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Methyl jasmonate-induced rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures

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Summary. A dramatic increase in rosmarinic acid (RA) content in cultured cells of *Lithospermum erythrorhizon* was observed after their exposure to methyl jasmonate (MJ). Preceding the induced RA accumulation, phenylalanine ammonia-lyase (PAL) and 4-hydroxyphenylpyruvate reductase (HPR) activities increased rapidly and transiently, whereas tyrosine aminotransferase (TAT) activity showed only a slight increase. The elicitation activity of MJ was much higher than that of yeast extract (YE) in terms of the induction of PAL and HPR activities, RA accumulation and incorporation of both ^{14}C-phenylalanine and ^{14}C-tyrosine into RA. However, the response of the cultured cells to MJ-treatment was slower than that to YE-treatment.

Key words: Rosmarinic acid - Biosynthesis - Elicitation - Methyl jasmonate - *Lithospermum erythrorhizon*

Abbreviations: 2,4-dichlorophenoxyacetic acid, 2,4-D; Linsmaier and Skoog, LS; 4-hydroxyphenylpyruvate reductase, HPR; phenylalanine ammonia-lyase, PAL; tyrosine aminotransferase, TAT; methyl jasmonate, MJ; yeast extract, YE.

INTRODUCTION

Rosmarinic acid (α-O-caffeoyl-3,4-dihydroxyphenyllactic acid; RA) is a common hydroxycinnamoyl ester accumulated in plants belonging to the families Boraginaceae and Lamiaceae as well as in the cultured cells derived therefrom. RA is biosynthesized by condensation of 4-coumaroyl CoA with 4-hydroxyphenyllactic acid, catalyzed by RA synthase (Petersen 1991). 4-Coumaroyl CoA is synthesized through the phenylpropanoid pathway, the initial step of which is catalyzed by phenylalanine ammonia-lyase (PAL), while the 4-hydroxyphenyllactic acid moiety is derived from L-tyrosine (Ellis and Towers 1970). An entry point enzyme of the tyrosine-derived pathway was shown to be tyrosine aminotransferase (TAT) (De-Eknamkul and Ellis 1987), which produces 4-hydroxyphenylpyruvic acid from L-tyrosine. 4-Hydroxyphenylpyruvate is then converted to 4-hydroxyphenyllactic acid by hydroxyphenylpyruvate reductase (HPR) (Häusler et al. 1991). RA formation provides an excellent model to investigate regulatory mechanisms of secondary metabolism because two parallel and presumably concertedly regulated pathways are involved in its biosynthesis.

The enzymology and regulation of RA biosynthesis have been actively investigated by using cell cultures of *Anchusa officinalis* (Boraginaceae) (Mizukami and Ellis 1991, and literature cited therein) and of *Coleus blumei* (Lamiaceae) (Petersen et al. 1992, and literature cited therein). However, detailed analysis of the regulatory mechanisms of RA biosynthesis would be facilitated by development of a cell culture system where RA biosynthesis can be rapidly induced by manipulating the cell environment since either *Anchusa* or *Coleus* cells display substantial levels of constitutive RA biosynthesis. In a previous paper (Mizukami et al. 1992), we reported that RA biosynthesis was rapidly and transiently induced by addition of yeast extract (YE) to *Lithospermum erythrorhizon* cell suspension cultures. In *L. erythrorhizon* cells, changes in PAL levels were shown to be closely linked to the elicitation of the phenylpropanoid pathway by YE treatment. However, the enzyme(s) involved in activation of the tyrosine derived-pathway remains uncertain, since TAT activity did not respond to YE elicitation. Similar induction of RA biosynthesis was also described in *Orthosiphon aristatus* cell cultures (Sumaryono et al. 1991).

Recently jasmonic acid and its methyl ester, methyl jasmonate(MJ), have been proposed to be key signal compounds in the process of elicitation leading to the accumulation of various secondary metabolites (Gundlach et al. 1992). The jasmonates have been shown to induce anthocyanin accumulation in *Glycine max* (Franceschi and Grimes 1991), and to control a number of plant development processes (Staswick 1992; Weiler et al. 1993). They have also been reported to play an important role in a signal transduction process that regulates defense genes in plants (Farmer and Ryan 1990).

In order to examine the possibility that MJ is involved in a signal transduction pathway leading to elicitation of RA biosynthesis, we examined the stimulatory effect of MJ on RA biosynthesis in cell suspension cultures of *Lithospermum erythrorhizon*. We also describe here that the associated changes in PAL and HPR activities are consistent with these enzymes serving as regulatory points in the phenylpropanoid pathway and tyrosine-derived pathway, respectively.

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MATERIALS and METHODS

Plant material: Suspension cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) were established from seedling-derived callus tissues grown on LS agar medium (Linsmaier and Skoog 1965). Cultures were maintained in LS liquid medium supplemented with 1 μM 2,4-D and 1 μM kinetin. Cell suspension (5 ml) was inoculated into 25 ml fresh medium in a 100 ml Erlenmeyer flask at 14-day intervals and cultured at 110 rpm on a gyratory shaker at 25°C in the dark.

Chemicals: Methyl jasmonate and yeast extract was purchased from Tokyo Kasei Co. and Difco, respectively. Sephadex G-25 was a product of Pharmacia and dye-reagent for protein assay was obtained from Bio-Rad. L-[U-^{14}C]Phenylalanine and L-[U-^{14}C]tyrosine were purchased from Amersham and silica gel 60F TLC plates from Merck Co. All other chemicals were of reagent grade.

Elicitor treatment: MJ was dissolved in ethanol and added to the suspension cultures at a final concentration of 100 μM. YE was dissolved in water and added to the cultures at a final concentration of 5g/l, the optimal concentration for induction of RA biosynthesis (Mizukami et al. 1992). The suspension cultures were treated with the elicitors 6 days after cell inoculation. The cells were collected at fixed time intervals by vacuum filtration, frozen in liquid nitrogen and stored at -70°C.

Enzyme extraction: PAL and TAT were extracted as reported earlier (Mizukami and Ellis 1991). For extraction of HPR the frozen cells (about 1.5g) were mixed with 0.5g polyvinylpolypyrrolidone and 6.0 ml of 0.1M potassium phosphate buffer, pH 7.0, containing 1 mM dithiothreitol. The buffer was flushed with nitrogen for 5 min immediately before use. The cells were thawed in the buffer with continuous stirring for 30 min and the slurry was centrifuged at 11,000 x*g* for 5 min. HPR was precipitated from the supernatant with 40-70% ammonium sulfate saturation. Precipitated protein was dissolved in the extraction buffer containing 0.3M NaCl. The solution was desalted on a Sephadex G-25 column and used as an enzyme preparation. All treatments in buffer were carried out at 4°C. Protein content in the enzyme extract was determined by the method of Bradford (1976).

Enzyme assay: PAL and TAT were assayed as described elsewhere (Mizukami and Ellis 1991). HPR assay was carried out according to the method decribed by Häusler et al. (1991) with slight modifications. Enzyme preparation (50 μl) was added to 250 μl aliquots of reaction mixture containing 100 mM potassium phosphate (pH 7.0), 1.0 mM 4-hydroxyphenylpyruvic acid, 2.0 mM NADH and 0.04 mM sodium ascorbate, 50 μl enzyme preparation was added and incubated for 60 min at 30°C. The reaction was terminated by adding 50 μl 5 M HCl. The mixture was extracted with 0.3 ml ethyl acetate three times. The combined ethyl acetate extract was evaporated and the residue was dissolved in 100 μl of methanol-water (adjusted to pH 3 with phosphoric acid) (67:33). The amount of 4-hydroxyphenyllactic acid was quantified by HPLC. Conditions for HPLC were as follows: Column; YMC-Pack-ODS (YMC Co.) 150 x 4.6 mm; solvent system, water-methanol-phosphoric acid (80:20:0.01); flow rate, 1.0 ml/min; detection, 280 nm.

Determination of RA content: The extraction and quantification assay of RA were carried out according to the method described in the previous paper (Mizukami et al. 1992).

Feeding experiments: L-[U-^{14}C]Phenylalanine (0.5μCi in 0.3μmol) and L-[U-^{14}C]tyrosine (1 μCi in 0.3μmol) were separately added to 6-day-old suspension cultures 2 hr before addition of MJ or YE. After 48 hr incubation under normal culture conditions, the cells were harvested and 0.5 g portions were extracted with 1.25 ml methanol for 60 min at 70°C with vigorous shaking. The slurry was centrifuged at 11,000 x*g* for 5 min. A 50 μl aliquot of the supernatant was applied to a silica gel 60F plate, which was then developed with ethyl acetate-methanol-water (100:25:15). The band corresponding to RA was detected under UV (254 nm), scraped off and the radioactivity was measured by liquid scintillation counting. Uptake of the ^{14}C-labeled aromatic amino acids was calculated from the radioactivity remaining in the culture medium after the cells were harvested.

RESULTS

MJ (100 μmol/l) and YE (5 g/l) were separately added to *Lithospermum erythrorhizon* suspension cultures 6 days after cell inoculation, and RA accumulation in the cells was monitored for 3 days (Fig. 1). In the YE-treated cells, RA content increased after a lag of 8 hr and reached a maximum 24 hr after treatment, which is consistent with

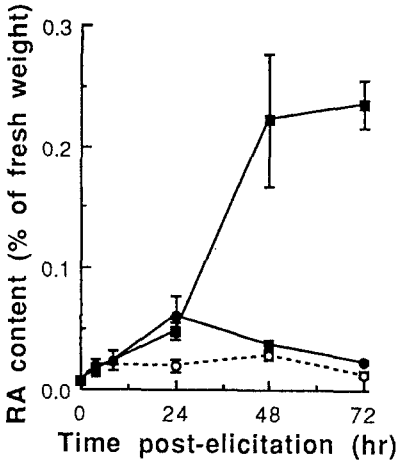

Fig. 1 Changes in RA content in cultured cells of *L. erythrorhizon* treated with methyl jasmonate (MJ) (closed squares) or yeast extract (YE) (closed circles). Open circles with dotted lines represent the control cells treated with water. MJ was added in 100 μl ethanol per culture. Cells treated with ethanol (100μl) behaved in similar manner to cells treated with water. Each point indicates an average from duplicate cultures with a standard error. The entire experiment was repeated twice with essentially the same result, and one representative data set is shown here.

the results described previously (Mizukami et al. 1992). The extent of the increase in the RA content was about 3-fold, relative to the content of the control cells. In contrast, MJ strongly induced RA accumulation after a lag of 24 hr, reaching a maximum 48-72 hr after treatment. RA accumulated to a maximum amount of 0.22 % of cell fresh weight in the MJ-treated cells, which is 10-fold higher than that in the control cells. The amounts of RA in the culture medium were negligible in either elicitor-treated or control cultures.

Elicitor-induced changes in the activity of the biosynthetic pathway leading to RA were evaluated by feeding experiments using radiolabelled phenylalanine and tyrosine

(Table 1). Addition of MJ or YE to *L. erythrorhizon* cell cultures enhanced incorporation of the radioactivity into RA

Table 1 Incorporation of radiolabelled phenylalanine and tyrosine into RA in cultured *L. erythrorhizon* cells treated with elicitors.

		% incorporation to RA		
Precursors	Treatment	Culture 1	Culture2	Average
Phenylalanine	MJ	13.5	16.1	14.8
	YE	6.2	7.1	6.7
	water	3.1	3.1	3.1
Tyrosine	MJ	17.7	22.1	19.9
	YE	8.4	8.2	8.3
	water	3.4	3.5	3.5

L-[U-^{14}C]Phenylalanine(0.5μCi)and L-[U-^{14}C]tyrosine (1μCi) were added to 6-day-old cultures 2 hr before addition of elicitors and incubated for further 48 hr. Incorporation rates were represented as % of the radioactivity taken by cells. The data are from duplicate cultures.

from either precursor. The rates of incorporation of ^{14}C-phenylalanine and ^{14}C-tyrosine in the MJ-treated cells were about 5-fold higher than the incorporation in the control cells, whereas the YE-treated cells showed about 2-fold higher incorporation of ^{14}C-precursors compared with the control cells. Uptake of the radiolabelled aromatic amino acids by the cultured cells was about 95% and did not differed between elicitor and control treatments.

The activity of PAL, the entry point enzyme of the phenylpropanoid pathway, rapidly and transiently increased after addition of MJ or YE, which correlated well with the accumulation of RA in the elicitor-treated cells (Fig. 2A). In the YE-treated cells, PAL activity reached a maximum 8 hr after addition, whereas maximal PAL activity was observed 24 hr after elicitation in the MJ-treated cells, later than the increase in the YE-treated cells. However, the extent of the increase in PAL activity was about 2-fold higher in the MJ-treated cells than that in the YE-treated cells.

Among the enzymes involved in the tyrosine-derived pathway, however, the activity of TAT, the entrypoint enzyme of the pathway, was only slightly enhanced by either MJ- or YE-treatment to the same extent (Fig. 2B). In contrast, the activity of HPR, which catalyzes the second step of the pathway, was rapidly and transiently increased by both elicitor treatments (Fig. 2C). In the MJ-treated cells, the peak activity of HPR was reached later, but was about 2-fold higher than that in the YE-treated cells, and had a profile similar to that of the PAL activity.

Both PAL activity and RA accumulation increased in a dose-dependent manner, with maximal induction at 100 μM MJ, and then decreased at higher levels of MJ (Fig. 3).

DISCUSSION

Addition of MJ to *Lithospermum erythrorhizon* cell suspension cultures at a concentration of 100 μM induced drastic accumulation of RA in the cultured cells. The extent of the increase in RA content in the MJ-treated cells was much higher (about four times) than that observed in the cells elicited by YE at a concentration of 5g/l. The increased RA accumulation in the elicited cells was apparently due to the activation of both the phenylpropanoid pathway and tyrosine-derived pathway since the incorporation of both radiolabelled phenylalanine and tyrosine into RA were enhanced to the same extent by MJ treatment. A transient and rapid increase in PAL activity in the MJ-treated cells correlated well with the induced RA accumulation, indicating again that this enzyme plays an important role in regulation of phenylpropanoid metabolism in cultured cells of *L. erythrorhizon*. With respect to the enzymes involved in the tyrosine-derived pathway, TAT activity responded only weakly to either MJ or YE, whereas a strong transient increase in HPR activity, consistent with

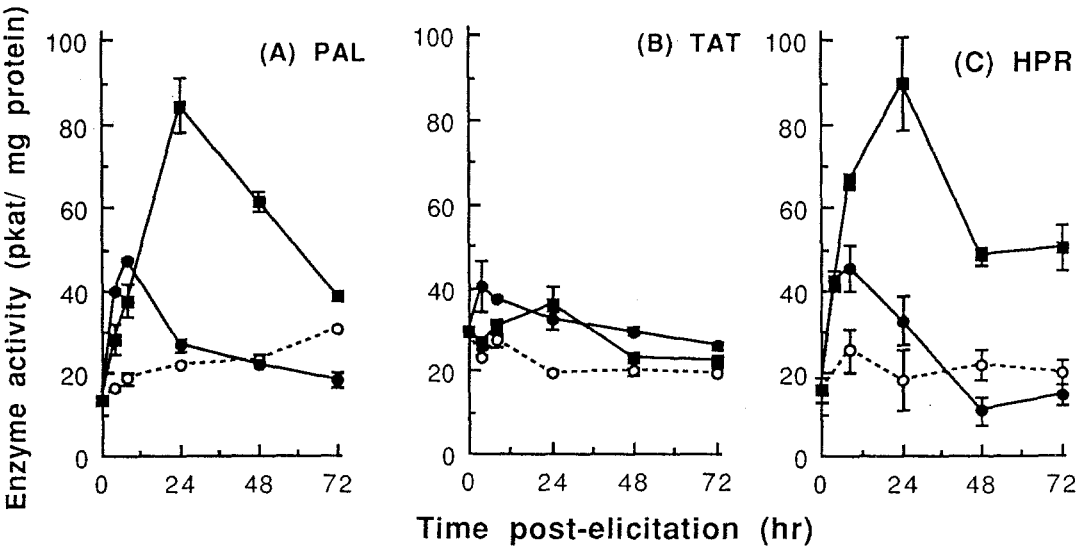

Fig. 2 Changes in PAL (left), TAT (center) and HPR (right) activities in cultured cells of *L. erythrorhizon* treated with methyl jasmonate (MJ) (100 μM) (closed squares) and yeast extract (YE) (5g/L) (closed circles) 6 days after cell inoculation. Open circles with dotted lines represent the control cells treated with water. Each point indicates an average from duplicate cultures with a standard error. The entire experiment was repeated twice with essentially the same result, and one representative data set is shown here.

the changes of PAL activity and parallel with RA accumulation, was observed in either MJ-treated or YE-treated cells. The extents of the increases in PAL and HPR activities induced by MJ treatment were about 2-fold greater than the increases induced by YE treatment, which was consistent with the higher RA accumulation and incorporation rates of the radiolabelled precursors into RA.

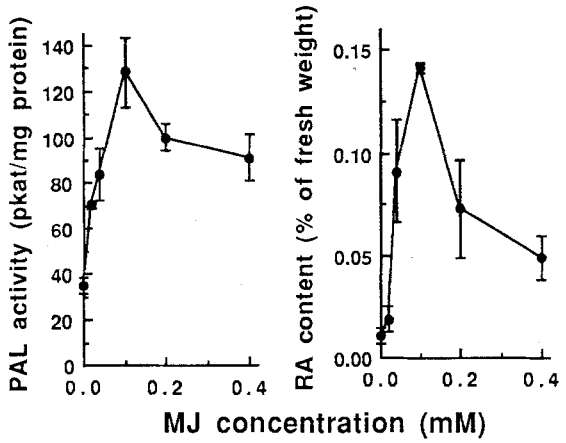

Fig. 3 Effects of methyl jasmonate (MJ) on PAL activity (left) and RA accumulation (right) in cultured cells of *L. erythrorhizon*. PAL activity and RA content were measured 24 hr and 48 hr, respectively, after addition of MJ to 6-day-old cell cultures. Each concentration of MJ was added in 100 µl EtOH per culture. Each point indictes an average from duplicate cultures with a standard error.

It is interesting to note that in spite of the greater effectiveness of MJ, the response of *L. erythrorhizon* cells to this elicitor was slower than that to YE-treatment. This may indicate that two independent elicitation pathways are involved, or it may reflect a requirement for conversion of exogeneously applied MJ to jasmonic acid or other metabolites, which then trigger the elicitation process within the cells. In *Rauvolfia canescens* suspension cultures challenged with a yeast cell wall preparation, a transient and drastic increase in the intracellular concentration of jasmonic acid was reported with a peak 45 min after challenge, compared with a slight increase in intracellular MJ levels 100 min after elicitation (Gundlach et al. 1992). Furthermore, it has recently been shown (Nojiri et al. 1992) that MJ was not detected in any tissue of bulbing onion plants even using an RIA which is 100-fold more sensitive than previously reported RIAs while jasmonic acid at 0.75-2.3 pmol/g fresh weight was found in various tissues of this plant.

The present results suggest that MJ may be involved in the signal transduction process in YE-induced RA biosynthesis in *Lithospermum erythrorhizon* cells, and are consistent with the proposal that the jasmonates play the integral role as a second messenger in the elicitation process leading to the accumulation of secondary metabolites (Gundlach et al. 1992). The data also indicate that HPR may catalyze a key regulatory step in the elicitor-induced activation of tyrosine-derived pathway in *L. erythrorhizon* cultured cells.

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