Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 2389–2393, March 1992
Plant Biology

Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures

(*Eschscholtzia californica*/*Rauvolfia canescens*/*Glycine max*/benzophenanthridine alkaloid/flavonoid)

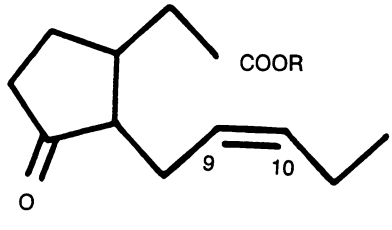
HEIDRUN GUNDLACH, MARTIN J. MÜLLER, TONI M. KUTCHAN, AND MEINHART H. ZENK*

Lehrstuhl für Pharmazeutische Biologie, Universität München, Karlstrasse 29, D-8000 Munich 2, Federal Republic of Germany

Communicated by Eric E. Conn, December 6, 1991 (received for review October 29, 1991)

ABSTRACT **To deter pathogenic microorganisms and herbivores, plants have developed an inducible chemical defense system. It is known that the induced synthesis of low molecular weight compounds can be provoked by exposing cultured cells to fungal cell wall fragments. In this study we show that endogenous jasmonic acid and its methyl ester accumulate rapidly and transiently after treatment of plant cell suspension cultures of *Rauvolfia canescens* and *Eschscholtzia californica* with a yeast elicitor. Thirty-six plant species tested in cell suspension culture could be elicited with respect to the accumulation of secondary metabolites by exogenously supplied methyl jasmonate. Addition of methyl jasmonate initiates *de novo* transcription of genes, such as phenylalanine ammonia lyase, that are known to be involved in the chemical defense mechanisms of plants. These data demonstrate the integral role of jasmonic acid and its derivatives in the intracellular signal cascade that begins with interaction of an elicitor molecule with the plant cell surface and results, ultimately, in the accumulation of secondary compounds.**

Plant defense against pathogens or herbivores involves the induced synthesis of low molecular weight compounds called phytoalexins (1, 2). Fungal cell walls and fragments thereof (biotic elicitors) (3) trigger this defense response even in plant cell suspension cultures (4). The elicitor molecule interacts with a plant membrane receptor (5) that through an unknown mechanism activates specific genes (6), resulting in the synthesis of almost all chemical classes of secondary plant products (7). Jasmonic acid and its methyl ester have been



For jasmonic acid, R is H; for methyl jasmonate, R is CH_3.

shown to be natural hormonal regulators controlling plant senescence (8, 9), to induce proteinase inhibitor proteins in response to wounding and pathogen attack (10), and to trigger tendril coiling upon mechanical stimulation (11).

It has been recently suggested that jasmonic acid could be an integral part of a general signal transduction system regulating inducible defense genes in plants (10). In search of the signal chain between the elicitor–receptor complex and the gene activation process, we have identified that, in suspension cultures of plant species representing a wide taxonomic distribution, jasmonic acid and its established precursors [e.g., α-linolenic acid (12)] have a position in this molecular cascade of events. Jasmonate is rapidly synthesized in response to treatment with a fungal elicitor in suspension cultures of *Rauvolfia* and *Eschscholtzia*, and exogenously applied methyl jasmonate induces, in the absence of elicitors, in all the plant cell suspension cultures tested, the synthesis of specific low molecular weight compounds. Moreover, exogenously applied methyl jasmonate induces *de novo* transcription of the gene (13) of the key enzyme of the phenylpropanoid pathway, phenylalanine ammonia lyase (PAL) (14), resulting in elevated levels of active enzyme in soybean (*Glycine max*) cell suspension cultures. The jasmonates are, therefore, key signal compounds in the elicitation process leading to *de novo* transcription and translation and, ultimately, to the biosynthesis of secondary metabolites in plant cell cultures.

MATERIALS AND METHODS

Plant Cell Cultures. Cell suspensions of *Corydalis claviculata, Crotolaria cobalticola, Eschscholtzia californica, G. max, Lactuca sativa, Lycopersicum esculentum, Rauvolfia canescens, Rubia tinctorum, Ruta chalepensis*, and *Sarcocapnos crassifolia* from the departmental culture collection were grown in Linsmaier and Skoog medium (15) on a gyratory shaker (100 rpm) at 24°C in continuous light (650 lux) for 7 days. Cells were harvested under sterile conditions by filtration, 200 g (fresh weight) was suspended in 1.0 liter of fresh medium in Fernbach flasks, and growth was continued for 3 days. For jasmonate analysis, cells were elicited (10–100 μg of yeast elicitor added per ml of cell suspension) and 60 ml was removed every 10 min for 2 hr. Control cells were treated in an identical manner except that 10 ml of H_2O was added instead of elicitor.

Most secondary metabolite elicitation experiments were conducted in 24-well Nunc multiculture dishes, each well containing 1 ml of cell suspension under sterile conditions, agitated on a reciprocal shaker at 140 strokes per min (16). Cells were harvested and the extracts were obtained as described (16). Secondary compounds were identified by comparison of HPLC retention times to those of reference compounds and/or by mass spectral and/or NMR analyses. Metabolites from *Glycine* and *Rubia* were analyzed as their aglycones. The yeast elicitor was prepared as described (17, 18).

Enzyme and RNA Analysis. PAL activity was measured spectrophotometrically (19) and the berberine bridge enzyme activity was assayed as described (20). RNA was isolated from 5 g (fresh weight) of *G. max* or *E. californica* cell suspension cultures and was fractionated by standard techniques (21). Northern blots of RNA from *G. max* and *E. californica* were hybridized to PAL cDNA (from *Petroseli-*

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Abbreviations: PAL, phenylalanine ammonia lyase; GC/MS, gas chromatography/mass spectrometry.

*To whom reprint requests should be addressed.

num crispum) and to berberine bridge enzyme cDNA (22), respectively; each DNA was labeled by nick-translation.

Jasmonate Determinations. Suspension cells (60 ml) grown under the conditions described above were removed under sterile conditions, rapidly filtered, and shock-frozen in liquid N_2. The frozen cells were thawed in 30 ml of ethanol, to which was immediately added 100 ng of 9,10-dihydrojasmonic acid methyl ester and 100 ng of 9,10-dihydrojasmonic acid (synthesized from jasmonic acid or its methyl ester by catalytic hydrogenation with Pd/charcoal) as internal standards. Samples were sonicated for 1 min; the tissue was separated and extracted a second time with 30 ml of ethanol. The ethanolic extracts were evaporated at 40°C to dryness and the residue was dissolved in 50 ml of H_2O. The solution was acidified with 3 ml of 12 M HCl and was extracted with three 50-ml volumes of $CHCl_3$. The organic phase was dried over Na_2SO_4 and evaporated. The residues were dissolved in 100 μl of methanol and an excess of diazoethane in ether (1 ml) was added. After 30 min at room temperature, the sample was evaporated to dryness, the residue was dissolved in 1 ml of *n*-hexane and was applied to a silica solid-phase extraction column (J. T. Baker Bakerbond SPE silica gel, 500 mg, 3 ml). The column was washed with 5 ml of *n*-hexane and eluted with 7 ml of *n*-hexane/diethyl ether, 2:1 (vol/vol). The sample was taken to dryness, dissolved in 20 μl of methanol and 3 μl was analyzed by gas chromatography/mass spectrometry (GC/MS) [Varian 3400 gas chromatograph linked to a Finnigan MAT quadrupole SSQ 700 mass spectrometer; column, J & W Scientific DB-5 (30 × 0.25 mm); linear He flow at 23 cm/s; column temperature step gradient 50°C for 1 min, 50–160°C at 30°C/min, 160–200°C at 5°C/min, 200–290°C at 30°C/min, 290°C for 5 min; electron potential, 70 eV]. Retention times were as follows: methyl jasmonate, 11.00 min; methyl 9,10-dihydrojasmonate, 11.05 min; ethyl jasmonate, 12.00 min; ethyl 9,10-dihydrojasmonate, 12.05 min; α-linolenic acid ethyl ester, 16.03 min.

Methyl jasmonate was obtained from Serva. Jasmonic acid was prepared by alkaline hydrolysis of the methyl ester. Both compounds were analyzed by GC/MS prior to application to cell suspension cultures (5 μl of an ethanolic solution was added per ml of cell suspension).

RESULTS

To establish the presence of jasmonic acid and/or its methyl ester in plant cell suspension cultures, six species in culture [four taxonomically distant (*E. californica, Rauvolfia canescens, G. max*, and *Lycopersicum esculentum*) and two taxonomically related (*Corydalis claviculata* and *S. crassifolia*)] were analyzed and both compounds were found in each culture at an average concentration of 25 ng/g (dry weight) of cells. *Rauvolfia canescens* (Apocynaceae), a strain showing for over a decade consistent growth and response to elicitation with increased indole alkaloid formation, was used in an analysis of the effect of elicitation on endogenous jasmonate concentration.

Twenty to 30 min after challenging cell suspension cultures of *Rauvolfia canescens* with a yeast cell wall preparation (100 μg/ml of medium), a transient increase in the concentration of jasmonic acid was observed (Fig. 1*A*). A peak concentration of the free acid [1370 ng/g (dry weight)] was reached 45 min after application of elicitor. The concentration of jasmonic acid gradually declined, showing a slight, but consistant, increase at ≈100 min after elicitation. The endogenous concentration of methyl jasmonate increased [108 ng/g (dry weight)] only at 100 min, remaining level throughout the rest of the period monitored. Unelicited control cultures showed no fluctuation in jasmonate concentration within experimental error (Fig. 1*B*). Using the same methodology, a similar induction profile was obtained for jasmonic acid and its ester

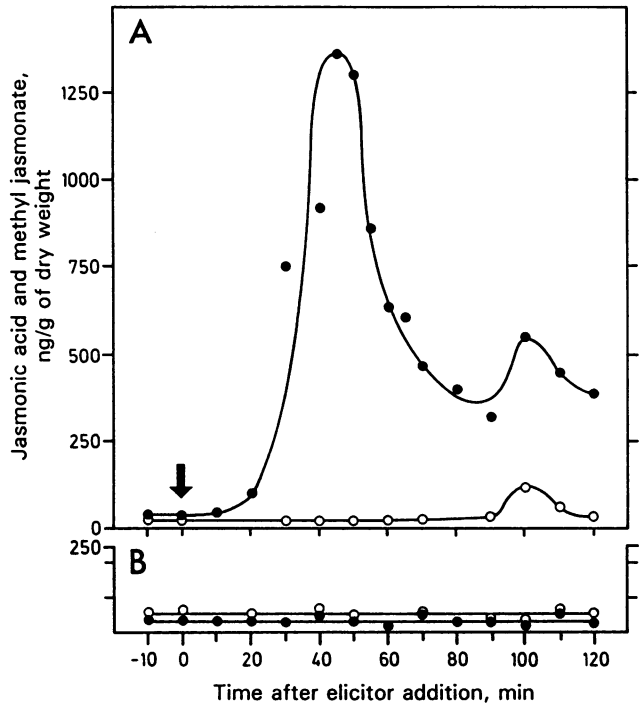

FIG. 1. Induction of endogenous jasmonates by addition of yeast cell wall elicitor to cell suspension cultures of *Rauvolfia canescens*. Changes in the abundance of jasmonic acid (●) and methyl jasmonate (○) in elicited (*A*) and nonelicited (*B*) cells are shown. Arrow indicates the point of elicitor addition.

in cell suspension cultures of *E. californica* (Papaveraceae) for which the elicitation of benzo[*c*]phenanthridine alkaloid accumulation has been reported (16). Sixty minutes after induction by a yeast cell wall preparation (10 μg/ml of medium), jasmonic acid, methyl jasmonate, and α-linolenic acid [590, 610, and 6600 ng/g (dry weight), respectively] reached their maxima. This peak was, as found for *Rauvolfia canescens*, accompanied by a subsequent, rapid decline. In both cell culture systems, induced jasmonic acid was unequivocally identified by GC/MS as the ethyl ester MS (electron impact) *m/z* 238 (35) (M^+) 193 (15), 170 (17), 151 (60), 135 (20), 109 (35), 95 (46), 83 (100), 67 (62), 55 (100). Analogously, methyl jasmonate MS (electron impact) *m/z*

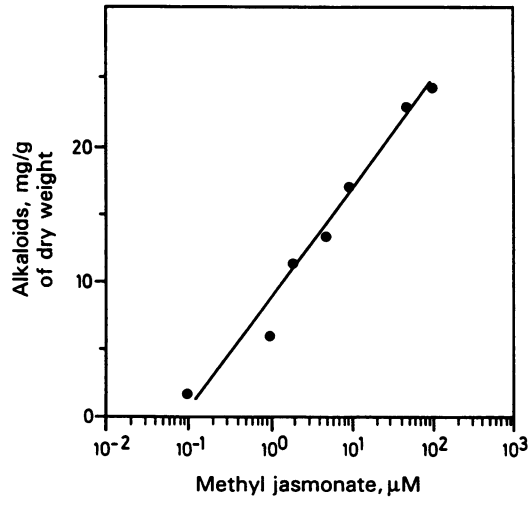

FIG. 2. Induction of total benzo[*c*]phenanthridine alkaloids in cell suspension cultures of *E. californica* 120 hr after the addition of methyl jasmonate. Each concentration of methyl jasmonate was added to the cultures in 5 μl of ethanol per ml of culture medium.

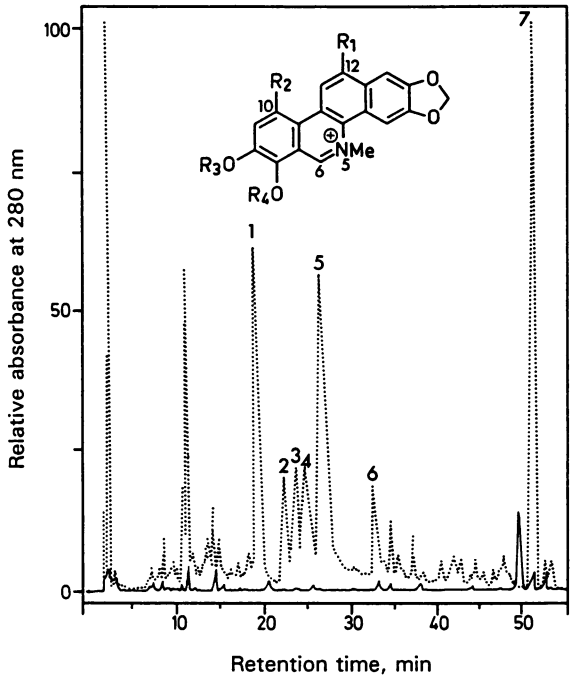

FIG. 3. HPLC profile of an alkaloid-containing extract 136 hr after addition of methyl jasmonate (100 μM) to *E. californica* cell suspension cultures. Induced cultures, dotted line; untreated cultures, solid line. Peaks in the HPLC profile: 1, sanguinarine ($R_1 = R_2 = H$; $R_3 + R_4 = -CH_2-$); 2, 12-hydroxychelirubine ($R_1 = OH$; $R_2 = OCH_3$; $R_3 + R_4 = -CH_2-$); 3, 10-hydroxychelerythrine ($R_1 = H$; $R_2 = OH$; $R_3 = R_4 = CH_3$); 4, chelerythrine ($R_1 = R_2 = H$; $R_3 = R_4 = CH_3$); 5, chelirubine ($R_1 = H$; $R_2 = OCH_3$; $R_3 + R_4 = -CH_2-$); 6, macarpine ($R_1 = R_2 = OCH_3$; $R_3 + R_4 = -CH_2-$); 7, 5,6-dihydrochelirubine ($R_1 = H$; $R_2 = OCH_3$; $R_3 + R_4 = -CH_2-$; C_5-C_6 saturated).

224 (M^+) and the ethyl ester of α-linolenic acid MS (chemical ionization, isobutane) m/z 307 $(M+H)^+$ yielded spectra identical to authentic standards. The here observed induction of

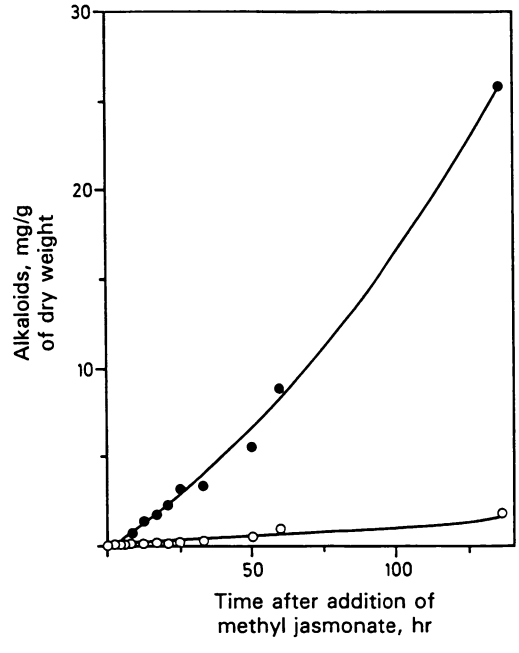

FIG. 4. Time course of the induction of benzo[*c*]phenanthridine alkaloid accumulation in methyl jasmonate (100 μM)-treated cell suspension cultures of *E. californica*. ●, Methyl jasmonate-induced cultures; ○, untreated cultures.

jasmonic acid is the most rapid transient accumulation of an apparent organic signal molecule in the elicitation process reported.

To further demonstrate that the accumulation of jasmonic acid and methyl jasmonate is indeed triggering the biosynthesis of secondary defense compounds in plant cell suspension cultures, a culture of *E. californica*, which was known to respond to fungal elicitors with the formation of a variety of hydroxylated and methoxylated benzo[*c*]phenanthridine alkaloids (16, 23), was exposed to methyl jasmonate. This ester is believed to be, at least, partially hydrolyzed by endogenous esterases to free jasmonic acid within the plant tissue. Benzo[*c*]phenanthridine alkaloids accumulated in a dose-dependent manner, with maximal induction at 0.1 mM jasmonate (Fig. 2). By considering that the physiologically active epimer of jasmonate [(+)-epijasmonate] represents only 5% of the total jasmonate in natural and synthetic samples (24, 25) due to rapid epimerization and that exogenously applied jasmonate is rapidly metabolized in plants (26), the physiological concentration measured after elicitation (Fig. 1) and the optimal exogenously applied concentration of the correct stereoisomer were both in the range of 1–10 μM. The HPLC profile of the alkaloid fraction of control and jasmonate-elicited cultures showed a general induction of alkaloid accumulation in the *E. californica* culture (Fig. 3). This resembled the profile obtained when *E. californica* cultures were challenged with a yeast cell wall preparation (16). Control cultures contained total benzo[*c*]phenanthridine alkaloids at 18 mg/liter of culture medium [1.3 mg/g (dry weight)], yeast-elicitor-treated optimally stimulated cultures contained 265 mg/liter of medium [26 mg/g (dry weight)], and methyl jasmonate (100 nmol/ml)-exposed cultures contained 271 mg/liter of medium [27 mg/g (dry weight)]. The exposure of *E. californica* cultures to 100 μM methyl jasmonate caused a maximal induction of alkaloids that could be detected within 6 hr after the addition of the signal substance to the culture medium. Alkaloids continued to accumulate at an almost linear rate for at least 136 hr (Fig. 4). Cell cultures not treated with jasmonate showed only a marginal accumulation of alkaloids.

The induction of secondary metabolism by jasmonate was not restricted to select cell cultures (Table 1). A number of suspension cultures from the departmental culture collection,

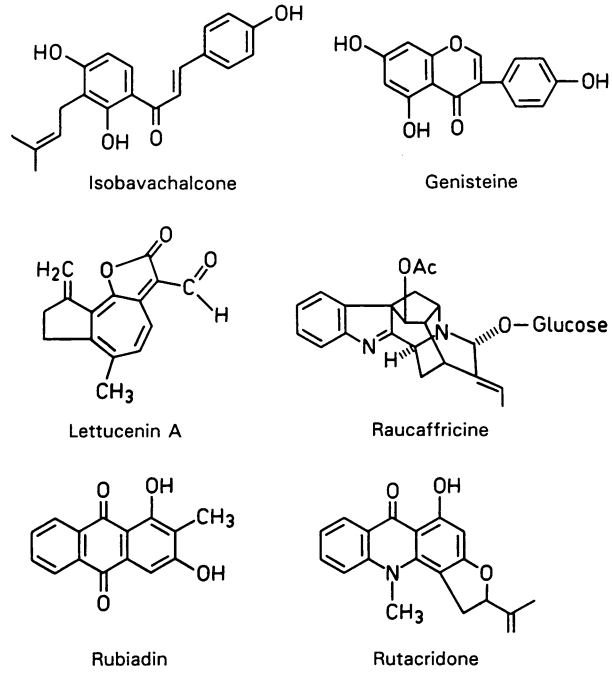

Table 1. Main secondary metabolite of known structure induced by methyl jasmonate administered to various cell cultures

Species	Metabolite induced	Metabolite, mg/liter of medium: Control culture	Metabolite, mg/liter of medium: Induced culture
Crotolaria cobalticola	Isobavachalcone	4	50
G. max	Genisteine	2	49
Lactuca sativa	Lettucenin A	0.2	6
Rauvolfia canescens	Raucaffricine	7	203
Rubia tinctorum	Rubiadin	7	78
Ruta chalepensis	Rutacridone	9	83

Methyl jasmonate (250 μM) was added to cultures 96 hr prior to cell harvest. The metabolites were quantitated by HPLC.

for which the main metabolite had been identified, were exposed to methyl jasmonate and the resultant concentrations of secondary compounds were identified and quantitated by HPLC. Table 1 shows six taxonomically distantly related cell cultures, producing in each case a unique major secondary compound of defined chemical structure. It is clearly seen that methyl jasmonate induced these metabolites by a factor of 9–30 over the control values in these systems. Induction by jasmonate does not appear to be specific to any one type of secondary metabolite but rather general to a wide spectrum of low molecular weight substances ranging from (in this limited survey) flavonoids, guaianolides, and anthraquinones to various classes of alkaloids (Table 1). A total of 36 species in culture belonging to mono- and dicotyledonous plants responded to treatment with various concentrations (0.1–500 μM) of methyl jasmonate as judged by the formation of low molecular weight compounds (HPLC analysis; detection at 280 nm). Thus far, all plant cell suspension culture systems tested responded to exposure to jasmonate with the accumulation of low molecular weight compounds.

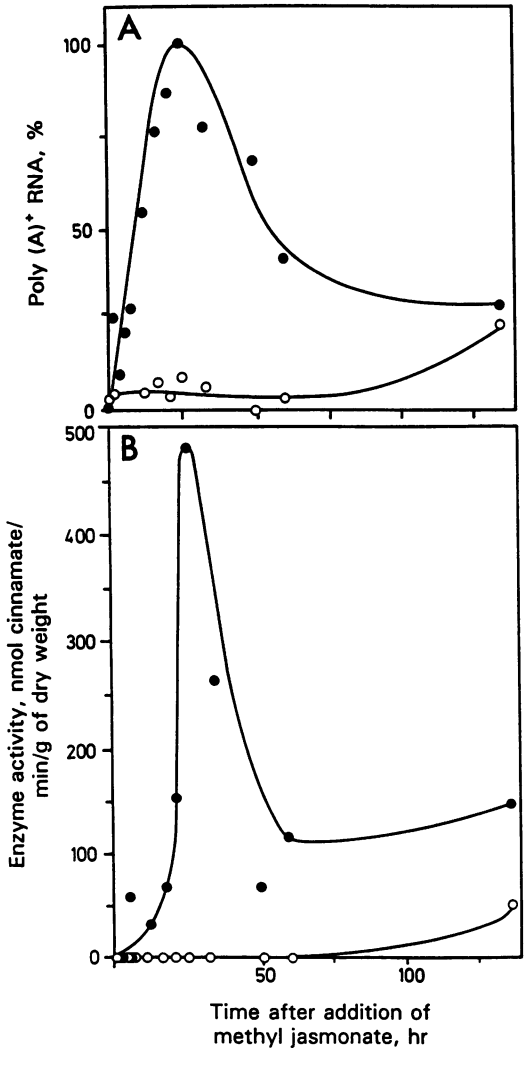

FIG. 5. Induction of PAL in *G. max* cell suspension cultures in response to treatment with methyl jasmonate (250 μM). Changes in the abundance of PAL poly(A)$^+$ RNA (*A*) and PAL enzyme activity (*B*) are shown. Both determinations were done from one cell culture harvested at each time point. ●, Methyl jasmonate-induced cultures; ○, untreated cultures.

Final proof that the exposure of plant cell cultures to methyl jasmonate leads to an increase in expression of the genes involved in secondary metabolism was provided by the measured increase in PAL poly(A)$^+$ RNA (27) followed by an increase in PAL enzyme activity, indicative of *de novo* transcription, translation, and flavonoid biosynthesis, in elicited soybean cell suspension cultures. After addition of 250 μM methyl jasmonate, this culture produced total flavonoids at 75 mg/liter as compared to 4 mg/liter in the control culture. PAL poly(A)$^+$ RNA and enzyme activity were strongly induced, reaching maximal values at 25 hr (Fig. 5*A*) and 33 hr (Fig. 5*B*), respectively, after the addition of methyl jasmonate. This inductive effect was also shown to proceed with *de novo* transcription and translation along the benzo[*c*]phenanthridine alkaloid biosynthetic pathway in *E. californica* cell suspension cultures by the increase in the central enzyme, the berberine bridge enzyme (20, 22), poly(A)$^+$ RNA (maximum at 6 hr after methyl jasmonate addition, a 5-fold increase over control) that preceded an increase in berberine bridge enzyme activity (maximum at 17 hr after methyl jasmonate addition, a 12-fold increase over control). The profiles obtained with methyl jasmonate induction were similar to those obtained with yeast-cell-wall-elicited cultures (22).

DISCUSSION

Exposure of cell suspension cultures of *Rauvolfia canescens* and *E. californica* to a yeast cell wall elicitor leads to the rapid transient induction of endogenous jasmonic acid and methyl jasmonate. Elicitation of cell cultures with fungal cell wall components has long been known to result in the synthesis of low molecular weight compounds (4). The biosynthetic pathways leading to these compounds have, in the case of flavonoids and benzo[*c*]phenanthridine alkaloids, been completely elucidated (28, 29), and the principal genes and certain elements governing their regulation have been identified (13). This elicitation process is now known to proceed with gene activation leading, ultimately, to the synthesis of biosynthetic enzymes and the metabolites they form. Jasmonic acid and its derivatives have an integral role in the cascade of events that occur in the elicitation process, causing either directly or indirectly the activation of the genes of secondary metabolism. There is one report (30) of the induction of secondary plant product accumulation (anthocyanin) in response to treatment of germinating soybean seedlings with methyl jasmonate, indicating that this defense mechanism is operative not only in plant cell suspension cultures but also in differentiated plants. In agreement with previously presented proposals for other plant systems (10, 11), the results reported here support the following hypothetical mechanism: an elicitor–receptor complex activates a lipase, thereby releasing α-linolenic acid, which is then transformed by constitutive enzymes (12) to jasmonic acid and methyl jasmonate. Whether the elicitation of phytoalexin biosynthesis also involves the recently discovered peptide systemin (31) is yet to be investigated.

The fact that exogenously supplied methyl jasmonate activates, in different plant systems, a multitude of "jasmonate-induced proteins" (32) can now be explained in a satisfactory manner by the fact that, in each plant, a multitude of species-specific genes involved in the formation of high (10) and low molecular weight compounds are expressed in response to these signal-transducer molecules. Without knowledge of its function in plants, jasmonic acid has been compared to the prostaglandins, chemically similar mammalian hormones (33). This hypothesis may now prove to be correct in light of the induction of proteinase inhibitors (10), storage proteins (30), and tendril coiling (11) and the results presented here.

We thank Prof. K. Hahlbrock (Cologne, F.R.G.) for providing us with the *P. crispum* PAL cDNA clone. This work was supported by SFB 145 of the Deutsche Forschungsgemeinschaft, Bonn, by the Bundesminister für Forschung und Technologie, Bonn, and by the Körber Foundation, Hamburg.

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