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The Role of Octadecanoids and Functional Mimics in Soybean Defense Responses

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Oxylipins of the jasmonate pathway and synthetic functional analogs have been analyzed for their elicitor-like activities in an assay based on the induced accumulation of glyceollins, the phytoalexins of soybean (*Glycine max* L.), in cell suspension cultures of this plant. Jasmonic acid (JA) and its methyl ester showed weak phytoalexin-inducing activity when compared to an early jasmonate biosynthetic precursor, 12-oxo-phytodienoic acid (OPDA), as well as to the bacterial phytotoxin coronatine and certain 6-substituted indanoyl-L-isoleucine methyl esters, which all were highly active. Interestingly, different octadecanoids and indanoyl conjugates induced the accumulation of transcripts of various defense-related genes to different degrees, indicating distinct induction competencies. Therefore, these signaling compounds and mimics were further analyzed for their effects on signal transduction elements, such as the transient enhancement of the cytosolic Ca^{2+} concentration and MAP kinase activation, which are known to be initiated by a soybean pathogen-derived β-glucan elicitor. In contrast to the β-glucan elicitor, none of the other compounds tested triggered these early signaling elements. Moreover, endogenous levels of OPDA and JA in soybean cells were shown to be unaffected after treatment with β-glucans. Thus, OPDA and JA, which are functionally mimicked by coronatine and a variety of 6-substituted derivatives of indanoyl-L-isoleucine methyl ester, represent highly efficient signaling compounds of a lipid-based pathway not deployed in the β-glucan elicitor-initiated signal transduction.

Key words: Ethyl-indanoyl-L-isoleucine methyl ester / Glyceollins / *Glycine max* L. / Jasmonate / Plant defense / Signal transduction.

Introduction

Octadecanoid-derived compounds, *i.e.* jasmonates and their biosynthetic C_{18} precursors, are ubiquitously occurring linolenic acid-derived oxylipins and represent plant compounds that may function as regulators in various physiological processes (Creelman and Mullet, 1997; Weiler, 1997). Among other processes, a role of jasmonates or certain octadecanoids in the induction of wound- and defense-related cellular reactions such as the elicitation of proteinase inhibitors, volatile compounds, secondary metabolites, and defense genes has been reported (Creelman and Mullet, 1997; Weiler, 1997). Initially, octadecanoids have been described as wound-inducible compounds elicited by herbivorous attack and proposed to act as cellular messengers during defense responses in plants (Farmer and Ryan, 1990, 1992).

In addition to intact plants, cell suspension cultures of various species were used to investigate the role of these putative signal compounds in the elicitation process. Using a yeast-derived cell wall preparation, the transient accumulation of jasmonic acid (JA) and methyl jasmonate (JAMe) has been reported concomitantly with the production of benzo[c]phenanthridine alkaloids in cell cultures of *Eschscholtzia californica* (Gundlach *et al.,* 1992). The postulated role as a signal transducer in elicitor-induced defense reactions of plant cell cultures (Gundlach *et al.,* 1992; Müller *et al.,* 1993) was corroborated by similar results obtained with rice (*Oryza sativa* L.) cell suspension cultures elicited with *N*-acetylchitoheptaose (Nojiri *et al.,* 1996) and tobacco (*Nicotiana tabacum* L.) cell cultures challenged with a species-specific elicitor from the tobacco pathogen *Phytophthora parasitica* var. *nicotianae* (Rickauer *et al.,* 1997). Interestingly, in this latter study the authors pointed out that the elicitor and JAMe, respectively, are differentially involved in the onset of various defense responses. In a first survey, a total of 36 species including soybean (*Glycine max* L.), were shown to respond to the exogenous application of jasmonates with the accumulation of secondary metabolites (Gundlach *et al.,* 1992). Other groups extended these results using additional plant cell cultures, *e.g.* parsley (*Petroselinum crispum*; Dittrich *et al.,* 1992), *Taxus cuspidata* (Mirjalili and Linden, 1996), rice (Norjiri *et al.,* 1996) or *Lithospermum erythrorhizon* (Mühlenweg *et al.,* 1998). Remarkably, the treatment with JA did not result for all species in the production of phytoalexins, inducible low molecular weight compounds with antimicrobial properties correlating with plant defense. In the case of soybean, elevated levels of genistein, a constitutively occurring isoflavone, were detected whereas the production of the pterocarpan-type phytoalexin glyceollin was not reported (Gundlach *et al.,* 1992). Therefore, a putative generalized role of octadecanoids as signaling compounds in the induction of plant defense besides wounding-induced processes has yet to be demonstrated.

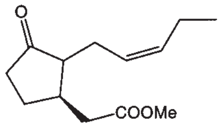
Soybean cell suspension cultures are known to respond to elicitation with carbohydrate elicitors derived from *Phytophthora sojae* cell walls in a manner comparable to soybean plants challenged with this phytopathogenic oomycete. The elicitors, (1,3)-(1,6)-β-glucooligosaccharides, initiate signaling events involving ion currents on the plasma membrane, alteration of the cytosolic Ca^{2+} concentration, activation of protein kinases, and the production of reactive oxygen species, as well as gene activation (Ebel and Mithöfer, 1998). In soybean, these cellular reactions finally lead to the accumulation of glyceollins, representing one important part of a set of inducible defense reactions (Ebel, 1998).

Extensive studies on structure-activity relationships of compounds active in plant mechanotransduction involved in tendril coiling in *Bryonia dioica* Jacq. (Blechert *et al.,* 1999), in the induction of volatile biosynthesis in lima bean (*Phaseolus lunatus*) (Koch *et al.,* 1999), and in transcript profiles in *Arabidopsis thaliana* (Stintzi *et al.,* 2001), respectively, suggested that JA and its biosynthetic precursor 12-oxo-phytodienoic acid (OPDA) very likely represent compounds differentially involved in signaling. In order to cover both possible signaling pathways, in the present study we comparatively investigated the impact of the naturally occurring JA, JAMe, OPDA, and coronatine, and of synthetic functional mimics of these compounds on inducible defense reactions in soybean cell cultures. The obvious experimental advantage of using suspension cultures is that wound-induced endogenous jasmonate production can be avoided. Our results demonstrate that the various signaling compounds tested largely differ in their efficiencies to induce phytoalexin production and, moreover, that octadecanoids are not required in the signaling cascade responsible for glyceollin accumulation induced by the *P. sojae* β-glucan elicitor.

Results

Induction of Glyceollins in Soybean Cell Cultures Treated with Jasmonates, 12-oxo-Phytodienoic Acid, and Mimics

Based on the different pattern of biological responses induced, the jasmonates, JA and JAMe, and their precursor OPDA were classified into different groups of signaling intermediates (Blechert *et al.,* 1999; Koch *et al.,* 1999). The phytotoxin coronatine, produced by several pathovars of *Pseudomonas syringae*, induced reactions similar to OPDA (Blechert *et al.,* 1999; Koch *et al.,* 1999). Therefore, coronatine is suggested to represent a structural analogue of OPDA (Weiler *et al.,* 1994). Deduced from the structure of coronatine, new synthetic analogs of OPDA have been designed which were expected to mimic OPDA and/or jasmonate activities. These analogs in-



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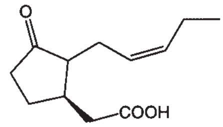

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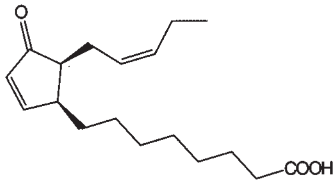

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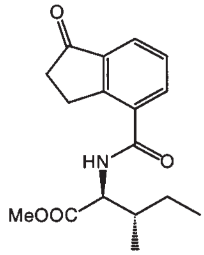

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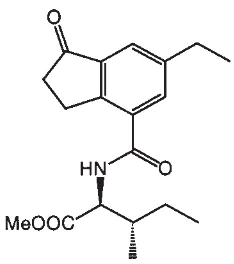

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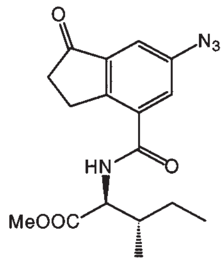

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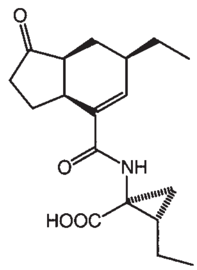

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Fig. 1 Structures of Octadecanoids and Functional Mimics Used in This Study.
(1) (3-Oxo-2-pent-2-enyl-cyclopentyl)-acetic acid methyl ester, jasmonic acid methyl ester (JAMe); (2) (3-oxo-2-pent-2-enyl-cyclopentyl)-acetic acid, jasmonic acid (JA); (3) 8-(4-oxo-5-pent-2-enyl-cyclopent-2-enyl)-octanoic acid, 12-oxo-phytodienoic acid (OPDA); (4) 3-methyl-2-[(1-oxo-indane-4-carbonyl)-amino]-pentanoic acid methyl ester, indanoyl-L-isoleucine methyl ester (Ind-IleMe); (5) 2-[(6-ethyl-1-oxo-indane-4-carbonyl)-amino]-3-methyl-pentanoic acid methyl ester, (IndEt-IleMe); (6) 2-[(6-azido-1-oxo-indane-4-carbonyl)-amino]-3-methyl-pentanoic acid methyl ester, (IndAz-IleMe); (7) 2-ethyl-1-[(6-ethyl-1-oxo-2,3,5,6,7,7a-hexahydro-1H-indene-4-carbonyl)-amino]-cyclopropanecarboxylic acid, coronatine.

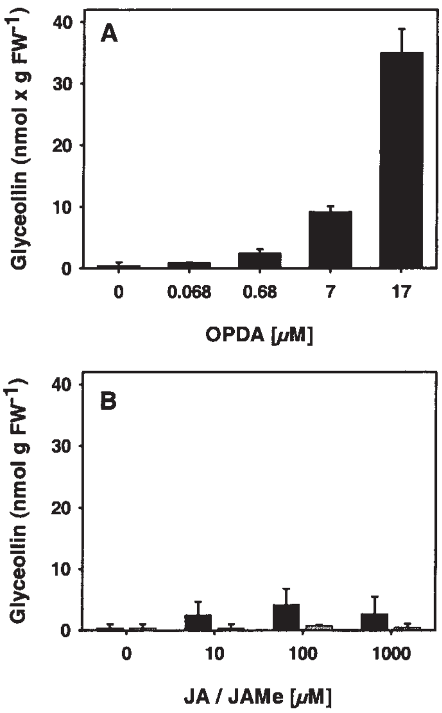

Fig. 2 Induction of Phytoalexin Accumulation by Octadecanoids.
Accumulation of glyceollins in soybean cell cultures upon treatment with increasing concentrations of the naturally occurring plant compounds OPDA (A), JA and JAMe (B); left bars: JA, right bars: JAMe. The culture medium was extracted after 48 h of incubation with ethyl acetate, the extract concentrated, and analyzed by HPLC for the glyceollin contents as described in the Materials and Methods section. The results are the mean ± SD (n = 3 – 10).

clude the 1-oxo-indanoyl isoleucine methyl ester (Ind-IleMe) conjugate (Krumm *et al.,* 1995) as well as the newly developed 6-ethyl-1-oxo-indanoyl isoleucine methyl ester (IndEt-IleMe), and 6-azido-1-oxo-indanoyl isoleucine methyl ester (IndAz-IleMe) derivatives (Schüler *et al.,* 1999). The structures of all compounds used in this study are given in Figure 1.

The accumulation of pterocarpane-type phytoalexins, the glyceollins, was monitored in soybean cell cultures after treatment with octadecanoids of plant origin. OPDA was much more active than JA or JAMe in eliciting glyceollin production, as illustrated by dose-response analyses (Figure 2A, B). An OPDA concentration of about 10 μM clearly resulted in a many fold higher level of glyceollin accumulation in comparison to those obtained with JA or JAMe (Figure 2B). The latter compound was completely inactive up to a concentration of 1 mM.

Similar to OPDA, the bacterial phytotoxin coronatine and the synthetic 1-oxo-indanoyl-isoleucine derivatives IndEt-IleMe and IndAz-IleMe also proved to be potent elicitors of phytoalexin production in soybean cells (Figure 3A – C), again yielding much higher glyceollin concentrations than the genuine lipid-derived jasmonates. Compared to IndEt-IleMe and IndAz-IleMe, the 6-non-substituted Ind-IleMe showed very little activity (Figure 3D), resembling the activity of JA. Obviously, in the experimental system used here compounds structurally related to OPDA and coronatine represent biologically highly active substances whereas the jasmonates and Ind-IleMe showed weak activity.

Regulation of Defense-Related Gene Expression

Steady-state transcript levels of a range of defense-related genes were analyzed after treatment with various octadecanoids. Soybean cell cultures were treated for 4 h and 9 h, respectively, with 10 μM of the most active compounds OPDA, IndAz-IleMe, IndEt-IleMe, coronatine, and, for comparison, with jasmonate. β-Glucan elicitor (200 μg ml^{-1}) served as an internal positive control demonstrating a transcript pattern resembling cells actively engaged in the phytoalexin defense response (Figure 4). Genes encoding early and late enzymes in the biosynthetic pathway of glyceollin were induced by β-glucan elicitor in a time-dependent manner as reported earlier (Schopfer *et al.,* 1998; Mithöfer *et al.,* 2001). The mRNAs for chalcone synthase (*CHS*) and chalcone reductase (*CHR*), the first committed step for 5-deoxy flavonoid and/or isoflavonoid synthesis, were strongly but transiently induced at 4 h after β-glucan elicitation. In contrast, the expression of the gene encoding dihydroxypterocarpan 6a-hydroxylase (*D6aH*), which is specific for the final branch of the glyceollin biosynthetic pathway, was up-regulated by β-glucan elicitor not until 9 h, a time point where the transcript levels for *CHS* and *CHR* had nearly decreased to the initial state.

Remarkably, the jasmonate-precursor OPDA, which was distinguished from other compounds by the highest biological activity concerning the induction of glyceollins, showed a much lower capacity to induce glyceollin-biosynthetic genes, when compared to β-glucan elicitor. In contrast to β-glucan elicitation, the transcripts for *CHS* and *CHR* increased only moderately during the first 4 h of induction. Transcripts for *D6aH* were detectable at 9 h, albeit at a lower level in comparison to β-glucan elicitation. Among the compounds mimicking the activity of OPDA, two groups of substances could be distinguished based on the timing of defense gene expression: after 4 h of elicitation, the transcript profiles induced by coronatine and IndEt-IleMe resembled that of the β-glucan elicitor, whereas at 9 h, JA and IndAz-IleMe mimicked the signaling activity of the β-glucan elicitor. Obviously, at different time points, the distinct octadecanoids displayed different impacts on gene regulation.

Furthermore, some of the compounds had broader, others more restricted effects on the expression of the group of genes selected for this study. For example, the influence of the octadecanoids on the expression of genes encoding enzymes serving a range of functions was tested. Flavonoid 6-hydroxylase (F6H), an enzyme catalyzing a side branch of flavonoid biosynthesis leading to trihydroxyflavanones (Latunde-Dada *et al.,* 2001),

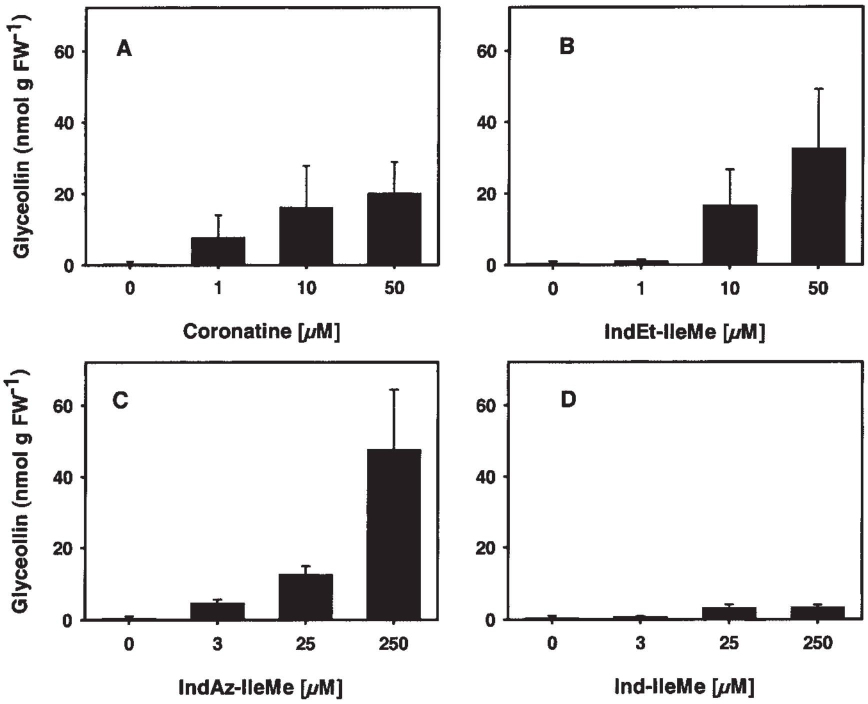

Fig. 3 Induction of Phytoalexin Accumulation by Octadecanoid Mimics.
Accumulation of glyceollins in soybean cell cultures upon treatment with increasing concentrations of the phytotoxin coronatine (A) and the synthetic compounds IndEt-IleMe (B), IndAz-IleMe (C), and Ind-IleMe (D). The culture medium was extracted after 48 h of incubation with ethyl acetate, the extract concentrated, and analyzed by HPLC for glyceollin content as described in Materials and Methods. The results are the mean ± SD (n = 3 – 10).

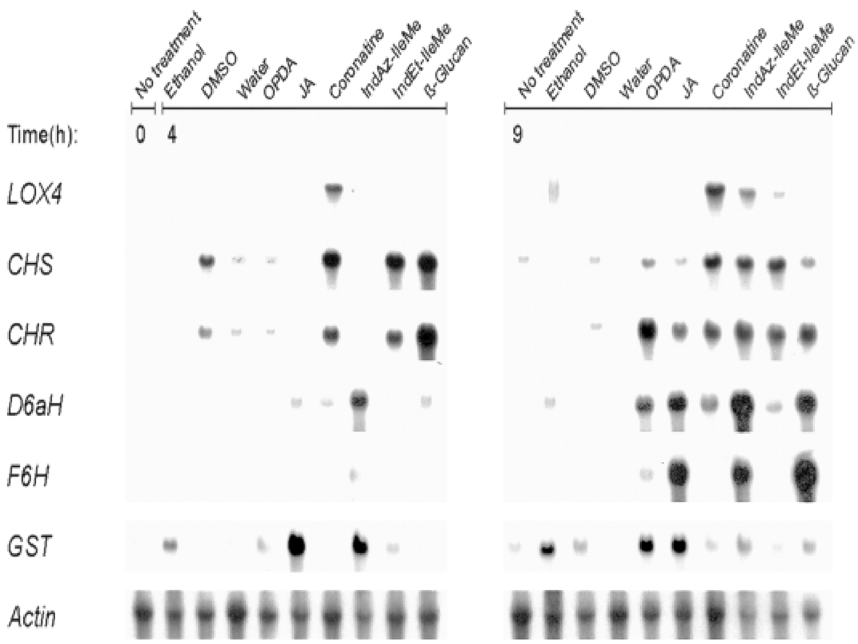

Fig. 4 Northern Blot Analyses of Defense-Related Gene Expression.
Effect of JA, OPDA, coronatine, IndAz-IleMe, IndEt-IleMe (10 μM each), and β-glucan elicitor (200 μg ml^{-1}) on the transcript levels for defense-related genes in soybean cell cultures treated for the indicated times (4, 9 h). Equal loading (15 μg RNA per lane) of the blots was confirmed by control hybridization with an actin probe. Control treatments were performed using the same amounts of the solvents (80 μl each of DMSO and ethanol, 160 μl water) as used for the application of the effectors. *CHS* = chalcone synthase; *CHR* = reductase co-acting with *CHS*; *D6aH* = 3,9-dihydroxypterocarpan 6a-hydroxylase; *F6H* = flavonoid 6-hydroxylase; *GST* = glutathione *S*-transferase, *LOX4* = lipoxygenase *Lox1:Gm:4*.

was investigated in comparison to D6aH, an enzyme dedicated to glyceollin biosynthesis. The gene encoding F6H was induced by β-glucan elicitor, JA, and IndAz-IleMe only, indicating a more restricted pattern of expression (Figure 4). Two additional gene families were tested, one coding for general stress-related enzymes, glutathione *S*-transferases (GST), and the other coding for lipoxygenases (LOX4). Interestingly, the expression of both gene families was not affected by treatment of the cell cultures with the β-glucan elicitor (Figure 4). In contrast, JA was a very potent inducer of a prolonged *GST* gene expression. Treatment with OPDA and IndAz-IleMe for 4 and 9 h, respectively, resulted in the elevation of *GST* mRNA levels as well. The effect on *LOX4* transcript levels was more selective: coronatine was the only compound which was able to elicit the accumulation of this mRNA after 4 and 9 h of treatment, while IndAz-IleMe and, to a lesser extent, IndEt-IleMe, provoked an increase of *LOX4* transcripts only at 9 h. In summary, the results presented above demonstrated differential regulation capacities of the β-glucan elicitor in comparison to genuine and artificial lipid-derived octadecanoids and within this latter group of compounds.

Activation of Early Signaling Elements

Early events take place as part of the signal transduction upon elicitation preceding defense activation, as outlined earlier (Ebel and Mithöfer, 1998). For instance, a transient increase of the cytosolic Ca^{2+} concentration has been proven to be a necessary signaling element in the β-glucan elicitor-dependent stimulation of phytoalexin biosynthesis in soybean (Mithöfer *et al.,* 1999). Using the transgenic, aequorin-expressing soybean cell line 6.6.12 (Mithöfer *et al.,* 1999), we tested the effects of JA, JAMe, OPDA, coronatine and the synthetic mimics IndEt-IleMe and IndAz-IleMe, respectively, on the cytosolic Ca^{2+} concentration at 10 μM each. None of these compounds was capable to alter the cytosolic Ca^{2+} level. In a positive control, the β-glucan elicitor caused an elevation of the cytosolic Ca^{2+} concentration, demonstrating the responsiveness of the cell culture as reported earlier (Mithöfer *et al.,* 1999) (data not shown).

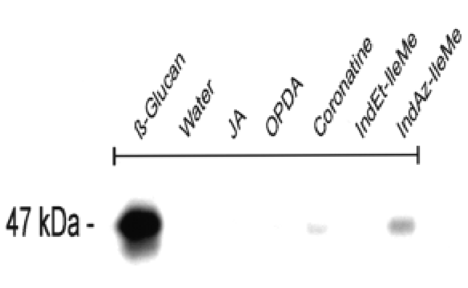

Fig. 5 In-Gel Kinase Assay of MAP Kinase Activity.
Activation of MAP kinases in soybean cell cultures treated with JA, OPDA, coronatine, IndEt-IleMe, IndAz-IleMe (10 μM each), and β-glucan elicitor (200 μg ml^{-1}). After 20 min, the incubation was stopped and MAP kinase activity was assayed in an in-gel kinase assay using myelin basic protein and [γ-^{32}P]-ATP as substrates. For details see the Materials and Methods section.

Protein phosphorylation cascades, such as the mitogen-activated protein (MAP) kinase cascade, are also thought to be elements of elicitor signal transduction being located downstream of the Ca^{2+} transients in the β-glucan signaling pathway (Ebel and Mithöfer, 1998). In soybean suspension cells, a β-glucan elicitor-dependent activation of a MAP kinase was demonstrated which correlated with the inducible biosynthesis of phytoalexins (A. Daxberger, unpublished). As shown in Figure 5, a strong increase of the activity of this 47 kDa-MAP kinase was elicited by β-glucan treatment but not by JA, OPDA, coronatine, or the synthetic mimics.

Quantification of Endogenous JA, OPDA, and SA Levels in Soybean Cell Cultures

Having shown that OPDA and several related compounds are very active inducers of glyceollin synthesis in soybean cell suspension cultures, we examined whether JA and/or OPDA production was a prerequisite for the β-glucan-induced phytoalexin accumulation. In two independent experiments, cell cultures were treated with 200 μg ml^{-1} β-glucan each and JA and OPDA concentrations were determined at different times after elicitation (0, 10, 20, 25, 45 min, 3 h, and 6 h). Neither the JA nor the OPDA contents showed a significant increase or decrease compared to non-treated controls. Irrespective of the treatment, the levels of these compounds were rather low representing 15 to 50 ng g^{-1} fw for JA, and 1 to 3 μg g^{-1} fw for OPDA. To ensure the responsiveness of the cells, we compared in a third experiment JA, OPDA as well as glyceollin levels in β-glucan-elicited soybean cell cultures after 90 min and 6 h of treatment (JA, OPDA) and after 48 h of treatment (glyceollin). In the water-treated control cells, we detected 48.8 ng JA g^{-1} fw and 1.3 μg OPDA g^{-1} fw after 90 min, and 17.2 ng JA g^{-1} fw and 3.0 μg OPDA g^{-1} fw after 6 h, respectively. Cell cultures treated with β-glucan elicitor contained 33.7 ng JA g^{-1} fw and 3.5 μg OPDA g^{-1} fw after 90 min. After 6 h of elicitation, 15.3 ng JA g^{-1} fw and 3.2 μg OPDA g^{-1} fw were found. The amount of glyceollins was determined to be 0.1 nmol g^{-1} fw in the control, while in the β-glucan-treated cells a glyceollin production, resulting in 12.1 nmol g^{-1} fw, was detected. A similar result was obtained by using a crude yeast-derived cell wall elicitor fraction (Gundlach *et al.,* 1992) at 10 mg ml^{-1}: no increase of JA or OPDA but an increase of the glyceollin level (11.2 nmol g^{-1} fw) was detected. Because plant defense might not be regulated by the JA pathway only but through a crosstalk of signaling cascades including salicylic acid (SA) (Engelberth *et al.*, 2001), we also analyzed the endogenous concentration of this signaling compound, in parallel to JA and OPDA. No significant increase of the SA concentration above the resting level of 30 to 80 ng SA g^{-1} fw could be detected, independent of the treatment.

JAMe and β-Glucan-Induced Glyceollins in Wounded Soybean Cotyledons

As shown in Figure 2, JAMe was the only natural octadecanoid-derived compound tested that was nearly inactive in eliciting the glyceollin accumulation in soybean cell cultures. In order to investigate whether wounding may potentiate a JAMe-mediated effect by creating a competency state of the cells (Graham and Graham, 1996), the soybean cotyledon assay was chosen for further investigations. Concentrations of up to 100 µM JAMe were not capable to induce the synthesis of phytoalexins when applied to the surface of cut cotyledons (Table 1). In contrast, the β-glucan elicitor strongly induced glyceollins showing that the cotyledons used in these experiments were able to respond upon treatment with elicitors. Maximal response with this elicitor was reached with a concentration of about 100 µg ml^{-1} β-glucan (Table 1). Increasing concentrations of JAMe used in combination with a fixed concentration of β-glucan elicitor (2 µg ml^{-1}) decreased the amount of accumulated glyceollins in a concentration-dependent manner (Table 1). At 100 µM JAMe, the phytoalexin concentration dropped to only 37% of the concentration measured in the control experiment without JAMe. By using 200 µg ml^{-1} β-glucan elicitor, this effect was less distinct (data not shown). *Vice versa*, when a fixed concentration of JAMe was used with increasing concentrations of β-glucan elicitor, the opposite effect was observed. A higher β-glucan elicitor concentration was necessary to induce similar quantities of glyceollins compared with the experiments without JAMe treatment (Table 1). In the presence of 100 µM JAMe, a 10-fold higher concentration of β-glucan elicitor was necessary (100 µg ml^{-1} instead of 10 µg ml^{-1}) to induce the same amount of glyceollins (36.8 nmol $cotyledon^{-1}$) as in the absence of JAMe (38.6 nmol $cotyledon^{-1}$). These results suggest that the presence of JAMe may activate biosynthetic reactions in flavonoid metabolism competing with the glyceollin pathway for the same substrates. Since OPDA was an efficient inductor of glyceollin in soybean cell cultures (Figure 2A) it was used in the cotyledon assay as well. Surprisingly, up to a concentration of 50 µM this compound showed no elicitor activity. Moreover, in combination with a fixed concentration of β-glucan (2 µg ml^{-1}) OPDA did not affect the glyceollin accumulation (Table 2).

Table 1 JAMe- and β-Glucan-Induced Accumulation of Glyceollins in Soybean Cotyledons.

Treatment	Glyceollins
	nmol $cotyledon^{-1}$
Control (H_2O)	8.9 ± 5.3
JAMe (10^{-7} M)	7.0 ± 0.6
JAMe (10^{-6} M)	7.8 ± 1.4
JAMe (10^{-5} M)	9.1 ± 0.8
JAMe (10^{-4} M)	7.0 ± 2.3
β-Glucan (2 µg ml^{-1})	35.4 ± 14.3
β-Glucan (10 µg ml^{-1})	38.6 ± 8.7
β-Glucan (100 µg ml^{-1})	47.9 ± 10.0
β-Glucan (2 µg ml^{-1} JAMe (10^{-7} M)	38.0 ± 14.0
β-Glucan (2 µg ml^{-1}) + JAMe (10^{-6} M)	30.3 ± 6.2
β-Glucan (2 µg ml^{-1}) + JAMe (10^{-5} M)	25.3 ± 9.2
β-Glucan (2 µg ml^{-1}) + JAMe (10^{-4} M)	17.6 ± 2.9
JAMe (10^{-4} M) + β-Glucan (10 µg ml^{-1})	17.5 ± 2.6
JAMe (10^{-4} M) + β-Glucan (100 µg ml^{-1})	36.8 ± 15.7

For each determination, ten soybean cotyledons (cv. 9007) were cut and incubated with 60 µl solution containing the indicated effector composition on the wound surface. After 22 h the wound droplets were collected and analyzed by HPLC for their glyceollin contents. The results are given as the mean ± SD of 3–4 experiments.

Table 2 OPDA- and β-Glucan-Induced Accumulation of Glyceollins in Soybean Cotyledons.

Treatment	Glyceollins
	nmol $cotyledon^{-1}$
Control (H_2O)	2.35 ± 2.9
OPDA (3.4×10^{-7} M)	0.95 ± 0.7
OPDA (10^{-5} M)	0.47 ± 0.3
OPDA (5×10^{-5} M)	0.49 ± 0.2
β-Glucan (2 µg ml^{-1})	10.78 ± 5.7
β-Glucan (100 µg ml^{-1})	17.30 ± 8.1
OPDA (3.4×10^{-7} M) + β-Glucan (2 µg ml^{-1})	11.07 ± 2.8
OPDA (10^{-5} M) + β-Glucan (2 µg ml^{-1})	9.81 ± 2.4

For each determination, ten soybean cotyledons (cv. Harosoy) were cut and incubated with 60 µl solution containing the indicated effector composition on the wound surface. After 22 h the wound droplets were collected and analyzed by HPLC for their glyceollin contents. The results are given as the mean ± SD of 3–5 experiments.

Discussion

During plant pathogenesis, octadecanoid-derived compounds are thought to mediate signals inside and outside the cell. First described as wound-induced volatile signaling compounds activating proteinase inhibitors to ward off herbivores (Farmer and Ryan, 1990, 1992), they soon were discussed as signal transducers generally integrated in elicitation processes that might commonly occur in diverse plant defense reactions (Gundlach *et al.,* 1992; Creelman and Mullet, 1997; Weiler, 1997). Using mostly soybean cell cultures as the experimental system, we analyzed for the first time the activities of both, naturally occurring octadecanoids as well as synthetic functional mimics, in the induction of defense responses, mainly phytoalexin accumulation. Moreover, we aimed to elucidate a possible role of octadecanoids as mediators in the *P. sojae*-derived β-glucan elicitor-induced general defense response.

Methyl jasmonate, a volatile signal transmitter that can be elicited by herbivorous attack, proved to be unable to induce the production of glyceollins in soybean (Figure 2B, Table 1). In the cotyledon assay, increasing concentrations of JAMe inhibited the β-glucan-induced production of the phytoalexins, demonstrating even a negative effect of this compound in soybean (Table 1). However, the biosynthetic precursor of JA, OPDA, was ineffective in the cotyledon assay (Table 2) in contrast to the soybean cell cultures (Figure 2A). The reason for this result is unknown but could be explained by a specific tissue sensitivity. Moreover, as responses in wounded tissues are more complex compared with unwounded tissues (Graham and Graham, 1996) non-differentiated cell suspension cultures seem to be a more simple biological system. JA was capable of inducing an increase of glyceollin content in soybean cell cultures to concentrations approaching the values routinely obtained with a saturating dose of β-glucans (200 μg ml^{-1}). Coronatine, a toxin produced by phytopathogenic bacteria, as well as certain structural mimics of coronatine and OPDA were much more active than JA in eliciting glyceollin accumulation in soybean cell cultures (Figure 2, 3). This result was somewhat surprising because the concomitant transcriptional regulation of genes representing crucial enzymes for the biosynthesis of glyceollins (CHS, CHR, D6aH) did not show an expression above the levels found, for example, with JA (Figure 4). OPDA, the most efficient elicitor of glyceollin production, failed to induce high transcript levels for genes encoding early enzymes of the biosynthetic pathway, *CHS* and *CHR*, and induced only intermediate mRNA levels for the late *D6aH*. The comparatively high glyceollin accumulation after treatment of the cells with OPDA, coronatine, and IndEt-IleMe, as opposed to JA and IndAz-IleMe, may result from the inability of the former group of substances to induce other flavonoid pathway enzymes, such as F6H, which channels the common flavonoid intermediates into an alternative branch pathway, yielding trihydroxyflavanones (Latunde-Dada *et al.,* 2001). Therefore, any conclusions from the transcript profiles shown here must remain rather tentative. Additional experiments designed to describe dose- and time-courses of the regulation of octadecanoid-dependent genes as well as a product profiling may result in a more comprehensive picture. Moreover, jasmonates were reported to regulate at the transcriptional, post-transcriptional, and translational level (Sembdner and Parthier, 1993), a threshold of complexity not yet tackled in this context.

Results presented in a recent publication on metabolic profiling demonstrated the ability to differentiate between two LOX-dependent pathways (Göbel *et al.,* 2001). Elicitor treatment of potato (*Solanum tuberosum* cv. Desirée) cultures led to the transcriptional activation of both lipoxygenase types (linoleate 9-LOX and linoleate 13-LOX), but only products formed by the 9-LOX reaction accumulated in the cells, leading to suspected antibiotic metabolites other than JA, and thus providing evidence for an octadecanoid-independent elicitor-signaling pathway (Göbel *et al.,* 2001). Our results concerning the activation of the *LOX4* gene (Figure 4) can be interpreted in a similar manner. Recently, the enzymatic specificity of this soybean LOX isoform (LOX4, synonymous to VLXA, vegetative lipoxygenase, isozyme A; gene family member *Lox1:Gm:4*) has been determined to preferentially produce (13*S*)-hydroxy linoleic acid (Fuller *et al.,* 2001). The soybean *LOX* gene family consists of at least eight members (Siedow, 1991; Bunker *et al.,* 1995). Since those isozymes that share the same substrate specificity are also structurally related (Fuller *et al.,* 2001), one could speculate that the *LOX4* probe would have detected transcripts of other 13-specific *LOXs* in our Northern blotting experiments. Since this was not the case for β-glucan-treated cells, and since we also were unable to detect one of the final products derived from the 13-LOX reaction, JA, the participation of octadecanoids in soybean β-glucan elicitor signaling appeared to be unlikely. Nevertheless, the ability of externally applied octadecanoids to influence both, genes encoding biosynthetic enzymes and the production of antibiotic phytoalexins argued in favor of a role as intracellular signal transmitters in soybean defense reactions. However, none of the selected members of the proposed β-glucan elicitor-dependent signaling cascade, neither the elevation of the intracellular Ca^{2+} concentration nor an activation of MAP kinases, showed a response to octadecanoid treatment (Figure 5 and data not shown). As mentioned above, the involvement of the octadecanoids in the β-glucan-elicited communication downstream of the cytosolic Ca^{2+} level or the MAP kinase activation could be ruled out because a β-glucan-mediated elevation in the intracellular concentrations of JA or OPDA was not detectable. Consequently, parallel signaling pathways might be postulated, a situation recently reported as well for other plants.

Jasmonates have been shown to be insufficient for the activation of a full elicitor response in cultured parsley and tobacco cells (Ellard-Ivey and Douglas, 1996; Rickauer *et al.,* 1997). In potato discs, distinct effects on the activation of single members of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase gene family were reported for JAMe *versus* the fungal elicitor, arachidonic acid, leading to different metabolite profiles suggesting distinct signaling pathways (Choi *et al.,* 1994). In barley (*Hordeum vulgare* cv. Salome), different sets of JA-responsive genes have been described, depending on endogenous or exogenous sources of these signaling molecules (Kramell *et al.,* 2000). Moreover, different octadecanoids clearly showed differences in their abilities to influence a variety of plant responses. Examples include the mechanosensing in *B. dioica* tendrils or the composition of volatiles, emitted after treatment of lima bean leaves (Blechert *et al.,* 1999; Koch *et al.,* 1999). Early intermediates (linolenic acid, OPDA) elicited the biosynthesis of diterpenoids whereas the products of this pathway, especially JA, triggered the synthesis of mono- and sesquiterpenes (Koch *et al.,* 1999), confirming the hypothesis of 'two independent centers of biological activity' within the signaling pathway (Blechert *et al.,* 1999; Koch *et al.,* 1999). Recently, this concept received a remarkable extension. By exploiting the *opr3* mutant of *A. thaliana*, the participation of biosynthetic precursors of JA in disease resistance was evaluated (Stintzi *et al.,* 2001). Surprisingly, JA-deficient *opr3* plants developed wildtype levels of *COII*-dependent resistance against an insect as well as a fungal pathogen, suggesting that defense pathways can be triggered by OPDA in the absence of JA (Stintzi *et al.,* 2001). Likewise, the resistance of *A. thaliana* to *Phytophthora porri* was reported to be independent of the presence of JA as signal transmitter, although the pathway itself was activated (Roetschi *et al.,* 2001). By monitoring a range of activities of precursors and mimics of JA in soybean cell cultures we were, thus, able to substantiate the emerging picture of octadecanoid-independent signaling pathways in plant defense responses.

Materials and Methods

Substances

Coelenterazine was from Molecular Probes Inc., Leiden, The Netherlands. Myelin basic protein (MBP) was purchased from Sigma-Aldrich, Deisenhofen, Germany. Coronatine was generously provided by Dr. M. Ullrich, Max-Planck-Institute, Marburg, Germany. Jasmonic acid methyl ester was from Givaudan S.A., Vernier, Switzerland, free jasmonic acid was obtained from the methyl ester by saponification. 12-Oxo-phytodienoic acid was from Cayman Chemicals, Hornby, Canada. 1-Oxo-indanoyl isoleucine methyl ester, 6-azido-1-oxo-indanoyl isoleucine methyl ester, and 6-ethyl-1-oxo-indanoyl isoleucine methyl ester were synthesized as described (Schüler *et al.,* 1999, 2001). Oomycete β-glucans from *Phytophthora sojae* Kaufmann and Gerdemann were prepared from purified cell walls as described earlier (Schmidt and Ebel, 1987).

Plant Material

Cell suspension cultures of soybean (*Glycine max* L. cv. Harosoy 63) were grown as described previously by Hille *et al.* (1982). The cultures were incubated in the dark at 26 °C on a rotary shaker at 110 rpm and were subcultured in fresh medium every 7 d. The transgenic soybean suspension cell line 6.6.12, carrying the stably integrated plasmid *pGNAequ/neo2* and expressing apoaequorin, was grown as described by Mithöfer *et al.* (1999). Soybean seeds were obtained from Pioneer, Buxtehude, Germany (*G. max* L. cv. 9007) and Agriculture Canada, Harrow, Canada (*G. max* L. cv. Harosoy) and grown as described (Schmidt and Ebel, 1987).

Aequorin Dependent Luminescence Measurements

Transgenic 6.6.12 cell lines were used to reconstitute aequorin *in vivo* with 10 μM synthetic coelenterazine on a shaker (125 rpm) in the dark for 24 to 48 h. Ca^{2+}-specific luminescence (470 nm) was measured and the cytosolic Ca^{2+} concentration was calculated as described (Mithöfer *et al.*, 1999). None of the compounds used interfered with the aequorin-dependent luminescence which was tested by the addition of ethanol and $CaCl_2$ to final concentrations of 10% (v/v) and 1 M, respectively.

In-Gel Protein Kinase Assay

The analysis of inducible protein kinases was performed as described (Suzuki and Shinshi, 1995) with minor modifications. Briefly, soybean cell suspension cultures (cv. Harosoy 63, 5 d-old) were subcultured in fresh medium (4 g fresh mass per 40 ml medium) in the dark for 2 h with shaking (110 rpm) and 1 h without. Aliquots of 500 μl were transferred into Eppendorf tubes and allowed to settle. The supernatant was removed and the cells were treated by addition of elicitors. After 20 min, the medium was removed by use of a syringe and the cells were frozen in liquid nitrogen. The homogenate was prepared by grinding the cells with a small mortar in extraction buffer (25 mM Tris/HCl, pH 7.8, 10 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 1 mM DTT, 1 mM NaF, 0.5 mM Na_3VO_4, 30 mM glycerophosphate, 0.1% Tween 20, 0.5 mM phenylmethylsulfonyl fluoride) and cleared by centrifugation at 10 000 g for 10 min in the cold. The supernatants were used for the in-gel kinase assay using 30 μg of protein and 0.2 mg ml^{-1} MBP embedded in the resolving gel of a 10% SDS-polyacrylamide gel according to Suzuki and Shinshi (1995). Further treatment of the gel was exactly as described (Suzuki and Shinshi, 1995) and the kinase activities were visualized by phosphorimaging of the dried gels.

Northern Blot Analyses

Soybean cell suspension cultures (cv. Harosoy 63, 5 d-old) were subcultured in fresh medium (4 g fresh mass per 40 ml medium) in the dark for 12 h with shaking (110 rpm) before the treatment. Total RNA of treated soybean cells was prepared according to Schröder *et al.* (1979) and 15 μg per lane were blotted onto Biodyne-A membranes (Pall, Dreieich, Germany). Hybridization probes used were: 3 kbp soybean actin gene, bean (*Phaseolus vulgaris*) chalcone synthase 1 (*CHS*) cDNA (Ryder *et al.,* 1984), soybean reductase co-acting with CHS (*CHR*) partial cDNA clone p1 (Welle *et al.,* 1991), soybean flavonoid 6-hydroxylase (*F6H*) gene-specific cDNA fragment (Schopfer and Ebel, 1998; Latunde-Dada *et al.,* 2001), soybean 3,9-dihydroxypterocarpane 6a-hydroxylase (*D6aH*) gene-specific probe generated by PCR from the cDNA (Schopfer *et al.,* 1998), soybean *Gmhsp-26A* glutathione *S*-transferase (*GST*) cDNA (Czarnecka *et al.,* 1988, kindly provided by R. Tenhaken, University of Kaiserslautern, Germany), and partial soybean lipoxygenase 4 (*LOX4*, corresponding to swissprot accession P38417) cDNA (J. Fliegmann, unpublished). Probes were radioactively labeled by use of the random prime procedure (Prime-a-Gene, Promega, Heidelberg, Germany). Hybridization and washing conditions of Biodyne-A membranes (Pall, Dreieich, Germany) were according to Fliegmann and Sandermann (1997). Hybridization signals were visualized by either phosphorimaging or exposure to X-ray film at – 80 °C.

Determination of Glyceollins

Soybean cell suspension cultures were subcultured as described above for Northern blot analyses. Treatment was done in 8 ml fractions of the cell suspension on a rotary shaker at 110 rpm and 26 °C in the dark. The β-glucan elicitor concentration was 200 μg ml^{-1}. Cells were harvested after 48 h by filtration under reduced pressure on a sintered-glass funnel. The medium was extracted twice with 8 ml acetic acid ethyl ester each. The organic phase was dried over Na_2SO_4 and removed under reduced pressure. The residue was dissolved in 200 μl ethanol and analyzed by HPLC (LiChrosorb RP-18, 4×250 mm; flow rate 1 ml min^{-1}; linear gradient from 40 – 65% (v/v) methanol in 20 min). Glyceollins were identified and quantified by using reference substances.

Cotyledon Assay

Detached cotyledons from 5 d-old greenhouse seedlings of the soybean cultivars 9007 and Harosoy, respectively, were cut and aliquots of solutions (60 µl) were placed on wounded areas (Ayers *et al.,* 1976). The cotyledons were incubated at 27 °C on moist filter paper in Petri dishes in the dark. After 22 h, the droplets collected from the wounded surface of the cotyledons were extracted twice with an equal volume of acetic acid ethyl ester for glyceollin determination. For each determination ten cotyledons were used.

Quantification of Endogenous JA, OPDA, and SA

Soybean cell suspension cultures were subcultured and treated as described above. The cells were harvested at the times indicated and frozen immediately in liquid nitrogen. Quantification of endogenous JA and SA followed a procedure originally established for JA by McCloud and Baldwin (1997) and extended for simultaneous quantification of JA and SA by Engelberth *et al.* (2001). Frozen cells (1 g fw) were ground under liquid nitrogen and extracted twice with 35 ml of diethyl ether: 50 mM citric acid (70:30, v/v) solution. As internal standards [9,10-$^2H_2$]-9,10-dihydro-JA (146 ng) and [3,4,5,6-$^2H_4$]SA (500 ng) were added. The pooled extracts were purified using a solid-phase extraction cartridge (500 mg sorbent, aminopropyl; Macherey und Nagel, Düren, Germany). After washing with 5 ml of a trichloromethane:2-propanol (2:1, v/v) mixture, bound JA, SA and the corresponding standards 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 and analyzed by GC/MS. Spectra were recorded in the selected ion mode, for JA determination by monitoring the fragment ion at m/z = 83 amu corresponding to the base peaks of JA and [9,10-$^2H_2$]-9,10-dihydro-JA, and in case of SA determination at m/z = 120 and 124 amu corresponding to the base peaks of SA and [3,4,5,6-$^2H_4$]SA, respectively. The amounts of endogenous JA and SA were calculated from the peak areas of the particular compounds compared with the corresponding standards using calibration curves that were determined independently.

For quantification of endogenous OPDA, cells (1 g fw) were frozen, ground under liquid nitrogen and the resulting powder was extracted twice with 25 ml peroxide-free ether. The pooled extracts were loaded onto a solid-phase extraction cartridge (500 mg sorbent, aminopropyl). After loading, the cartridges were washed with 5 ml of a solvent mixture of trichloromethane:2-propanol (2:1, v/v). Bound OPDA was eluted with 10 ml diethylether:acetic acid (98:2, v/v). After evaporation of solvents and esterification of the residue with excess diazomethane, this residue was mixed with 10 µl *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide and heated for 30 min at 40 °C. The sample was diluted with 10 µl dichloromethane and analyzed by GC/MS (column: Alltech EC5 10×0.25 mm, 0.25 µm; injected volume: 2 µl; splitless mode; constant pressure: 30 kPa; injector temp: 280 °C; temp profil: 50 °C to 100 °C with 10 °C/min followed by 5 °C/min to 300 °C, 5 min hold). The quantification of endogenous OPDA was achieved by comparison of the peak area with a calibration curve determined independently with authentic material.

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