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Colloquium Paper

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The octadecanoic pathway: Signal molecules for the regulation of secondary pathways

(phytoalexins/jasmonic acid/12-oxophytodienoic acid/oxylipid cascade/signal transduction)

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ABSTRACT **Plant defense against microbial pathogens and herbivores relies heavily on the induction of defense proteins and low molecular weight antibiotics. The signals between perception of the aggression, gene activation, and the subsequent biosynthesis of secondary compounds are assumed to be pentacylic oxylipin derivatives. The rapid, but transient, synthesis of *cis*-jasmonic acid was demonstrated after insect attack on a food plant and by microbial elicitor addition to plant suspension cultures. This effect is highly specific and not caused by a number of environmental stresses such as light, heavy metals, or cold or heat shock. Elicitation of *Eschscholtzia* cell cultures also led to a rapid alkalinization of the growth medium prior to jasmonate formation. Inhibition of this alkalinization process by the protein kinase inhibitor staurosporine also inhibited jasmonate formation. The induction of specific enzymes in the benzo[*c*]phenanthridine alkaloid pathway leading to the antimicrobial sanguinarine was induced to a qualitatively and quantitatively similar extent by fungal elicitor, methyl jasmonate, and its linolenic acid-derived precursor 12-oxophytodienoic acid. It is herein proposed that a second oxylipid cascade may exist in plants starting from linoleic acid via 15,16-dihydro-12-oxophytodienoic acid to 9,10-dihydrojasmonate. Experiments with synthetic trihomojasmonate demonstrated that β-oxidation is not a prerequisite for biological activity and that 12-oxophytodienoic acid and derivatives are most likely fully active as signal transducers. Octadecanoic acid-derived compounds are essential elements in modulating the synthesis of antibiotic compounds and are thus integral to plant defense.**

Plants contain an overwhelming number of so-called secondary compounds, sophisticated organic chemicals that are species specific and not necessary for the normal life function (1). The modern view (2) is that some of these chemicals serve to attract pollinating insects or symbiotic organisms, but the majority serve in self-defense of plants against pathogens and herbivores. This self-defense arsenal of low molecular weight molecules is supplemented by high molecular weight proteins such as proteinase inhibitors (3). The low molecular weight defense compounds are called phytoalexins (1, 4), have antibiotic properties, and are one of the dominant survival factors for plants in the ecosystems. Recent research using transgenic plants showed unequivocally that if genes involved in phytoalexin biosynthesis are transferred interspecifically, this results in decreased susceptibility of the host plant toward fungal attack (5, 6). Conversely, if one of the biosynthetic enzymes leading to the phytoalexin is suppressed by antisense technology, this plant is more disease susceptible than the wild type, indicating that secondary products contribute to disease limitation (7). While many of the secondary compounds such as the potent cyanogenic glucosides (8) are formed constitutively, some are specifically induced only after the plant has been attacked by a hostile organism (9). The constitutive synthesis of self-defense compounds is easily understood; it bears, however, the disadvantage that the chemicals are normally present throughout the ontogeny of the organism and thus allows certain organisms to break through this chemical defense line by slowly adapting resistance to these compounds. The more sophisticated way is induced synthesis—that is, defense chemicals are biosynthesized only after the plant has already been attacked. This process has to rely, however, on a complex signaling system. The aggression by fungus or the wounding caused by an herbivore has first to be sensed; then a signal has to activate the silent defense genes to produce, through transcription, enzymes of the secondary pathway, which will ultimately lead to the synthesis of phytoalexins, either located in the vicinity of the attack or systemically throughout the plant.

In a series of most impressive experiments, Farmer and Ryan (summarized in refs. 10 and 11) showed that the signal involved in the induction of (high molecular weight) proteinase inhibitors involved a lipid-based transduction system, which yielded jasmonic acid and its methyl ester (see Fig. 5*A*). The model proposed (10, 11) that wounding by insects or microbial pathogen attack led to an interaction of elicitors with receptors, thus initiating the octadecanoic-based pathway from the C_{18} fatty acid linolenic acid to jasmonic acid (12). Synthetic jasmonic acid indeed acted as a powerful inducer of *de novo* defense protein synthesis, simulating a wound response.

Stimulated by this work (10, 11) and by the fact that jasmonic acid and its biosynthetic precursors were also the factors inducing tendril coiling in *Bryonia* (13, 14), we explored whether the jasmonate family of compounds could also be involved in low molecular weight defense substance (phytoalexin) induction.

Indeed, exposure of plant cell suspension cultures to a fungal cell wall elicitor led within minutes to a drastic, but transient, accumulation of intracellular jasmonic acid (15, 16). Thirty-six different plant species tested in cell suspension culture could

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Abbreviations: 12-oxo-PDA, 12-oxophytodienoic acid; trihomojasmonate, 2-(3-oxo-2-pent-2-en-cyclopentyl)ethoxyacetic acid; hexahomojasmonate, 5-(3-oxo-2-pent-2-en-cyclopentyl)pentoxyacetic acid; BBE, berberine bridge enzyme.

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be elicited with respect to the accumulation of secondary metabolites by exogenously supplied jasmonates and their biosynthetic precursor, 12-oxophytodienoic acid (12-oxo-PDA). Numerous phytoalexin types were synthesized such as terpenoids, flavonoids, anthraquinones, and different alkaloid classes (15–17) as a consequence of this exposure. Furthermore, it was shown that elicitation of plant cell cultures with a fungal cell wall preparation led to a rapid release of α-linolenic acid, the ultimate precursor (12) of the members of the biologically active pentacyclic acids, from the lipid pool of the plant cell.

MATERIALS AND METHODS

Plant Material. Cell suspension cultures of *Agrostis tenuis, Eschscholtzia californica, Rauvolfia serpentina*, and *Phaseolus vulgaris* were grown in Linsmaier and Skoog medium (18) on a gyratory shaker (100 rpm) at 23°C in continuous light (750 lx) for 5–7 days. *Petroselinum crispum* cultures were grown in complete darkness. Cells were harvested under sterile conditions by suction filtration, resuspended in either 1-liter flasks containing 300 ml of medium (16) or 300-ml flasks containing 75 ml of medium, and after a growth period of 3 days were used directly for the various experiments. Structure–activity analyses were done in 24-well multiculture dishes containing 1-ml suspension cultures of *E. californica* cells incubated under sterile conditions at 140 rpm for 4 days. Cells were harvested by centrifugation and extracted with ethanol/0.1 M HCl at 60°C for 2 h. Aliquots of these extracts were subjected to resolution and quantitation by HPLC chromatography (19) with UV detection or to quantitation of total benzo[*c*]phenanthridine alkaloids by extinction measurements.

Three-hundred-milliliter flasks each containing 75 ml of cell suspension culture were subjected to various environmental stresses consisting of exposure to a 10,000-lx incandescent light source, 50 μM $CuCl_2$ or 150 μM $CdCl_2$, or 4°C or 37°C. At each time point, the cells of one flask were harvested, extracted, and analyzed for jasmonates (20).

Vicia faba plants were grown in a greenhouse under natural daylight conditions for 3 weeks. Plants were exposed to attack by 2- to 3-week-old caterpillars (*Spodoptera littoralis*). Within 10 min, these spodopterans had typically eaten a leaf area of 1.5 cm^2, which resulted in a 4-cm length of wounded tissue. The entire leaf, 14 cm^2, was shock frozen in liquid N_2 and analyzed for jasmonate content (20).

Extracellular Alkalinization. *Phaseolus* cells at a density of 5 g of fresh weight in 50 ml of medium were incubated in small aliquots in a beaker on a shaker at 150 rpm. The extracellular pH was monitored with a glass electrode for 15 min, after which the pH value was 4.5–4.8. Yeast cell wall elicitor (19) (250 μg/ml) was added, and the pH value was recorded for 60 min. In the case of the staurosporine experiments, this inhibitor was added 10 min prior to the addition of elicitor to a final concentration of 10 μg/ml. Parallel experiments were conducted under the conditions indicated for jasmonate analysis, but in these cases, 60 ml of suspension was used.

Enzyme and RNA Analyses. Activities of the enzymes of the sanguinarine pathway were analyzed as summarized in ref. 21. Total RNA was isolated from *E. californica* cell suspension cultures, fractioned on a formaldehyde gel, blotted onto a Hybond N filter, and hybridized to ^{32}P-labeled berberine bridge enzyme (BBE; EC 1.5.3.9) cDNA exactly as described (22). Experiments using flax seedling (*Linum usitatissimum*) extracts were carried out according to ref. 23.

Jasmonate Determinations. Suspension cells (60 ml, 6 g fresh weight) grown as described above were removed under sterile conditions, rapidly filtered, and shock frozen in liquid N_2. The cells were processed with 9,10-dihydrojasmonic acid as internal standard as described (20). Jasmonates were determined by capillary GC/negative chemical ionization/MS using a Varian 3400 gas chromatograph interfaced to a Finnigan-MAT (San Jose, CA) quadrupole SSQ 700 mass spectrometer. The detection limit for jasmonate with this procedure was 1 pg (≈5 fmol) (20).

Chemical Syntheses. The oxahomojasmonate compounds were synthesized (S.B. and S.H.) from 2-(6-pent-2-enyl-1,4-dioxa-spiro[4.4]non-7-yl)ethanol in either four or six steps. The four-step protocol yielded 2-(3-oxo-2-pent-2-en-cyclopentyl)-ethoxyacetic acid (trivial name: trihomojasmonate): ^1H NMR (400 MHz, C^2HCl_3) δ 5.42 (dtt, 11, 7, 1.5, 1H), 5.25 (dtt, 11, 7, 1.5, 1H), 4.13 (s, 2H), 3.65 (t, 6.5, 2H), 2.38 (m, 3H), 2.25–2.15 (m, 1H), 2.22–1.95 (m, 5H), 1.88–1.80 (m, 1H), 1.62–1.50 (m, 1H), 1.50–1.38 (m, 1H), 0.95 (t, 7, 3H); mass spectrum *m/e* (rel intensity) 254 (M^+, 20), 186 (18), 151 (56), 83 (56), 69 (100). The six-step synthesis yielded 5-(3-oxo-2-pent-2-en-cyclopentyl)pentoxyacetic acid (trivial name: hexahomojasmonate): ^1H NMR (400 MHz, C^2HCl_3) δ 5.42 (dtt, 10.5, 7.5, 1.5, 1H), 5.23 (dtt, 10.5, 7.5, 1.5, 1H), 4.13 (s, 2H), 3.58 (t, 6.5, 2H), 2.41–2.32

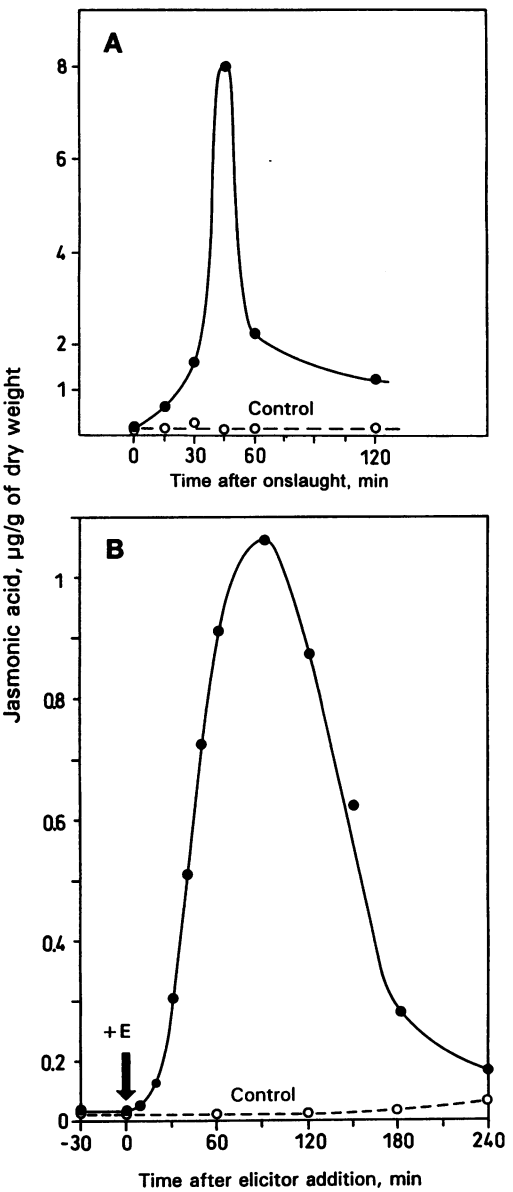

FIG. 1. (*A*) Induction of jasmonate by the feeding of a caterpillar of *S. littoralis* on a leaf of *V. faba*. ●, Attacked leaf tissue; ○, control tissue. (*B*) Induction of jasmonate by addition of yeast cell wall elicitor (250 μg/ml) to cell suspension cultures of *A. tenuis*. ●, Elicitor-treated culture; ○, untreated culture.

(m, 3H), 2.19–2.0 (m, 4H), 1.91–1.80 (m, 2H), 1.74–1.62 (m, 3H), 1.51–1.21 (m, 6H), 0.96 (t, 7.5, 3H); mass spectrum *m/e* (rel intensity) 296 (M^+, 10), 278 (18), 151 (50), 83 (100). The experimental details will be published elsewhere. The known C_{18} and C_{12} oxylipid derivatives were synthesized (Z.-Q.X. and W.B.), and the structures (14, 24) were verified by GC/MS using the conditions given above. All compounds were converted to the corresponding methyl ester by reaction with diazomethane in ether at room temperature and were subsequently purified by TLC.

RESULTS AND DISCUSSION

Induction of Endogenous Jasmonate. The induction of proteinase inhibitors by mechanical wounding of leaves as well as the simulation of this effect by methyl jasmonate on both the transcriptional and translational levels has been amply demonstrated (10, 11). To establish the induction of jasmonic acid in differentiated leaf tissue as the result of the onslaught of an insect, *V. faba* leaves were exposed to a starved larvae of *S. littoralis*. Jasmonic acid levels in leaf tissue rose immediately after feeding began, reached a maximal concentration after 45 min, and declined steadily thereafter (Fig. 1*A*). The jasmonate induction curve followed a transient pattern, which would be expected for an endogenous signal or hormonal substance.

A qualitatively similar induction of jasmonic acid was observed by exposing plant cell cultures to a yeast cell wall elicitor (15, 16). Suspension culture cells of the grass *A. tenuis* were elicited with the fungal cell wall preparation whereupon a dramatic increase of jasmonate was observed (Fig. 1*B*). Within 5–15 min after elicitation, the endogenous level of jasmonic acid rose noticeably over the nonelicited control culture. The maximal jasmonate concentration was found 90 min after exposure to the elicitor. Thereafter, the intracellular jasmonic acid level decreased, returning to background levels 4 h after the initial challenge. No pentacyclic compounds were found in the culture medium of this cell suspension. One has to assume, therefore, that the lipid-derived signal compound, jasmonic acid, is intracellularly induced and subsequently metabolized within this system. The jasmonic acid liberated in these experiments has been proven unequivocally by capillary GC/negative chemical ionization/MS to be (3*R*,7*S*)-(+)-7-*iso*-jasmonic acid (20). Since this jasmonic acid diastereomer rearranges rapidly to the (−)-*trans* isomer (20), it is the resulting diastereomeric mixture (≈93:7) that is called jasmonic acid.

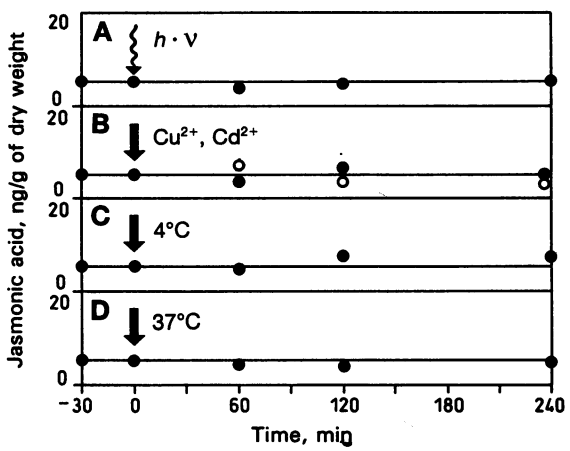

FIG. 2. (*A*) Induction of jasmonate by treatment of dark-grown suspension cultures of *P. crispum* with light (10,000 lx). (*B–D*) Induction of jasmonate in *R. serpentina* suspension cultures with 50 μM $CuCl_2$ (●) or 150 μM $CdCl_2$ (○) (*B*), cold shock at 4°C (*C*), or heat shock at 37°C (*D*).

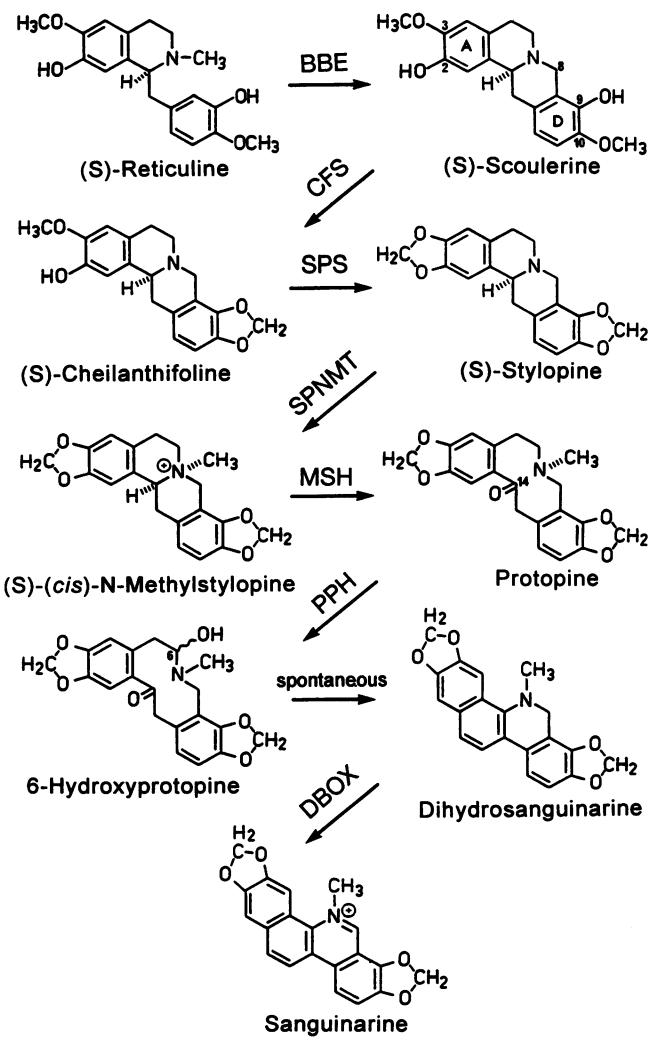

FIG. 3. Enzymatically verified biosynthetic pathway from (*S*)-reticuline to sanguinarine. CFS, (*S*)-cheilanthifoline synthase; SPS, (*S*)-stylopine synthase; SPNMT, *S*-adenosyl-L-methionine:(*S*)-tetrahydroprotoberberine *cis*-*N*-methyltransferase; MSH, (*S*)-*cis*-*N*-methylstylopine 14-hydroxylase; PPH, protopine 6-hydroxylase; DBOX, dihydrobenzophenanthridine oxidase.

Next we checked whether the synthesis of jasmonic acid could be induced by various types of environmental stresses. To this end, dark-grown cell cultures of *P. crispum* were exposed to strong light, or *R. serpentina* cell cultures were exposed to heavy metal stress involving Cu^{2+} and Cd^{2+} ions and to cold (4°C) and heat (37°C) shock. None of these treatments induced jasmonic acid within these cultures (Fig. 2). This indicated that jasmonate signaling is rather restricted toward attack by various microbial and animal pests.

Structure–Function Relationship. The role of jasmonates in provoking the synthesis of low molecular weight defense compounds was well established in more than 36 different plant species in cell culture and in differentiated plants (15, 16, 25). It had previously been noticed that anthocyanin was induced as a result of exposure of soybean seedlings to methyl jasmonate (26). More recently, a potent effect of jasmonates on indole glucosinolates in oil seed rape and mustard (27), on the synthesis of antimicrobial isoprenoids and the associated 3-hydroxy-3-methylglutaryl-CoA reductase genes in potato (28), and on alkaloid content of *Catharanthus* and *Cinchona* seedlings (29) has been observed.

In all of these cases, commercially available methyl jasmonate was used. It would be of considerable importance to

test the various jasmonate precursors and analogues for the ability to induce low molecular weight compounds in plant systems. From the original observation (19) that infection of a colorless callus of the California poppy *E. californica* by a *Penicillium* fungus led to an elevated synthesis of a family of bright red colored benzo[*c*]phenanthridine alkaloids (19, 30), a sensitive assay for the induction of these alkaloids using multiwell plates (19) has been developed. The complete biosynthetic pathway for the antibacterial (31) and antifungal (ref. 32; E. Liebing and M.H.Z., unpublished results) benzo[*c*]-phenanthridines from the primary metabolite, L-tyrosine, to the most highly oxidized alkaloid, macarpine, was eventually worked out completely at the enzyme level, and 19 enzymes comprising this pathway were discovered and characterized (21). The biosynthesis of the first and most simple benzo[*c*]-phenanthridine alkaloid, sanguinarine, starting from (*S*)-reticuline, the branchpoint intermediate in isoquinoline alkaloid biosynthesis, is depicted in Fig. 3. The cDNA encoding the first enzyme of the sanguinarine part of the pathway, BBE, has been cloned and heterologously expressed in catalytically active form (22). A systematic effort was made to analyze all 7 enzymes involved in this portion of the pathway under identical conditions and, thereby, to identify which of the enzymes were induced under the influence of the yeast cell wall elicitor, methyl jasmonate, and 12-oxo-PDA. In experiments not shown here, all of the cytosolic enzymes between L-tyrosine and (*S*)-reticuline were shown not to be significantly induced by these three compounds. However, a very strong induction was observed for all five membrane-associated enzymes of the sanguinarine pathway. This induction ranged from a 6.5- to an almost 16-fold stimulation of BBE and of four cytochrome P450-dependent enzymes over unelicited control cultures (Fig. 4). Interestingly, the cytosolic enzymes, stylopine *cis-N*-methyltransferase and the copper-containing dihydrobenzophenanthridine oxidase (33, 34) were only minimally induced. As a control enzyme of primary metabolism, glucose-6-phosphate dehydrogenase was measured under the elicitation regimes and compared to the control. This enzyme was unaffected by the fungal elicitor as well as the pentacyclic compounds. As shown previously (35) by analyzing levels of BBE transcripts in elicited cell cultures, *de novo* transcription was demonstrated to be the likely mode of jasmonate-based gene activation. In analogy, it has to be assumed that all

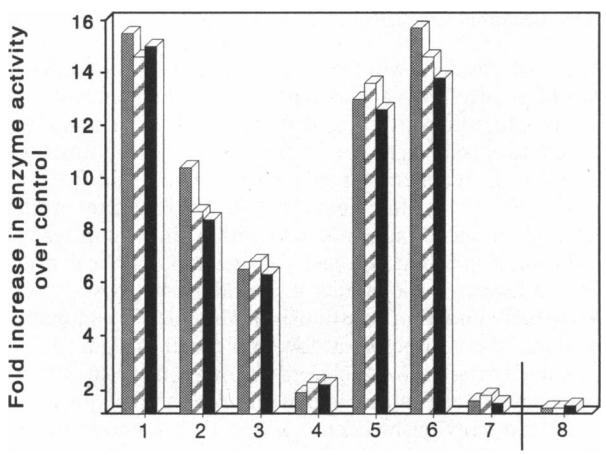

FIG. 4. Induction of the enzymes of the sanguinarine pathway in *E. californica* cell suspension cultures in response to the addition of yeast cell wall elicitor (250 μg/ml; stippled bar), methyl jasmonate (100 μM; hatched bar), or 12-oxo-PDA (100 μM; black bar). Bars: 1, BBE; 2, (*S*)-cheilanthifoline synthase; 3, (*S*)-stylopine synthase; 4, *S*-adenosyl-L-methionine: (*S*)-tetrahydroprotoberberine *cis-N*-methyltransferase; 5, (*S*)-*cis-N*-methylstylopine 14-hydroxylase; 6, protopine 6-hydroxylase; 7, dihydrobenzophenanthridine oxidase; 8, glucose-6-phosphate dehydrogenase.

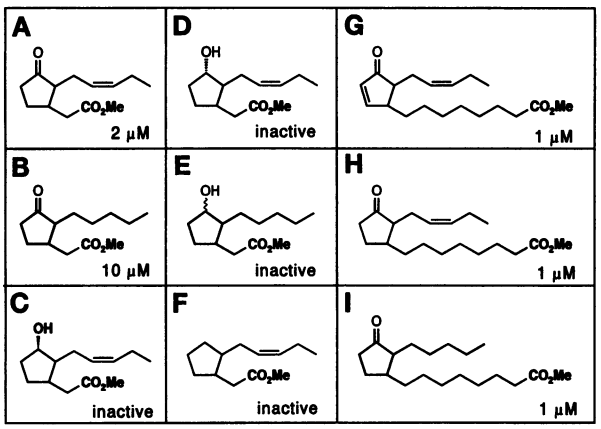

FIG. 5. Induction of benzo[*c*]phenanthridine alkaloid biosynthesis in cell suspension cultures in *E. californica* by pentacyclic oxylipin derivatives. Biological activity is reported as the concentration that resulted in a half-maximal response. Methyl esters of jasmonic acid (*A*), 9,10-dihydrojasmonic acid (*B*), 6-*epi*-cucurbic acid (*C*), cucurbic acid (*D*), racemic 6-hydroxy-9,10-dihydrojasmonic acid (*E*), desoxyjasmonic acid (*F*), 12-oxo-PDA (*G*), 10,11-dihydro-12-oxo-PDA (*H*), and 10,11,15,16-tetrahydro-12-oxo-PDA (*I*) are shown.

membrane-associated enzymes of the sanguinarine pathway, the flavin-containing BBE, and the four highly substrate-specific cytochrome P450-dependent enzymes are induced by transcriptional activation of the corresponding genes. Within experimental error, all three compounds tested induced the membrane-associated enzymes in an equivalent pattern and to an equal extent (Fig. 4). In addition, the alkaloid composition produced under the influence of each of the three potential signal compounds was, within experimental error, the same.

Several pentacyclic jasmonate derivatives were synthesized, and their action on the synthesis of alkaloids in *E. californica* suspension cells was studied. Up to now, variously substituted members of the jasmonate family have been studied only by

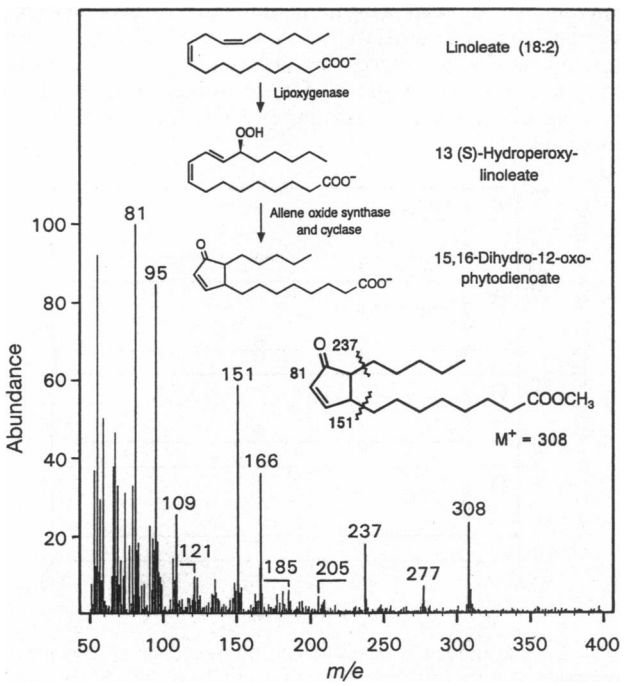

FIG. 6. Proposed biosynthesis of 15,16-dihydro-12-oxo-PDA from linoleic acid and mass spectrum of the target compound (electron impact ionization, mass selective detector).

Weiler *et al.* (14) with regard to their ability to induce tendril coiling. As shown in Fig. 5, C_{18} and C_{12} methyl esters of the jasmonate cascade (12) were chemically modified and tested for their ability to induce alkaloid biosynthesis in *E. californica* suspension cells. As a case of warning, all jasmonate derivatives have to be rigorously purified of potentially highly biologically active contaminants. Thus, the recently synthesized 7-methyl-7-*epi*-jasmonic acid, which was claimed to have shown strong biological activity in the tendril coiling assay (36), was synthesized by an independent route that avoided methyl jasmonate as the starting material and was subsequently shown to be biologically inactive (37). 9,10-Dihydrojasmonate was active; however, it was less so than jasmonate, which was in contrast to the *Bryonia* tendril system where the same dihydro compound was completely inactive (14). The *cis*- and *trans*-6-hydroxy analogues of jasmonate, 6-hydroxy-9,10-dihydrojasmonate and 6-desoxyjasmonate, were totally inactive in inducing secondary product formation as well as in triggering tendril coiling (13), thereby demonstrating the necessity of a 6-oxo group for biological activity. The methyl esters of 12-oxo-PDA and of 10,11-dihydro-12-oxo-PDA, however, were highly active in the California poppy system. 10,11,15,16-Tetrahydro-12-oxo-PDA, a compound that was found to be inactive in the tendril system (13), was active in inducing alkaloid biosynthesis, which demonstrates considerable differences in these two biological responses. The pentenyl side chain of compounds of the linolenic cascade was not necessary for activity in secondary product induction but was required for tendril coiling. This may be a reflection of different receptor systems for these signal compounds.

The biological activity of the 10,11,15,16-tetrahydro-12-oxo-PDA, which formally could arise from linoleic acid, caused us to investigate whether this 18:2 fatty acid could function as a jasmonate precursor in flax seedling extracts (38). Previously, it had been claimed that other polyunsaturated fatty acids in addition to linolenic acid could be utilized as substrates in the synthesis of pentacyclic fatty acids, provided that they were unsaturated at C3, C6, and C9 (23, 39). This excluded linoleic acid, which proved in that particular experiment not to be a substrate (23). Repetition of this experiment, however, demonstrated that linoleic acid could indeed be used as a substrate by flax and clearly resulted in the synthesis of 15,16-dihydro-12-oxo-PDA as shown in the mass spectrum in Fig. 6. The spectrum is consistent with the presence of an unsaturated cyclopentenone ring as well as a pentyl side chain. Reduction of this acid with BH_4^- and reoxidation with Jones reagent yielded 3-oxo-2-pentylcyclopentane octanoic acid. The fact that this series of compounds derived from linoleic acid was active in the induction of low molecular weight defense compounds may cause us to change our picture of the assumed exclusiveness of the linolenic acid cascade in the defense system of higher plants. The fact that linoleic acid was almost as effective in inducing accumulation of proteinase inhibitor proteins in tomato leaves as was wounding (10) may strengthen the case for a linoleic acid cascade. This cascade would furnish, as an end product, 9,10-dihydrojasmonate, which has been found to occur in higher plants and fungi (40, 41). A secondary reduction of the pentenyl side chain of jasmonic acid has, up to now, not been found to occur (42).

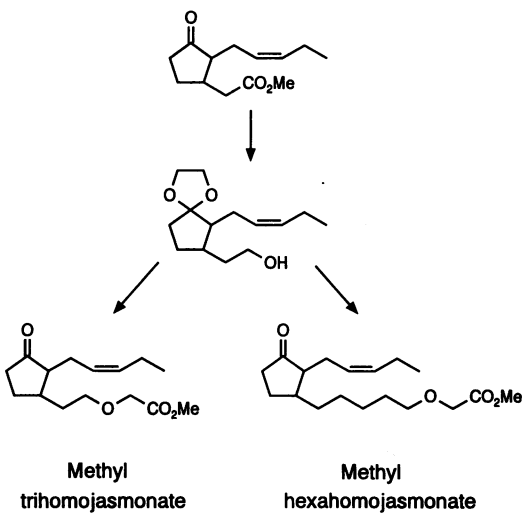

FIG. 7. Scheme for the synthesis of the methyl esters of trihomojasmonate and hexahomojasmonate, molecules in which metabolic β-oxidation is prevented.

Other potential signal compounds such as salicylic acid (at pH 5 up to toxic levels) and systemin were completely ineffective in the *Eschscholtzia* suspension culture test system. However, the microbial product, coronatine, which reveals a striking structural similarity to 12-oxo-PDA, did prove to be effective in the induction of low molecular weight defense compounds (43).

Is β-Oxidation Necessary for Activity? Undoubtedly jasmonic acid is derived from 12-oxo-PDA, which is metabolized via 10,11-dihydro-12-oxo-PDA, which undergoes three cycles of β-oxidation at the carboxyl containing side chain (12). The complete metabolic pathway consists of 15 intermediate steps and demonstrates the complexity of the signal transduction process.

The question, however, remained whether 12-oxo-PDA was genuinely active as a signal substance *per se* or only after β-oxidation to jasmonate. To answer this question, oxa analogues of 12-oxo-PDA that were unable to undergo side chain β-oxidation were synthesized. A similar approach had previously been taken for the mammalian signal molecules, the prostacyclins (44). The synthesis of a 10,11-dihydro-12-oxo-PDA analogue, a hexahomojasmonate, and also of a trihomojasmonate in which the β-carbon had been replaced by an oxygen atom was achieved by the route shown in Fig. 7. Both compounds were carefully freed of any contaminants. Biological activity was tested by exposing *E. californica* cell suspensions to the methyl esters of these substances. The results are depicted in Fig. 8. At a saturating concentration of 100 μM each, methyl jasmonate, 12-oxo-PDA, 10,11-dihydro-12-oxo-PDA, and 10,11,15,16-tetrahydro-12-oxo-PDA were about equal or superior to the yeast cell wall elicitor in provoking

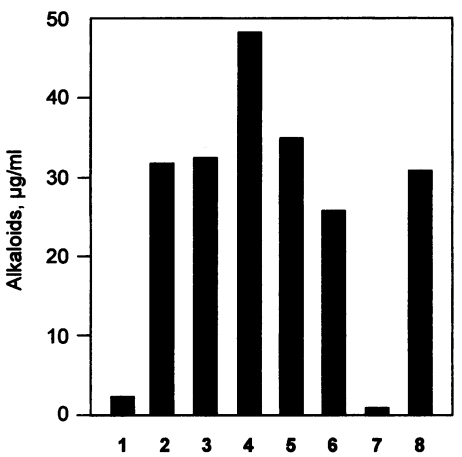

FIG. 8. Benzo[*c*]phenanthridine alkaloid-inducing potential of various synthetic methyl esters of pentacyclic oxylipin derivatives at saturating concentration (100 μM) as compared to yeast cell wall elicitor in *E. californica* cell cultures. Bars: 1, control; 2, yeast cell wall elicitor; 3, methyl jasmonate; 4, 12-oxo-PDA; 5, 10,11-dihydro-12-oxo-PDA; 6, 10,11,15,16-tetrahydro-12-oxo-PDA; 7, hexahomojasmonate; 8, trihomojasmonate.

alkaloid synthesis. However, hexahomojasmonate proved lethal to the suspension cells. Even at lower concentrations, there was only a marginal induction of alkaloids by this compound, accompanied by a toxic effect on the cells. In contrast, the trihomojasmonate was fully active, and the alkaloid pattern was quantitatively and qualitatively the same as that induced by the jasmonate family (Fig. 8). To substantiate these results, the BBE transcript level was monitored after induction with each of the oxa compounds and with jasmonate for comparison. Methyl jasmonate strongly induced BBE poly(A)$^+$ RNA, as did trihomojasmonate at 10-fold higher concentration (Fig. 9). The toxicity of the 12-oxo-PDA oxa analogue suggested that the natural C_{18} compounds, aside from being important signal transducers, may play a role even in poorly understood primary metabolic processes, or they may selectively activate a ribosome-inactivating protein (45), thus leading to cell death. However, the trihomojasmonate oxa compound, which cannot undergo side chain degradation, was biologically active as a defense-inducing signal substance, which clearly demonstrated that β-oxidation is *not* required for physiological activity. We assume, therefore, that 12-oxo-PDA and 10,11-dihydro-12-oxo-PDA are primary signal transducers in the elicitation process. This assumption is in agreement with previous claims (14, 17, 35, 43). Jasmonic acid is, of course, also biologically active in this same signal transduction chain. It potentially is able to interact with the same hypothetical receptor or transactivators, but it should be borne in mind that it is a degradation product of biologically active C_{18} compounds, which can be further inactivated in the cell by conjugation (46).

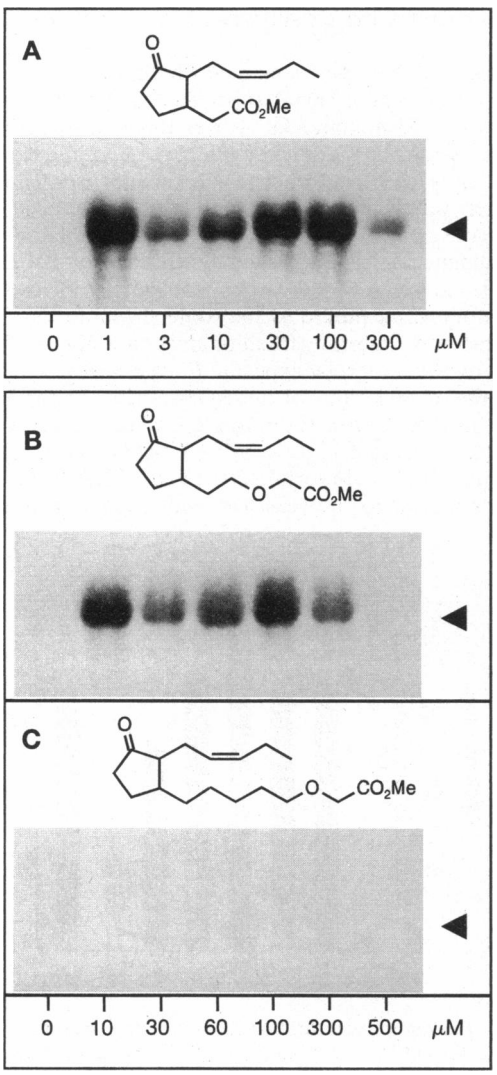

FIG. 9. Dose-dependent response of the induction of BBE poly(A)$^+$ RNA in *E. californica* cell suspension cultures to treatment with methyl jasmonate (*A*), trihomojasmonate (*B*), and hexahomojasmonate (*C*). Arrowheads indicate the position of the BBE transcript.

Release of Signal Transducers. One of the earliest events in the defense gene signaling mechanism is protein phosphorylation, which occurs within seconds after the plant has been treated with a carbohydrate elicitor (47–50). Concomitantly, a rapid basification of the growth medium of these suspension cells was observed (49, 50).

This rise in pH value has been interpreted as the phosphorylation of the protein component of a plasma membrane H$^+$-ATPase (51) or even as an unidentified H$^+$ channel protein (52). The presence of the antibiotic staurosporine was found to eliminate both the extracellular alkalinization and the protein phosphorylation (49). In our continuing interest in elucidating the involvement of lipid-based signaling compounds of the jasmonate family in the elicitation process, we investigated the action of elicitor in the presence of staurosporine on the alkalinization of the growth medium and on jasmonate production. *Phaseolus* cell cultures were used due to good reproducibility of the alkalinization effect. As shown in Fig. 10, the rapid alkalinization process after addition of elicitor (49, 50) was reproducible with this culture. The extracellular pH rose in a biphasic mode immediately after elicitor addition. An increase of almost 1.5 pH units was reached within 20 min after elicitor addition. Simultaneously, jasmonic acid was produced by the *Phaseolus* culture as had been previously observed in this and in various other systems (15,

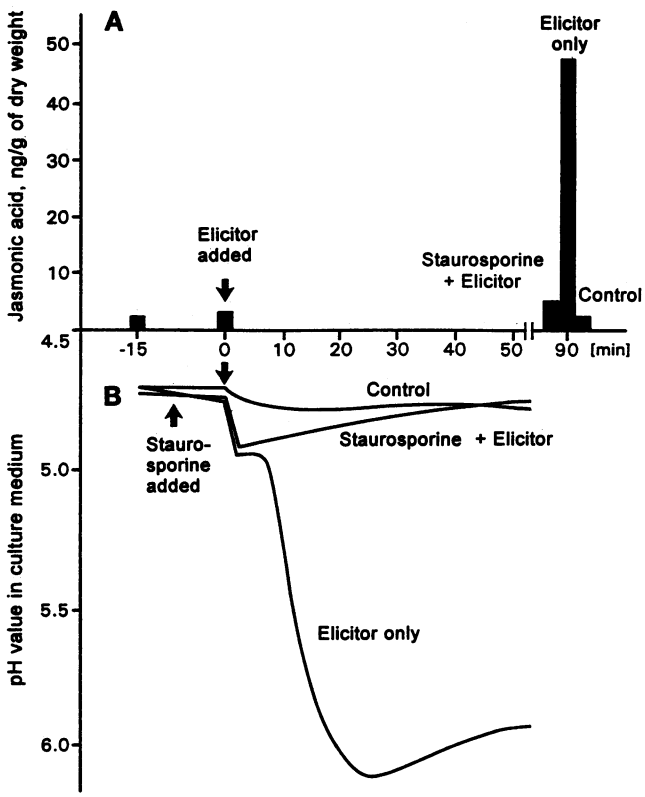

FIG. 10. Effect of staurosporine on elicitor-induced intracellular jasmonic acid accumulation and on extracellular alkalinization of *Phaseolus* cell suspension cultures. Elicitor (250 μg/ml), with or without prior addition (at time −10 min) of staurosporine (10 μg/ml) to the cultures, was added at time zero. (*A*) After 90 min, the intracellular jasmonic acid concentration was measured. (*B*) Extracellular pH was monitored over a period of 75 min.

16). This rise in pH value could be almost completely abolished in the presence of staurosporine (Fig. 10). Staurosporine also completely repressed the formation of jasmonate. Raising the pH of the cell culture medium by addition of base (KOH, NaOH, or NH_4OH) led neither to jasmonate release nor to phytoalexin induction. These experiments may indicate that elicitor-based acidification of the cytosol via a proton pump mechanism leads to the enzyme-catalyzed liberation of linolenic acid from the plasma membrane, as has been observed during the elicitation process (16). α-Linolenic acid, in turn, can be transformed via 12-oxo-PDA and 10,11-dihydro-12-oxo-PDA to jasmonic acid (12).

These correlations, together with previously reported data (20), point toward a signaling sequence in which acidification of the cytosol is followed by liberation of 18:3 or 18:2 fatty acids, which yield lipid-based C_{18} cyclopentanone compounds. These compounds, in turn, mediate signal transduction between the elicitor–receptor complex (53) via transcriptional activation of defense genes to the synthesis of low molecular weight, antibiotic chemicals. These C_{18} signal molecules are subsequently degraded to biologically active jasmonates, which transiently accumulate in the tissue, but are eventually inactivated by conjugation. The foresighted prediction by Vick and Zimmerman (12) for this class of compounds with a then unknown function has been fulfilled in the tremendously important process of plant survival in the ecosystem: "The octadecanoids of plants behave as potent metabolic regulators."

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