Treatment with JA does not stimulate the biosynthesis of the diterpenoid-derived homoterpene TMTT. No volatiles were formed in control experiments using tap water. Identification of compounds: 1, hexenyl acetate; 2, β-ocimene; 3, linalool; 4, DMNT; 5, $C_{10}H_{14}$; 6, $C_{10}H_{16}O$; 7, indole. IS, Internal standard (1-bromodecane).

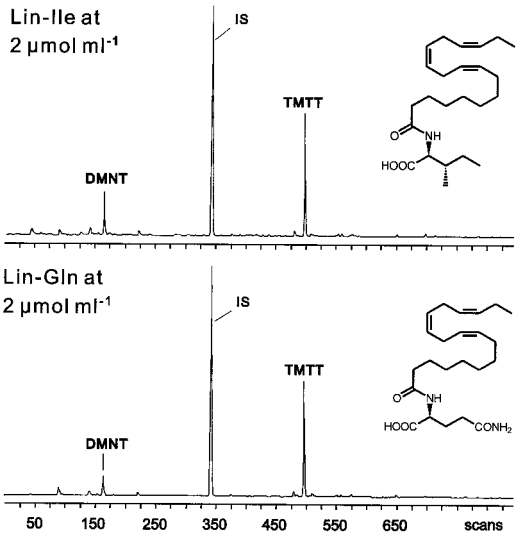

Figure 5. GC profiles of the induced volatiles after treatment of lima bean leaves with the amino acid conjugates Lin-Ile and Lin-Gln. Both conjugates selectively induced the biosynthesis of the two homoterpenes DMNT and TMTT and this resembled the effects after PDA treatment. IS, Internal standard.

Induction of Volatile Biosynthesis by Amino Acid Conjugates of Linolenic Acid

In addition to the amino acid conjugates coronatine and In-Ile, Lin-Gln was also assayed for its ability to induce volatile biosynthesis. Lin-Gln was used as a nonhydroxylated analog of volicitin, previously isolated as the active principle from caterpillar saliva (Alborne et al., 1997; Paré et al., 1998). Indeed, treatment of lima bean leaves with either Lin-Gln or Lin-Ile elicited volatile biosynthesis. Only the emission of the two homoterpenes DMNT and TMTT was observed (see Fig. 5), as was the case after treatment with a high concentration of free linolenic acid or a low concentration of PDA (Fig. 2).

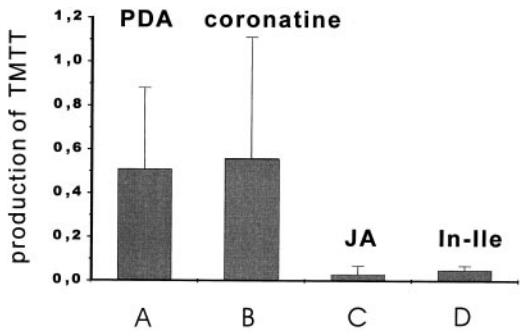

Figure 4. Induction of the biosynthesis of the diterpenoid-derived homoterpene TMTT by different low-M_r elicitors (for concentrations of the test solutions, see "Materials and Methods"). PDA (A) and coronatine (B) induced the biosynthesis of TMTT with similar efficiency. The inducing capacity of JA (C) and In-Ile (D) were insignificant. The homoterpenes were quantified via their peak areas relative to the peak area of the internal standard. PDA and coronatine, three replicates; JA and In-Ile, five replicates. Error bars indicate SD.

Quantification of Endogenous JA after Elicitation of Volatile Biosynthesis with Cellulysin and Amino Acid Conjugates

To demonstrate the activation of the octadecanoid pathway for the induction of volatile biosynthesis after treatment with the different elicitors, endogenous JA was quantified according to the procedure of Baldwin et al. (1997). Treatment of lima bean leaves with cellulysin, a macromolecular elicitor acting via octadecanoid-signaling, allowed us to follow the concentration/time course of endogenous JA (Piel et al., 1997). Samples were taken at defined intervals after placing a freshly detached leaf into an aqueous solution of cellulysin. After extraction and purification, the endogenous JA level was determined by GC-MS. Figure 6a shows that the level of endogenous JA started to rise 10 to 20 min after the beginning of the experiment, reached a transient maximum of approximately 350 ng/g fresh weight after 30 min, and then leveled off within 2 h to the initial concentration of about 30 to 50 ng/g fresh weight. Control experiments using tap water without additives also resulted in a moderate increase of endogenous JA, but the maximum level was much lower (approximately 70 ng/g fresh weight) and was apparently due to the wounding by cutting the stem (Fig. 6b).

The low level of endogenous JA caused by mechanical damage was not sufficient to induce volatile biosynthesis, but the JA level following cellulysin treatment reproducibly resulted in the emission of a volatile profile identical to that after JA treatment (Piel et al., 1997). Interestingly, when leaves of lima bean were treated with coronatine at levels as low as 10 to 20 μM, strong volatile production was observed, but the endogenous JA level was not altered. Aside from a transient increase within the first 2 h due to mechanical damage (see above), no increase in JA was

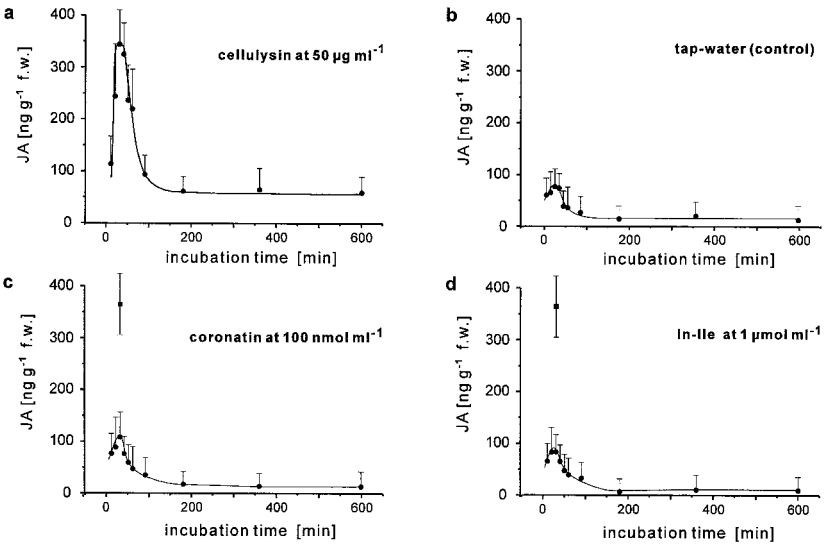

Figure 6. Quantification of endogenous JA level after treatment of lima bean leaves with the test solutions indicated. a, Treatment with cellulysin at 50 μg mL^{-1}. b, Tap water control. c, Treatment with coronatine at 100 nmol mL^{-1}. d, Treatment with In-Ile at 1 μmol mL^{-1}. In each case the endogenous JA level was followed over a period of 10 h after the start of the experiment. The transient increase of the endogenous JA level following the treatment with coronatine and In-Ile was comparable to that of the control and may have been due to mechanical wounding caused by cutting the stem. f.w., Fresh weight.

observed (Fig. 6c). The resting and recovery level of JA was somewhat lower than after cellulysin treatment. Not surprisingly, the coronatine analog In-Ile also failed to increase the endogenous JA level (Fig. 6d), although a significant volatile production was observed. In another set of experiments, the leaves were pretreated with inhibitors of the octadecanoid pathway (phenidone, diethyldithiocarbamic acid [DIECA], and *n*-propyl gallate), but the two amino acid conjugates coronatine and In-Ile nevertheless induced volatile patterns resembling those after PDA or JA treatment (compare with Figs. 2 and 3). Control experiments with inhibitor pretreatment and subsequent treatment with cellulysin exhibited no volatile production and therefore corroborated the efficiency of inhibition. If plants were elicited with Lin-Ile or Lin-Gln following a pretreatment with phenidone or *n*-propyl gallate, no volatile production was observed.

DISCUSSION

The induction of volatile biosynthesis by microorganisms and insect herbivores has been demonstrated for several systems in recent years (Dicke et al., 1990; Turlings et al., 1990; Croft et al., 1993). Of particular importance is the observation that the volatile profiles released from individual plants may vary depending on the attacking organisms (Takabayashi et al., 1991; De Moraes, 1997). Such findings clearly demand a differential recognition of the attacking organism by the plant, followed by an internal signaling network, and resulting in a species-related response manifested in the differential activation of a network of metabolic pathways.

Our observation that different members of the octadecanoid pathway are able to induce different volatile patterns in leaves of lima bean represents an important clue in understanding the origin of species-related volatile blends. Focusing on the type of induced terpenoids, it becomes obvious that early intermediates of the octadecanoid pathway have the ability to trigger the biosynthesis of TMTT, which is of diterpenoid origin. In contrast, late intermediates, especially JA, trigger the biosynthesis of mono- and sesquiterpenes, but lack the ability to induce the biosynthesis of diterpenoids. Taking into account that pretreatment of the leaves with phenidone as an inhibitor of lipoxygenase activity prevents the induction of volatile biosynthesis by linolenic acid, it is evident that the first active compound has to be located downstream of linolenic acid. Since DIECA, which rapidly reduces hydroperoxides to alcohols, also suppresses the induction of volatile biosynthesis (Piel et al., 1997), the active signal is also downstream of the (13*S*)-hydroperoxide (see Fig. 1). As a matter of fact, the volatile pattern induced by very low levels of PDA perfectly matched the profile of linolenic acid-induced volatiles (Fig. 2). Products downstream of PDA, e.g. dihydro-PDA and the subsequent β-oxidation products representing 16- and 14-carbon-atom homologs of JA, proved to be largely inactive in the lima bean system (Boland et al., 1998, 1999; Hopke, 1998).

The profile of JA-induced volatiles, with its lack of diterpenoid-derived TMTT, suggested that JA and related compounds apparently represent a second center of biologically active chemicals within the signaling pathway. These findings are in accord with previous studies on the signal transduction following mechanical stimulation of touch-sensitive tendrils of *Bryonia diocia* (Falkenstein et al., 1991). Based on the endogenous levels of PDA and JA following mechanical stimulation, PDA has been recognized as the more active compound, and these conclusions were confirmed by dose-response studies with individual signaling compounds (Weiler et al., 1994; Blechert et al., 1999).

The lima bean, however, is unique in that the identity of the major signaling compounds can be easily identified by the pattern of the emitted volatiles. The increasing complexity of volatile blends released after treatment of leaves with a higher concentration of PDA (compare Figs. 2 and 3) might be due to a concentration-dependent induction of different metabolic pathways, as is the case for JA (Hopke,

1998), or it may reflect the effect of an increasing level of endogenous JA resulting from processing of PDA along the octadecanoid pathway. A combination of both effects cannot be excluded either.

Another analogy to the touch-sensitive tendrils of *B. dioica* is the high activity of the amino acid conjugate coronatine (Ichihara et al., 1977; Boland et al., 1995). In mechanotransduction, the compound is active without raising the internal level of octadecanoids, and it has been claimed on the basis of structure-mapping calculations that coronatine may represent an analog of PDA (Weiler et al., 1994; Blechert et al., 1999). Although this conclusion still lacks direct evidence, it is supported by our observations that both PDA and coronatine induce the biosynthesis of the degraded diterpene TMTT. As shown in Figure 6c, coronatine failed to induce JA biosynthesis in the lima bean, although it did induce volatiles at threshold concentrations as low as 10 μM.

More interesting still was the observation that treatment of lima bean leaves with the coronatine analog In-Ile resulted in the emission of a volatile pattern closely resembling that seen after JA treatment. This finding clearly tells us that at least two structurally well-defined processing systems (receptors and/or binding proteins) that are selective for either PDA or JA must exist and can be addressed independently by different low-M_r compounds. Since coronatine and In-Ile are only active as intact molecules (Krumm et al., 1995), their ability to induce volatile biosynthesis is not prevented by inhibition of the octadecanoid-signaling cascade (Table I). The actual receptors or macromolecular targets involved in this signaling processes remain to be established.

The two amino acid conjugates of linolenic acid tested in this study, Lin-Gln and Lin-Ile, proved to be significantly more active than the free acid, but produced the same pattern of volatiles as linolenic acid. Whether the conjugation with an amino acid is only important to facilitate transport across membranes into specialized compartments, or if, like coronatine, it facilitates selective binding to the processing unit for PDA remains to be established. Since Lin-Gln was recently identified as a minor component in the salivary secretion of the generalist herbivore *Spodoptora exigua* (Paré et al., 1998), it is obvious that induction of herbivore-specific blends of volatiles may be

Table I. *Effects of different elicitors and inhibitors of the jasmonate biosynthesis on the induction of volatile biosynthesis in lima bean leaves*

Elicitor/Inhibitor Treatment	DMNT	TMTT	Other Volatiles	Endogenous JA Level	Reference
Cellulysin					
50 μg mL^{-1}	+[a]	−	++[b]	↑[c]	Piel et al. (1997)
Phenidone	−	−	−	ND[d]	
DIECA	−	−	−	ND	
Linolenic acid					
2 mM	+	++	−	ND	
Phenidone	−	−	−	ND	
n-Propyl gallate	−	−	−	ND	
PDA					
0.1 mM	+	+++[e]	−	ND	
1.0 mM	+	+++	+	ND	
JA					
1.0 mM	+	−	++	ND	Boland et al. (1995)
Coronatine					
0.1 mM	++	+++	+++	(↑)[f]	Boland et al. (1995)
Phenidone	++	+++	+++	ND	
DIECA	++	+++	+++	ND	
In-Ile					
1.0 mm	+	−	++	(↑)	Krumm et al. (1995)
Phenidone	+	−	++	ND	
DIECA	+	−	++	ND	
Lin-Ile					
2 mM	+	+++	−	ND	
Phenidone	−	−	−	ND	
n-Propyl gallate					
Lin-Gln					
2 mM	+	+++	−	ND	
Phenidone	−	−	−	ND	
n-Propyl gallate	−	−	−	ND	
Water, control	−	−	−	(↑)	
Water, detergent	−	−	−	(↑)	

[a]+, Minor components (<5%). [b]++, Byproducts (5% → 20%). [c]↑, Transient upregulation. [d]ND, Not determined. [e]+++, main product(s) ⩾20% (usually base peak of the chromatogram). [f](↑), Minor increase due to mechanical wounding.

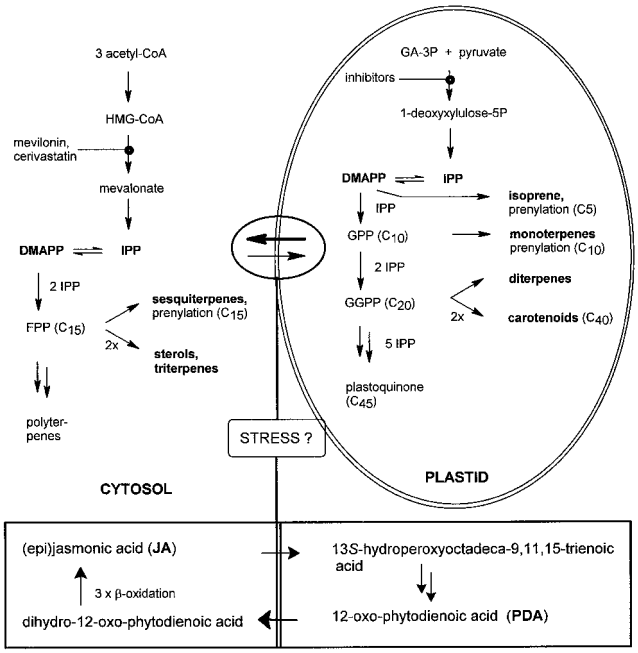

Figure 7. Localization of the mevalonate-dependent and the mevalonate-independent biosynthesis of terpenoids in cytosolic and plastidic compartments of the cell (Gershenzon and Croteau, 1993; Chappell, 1995; fig. modified after Lichtenthaler, 1998). The extent of precursor exchange (e.g. prenyl diphosphates) and the distribution of the signaling compounds of the octadecanoid cascade (Weiler, 1997; Beale and Ward, 1998; Müller, 1998) between the compartments are not known. DMAPP, Dimethylallyl diphosphate; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; HMG, 3-hydroxy3-methyl glutaryl; IPP, isopentenyl diphosphate.

due to the presence of different (low-M_r) elicitors in the secretions of plant-consuming insects. The finding that pretreatment of leaves with the inhibitors phenidone or *n*-propyl gallate prior to elicitation with Lin-Ile or Lin-Gln suppressed the induction of volatile biosynthesis may point toward amide hydrolysis and subsequent channeling of the free linolenic acid into the octadecanoid-signaling pathway. Additional experiments with hydrolysis-resistant analogs are necessary to clarify this important question.

It must be stressed, however, that the differential induction of volatiles in lima bean is not a general phenomenon, but has to be established for each plant species individually. For example, identical volatile patterns were obtained upon treatment of maize with coronatine and JA (Hopke, 1998), although leaves of this plant also emit both DMNT and TMTT (Hopke et al., 1994). Linking the events of octadecanoid biosynthesis and those of the induced terpenoid biosynthesis to specific cellular organelles leads to interesting conclusions. We have shown recently that the biosynthesis of TMTT is fueled from the novel deoxy-D-xylulose pathway (Rohmer et al., 1993; Arigoni et al., 1997; Lichtenthaler et al., 1997), while the sesquiterpenoid-derived DMNT is predominantly, but not exclusively, assembled from C_5 units originating from the mevalonate pathway, as outlined in Figure 7 (Boland et al., 1998; Piel et al., 1998). Since the first steps of the octadecanoid pathway, lipid peroxidation, formation of the unstable allene oxide, and cyclization to PDA, take place in the chloroplast (Gundlach and Zenk, 1998; Müller, 1998), as is the case for mono- and diterpenoid biosynthesis (Gershenzon and Croteau, 1993), it appears reasonable to assume that one processing unit for PDA-dependent activation of terpenoid biosynthesis has to be located in the plastid. Next, PDA is exported into the cytosol, reduced to 10,11-dihydro-PDA by a reductase (Schaller et al., 1998), and further degraded by β-oxidation in the cytosol-embedded peroxisomes. After release of JA to the cytosol, this compound may turn on the biosynthetic machinery for terpenoid assembly located here (e.g. sesquiterpenes). If the compound is able to reenter the plastid (Dathe et al., 1993), JA, like PDA, will also trigger the plastid-associated biosynthesis of (mono)terpenes. Alternatively, corresponding signaling systems have to exist across the membrane of the plastid. Whether or not such a simplified model of PDA-dependent diterpenoid biosynthesis (and predominately JA-dependent sesquiterpenoid biosynthesis) really exists must await the isolation and structural characterization of the macromolecules responsible for octadecanoid recognition and subsequent signal transduction to gene expression.

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