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Original Paper

Elicitor-induced activity of isorinic acid 3′-hydroxylase, an enzyme catalyzing the final step of rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures

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Abstract Isorinic acid 3′-hydroxylase (IA3′H) catalyzes a final step in the biosynthetic pathway leading to rosmarinic acid (RA). IA3′H activity was detected in a microsomal fraction prepared from cultured cells of *Lithospermum erythrorhizon*. Addition of yeast extract or methyl jasmonate to the cell suspension cultures drastically increased IA3′H activity in parallel with increased RA production, whereas addition of salicylic acid enhanced neither IA3′H activity nor RA production. These results are consistent with an integral role for IA3′H in biosynthesis of RA in response to elicitation with yeast extract or methyl jasmonate.

Key words: Elicitation, isorinic acid 3′-hydroxylase, *Lithospermum erythrorhizon*, rosmarinic acid.

Rosmarinic acid (α-*O*-caffeoyl-3,4-dihydroxyphenyllactic acid, RA) is a caffeoyl ester widely distributed in Boraginaceous and Lamiaceous plants. It also occurs in other evolutionarily diverse taxa belonging to the eudicots, monocots, ferns and hornworts (Petersen and Simmonds 2003). RA has been shown to exhibit anti-oxidative (Gao et al. 2005), anti-inflammatory (Osakabe et al. 2004) and anti-allergic (Sanbongi et al. 2004) activities in mammalian systems. Oral administration of RA to patients was shown to result in a significant decrease in responder rates for seasonal allergic rhinoconjunctivitis, accompanied by a reduction in the neutrophils and eosinophils in nasal lavage fluids of the patients (Osakabe et al. 2004). Thus RA can potentially be used as a medicine, a neutraceutical and a food additive.

Studies of RA biogenesis in various plant species (*Coleus blumei*, *Anchusa officinalis*, *Lithospermum erythrorhizon*) have defined the biosynthetic pathway shown in Figure 1. Condensation of 4-coumaroyl CoA derived from phenylalanine, with 4-hydroxyphenyllactic acid derived from tyrosine, results in formation of 4-coumaryl-4′-hydroxyphenyllactic acid (CHPL). CHPL is then converted to RA through two consecutive hydroxylations at the 3- and 3′-positions of the two aromatic rings. We have recently suggested that CHPL is first converted to caffeoyl-4′-hydroxyphenyllactic acid which would be converted to RA by 3′-hydroxylation (Ogata et al. 2004). In fact, the caffeoyl-4′-hydroxyphenyllactic acid intermediate was reported to occur naturally in fruits of *Helicteres isora* as the 4-*O*-β-glucoside derivative, and named isorinic acid (IA) (Satake et al. 1999).

RA production in *L. erythrorhizon* cell cultures is rapidly stimulated by addition of yeast extract (YE) or methyl jasmonate (MJ) (Mizukami et al. 1992, 1993). Such elicitation of RA biosynthesis has also been reported in suspension cultures of *Orthosiphon aristatus* (Sumaryono et al. 1991), *C. blumei* (Szebo et al. 1999) and *Lavendula officinalis* (Nitzsche et al. 2004), and in hairy root cultures of *Salvia mirritorhiza* (Chen et al. 2001), which provides a convenient system for analysis of the molecular events regulating RA biosynthesis. Addition of YE or MJ to the *L. erythrorhizon* cell cultures up-regulates the activities of both phenylalanine ammonia-lyase (PAL) and 4-hydroxyphenylpyruvate reductase (HPR), the entrypoint enzymes for the phenylpropanoid pathway and the tyrosine-derived

Abbreviations: C4H, cinnamic acid 4-hydroxylase; CHPL, 4-coumaryl-4′-hydroxyphenyllactic acid; HPR, hydroxyphenylpyruvate reductase; IA, isorinic acid; IA3′H, isorinic acid 3′-hydroxylase; MJ, methyl jasmonate; PAL, phenylalanine ammonia-lyase; RA, rosmarinic acid; RAS, rosmarinic acid synthase; TAT, tyrosine aminotransferase; YE, yeast extract.
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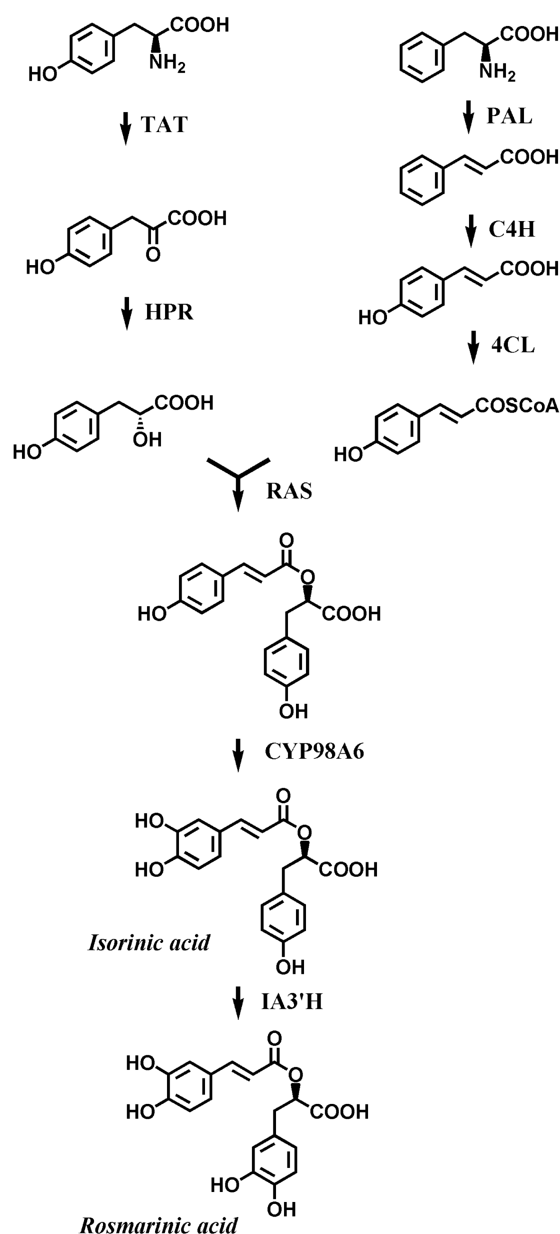

Figure 1. The proposed biosynthetic pathway leading to rosmarinic acid in *Lithospermum erythrorhizon*. PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaric acid:CoA ligase; TAT, tyrosine aminotransferase; HPR, 4-hydroxyphenylpyruvate reductase; RAS, rosmarinic acid synthase; IA3′H, isorinic acid 3′-hydroxylase.

pathway, respectively. Furthermore, YE or MJ treatment increases the transcript levels of CYP98A6, which catalyzes 3-hydroxylation of CHPL to yield IA (Matsuno et al. 2002). In contrast, neither the activities of tyrosine aminotransferase (TAT) (Mizukami et al. 1993) and rosmarinic acid synthase (RAS) (Ogata et al. 2004), nor the transcript levels of cinnamic acid 4-hydroxylase (C4H) (Yamamura et al. 2001) are affected by YE or MJ addition to *L. erythrorhizon* suspension cultures. However, nothing is known about the behaviour of isorinic acid 3′-hydroxylase (IA3′H), the enzyme catalyzing the final step to RA, in response to elicitor treatment, although the IA3′H activity was first detected in *C. blumei* cell suspension cultures (Petersen et al. 1997).

We have now established that the IA3′H activity is drastically enhanced by elicitor treatment. The extent and pattern of this increase in IA3′H activity are well correlated with those of RA production, suggesting that IA3′H plays a key role in regulating biosynthesis of RA in elicited cells.

Materials and methods

Cell suspension culture and elicitor treatments

Suspension cultures of *Lithospermum erythrorhizon* were established from seedling-derived callus tissues and 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 Erlenmeyer flask at 14-day intervals and cultured on a rotary shaker at 25°C in the dark. Yeast extract (Difco) was dissolved in water, autoclaved at 121°C for 20 min, and aseptically added to the cell suspension at a final concentration of 5 mg ml^{-1}. Methyl jasmonate (Tokyo Kasei) and salicylic acid (Katayama Chemical) were dissolved in dimethylsulfoxide and added to the cultures through a membrane filter (0.22 μm) to give a final concentration of 100 μM. These elicitors were added to the cells 7 days after cell transfer and the elicitated cells were subsequently collected by vacuum filtration at defined times, immediately frozen in liquid nitrogen, and stored at −75°C until use.

Enzyme preparation

Preparation of soluble enzyme and microsomal fractions was carried out at 0–4°C. Briefly, frozen cells were homogenized using Polytron homogenizer in 50 mM tris-HCl buffer, pH 7.5, containing 1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM sorbitol and 50 mg ml^{-1} polyvinylpolypyrrolidone. The homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged at 12,000 g for 20 min. This supernatant was used as soluble enzyme preparation. The supernatant was centrifuged at 100,000 g for 60 min. The pellet was suspended in 100 mM sodium phosphate buffer, pH 7.5 and applied to a Sephadex G-25 column. The excluded fraction (flow-through) was then centrifuged again at 100,000 g for 60 min. The resulting pellet was re-suspended in the same buffer and used as the microsomal preparation. The protein content in the soluble and microsomal enzyme preparations was determined by the method of Bradford (1976).

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PAL assay

PAL activity was assayed by spectrophotometric determination of cinnamic acid formation. The reaction mixture containing 10 mM phenylalanine and 40 μg soluble protein in a 500 μl 50 mM borate buffer, pH 8.8, was incubated for 30 min at 30°C. The reaction was terminated by addition of 250 μl 2 M $HClO_4$ and the product was extracted into 500 μl ethyl acetate. The absorbance of the ethyl acetate extract was determined at 280 nm and the amount of cinnamic acid was calculated using a calibration curve (ε=21,500).

IA3′H assay

Isorinic acid (α-*O*-caffeoyl-4-hydroxyphenyllactic acid) was prepared from an extract of *Helicteres isora* fruits (Satake et al. 1999). The standard enzyme reaction mixture contained 67 μM IA, 667 μM NADPH and 20 μg microsomal protein in a total volume of 30 μl 100 mM sodium phosphate buffer, pH 7.5. This assay mixture was incubated for 30 min at 30°C, heated for 5 min reaction at 95°C and centrifuged at 13,000 *g* for 10 min. The supernatant was subjected to HPLC chromatography (Cosmosil 5C18-AR (Nacalai Tesque), 4.6×150 mm using elution with the following gradient: solvent A: 0.1% acetic acid; solvent B: 100% acetonitrile; 0–5 min, 10% solvent B; 5–20 min, 10–52% solvent B. Flow rate was 1.0 ml min^{-1}. The elution was monitored using a photodiode array detector.

Extraction and determination of RA

The frozen cells (about 0.25 g) were extracted with 2 ml methanol at 70°C for 60 min with vigorous shaking. The slurry was centrifuged at 20,600 *g* for 10 min and the supernatant was subjected to HPLC fractionation. The conditions for HPLC analysis have been described previously (Mizukami et al. 1992).

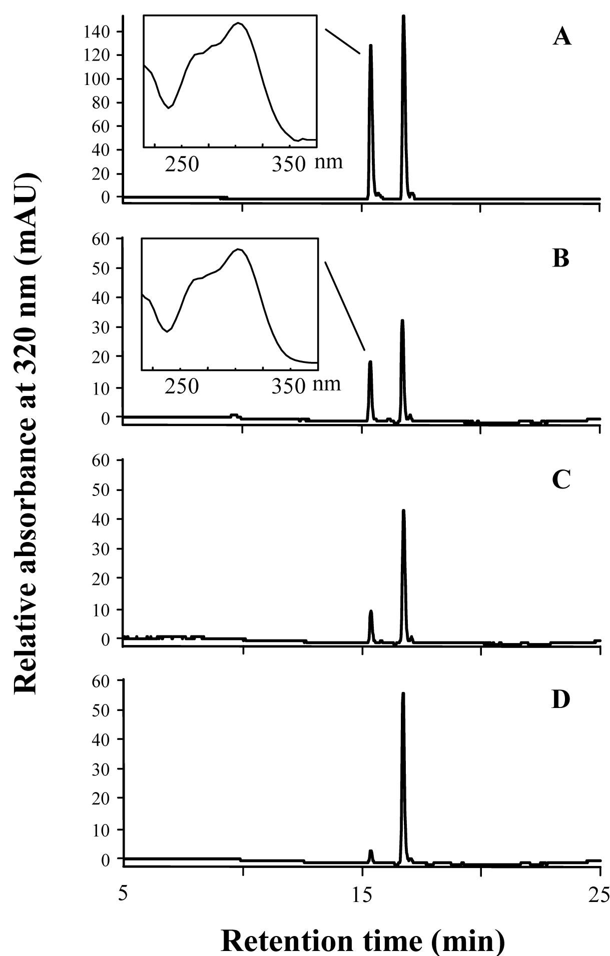

Figure 2. Analysis of isorinic acid 3′-hydroxylase activity in microsomes prepared from cultured cells of *Lithospermum erythrorhizon*. (A) The HPLC chromatogram of authentic standards of rosmarinic acid (RA, Rt=15.4 min) and isornic acid (IA, Rt=16.8 min). The enzymic assay was carried out with 10 μg (B) or 5 μg (C) microsomal protein from the methyl jasmonate-treated cells in the presence of 0.7 mM NADPH for 30 min. (D) The incubation was carried out as in (B) but for 5 min. The insets show the UV absorption spectra corresponding to rosmarinic acid (A) and the product peak of the enzyme assay (B).

Results

We earlier showed that conversion of CHPL to RA in *Lithospermum erythrorhizon* cells was enhanced by MJ treatment (Ogata et al. 2004), suggesting that the final biosynthetic reaction(s) must be activated by this treatment. When IA was incubated with NADPH and microsome fraction prepared from cells 24 h after addition of MJ (100 μM) to the suspension cultures, a product peak was detected whose HPLC retention time and UV-spectrum were identical with those of RA (Figure 2A and 2B). The RA formation was both protein concentration- (Figure 2B and 2C) and time-dependent (Figure 2B and 2D). The microsomes did not convert IA to RA in the absence of NADPH. Two inhibitors reported to be specific for cytochrome P450-dependent monooxygenases were examined for their effects on the 3′-hydroxylation activity of the microsomes (Figure 3).

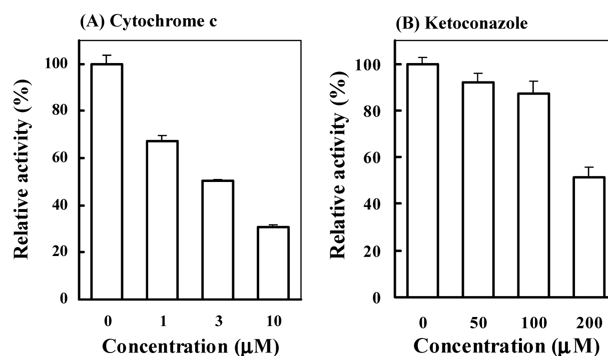

Figure 3. Effect of cytochrome P450 inhibitors on the isorinic acid 3′-hydroxylase activity in *Lithospermum erythrorhizon* cell cultures. The specific activity in the control incubation without inhibitors corresponding to 100% activity is 51.1 pkat mg^{-1} protein and 32.5 pkat mg^{-1} protein in the incubations with cytochrome c and ketoconazole, respectively. Each graph represents the mean of three experiments, with bars indicating standard error.

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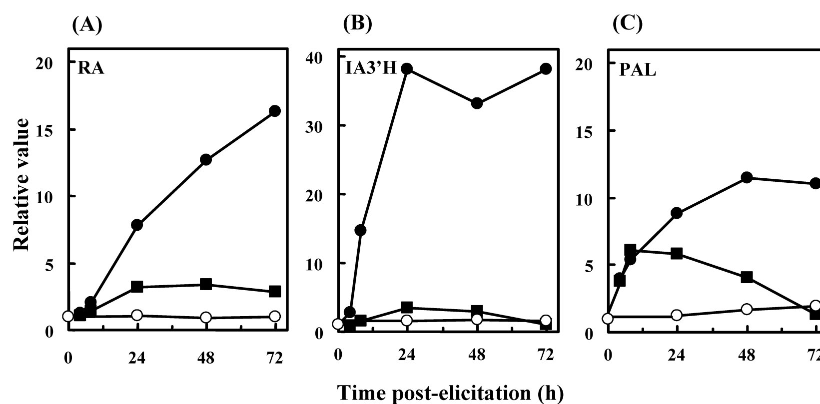

Figure 4. Changes in rosmarinic acid content (A), and isorinic acid 3′-hydroxylase (B) and phenylalanine ammonia-lyase (C) activities in cultured cells of *Lithospremum erythrorhizon* treated with methyl jasmonate (closed circles) or yeast extract (closed squares) 7 days after cell inoculation. The control cells (open circles) were treated with dimethylsulfoxide. Each point indicates a value relative to the value at 0 h (just before elicitation). Rosmarinic acid content, isorinic acid 3′-hydroxylase activity and phenylalanine ammonia-lyase activity at 0 h were 0.58 μmol mg^{-1} fresh weight, 0.99 pkat mg^{-1} protein and 5.1 pkat mg^{-1} protein, respectively. Each point represents an average of values from triplicate cultures. The entire experiment was repeated twice with the essentially same result, and one representative data set is shown here.

Cytochrome c strongly inhibited the 3′-hydroxylation activity at a concentration of as low as 1 μM. Ketoconazole (200 μM) also reduced the microsomal 3′-hydroxylation activity by 50%.

Addition of YE or MJ to the suspension cultures rapidly enhanced RA production in *L. erythrorhizon* cell cultures (Figure 4A), with the initial increase appearing within 4 to 8 h after elicitation. The extent of the increase in RA content by YE addition was about 3-fold, relative to the content of the control cells. A more drastic increase in RA accumulation by MJ addition was observed until 72 h post-elicitation, by which time the RA content reached 8.9 μmol per gram fresh weight, about 16-fold higher than in the control cells. The activity of PAL, an entry-point enzyme for the phenylpropanoid pathway rapidly increased immediately after YE or MJ addition, reaching a maximum activity between 8 and 24 h after YE addition and 48 h after MJ treatment. The peak of PAL activity was about 6-fold and 11-fold higher in the YE- and MJ-treated cells, respectively, than in the control cells (Figure 4C). The 3′-hydroxylation activity for IA was drastically induced by YE addition, and especially by MJ treatment, with a lag period of 4 h (Figure 4B). The peak IA3′H activity at 24 h post-elicitation was 2.5-fold and 40-fold higher in the YE-treated and MJ-treated cells, respectively, than that in the control cells. The activity gradually decreased in the YE-treated cells but remained high until at least 72 h post-elicitation in the MJ-treated cells. By contrast, the activities of PAL and IA3′H were unaffected by addition of salicylic acid up to a final concentration of 0.5 mM, nor was RA accumulation induced (data not shown).

Discussion

IA3′H activity was first detected in microsomal fraction of the cultured cells of *Coleus blumei* cells (Petersen 1997). However, no detailed study was carried out on the regulation of the activity by elicitor treatments. We showed for the first time that IA3′H activity in *Lithospermum erythrorhizon* cells in culture was drastically enhanced by the elicitor treatment in the coordinate fashion to elicitation of RA accumulation, indicating its pivotal role in the regulation of RA biosynthesis. Biosynthesis of plant secondary metabolites has been shown to be activated by both abiotic and biotic elicitors and the use of microbial elicitors has been one of the most effective strategies for improving the productivity of useful metabolites in plant cell cultures (Roberts and Shuler 1997). The present study demonstrates that such elicitation responses provide effective systems in order to explore the molecular basis of regulation of plant secondary metabolism, including the relevant signal transduction pathways, and to pursue the identification and characterization of the enzymes participating in the biosynthetic pathways of interest.

A large number of cytochrome P450s are involved in both biosynthetic and detoxification pathways in higher plants and many of these appear to play key regulatory roles in plant secondary metabolism (Schuler and Werck-Reichhart 2003). Three cytochrome P450s were presumed to be involved in RA biosynthesis. Cinnamic acid 4-hydroxylase (C4H) catalyzes 4-hydroxylation of *trans*-cinnamic acid to form 4-coumaric acid, and two additional cytochrome P450s catalyze the two final hydroxylation steps leading to RA from CHPL. C4H cDNAs have been isolated from the cultured cells of *L. erythrorhizon* and identified as CYP73A30 and

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CYP73A31 (Yamamura et al. 2001). An enzyme catalyzing hydroxylation of 3-position of CHPL to yield IA was also shown to be encoded by another cytochrome P450 gene, *CYP98A6*, in *L. erythrorhizon* (Matsuno et al. 2002). However, identification and characterization of the enzyme responsible for 3′-hydroxylation of IA to form RA in *L. erythrorhizon* remained unsolved. In the present investigation we have detected this enzyme activity in microsome preparations from MJ-treated cells of *L. erythrorhizon*. The conversion of IA to RA requires NADPH and is inhibited by both cytochrome c and ketoconazole. It is well known that cytochrome c is an electron acceptor competing for electrons transferred from NADPH: cytochrome P450 reductase to cytochrome P450, while ketoconazole inhibits cytochrome P450s by interacting with the prosthetic heme group (Muto et al. 1987). These results indicate that IA3′H of *L. erythrorhizon* is also a cytochrome P450-dependent monooxygenase, as suggested by earlier experiments in *Coleus blumei* cells (Petersen 1997). The relatively unreactive nature of the aromatic rings of 4-hydroxyphenyllactic acid compared with the hydroxycinnamic acids, and our preliminary finding that recombinant CYP98A6 did not catalyze 3′-hydroxylation of IA suggest that IA3′H is a distinctive cytochrome P450 species, whose further characterization will require cloning of the corresponding cDNA and expression of the encoded gene product. In view of the dramatic response of IA hydroxylase activity to MJ treatment, differential display or subtractive hybridization between MJ-treated and control cells might be a useful approach to cloning the corresponding cDNA, which is now under investigation.

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