Plant Physiol. (1997) 115: 1057–1064

Induction of 12-Oxo-Phytodienoic Acid in Wounded Plants and Elicited Plant Cell Cultures[1]

Stefanie Parchmann, Heidrun Gundlach, and Martin J. Mueller*

Institute of Pharmaceutical Biology, University of Munich, Karlstraβe 29, D-80333 Munich, Germany

Jasmonic acid (JA) is rapidly biosynthesized from α-linolenic acid in plants upon contact with pathogens or wounding, and triggers gene activation, leading to the synthesis of defensive secondary metabolites and proteins. Despite the recent finding that its precursor, 12-oxo-phytodienoic acid (PDA), is a more powerful inducer of gene activation, interest has focused so far almost exclusively on JA. A validated negative chemical ionization-gas chromatography-mass spectrometry method has been developed that allows the simultaneous quantification of endogenous 12-oxo-PDA and JA in plant tissues. In six out of eight plant species tested maximal levels of 12-oxo-PDA exceeded peak levels of JA by approximately 3- to 5-fold after elicitation with a yeast cell wall preparation or when plants were wounded. These experiments support the hypothesis that 12-oxo-PDA acts as the predominant jasmonate signal in most plants, whereas JA remains an active metabolite of its precursor. Furthermore, JA but not 12-oxo-PDA was shown to be secreted into the medium from cultured plant cells, suggesting that JA may also act as an intercellular signal.

Recent research on the oxidative metabolism of unsaturated fatty acids in plants has led to the discovery of a variety of potent signal compounds (Farmer, 1994). One of the most thoroughly studied mediators is JA. JA appears to play an important role in developmental processes such as flowering, tuberization, fruit ripening, storage, and tendril coiling (Hamberg and Gardner, 1992; Sembdner and Parthier, 1993). Furthermore, JA has attracted much attention because of its structural and biosynthetic similarity to the arachidonic acid-derived signals of the prostaglandin and leukotriene type in animals, suggesting a possible functional analogy in host defense reactions (Vick and Zimmerman, 1983; Bergey et al., 1996).

In 1990 Farmer and Ryan demonstrated that airborn JA methyl ester activates the expression of defensive genes (Farmer and Ryan, 1990). Since then it has been demonstrated that yeast cell wall preparations, bacterial antibiotics, plant cell wall-derived oligouronides, fungal cell wall-derived chitosan, *N*-acetylchitohepatose, wounding, and a variety of other stimuli lead to a rapid synthesis of endogenous JA (for review, see Mueller, 1997). This in turn activates defensive genes, eventually yielding a variety of jasmonate-induced proteins (Herrmann et al., 1989) and antibiotic secondary metabolites (Gundlach et al., 1992). Jasmonate-induced proteins fall into different categories. Some are defensive proteins such as proteinase inhibitors and proteolytic enzymes. Others are involved in the biosynthesis of defensive secondary metabolites. In addition, a variety of induced proteins have been identified that are associated with the signal transduction pathway, among them, lipoxygenase and calmodulin (Bergey et al., 1996).

A hypothesis suggesting a role of JA in plant defense has been proposed by Farmer and Ryan (1992). In the model, the JA signal pathway is initiated after activation of receptors on the plant cell membrane that sense an attack by insects and pathogens. This leads to the activation of a lipase releasing α-linolenic acid from an as-yet-unknown membrane lipid. Linolenic acid is converted by the presumably constitutive enzymes lipoxygenase, allene oxide synthase, and allene oxide cyclase to 9(*S*),13(*S*)-12-oxo-PDA. 12-Oxo-PDA is the first cyclopentenone in the jasmonate pathway and is subsequently converted to 3(*R*),7(*S*)-JA by reduction of the Δ10 double bond and three rounds of β-oxidation. 12-Oxo-PDA, along with its metabolite, JA (collectively termed jasmonates), either directly or indirectly triggers gene activation, which leads to a local defense response (Dittrich et al., 1992; Blechert et al., 1995) or tendril coiling (Weiler et al., 1993). Thus far, the relative contribution of 12-oxo-PDA and JA to gene activation within the plant is unknown (Fig. 1).

Since 12-oxo-PDA is the first bioactive jasmonate in the biosynthetic sequence and displays a greater potency than JA (Weiler et al., 1993; Blechert et al., 1995), it is necessary to measure the intracellular accumulation of 12-oxo-PDA together with JA in response to elicitors or wounding. So far, only the accumulation of the metabolite JA has been examined and utilized as an indicator of induction of the jasmonate pathway. However, there is a danger of underestimating or even overlooking a jasmonate-mediated effect by restricting the focus to the metabolite JA.

In animal cells lipid mediators are typically secreted from the producer cells into the extracellular medium, where they modulate the host defense response (i.e. the inflammatory response) via extracellular membrane receptors located on their target cells. Although plant-derived jasmonates are thought to be intracellular messengers, secretion of jasmonates by elicited cells and uptake by nonactivated neighboring cells could be a mechanism by which

[1] This work was supported by Sonderforschungsbereich 369 of the Deutsche Forschungsgemeinschaft (Bonn, Germany).

* Corresponding author; e-mail martin.mueller@lrz.uni-muenchen.de; fax 89–5902–611.

Abbreviations: JA, jasmonic acid; PDA, 10,15(Z)-phytodienoic acid; PFB, pentafluorobenzyl.

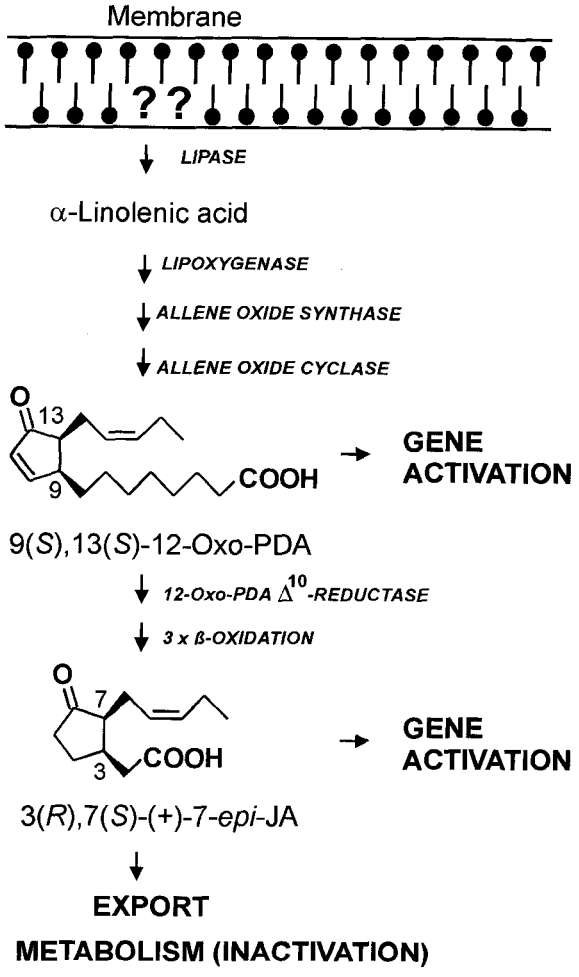

Figure 1. Model of the jasmonate pathway mediating plant defense responses.

a defense signal is spread locally in the tissue at the site of infection. To address this question, release of 12-oxo-PDA and JA into the culture medium of plant suspension cells was monitored after elicitation with a yeast cell wall preparation.

MATERIALS AND METHODS

JA methyl ester (a racemic mixture) was obtained from Serva (Heidelberg, Germany). JA was prepared by alkaline hydrolysis of the methyl ester and 9,10-dihydro-JA was synthesized from JA by catalytic hydrogenation, as previously described (Gundlach et al., 1992).

PFB bromide and *N,N*-diisopropylethylamine were from Aldrich. α-Linolenic acid was obtained from Sigma and [1-^{14}C]α-linolenic acid (specific activity of 52 mCi/mmol) was from Amersham. Solid-phase extraction glass columns (500 mg) were obtained from Macherey-Nagel (Düren, Germany). Lipid extracts from algae grown in the presence of $^{13}CO_2$ as a sole C source were a gift from Prof. A. Bacher, Technische Universität München, Germany.

Isolation of [U-^{13}C]α-Linolenic Acid from ^{13}C-Labeled Algae Lipid Extracts

Uniformly labeled [^{13}C]lipid from algae (100 mg) grown in the presence of $^{13}CO_2$ as the sole C source was dissolved in 5 mL of methanol containing 0.005% (w/v) butylated hydroxytoluene and an equal volume of aqueous KOH (15%, w/v). Lipids were hydrolyzed for 2 h at 60°C and the methanol was evaporated in vacuo. The residue was acidified with 1 M citric acid, extracted with hexane/ether (2:1, v/v), and passed through a silica solid-phase extraction column. The eluate was taken to dryness under a stream of N_2, reconstituted in 2 mL of methanol, and passed through a C18 endcapped solid-phase extraction column prior to preparative HPLC on an RP-18 column (250 × 25 mm, 7-μm particle size, LiChrosorb, Merck, Darmstadt, Germany).

Fatty acids were separated using a linear gradient from 70% solvent A (water/acetonitrile/phosphoric acid, 98:2:0.01, v/v) to 100% solvent B (water/acetonitrile/phosphoric acid, 2:98:0.01, v/v) in 60 min at a flow rate of 10 mL/min. Fatty acids were detected at 204 nm and identified by comparison with the retention times of authentic standards. After acidification of the fractions with 1 M citric acid, fatty acids were extracted with hexane/ether (2:1, v/v), taken to dryness under N_2, and stored in acetonitrile under Ar at −20°C until use. From 520 mg of algae lipid, 215.14 μmol of [U-^{13}C]α-linolenic acid was obtained. GC-MS analysis revealed that the labeled fatty acid represented a mixture of isotopomeres with an average of more than 97% ^{13}C atom excess for each of the 18 C atoms. There were no isotopomeres detectable with less than 14 ^{13}C atoms per molecule. The 100%-labeled isotopomere was used for quantification.

Synthesis of ^{13}C-Labeled 12-Oxo-PDA

A previously described enzymatic approach (Zimmerman and Feng, 1978) was used for the synthesis of labeled 12-oxo-PDA and was optimized to increase the yield. Linseed acetone powder (250 mg) was suspended in 50 mL of 100 mM K-P_i buffer, pH 7, and stirred in an ice bath for 30 min. After centrifugation (10 min, 2000*g*), 1 mg of [U-^{13}C]linolenic acid (97% ^{13}C atom excess) in 200 μL of MeOH was added to the supernatant and incubated for 30 min at 30°C. After the addition of 2 mL of 1 M citric acid, the products were extracted twice with ethyl acetate and subjected to semipreparative HPLC on an RP-C18 endcapped column (5 μm, 250 × 10 mm, LiChrospher, Merck). 12-Oxo-PDA was eluted with a linear gradient from 50 to 60% solvent B (solvents as described above) at 4.5 mL/min in 20 min and detected at 222 nm. GC-MS analysis using authentic unlabeled 12-oxo-PDA as an internal standard revealed an overall yield of 120 μg of [U-^{13}C]12-oxo-PDA (97% ^{13}C atom excess), chemical ionization GC-MS (direct inlet, isobutane): *m/z* $[M+H]^+$ 311.

[1-^{14}C]12-Oxo-PDA Synthesis

Radioactive 12-Oxo-PDA was synthesized from [1-^{14}C]α-linolenic acid (1 μCi) using 10 mg of linseed acetone pow-

der in a total volume of 1 mL, as described above. The synthesis yielded 0.18 μCi of [1-^{14}C]12-oxo-PDA with a specific activity of 52 mCi/mmol.

Plant Cell Cultures

Suspension cultures were obtained from the departmental culture collection. Cells were cultivated in Linsmaier-Skoog medium on a gyratory shaker (100 rpm) at 24°C in continuous light for 7 d and harvested under sterile conditions by suction filtration. Then, 160 g (fresh weight) was suspended in 800 mL of fresh medium in 1-L Fernbach flasks, and growth was continued for 3 d. For jasmonate analyses, cells were elicited with 250 μg of yeast elicitor per milliliter of cell suspension and 100 mL of suspension was removed at the times indicated. Before the addition of the elicitor, one-half of the cell suspension was separated and treated in an identical manner except that water was added instead of elicitor.

Sample Preparation and Derivatization for Jasmonate Determination

Aliquots of the suspension cells (100 mL, 0.8 g dry weight) were removed under sterile conditions at desired time points and were rapidly filtered. Cells and medium were separately shock-frozen in liquid N_2. For quantification, 100 ng of 9,10-dihydro-JA and 100 ng of [U-^{13}C]12-oxo-PDA (97% ^{13}C atom excess) were added as internal standards to the frozen cells and, in separate experiments, to the frozen medium. Sample preparation for GC-MS analysis was essentially the same as previously reported (Mueller and Brodschelm, 1994). Jasmonates were extracted from cells or culture fluid with ether using a high-performance disperser and the ether phase was passed through an aminopropyl solid-phase extraction column. The column was washed with chloroform/isopropanol (2:1, v/v), and eluted with ether/acetic acid (98:2, v/v). After derivatization of jasmonates with PFB bromide, the PFB esters were dissolved in hexane, loaded on a silica solid-phase extraction column, and eluted with hexane/ether (9:1, v/v). The sample was taken to dryness under a stream of N_2, reconstituted in 200 μL of methanol, and 1 μL of the solution was analyzed by GC-MS.

GC-MS Quantification

Samples were analyzed on a gas chromatograph (model 3400, Varian, San Fernando, CA) linked to a mass spectrometer (MAT quadrupole SSQ 700, Finnigan, San Jose, CA). The MS source was set at 150°C and the electron energy was set at 70 eV. JA and 12-oxo-PDA were analyzed using an FS-SE-54-CB-0.25 column (25 m × 0.25 mm; CS, Langerwehe, Germany) at a linear He flow of 23 cm/s. A column temperature step gradient of 100°C for 0.5 min, 100–190°C at 30°C/min, 190–225°C at 5°C/min, 225–280°C at 30°C/min, 280–300°C at 5°C/min and 300°C for 10 min was used for jasmonate analysis. The injector was set at 300°C. Negative ion chemical ionization-MS using isobutane showed one intense peak at *m/z* corresponding to the [molecular anion-PFB]$^-$ ions. Retention times of the PFB esters were as follows: (9*E*)-*trans*-JA, 7:56 min; (9Z)-*trans*-JA, 8:21 min; *trans*-9,10-dihydro-JA, 8:24 min; (9Z)-*cis*-JA, 8:43 min; *cis*-9,10-dihydro-JA, 8:46 min; (15Z)-*trans*-12-oxo-PDA, 13:52 min; (15Z)-*cis*-12-oxo-PDA, 14:08 min; and a (15Z)-12-oxo-PDA isomer having the ring double bond between C9 and C13, 14:23 min.

Jasmonate biosynthesis yielded exclusively 9(*S*),13(*S*)-12-oxo-PDA (Laudert et al., 1997) and 3(*R*),7(*S*)-JA (Mueller and Brodschelm, 1994) with the side chains in the *cis* configuration relative to the pentacyclic ring. During sample preparation and in the GC injector, partial isomerization of jasmonates leads to the formation of the *trans* isomers and, in the case of 12-oxo-PDA, to an isomerization of the ring double bond from the original 10,11 position to the 9,13 position. Therefore, the peaks of all isomers were usually integrated together. For routine quantitative analysis, negative fragment ions corresponding to [molecular anion-PFB]$^-$ were measured in the selected ion-monitoring mode at *m/z* = 209 (JA), 211 (9, 10-dihydro-JA), 291 (12-oxo-PDA), and 309 ([U-^{13}C]12-oxo-PDA).

RESULTS

Assay Validation

The method for quantification of 12-oxo-PDA in plant cell cultures and intact plants takes advantage of the recently developed procedure for JA measurement (Mueller and Brodschelm, 1994), which has been modified to allow the simultaneous quantification of both jasmonates. Dihydro-JA has previously been used as internal standard for the GC-MS quantification of JA. Thorough validation of the method revealed that no discrimination of the internal standard dihydro-JA and JA occurred during sample preparation or GC-MS analysis, thus demonstrating its usefulness for JA analysis. The absence of endogenous dihydro-JA in all tissues during elicitation or wounding was checked in separate experiments without the addition of internal standard. Dihydro-JA could not be used as the internal standard for 12-oxo-PDA analysis, since the workup procedure and the GC-MS method discriminate between 12-oxo-PDA and dihydro-JA. Therefore, [U-^{13}C]12-oxo-PDA was synthesized in vitro from [U-^{13}C]α-linolenic acid. Stable isotope-labeled 12-oxo-PDA mimics the chemical and physical properties of 12-oxo-PDA and represents the ideal internal standard for the quantification of 12-oxo-PDA.

Recovery of 12-oxo-PDA during sample preparation was monitored with radioactive [1-^{14}C]12-oxo-PDA (specific activity of 52 mCi/mmol). Cell cultures were spiked with [1-^{14}C]12-oxo-PDA, and 34 ± 4% of the added 12-oxo-PDA could be recovered after the full sample preparation procedure (Table I). For JA a recovery of 62 ± 6% has been documented after a very similar workup procedure (Mueller and Brodschelm, 1994).

Suitability of the method for isolation and determination of 12-oxo-PDA and JA by GC-MS was assessed by analyzing cells from *Tinospora cordifolia* (8 g fresh weight) spiked with 100 ng of the internal standards dihydro-JA and [U-

Table I. *Recovery of [1-^{14}C]12-oxo-PDA during the sample preparation (n = 3)*

Values are means ± SD.

Processing Step	Recovery
	%
Ether extraction	91 ± 1
Aminopropyl solid-phase extraction	76 ± 1
Derivatization to PFB-ester (1 h)[a]	65 ± 4
Silica solid-phase extraction	89 ± 10
Total workup procedure without cells	41 ± 4
Total workup procedure with cells[b]	34 ± 4

[a] Products were analyzed using a radio TLC scanner (hexane/ether, 2:1 [v/v]). [b] Eight grams (0.6 g dry weight) of *T. cordifolia* cells was spiked with 100,000 cpm [1-^{14}C]12-oxo-PDA.

^{13}C]12-oxo-PDA and various concentrations of 12-oxo-PDA and JA ranging from 25 to 1000 ng. Endogenous levels of 12-oxo-PDA and JA were 4 and 10 ng, respectively. A linear relationship between added and measured 12-oxo-PDA and JA demonstrated that no discrimination of the internal standards and the analytes occurred during the sample preparation (Fig. 2) (Mueller and Brodschelm, 1994).

The detection limit after the whole workup procedure was 1 pg for JA and 40 pg for 12-oxo-PDA, which is approximately 10^3 times lower than the endogenous baseline levels typically found in plant cell cultures (0.5–15 and 5–80 ng/g of dry weight for JA and 12-oxo-PDA, respectively).

Induction of 12-Oxo-PDA and JA in Plant Cell Cultures Elicited with Yeast Elicitor

Cell cultures of *Agrostis tenuis* (Poaceae), *Eschscholtzia californica* (Papaveraceae), *Mahonia nervosa* (Berberidaceae), *Phaseolus vulgaris* (Fabaceae), *Rauvolvia serpentina*, *Rauvolvia canescens* (Apocyanaceae), and *Tinospora cordifolia* (Menispermaceae) were elicited with a delipided cell wall preparation from yeast. All cell cultures showed a rapid increase of 12-oxo-PDA and JA, whereas unelicited control cells showed no change in the intracellular concentrations of 12-oxo-PDA and JA (Figs. 3 and 4).

Endogenous levels of 12-oxo-PDA began to increase as early as 7 to 30 min after the onset of elicitation, reaching peak levels at 22 to 75 min. Thereafter, different time courses of endogenous 12-oxo-PDA and JA levels were observed. In the case of *A. tenuis*, a spike pattern was measured, i.e. a transient accumulation of 12-oxo-PDA (680 ng/g of dry weight) 30 min after onset of elicitation was followed by a rapid return to baseline levels (5 ng/g of dry weight). JA induction was not only weaker (220 ng/g of dry weight) but also considerably later, reaching peak levels 90 min after the addition of elicitor. In many species, time curves of jasmonates are biphasic. After an initial peak of 12-oxo-PDA and JA a more or less pronounced, second increase of one or both of these molecules was observed (Figs. 3 and 4). In all cases, the increase of 12-oxo-PDA either preceded the increase of JA or accumulated concurrently, which is consistent with the model of de novo synthesis of JA from α-linolenic acid via 12-oxo-PDA. Moreover, maximal 12-oxo-PDA concentrations in cell cultures of *A. tenuis*, *E. californica* (data not shown), *M. nervosa*,

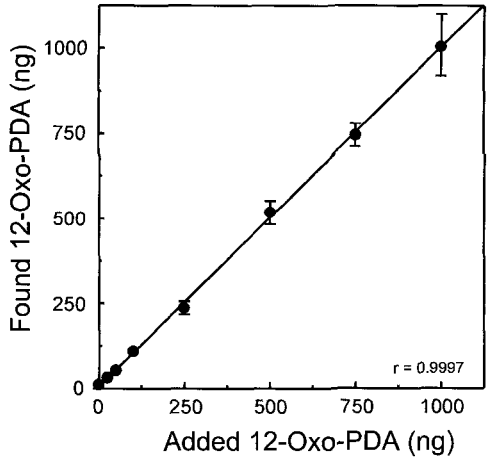

Figure 2. Accuracy and precision of the quantitative analysis of 12-oxo-PDA. 12-Oxo-PDA (25–1000 ng) and [U-^{13}C]12-oxo-PDA (100 ng; internal standard) were added to 8 g (0.6 g dry weight) of suspension culture cells from *T. cordifolia*. Jasmonates were extracted and analyzed as described in "Materials and Methods." Quantities of 12-oxo-PDA are expressed as nanograms of 12-oxo-PDA per gram dry weight. Error bars indicate means ± SD (n = 3).

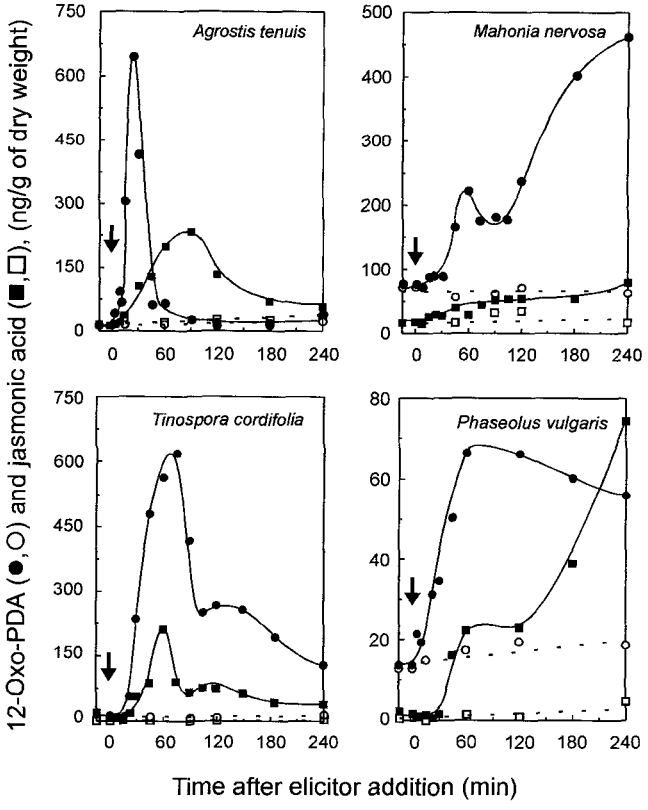

Figure 3. Induction of 12-oxo-PDA (●) and JA (■) by the addition of yeast cell wall elicitor (250 μg/mL) to different cell-suspension cultures. 12-Oxo-PDA (○) and JA (□) levels of untreated controls did not change during the duration of the experiment. Arrows indicate the point of elicitor addition.

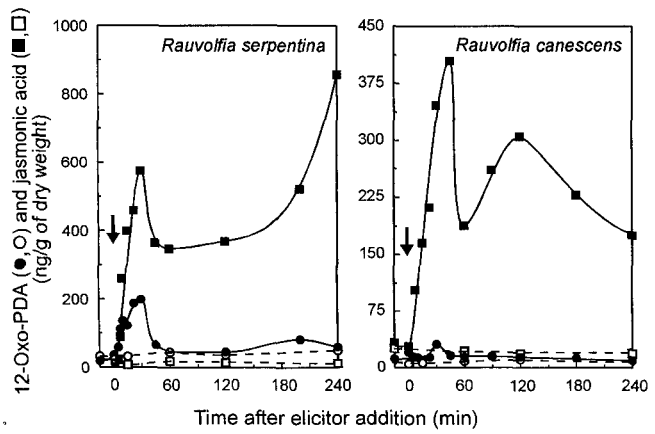

Figure 4. Induction of 12-oxo-PDA (●) and JA (■) by the addition of yeast cell wall elicitor (250 μg/mL) to *Rauvolfia* sp. cell-suspension cultures. See legend to Figure 3.

P. vulgaris, and *T. cordifolia* were at least 3-fold higher than maximal JA induction during the first 120 min (Fig. 3).

Some cell cultures such as *M. nervosa*, *E. californica*, and *P. vulgaris* show a weak accumulation of JA within the first 120 min after the onset of elicitation, which is in the range of 12-oxo-PDA baseline levels. However, these cultures still gave a pronounced 12-oxo-PDA response, reaching peak levels 3- to 5-fold over the maximum JA levels, which suggests that 12-oxo-PDA rather than JA acts as a defense signal.

Repetition of the experiments always gave a similar result with respect to jasmonate baseline levels, the induction pattern of jasmonates (e.g. spike or biphasic pattern), the time of peak maxima, and the relationship between 12-oxo-PDA and JA. However, the levels of accumulated 12-oxo-PDA and JA in response to yeast elicitor may vary within a certain range (up to ± 50%) depending on the condition of the plant tissue (developmental stage and other factors), which is not uncommon for these type of experiments.

Induction of 12-Oxo-PDA and JA in *Rauvolvia* sp.

It has been shown that *R. serpentina* and *R. canescens* respond to elicitation with yeast elicitor with a rapid and strong synthesis of JA similar to the induction pattern shown in Figure 4. In contrast to the cell cultures shown in Figure 3 a transient, much less pronounced increase of 12-oxo-PDA, which mimics the kinetics of JA, was observed (Fig. 4). Peak levels of JA exceeded maximum 12-oxo-PDA levels by a factor of 3- to 13-fold. Thus, in *Rauvolvia* sp. the predominant induced jasmonate species appears to be JA.

Induction of 12-Oxo-PDA and JA in Wounded Tomato Plants

Upper leaves (3–5 cm in length) from 3- to 4-week-old tomato (*Lycopersicon esculentum*, Solanaceae) plants were excised, wounded using a punch with 10 needles per square centimeter, incubated in a chamber with a water-saturated atmosphere, shock-frozen with liquid N_2, minced in a mortar, and worked up as described above. To serve as controls, some leaves were excised from the same plants but were not wounded and were incubated in a different chamber. A rapid increase of 12-oxo-PDA reaching a maximum value of 2020 ng/g dry weight between 30 and 50 min after wounding was observed, followed by an accumulation of JA to peak levels of 3540 ng/g dry weight 60 min after wounding (Fig. 5). Peak levels of 12-oxo-PDA and JA exceeded those of unwounded leaves by 11- and 177-fold, respectively.

Intracellular Levels of Jasmonates Other Than JA and 12-Oxo-PDA

Since the sample preparation does not discriminate between 12-oxo-PDA and JA, it is in principle possible to identify and quantify the intermediates in the pathway such as 9,10-dihydro-12-oxo-PDA and the carboxyl side-chain shortened derivatives. However, none of the metabolites in the biosynthetic pathway between 12-oxo-PDA and JA accumulated to exceed the limit of detection in elicited cell cultures or plants.

Release of 12-Oxo-PDA and JA into the Cell Culture Medium

Previously, it has been shown that exogenous JA and its methyl ester are readily taken up by plant cells, leading to gene activation (Farmer and Ryan, 1990; Gundlach et al., 1992). Consequently, the question arises of whether jasmonates can be released from the producer cells. Using the method described above, measurement of unconjugated jasmonates released from cultured suspension cells into the

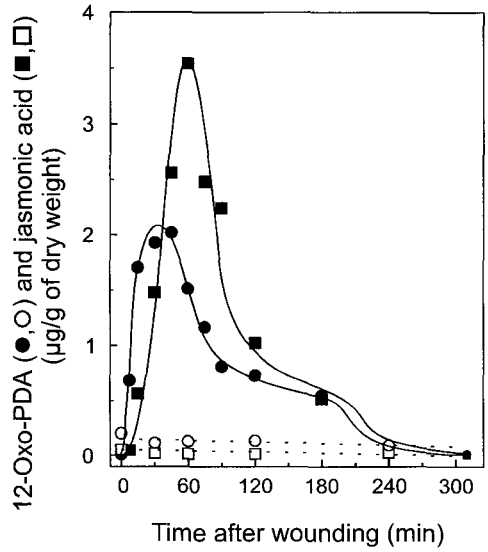

Figure 5. Time course of 12-oxo-PDA (circles) and JA (squares) accumulation in wounded (●, ■) and unwounded (○, □) tomato leaves. Leaves were excised, wounded, and incubated as described in "Results" for the times indicated. Leaves serving as controls were excised at time zero but were not wounded.

extracellular medium is straightforward. Accumulation of JA in the cell culture medium was observed in three out of six cell cultures from different plant families, whereas 12-oxo-PDA was always found to be exclusively cell associated. JA was secreted in substantial amounts from *A. tenuis*, *R. serpentina*, and *P. vulgaris* cells (Fig. 6). In *A. tenuis* intra- and extracellular JA concentrations reached a maximum at about 90 min and both concentrations decreased thereafter, suggesting that a rapid uptake and metabolism of JA had occurred. In *P. vulgaris* and *R. serpentina* intracellular JA levels remained at a high concentration and extracellular JA accumulated in the medium. Despite the possible reuptake of JA, excreted JA exceeded the total intracellular JA in *R. serpentina* by at least a factor of four following induction of JA over the entire time course.

Not all plant cell cultures that reacted with an intracellular JA accumulation were able to secrete JA. For example, cell cultures of *T. cordifolia*, *M. nervosa*, and *E. californica* did not accumulate any JA in the culture medium (Fig. 6; data not shown for *E. californica* and *M. nervosa*).

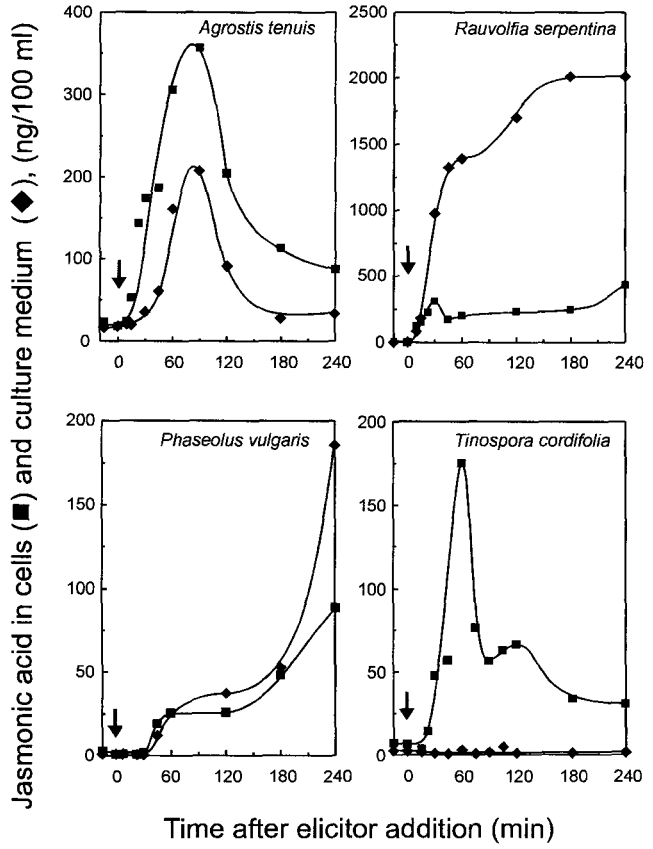

Figure 6. Time course of cell-associated JA (■) and JA in the culture medium (◆) after the addition of yeast cell wall elicitor (250 μg/mL). Aliquots of 100 mL were taken at the times indicated, followed by separation of cells and medium by suction filtration and jasmonate analysis. 12-Oxo-PDA could not be detected in the culture medium of any of the plant species tested. Arrows indicate the point of elicitor addition.

DISCUSSION

Recently, it has been well established that cultured plant cells and intact plants respond toward elicitors, wounding, and mechanical stimulation with a dramatic increase in production of endogenous 3(*R*),7(*S*)-JA (Creelman et al., 1992; Gundlach et al., 1992; Mueller et al., 1993; Weiler et al., 1993; Laudert et al., 1996). This has been regarded as an essential event in the signal transduction cascade, eventually leading to gene activation and secondary metabolite formation. However, induction of JA is not always very pronounced (Fig. 3; Mueller et al., 1993). Since 12-oxo-PDA appears to be a stronger signal in the induction of secondary metabolites and in the *Bryonia dioica* tendril coiling assay, it has been suggested that 12-oxo-PDA is the primary gene-activating signal (Weiler et al., 1993; Blechert et al., 1995). This hypothesis has been strengthened by the finding that analogs of 12-oxo-PDA, such as methyl trihomojasmonate, that cannot undergo β-oxidation to form JA are as active as JA in these bioassays (Blechert et al., 1995). Furthermore, coronatine, a metabolite of the phytopathogen *Pseudomonas syringae* pv. *atropurpurea*, a structural analog of 12-oxo-PDA, was highly active in the two bioassays mentioned above (Weiler et al., 1994). The secondary metabolite inducing activity of 12-oxo-PDA was recently compared with JA in 165 different cultured plant species. Applied at the same concentration as JA, 12-oxo-PDA almost always caused a greater increase in secondary metabolite formation in those cell cultures that responded to jasmonates (84% of all cultures) (H. Gundlach, and M.H. Zenk, unpublished results). Thus, evidence is accumulating that 12-oxo-PDA in itself is a gene-activating compound that does not need to be metabolized to JA to become biologically active.

The oxidative metabolism of fatty acids in mammalians has been well studied and has been often used as a guide in plant lipid research. In animals, bioactive prostaglandins and leukotrienes are generated from unsaturated fatty acids via hydroperoxides, which are subsequently inactivated by reactions such as β-oxidation, reduction of a double bond, reduction of a keto group, or conjugation. By analogy, from a biosynthetic point of view 12-oxo-PDA would be the equivalent of the mammalian prostaglandins in the plant jasmonate pathway (Mueller, 1997). In plants, however, reduction and β-oxidation of 12-oxo-PDA yields the bioactive molecule JA.

The time course of both mediators in elicited cells of different species suggests that the relative intracellular concentration of both signals and thus their expected contribution to defense gene activation differs between plant species. In five out of seven plant families analyzed, 12-oxo-PDA was the first jasmonate that was induced and represented the predominant intracellular pentacyclic acid exceeding the maximum levels of JA by at least a factor of three. In contrast, in the two *Rauvolvia* sp. analyzed, JA appeared to be the predominant jasmonate species that accumulated in response to the yeast elicitor. In a differentiated plant (*L. esculentum*), which was chosen to demonstrate that accumulation of 12-oxo-PDA is not restricted to elicited plant cell cultures, 12-oxo-PDA and JA were both

shown to be strongly induced upon wounding. None of the pentacyclic metabolites between 12-oxo-PDA and JA accumulated in the plant tissues over the limit of detection.

12-Oxo-PDA synthesis is localized in plastids, whereas the reduction of 12-oxo-PDA to 10,11-dihydro-12-oxo-PDA takes place in the cytosol (Schaller and Weiler, 1997). 10,11-Dihydro-12-oxo-PDA is readily subjected to β-oxidation, whereas 12-oxo-PDA is not. Thus, the export of 12-oxo-PDA from plastids and/or the subsequent cytosolic reduction appears to be a bottleneck in the JA pathway resulting in a transient accumulation of 12-oxo-PDA. Apparently, β-oxidation of 10,11-dihydro-12-oxo-PDA rapidly takes place in the peroxisome (or mitochondria) and is initiated by activation of the carboxylic acid function. The following three rounds of β-oxidation to JA probably proceed without liberation of the side-chain-shortened intermediates by a thioesterase. Therefore, β-oxidation intermediates may not accumulate as free acids under physiological conditions. However, it is possible to detect these intermediates by GC-MS after saturating the pathway with exogenous 12-oxo-PDA (Vick and Zimmerman, 1984). Thus, the differential induction of 12-oxo-PDA and JA in different plant species may indicate a certain flexibility in the rates of biosynthesis, intracellular transport, and metabolism in various plants.

In addition, secretion of jasmonates into the extracellular space may be another regulatory mechanism determining intracellular concentrations of the JA signal. This may even represent a possible intercellular signal transduction pathway similar to that of the well-known mammalian lipid signals.

We have shown that JA but not 12-oxo-PDA can be released in substantial amounts from cultured plant cells. Release of JA does not appear to be a general feature of elicited plant cells, since only three out of six species tested excreted JA. However, when JA release was observed, the excreted JA may be sufficient to induce gene activation in unelicited cells. It should be pointed out that because of the avid uptake of JA by plant cells the observed concentrations of JA in the culture medium most likely represent an underestimation of the actual release. Therefore, in vivo, release of JA may be even greater than these experiments indicate.

In an intact plant those cells having the first contact with a pathogen and thus being elicited first may be killed before they have the chance to implement their full defense program. Therefore, JA can be actively transferred to neighboring cells, which can synthesize all of the defensive proteins and antibiotic secondary metabolites required to fight off the infection.

The results presented here lend support to the concept that 12-oxo-PDA and JA are involved in mediating the plant defense response. While focusing on JA alone (i.e. neglecting 12-oxo-PDA), the jasmonate signal might be substantially underestimated. 12-Oxo-PDA levels increase earlier and in most instances to higher levels than JA. Considering the greater gene-activating potency of 12-oxo-PDA relative to JA, these findings suggest that endogenous 12-oxo-PDA concentrations may be more relevant for the induction of a defense response than JA.

For example, cell cultures of *M. nervosa*, *P. vulgaris*, and *E. californica* commonly display a rather weak and late increase of JA to yeast elicitor, which fails to significantly exceed the baseline levels of 12-oxo-PDA within the first 120 min (Fig. 3). However, 12-oxo-PDA kinetics show a rapid and distinct response very early in the time course. Since the induction of mRNA could be detected as early as 120 min after the onset of elicitation (Gundlach et al., 1992), it is plausible that 12-oxo-PDA rather than JA represents, at least in the cultures shown in Figure 3, the signal that activates directly or indirectly the expression of defensive genes.

ACKNOWLEDGMENTS

We express our sincere gratitude to Prof. A. Bacher (SFB 369), Technische Universität, München, Germany, for his generous supply of ^{13}C-labeled algae lipid extract. We also thank P. Bauer for excellent technical assistance and S. O'Sullivan for linguistic help in the preparation of the manuscript.

Received May 16, 1997; accepted July 31, 1997.

LITERATURE CITED

Bergey DR, Howe GA, Ryan CA (1996) Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. Proc Natl Acad Sci USA **93:** 12053–12058

Blechert S, Brodschelm W, Hölder S, Kammerer L, Kutchan TM, Xia Z-Q, Zenk MH (1995) The octadecanoic pathway: signal molecules for the regulation of secondary pathways. Proc Natl Acad Sci USA **92:** 4099–4105

Creelman RA, Tierney ML, Mullet JE (1992) Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc Natl Acad Sci USA **89:** 4939–4941

Dittrich H, Kutchan TM, Zenk MH (1992) The jasmonate precursor, 12-oxo-phytodienoic acid, induces phytoalexin synthesis in *Petroselinum crispum* cell cultures. FEBS **309:** 33–36

Farmer EE (1994) Fatty acid signalling in plants and their associated microorganisms. Plant Mol Biol **26:** 1423–1437

Farmer EE, Ryan CA (1990) Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc Natl Acad Sci USA **87:** 7713–7716

Farmer EE, Ryan CA (1992) Octadecanoid-derived signals in plants. Trends Cell Biol **2:** 236–241

Gundlach H, Müller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc Natl Acad Sci USA **89:** 2389–2393

Hamberg M, Gardner HW (1992) Oxylipin pathway to jasmonates: biochemistry and biological significance. Biochim Biophys Acta **1165:** 1–18

Herrmann G, Lehmann J, Peterson A, Sembdner G, Weidhase RA, Parthier B (1989) Species and tissue specificity of jasmonate-induced abundant proteins. J Plant Physiol **134:** 703–709

Laudert D, Henning P, Stelmach BA, Müller A, Andert L, Weiler EW (1997) Analysis of 12-oxo-phytodienoic acid enantiomers in biological samples by capillary gas chromatography-mass spectrometry using cyclodextrin stationary phases. Anal Biochem **24:** 211–217

Laudert D, Pfannschmidt U, Lottspeich F, Holländer-Czytko H, Weiler EW (1996) Cloning, molecular and functional characterisation of *Arabidopsis thaliana* allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. Plant Mol Biol **31:** 323–335

Mueller MJ (1997) Enzymes involved in jasmonic acid biosynthesis. Physiol Plant **100:** 653–663

Mueller MJ, Brodschelm W (1994) Quantification of jasmonic acid by capillary gas chromatography-negative chemical ionization-mass spectrometry. Anal Biochem **218:** 425–435

Mueller MJ, Brodschelm W, Spannagl E, Zenk MH (1993) Signaling in the elicitation process is mediated through the octadecanoid pathway leading to jasmonic acid. Proc Natl Acad Sci USA **90:** 7490–7494

Schaller F, Weiler EW (1997) Enzymes of octadecanoid biosynthesis in plants: 12-oxo-phytodienoate 10,11-reductase. Eur J Biochem **245:** 294–299

Sembdner G, Parthier B (1993) The biochemistry and the physiological and molecular actions of jasmonates. Annu Rev Plant Mol Biol **44:** 569–89

Vick BA, Zimmerman DC (1983) The biosynthesis of jasmonic acid: a physiological role for plant lipoxygenase. Biochem Biophys Res Commun **111:** 470–477

Vick BA, Zimmerman DC (1984) Biosynthesis of jasmonic acid by several plant species. Plant Physiol **75:** 458–461

Weiler EW, Albrecht T, Groth B, Xia Z-Q, Luxem M, Liβ H, Andert L, Spengler P (1993) Evidence for the involvement of jasmonates and their octadecanoid precursors in the tendril coiling response of *Bryonia dioica*. Phytochemistry **32:** 591–600

Weiler EW, Kutchan TM, Gorba T, Brodschelm W, Niesel U, Bublitz F (1994) The *Pseudomonas* phytotoxin coronatine mimics octadecanoid signalling molecules of higher plants. FEBS Lett **345:** 9–13

Zimmerman DC, Feng P (1978) Characterization of a prostaglandin-like metabolite of linolenic acid produced by a flaxseed extract. Lipids **13:** 313–316