Plant Biotechnology 21(5), 393–396 (2004)

Short Communication

Elicitor-induced rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures: Activities of rosmarinic acid synthase and the final two cytochrome P450-catalyzed hydroxylations

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Received September 13, 2004; accepted October 6, 2004 (Edited by K. Yazaki)

Abstract A drastic increase in rosmarinic acid (RA) accumulation is induced in cultured cells of *Lithospermum erythrorhizon* after their exposure to yeast extract or methyl jasmonate. Addition of the elicitors to the cell cultures only slightly enhanced the activity of rosmarinic acid synthase, which catalyzes formation of 4-coumaroyl-4′-hydroxyphenyllactic acid (CHPL), the first reaction specific to RA biosynthesis. In contrast, the two-step cytochrome P450-catalyzed hydroxylations of CHPL to form RA were dramatically up-regulated by the elicitor treatments, indicating that these hydroxylation activities are likely to play a key regulatory role in elicitation of RA biosynthesis.

Key words: *Lithospermum erythrorhizon*, rosmarinic acid biosynthesis, rosmarinic acid synthase, cytochrome P450-catalyzed hydroxylation.

Rosmarinic acid (RA), α-*O*-caffeoyl-3′, 4′-dihydroxyphenyllactic acid, is a well-known hydroxycinnamic acid ester that occurs across a wide range of plant families, including the Boraginaceae and Lamiaceae (Petersen and Simmonds 2003). The biosynthesis and production of RA in cultured plant cells have been extensively studied because RA exhibits various pharmacological activities, including potent antioxidative properties which make it a potentially useful compound as a medicine and a food additive. The biosynthetic pathway leading to RA is well defined, as shown in Figure 1. Condensation of 4-hydroxyphenyllactic acid (derived from tyrosine) with 4-coumaroyl CoA (derived from phenylalanine) is catalyzed by an acyltransferase named rosmarinic acid synthase (RAS) to yield 4-coumaroyl-4′-hydroxyphenyllactic acid (CHPL). CHPL is then converted to RA by two consecutive hydroxylation steps, each of which is catalyzed by distinct cytochrome P450s (3H and 3′H).

RA production in cultured plant cells was earlier shown to be enhanced by addition of elicitors such as yeast extract (YE) and methyl jasmonate (MJ), as exemplified in *Lithospermum erythrorhizon* cell cultures (Mizukami et al. 1992 and 1993). Elicitation by YE or MJ up-regulated the activities of both phenylalanine ammonia-lyase (PAL) and 4-hydroxyphenylpyruvate reductase (HPR), entrypoint enzymes for the phenylpropanoid pathway and tyrosine-derived pathway, respectively. Not all steps in the biosynthetic pathway respond to elicitation, since neither the expression level of cinnamic acid 4-hydroxylase (Yamamura et al. 2001) nor the activities of tyrosine aminotransferase (Mizukami et al. 1993) were affected by elicitor treatment in *L. erythrorhizon* cells. However, little is known whether the enzyme activities involved in the three final steps in the RA biosynthesis (RAS, 3H and 3′H) are affected by elicitors. In the present investigation we analyzed the effect of elicitor treatment on these three enzyme activities, and conclude that the two final hydroxylation steps are likely to play key roles in regulating elicitor-induced RA biosynthesis in *L. erythrorhizon* cell suspension cultures.

Suspension cultures of *L. erythrorhizon* were maintained in LS liquid medium (Linsmaier and Skoog 1965) supplemented with 1 μM 2,4-dichlorophenoxyacetic acid and 1 μM kinetin. Cell suspension (5 ml) was transferred into 25 ml fresh medium in a 100 ml

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Abbreviations: 3H, 4-coumaroyl-4′-hydroxyphenyllactic acid 3-hydroxylase; 3′H, caffeoyl-4-hydroxyphenyllactic acid 3′-hydroxylase; CHPL, 4-coumaroyl-4′-hydroxyphenyllactic acid; CaHPL, caffeoyl-4′-hydroxyphenyllactic acid; HPR, 4-hydroxyphenylpyruvate reductase; MJ, methyl jasmonate; PAL, phenylalanine ammonia-lyase; RA, rosmarinic acid; RAS, rosmarinic acid synthase; YE, yeast extract.

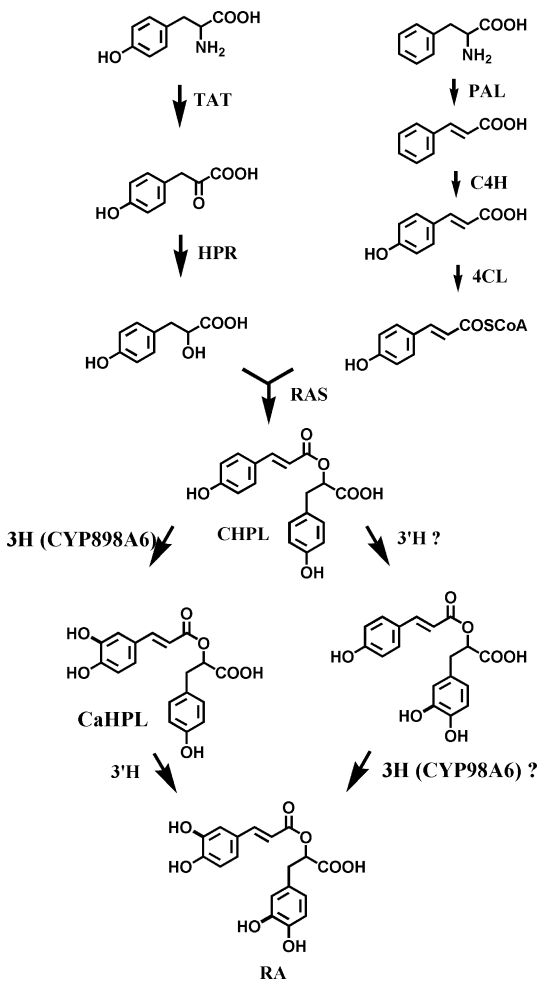

Figure 1. The proposed biosynthetic pathway leading to rosmarinic acid. PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaric acid:CoA ligase; TAT, tyrosine aminotransferase; HPR, 4-hydroxyphenylpyruvate reductase; RAS, rosmarinic acid synthase, 3H, 4-coumaroyl-4′-hydroxyphenyllactic acid 3-hydroxylase; 3′H, caffeoyl-4′-hydroxyphenyllactic acid 3′-hydroxylase; CHPL, 4-coumaroyl-4′-hydroxyphenyllactic acid; CaHPL, caffeoyl-4′-hydroxyphenyllactic acid; RA, rosmarinic acid.

Erlenmeyer flask at 14-day intervals and cultured on a rotary shaker at 25°C in the dark. YE (Difco) was dissolved in water, autoclaved at 120°C for 20 min, and aseptically added to the cell suspension at a final concentration of 5 g l^{-1}. MJ (Tokyo Kasei) was dissolved in dimethylsulfoxide and added to the cultures through a membrane filter to give a final concentration of 100 μM. These elicitors were added to the cells 7 days after cell transfer and the cells were collected by vacuum filtration at defined times, immediately frozen in liquid nitrogen, and stored at −75°C until use.

Preparation of soluble enzyme and microsome fractions was carried out at 0–4°C. The frozen cells were homogenized in 100 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT, 5 mM EDTA, 0.5 M sorbitol and 30 g l^{-1} polyvinylpolypyrrolidone. The homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged at 12,000 *g* for 20 min. PAL and RAS were precipitated from the supernatant with 80% ammonium sulfate saturation. Precipitated protein was dissolved in the extraction buffer containing 0.3 M sodium chloride. The solution was desalted on a Sephadex G-25 column and used as the soluble enzyme fraction.

For preparation of the microsome fraction, the supernatant from the 12,000 *g* centrifugation was centrifuged again at 100,000 *g* for 60 min. The pellet was suspended in 100 mM potassium phosphate buffer, pH 7.0, containing 3.5 mM 2-mercaptoethanol and 0.5 M sorbitol, and this slurry was applied to a Sephadex G-25 column. The fraction eluting at the void volume was collected and centrifuged again at 100,000 *g* for 60 min. This pellet was re-suspended in the same buffer and used as the microsomal fraction. The protein content in the soluble and microsomal fractions was estimated by the method of Bradford (Bradford 1976).

PAL was assayed by the spectrophotometric method (Edwards and Kessmann 1992), while the RAS assay was carried out according to the method described by Szabo et al. (1999) with slight modifications as follows. Soluble enzyme preparation (25 μl) was added to 25 μl aliquots of RAS reaction mixture (100 mM potassium phosphate (pH 7.0) containing 0.1 mM DTT, 1 mM ascorbic acid, 1 mM 4-coumaroyl CoA and 0.4 mM 4-hydroxyphenyllactic acid) and incubated for 30 min at 30°C. 4-Coumaroyl CoA was synthesized according to the method of Stöckigt and Zenk (1975). The RAS reaction was terminated by adding 4 μl 5 M HCl, and the acidified mixture was extracted with 50 μl ethyl acetate three times. The combined ethyl acetate extract was evaporated and the residue was dissolved in 50 μl methanol containing 0.1% (v/v) acetic acid. The amount of CHPL was estimated by HPLC. Conditions for HPLC are as follows: COSMOSIL 5C18-MS (Nacalai Tesque) 150×4.6 mm; solvent system, methanol-0.1% acetic acid (50 : 50); flow rate, 1.0 ml; detection; 310 nm.

To estimate the hydroxylation activity, the microsome preparation (50 μl) was added to 250 μl aliquots of hydroxylation reaction mixture (100 mM potassium phosphate, pH 7.0, containing 80 μM CHPL and 0.8 mM NADPH) and incubated for 30 min at 20°C. CHPL was prepared as described previously (Matsuno et al. 2001). The reaction was terminated by adding 20 μl 5 M HCl, and the acidified mixture was extracted with 600 ml ethyl acetate three times. The combined ethyl acetate extract was evaporated, and the residue was dissolved in 25 ml methanol containing 0.1% (v/v) acetic acid. The amount of RA formed by the enzymatic reaction was determined by HPLC according to the previously described method (Mizukami et al. 1992).

For quantitative determination of RA, frozen cells (about 0.2 g) were extracted with 2 ml methanol at

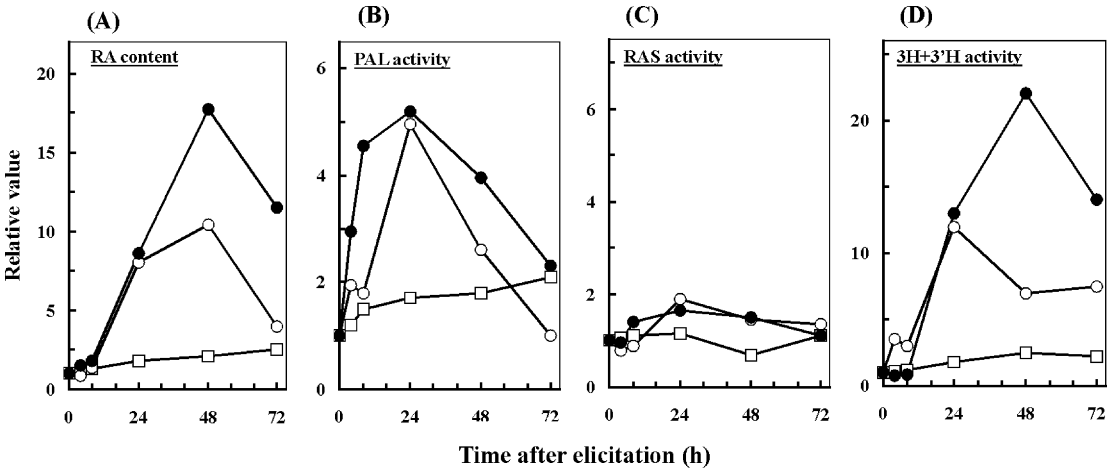

Figure 2. Changes in RA content (A), and PAL (B), RAS (C) and 3H plus 3′H (D) activities in cultured cells of *Lithospermum erythrorhizon* treated with methyl jasmonate (closed circles) or yeast extract (open circles) 7 days after cell inoculation. The control cells (open squares) were treated with dimethylsulfoxide. Each point indicates a value relative to the value at 0 h (just before elicitation). RA content, and PAL, RAS and 3H plus 3′H activities at 0 h were 0.02 % of the cell fresh weight, 29.4 pkat mg^{-1} protein, 5.2 pkat mg^{-1} protein and 0.15 pkat mg^{-1} protein, respectively. Each point represents an average from duplicate cultures. The entire experiment was repeated twice with the essentially the same result, and one representative data set is shown here.

65°C for 1 h with vigorous shaking. The slurry was centrifuged at 12,000 *g* for 5 min and the supernatant was subjected to HPLC analysis (Mizukami et al. 1992).

Addition of YE or MJ rapidly and transiently enhanced accumulation of RA in *L. erythrorhizon* suspension cultures (Figure 2A). In the YE-treated cells, the RA content reached a maximum between 24 and 48 h after treatment and the extent of the increase in the RA content was about 4-fold higher than the content of the control cells. In the MJ-treated cells RA accumulation reached a maximum 48 h after treatment and the RA content was about 10-fold higher than that in the control cells. The RA content was then decreased between 48 and 72 h after elicitor addition. RA might be metabolized to some other compounds although such peaks could not be detected under the present HPLC condition. These results are essentially consistent with those described previously (Mizukami et al. 1993).

A crude enzyme preparation from cultured *L. erythrorhizon* cells catalyzed condensation of 4-coumaroyl CoA with 4-hydroxyphenyllactic acid to form CHPL in a protein- and incubation time-dependent manner, thus indicating the presence of RAS activity. The activity of RAS was only slightly enhanced by either YE- or MJ-treatment, in contrast to PAL activity which was rapidly and transiently induced by both elicitor treatments (Figure 2B and 2C).

CHPL, a product of the reaction catalyzed by RAS, is converted to RA by two consecutive hydroxylation reactions. When CHPL was incubated with the microsome preparation from *L. erythrorhizon* cells in the presence of NADPH at 20°C for 30 min, accumulation of RA in the reaction mixture could be detected by HPLC. Shorter incubation for 5 to 10 min yielded, together with RA, an additional product whose retention time and UV-spectrum were consistent with those of caffeoyl-4′-hydroxyphenyllactic acid (CaHPL). The identity of the HPLC peak with CaHPL was further confirmed by measuring ^{1}H-NMR spectrum of the pooled eluate. The microsomes did not produce RA from CHPL in the absence of NADPH. Furthermore, both CaHPL and RA formation were completely inhibited by the addition of 2 mM miconazole, a cytochrome P450 inhibitor (data not shown).

The enzymatic formation of RA from CHPL was drastically and transiently induced by elicitor treatments as shown in Figure 2D. In the YE-treated cells the activity reached a maximum 24 h after YE-addition and was about 7-fold higher than that of the control. In the MJ-treated cells the peak activity was reached later (48 h after treatment) and was 10-fold higher than that of the control cells.

Since the first report that RA production could be enhanced by the addition of YE to the cell cultures of *Orthosiphon arisatus* (Sumaryono et al. 1991), biochemical and molecular biological characterization of elicitation of RA biosynthesis has been extensively investigated using *L. erythrorhizon* (Boraginaceae) and *Coleus blumei* (Lamiaceae) cell cultures. We have shown that PAL in the phenylpropanoid pathway and HPR in the tyrosine-derived pathway were responsible for the elicitor-enhanced RA production in *L. erythrorhizon* cells (Mizukami et al. 1993). These results were later confirmed using *C. blumei* cells (Szabo et al. 1999).

In the present investigation we have examined the effects of elicitor treatment on the enzyme activities involved in final three steps in RA biosynthesis using *L. erythrorhizon* cell cultures. The activity of RAS in

transferring the 4-coumaroyl moiety of 4-coumaroyl CoA to 4-hydroxyphenyllactic acid was only slightly enhanced by either YE- or MJ-addition to the cultures, even though this enzyme catalyzes the first reaction specific to RA biosynthesis. This result is consistent with an earlier report that RAS activity was largely unaffected by elicitor addition to *C. blumei* cells (Szabo et al. 1999).

The two consecutive hydroxylation steps that convert CHPL to RA were first shown to be catalyzed by two different cytochrome P450s (designated 3H and 3′H) in *C. blumei* cells (Petersen 1997). A cytochrome P450 (CYP98A6) cDNA clone coding for 3H was subsequently isolated from *L. erythrorhizon* and expression of the corresponding gene was shown to be drastically up-regulated by addition of YE or MJ to *L. erythrorhizon* cells (Matsuno et al. 2002). In the present study we estimated these hydroxylation activities by measuring the rate of RA production from CHPL by microsome preparations in the presence of NADPH, an approach that reports the total activity of 3H and 3′H. The 3H plus 3′H activity in *L. erythrorhizon* cells was dramatically increased by YE or MJ-addition. The extent of the increase in the hydroxylation activity was much higher than that seen for PAL and HPR activities and was proportional to the increase in RA content. This indicates that the microsomal hydroxylations of CHPL are likely to play key roles in regulation of elicitor-induced RA biosynthesis in *L. erythrorhizon*.

The order of introduction of the 3- and 3′-hydroxyl groups into CHPL remains unresolved, as does the question whether the activity and/or mRNA expression of both 3H and 3′H are both enhanced by elicitors. Our preliminary results from short-term incubation of CHPL with the microsomes, where we detected a product tentatively identified as CaHPL, as well as the recent report of the natural occurrence of CaHPL (isorinic acid) in plants (*Helicteres isora*) (Satake et al. 1999) suggests that the 3-hydroxyl group may be introduced first, converting CHPL to CaHPL, which would then be converted to RA by 3′-hydroxylation. Since CHPL was efficiently converted to RA by microsomes prepared from MJ-treated cells, without accumulation of the putative intermediate, it would appear that both 3H and 3′H activities are increased by elicitors. However, identification and characterization of 3′H is necessary to unambiguously resolve these questions.

Acknowledgement

One of the authors (HM) is grateful to late Dr. Mamoru Tabata, Professor Emeritus of Kyoto University, for his continuous encouragement to the author's research activities in the field of plant secondary metabolism. We also thank Professor Brian Ellis, University of British Columbia, for critical reading of the manuscript. This work was supported in part by a Grant-in-Aid for High-Tech Research Center Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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