CYP98A6 from *Lithospermum erythrorhizon* encodes 4-coumaroyl-4′-hydroxyphenyllactic acid 3-hydroxylase involved in rosmarinic acid biosynthesis[1]

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Abstract Rosmarinic acid is the dominant hydroxycinnamic acid ester accumulated in Boraginaceae and Lamiaceae plants. A cytochrome P450 cDNA was isolated by differential display from cultured cells of *Lithospermum erythrorhizon*, and the gene product was designated CYP98A6 based on the deduced amino acid sequence. After expression in yeast, the P450 was shown to catalyze the 3-hydroxylation of 4-coumaroyl-4′-hydroxyphenyllactic acid, one of the final two steps leading to rosmarinic acid. The expression level of *CYP98A6* is dramatically increased by addition of yeast extract or methyl jasmonate to *L. erythrorhizon* cells, and its expression pattern reflected the elicitor-induced change in rosmarinic acid production, indicating that *CYP98A6* plays an important role in regulation of rosmarinic acid biosynthesis.

Key words: Cytochrome P450; Rosmarinic acid biosynthesis; 3-Hydroxylation of phenolic ester; *Lithospermum erythrorhizon*

1. Introduction

A remarkably diverse array of cytochrome P450s is utilized in both biosynthetic and detoxification pathways in higher plants. The *Arabidopsis* genome project has revealed 273 cytochrome P450 genes clustered in 45 CYP families in this species (http://drnelson.utmem.edu/CytochromeP450.html), most of which have not yet been functionally characterized. P450 gene products often appear to form key regulatory steps in plant secondary metabolism, as exemplified during sesquiterpene biosynthesis in cotton [1].

Rosmarinic acid (α-*O*-caffeoyl-3,4-dihydroxyphenyllactic acid, RA) is a prominent hydroxycinnamic acid ester that accumulates in Boraginaceae and Lamiaceae plants. RA exhibits various pharmacological activities including prevention of oxidation of low density lipoprotein [2], inhibition of murine cell proliferative activity [3] and of cyclooxygenase [4], and anti-allergic action [5]. The biosynthesis and production of RA have been extensively studied using cell and organ culture systems because (1) RA is a potentially useful compound as a medicine and a food additive, and (2) RA biosynthesis represents an interesting model system in which two parallel biosynthetic pathways must be regulated in a coordinated manner. Earlier radiotracer studies established that the caffeic acid moiety and the 3,4-dihydroxyphenyllactic acid moiety are derived from phenylalanine and tyrosine, respectively [6]. RA synthase [7] catalyzed the condensation of 4-hydroxyphenyllactic acid, derived from tyrosine, with 4-coumaroyl-CoA formed through the phenylpropanoid pathway, to yield α-*O*-4-coumaroyl-4′-hydroxyphenyllactic acid (CHPL). CHPL is then converted to RA through two consecutive hydroxylation reactions as shown in Fig. 1. Of the three hydroxylation steps required to form RA, the first (hydroxylation of *trans*-cinnamic acid to form 4-coumaric acid) is catalyzed by cinnamic acid 4-hydroxylase (C4H). Two C4H cDNAs have been cloned from *Lithospermum erythrorhizon* (Boraginaceae) and characterized as CYP73A30 and CYP73A31 [8]. Much less is known about the final two hydroxylation steps. The relevant enzyme activity has been detected in microsomal preparation from *Coleus blumei* (Lamiaceae) [9] but has yet to be fully characterized.

RA formation in *L. erythrorhizon* cell suspension cultures is rapidly and transiently stimulated by addition of yeast extract (YE) or methyl jasmonate (MJ) [10–11], providing a convenient system for analysis of the molecular events that regulate RA biosynthesis. Elicitation by YE or MJ induced increased activities of both phenylalanine ammonia-lyase (PAL) and 4-hydroxyphenylpyruvate reductase (HPR), entrypoint enzymes for the phenylpropanoid pathway and tyrosine-derived pathway, respectively. Similar effects of YE or MJ treatment on RA biosynthesis have also been reported in *Orthosiphon aristatus* [12] and *C. blumei* [13] cell suspension cultures, and in hairy root cultures of *Salvia miltiorrhiza* [14].

In order to gain insight into the molecular mechanisms underlying RA biosynthesis, we have undertaken differential display analysis of genes induced during elicitation of RA biosynthesis after YE addition to *L. erythrorhizon* cultured cells. In the present paper we describe a cDNA encoding a cytochrome P450 protein (CYP98A6), whose encoded protein

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[1] The nucleotide sequence reported here will appear in the DDBJ/EMBL/GenBank Nucleotide Sequences Database under the accession number AB017418 (CYP98A6).

Abbreviations: RA, rosmarinic acid; CHPL, 4-coumaroyl-4′-hydroxyphenyllactic acid; PAL, phenylalanine ammonia-lyase; HPR, 4-hydroxyphenylpyruvate reductase; C4H, cinnamic acid 4-hydroxylase; YE, yeast extract; MJ, methyl jasmonate

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was found to catalyze the 3-hydroxylation of a phenolic ester, CHPL, which is a central intermediate in the RA biosynthetic pathway. We also confirmed that expression of this gene is rapidly induced in *L. erythrorhizon* cells by YE or MJ treatment, concomitantly with the induction of RA biosynthesis.

2. Materials and methods

2.1. Plant cell culture and elicitor treatment

Suspension cultures of *L. erythrorhizon* Sieb. et Zucc. were established from seedling-derived callus tissues and maintained in LS liquid medium [15] 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 120°C for 20 min, and aseptically added to the cell suspension at a final concentration of 5 g/l. 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 −80°C.

2.2. Isolation and analysis of RNA

Total RNA was extracted from the frozen cells using TRIzol (Invitrogen) according to a protocol supplied by the manufacturer. Poly(A)$^+$ RNA was prepared from the YE-treated cells 8 h after YE addition, using a Quick Prep mRNA Purification Kit (Amersham Pharmacia Biotech). The cDNA library was constructed using a λZAP-cDNA Synthesis/Gigapack Gold Packaging kits (Stratagene). For Northern hybridization, total RNA (20 μg) was fractionated in a formaldehyde–1.25% agarose gel and transferred onto Hybond N$^+$ membrane (Amersham Pharmacia Biotech). Hybridization with digoxigenin-labeled probes and chemiluminescent detection were carried out according to a protocol supplied by Roche Biochemicals.

2.3. Differential display

Differential display was performed essentially as described by Yoshida et al. [16] with some modifications. The DNase I-treated total RNA (200 ng) was reverse-transcribed in 10 μl reverse transcription buffer containing 0.9 mM $MnCl_2$, 0.25 mM dNTP, 0.8 μM 12-mer arbitrary primer (Wako Pure Chemical Industries) and 1 U *Tth* DNA polymerase (Roche Biochemicals) at 40°C for 30 min. PCR was then performed in 50 μl reaction mixture containing 10 μl of the reverse transcription mixture, 40 μl PCR buffer (Roche Biochemicals) and 0.75 mM EGTA. In the present protocol, a single arbitrary primer was used for both reverse transcription and PCR. PCR was carried out in a Perkin Elmer Model 2400 Thermal Cycler using the following parameters: denaturing at 94°C for 3 min, followed by 40 cycles at 94°C for 1 min, 40°C for 1 min and 72°C for 2 min, and then a final extension at 72°C for 5 min. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Differential displayed cDNA bands were recovered from the gel, re-amplified with the A07A primer (5′-GATGGATTTGGG-3′) and cloned into pGEM-T vectors (Promega). The pGEM-T clone inserts were sequenced for both strands using the Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech). The inserts were digoxigenin-labeled with the PCR DIG Probe Synthesis Kit (Roche Biochemicals) and used as a probe for Northern hybridization and library screening.

2.4. Expression of CYP98A6 in Saccharomyces cerevisiae

For construction of the pYES2-CYP98A6 expression vector, the open reading frame of *CYP98A6* was amplified by PCR using *Pfu* DNA polymerase (Promega) with primers 5′-CGGGGTACCATGGCCCTGCCAGCCATACCCCTA-3′ and 5′-GGTCTAGATTACATGTCCGACTCGACTCGTT-3′. These primers correspond to the 5′ and 3′ ends of the open reading frame and introduce a *Kpn*I site upstream of the start codon and a *Xba*I site downstream of the stop codon. PCR was carried out as recommended by Promega. Upon completion of the PCR cycle, the PCR mixture was incubated for another 2 min at 72°C with 3 U of *Taq* DNA polymerase (Roche Biochemicals). The resulting 1.5-kb PCR product was ligated into a pGEM-T vector (Promega), sequenced to ensure that no mutation was incorporated, and subcloned into *Kpn*I–*Xba*I-digested pYES2 (Riken DNA Bank, Japan). The resulting yeast expression vector was transformed into *S. cerevisiae* strain INVSc1 (Invitrogen) by electroporation. The transformed yeast cells were grown as described previously [8], and microsomes were prepared according to the method of Guengerich et al. [17] 16–22 h after induction with galactose. Protein content in the microsomal fraction was estimated using the method of Bradford [18].

2.5. Enzyme assay

The standard assay mixture for CHPL 3-hydroxylase activity contained in a total volume of 400 μl of 100 mM sodium phosphate buffer, pH 7.5, 0.56 mg microsomal protein from yeast, and 12 nmol (±)-CHPL and 24 nmol NADPH. (±)-CHPL was synthesized as described elsewhere [19]. The reaction was incubated at 25°C for 30 min and terminated by addition of 40 μl acetic acid. Reaction products were extracted three times with 800 μl ethyl acetate. The pooled ethyl acetate extract was evaporated under vacuum, and the residue was dissolved in 30 μl MeOH–0.1% acetic acid (1:1) and subjected to HPLC analysis. HPLC conditions: column, Cosmosil 5C18-MS (4.6×150 mm I.D.; Nakarai Tesque); elution, MeOH–0.1% acetic acid (1:1) at a flow rate of 1 ml/min. The absorbance of the eluate was monitored with a diode array detector. The retention times of the product and the substrate were 5.0 min (for CHPL) and 4.2 min (for the enzyme product), respectively. For identification of the product,

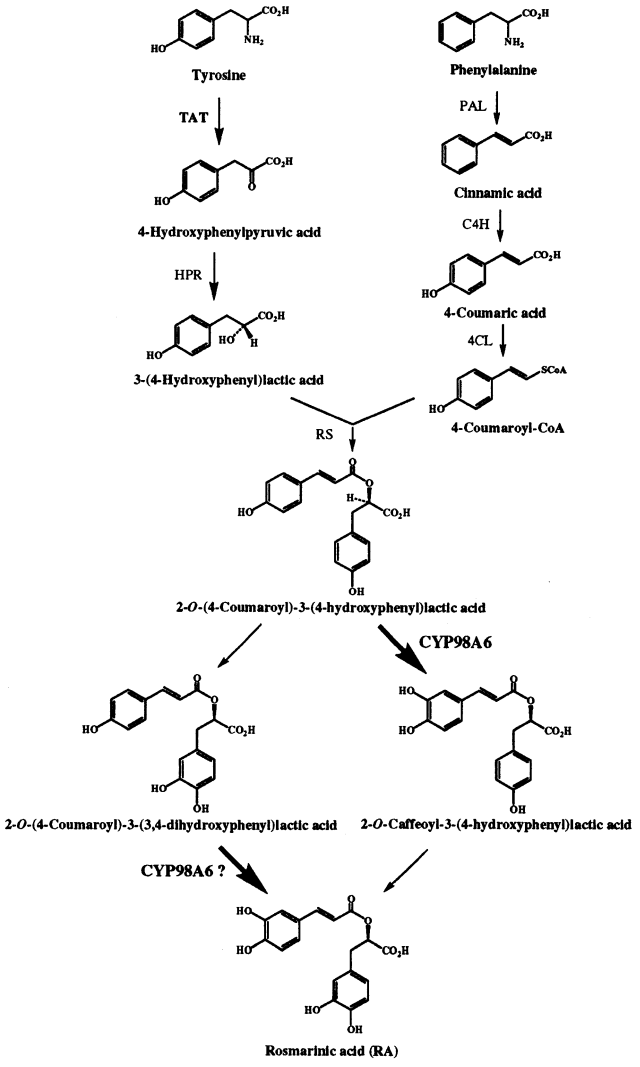

Fig. 1. The proposed biosynthetic pathway leading to rosmarinic acid. PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaric acid:CoA ligase; TAT, tyrosine aminotransferase; HPR, 4-hydroxyphenylpyruvate reductase; RS, rosmarinic acid synthase.

HPLC eluate corresponding to the product peak was pooled, evaporated and analyzed by the FAB-MS and ^{1}H-NMR spectra using a JEOL JMS SX-102 spectrometer and a JEOL JNM Lambda 400 (400 MHz) spectrometer, respectively. The CD spectra of the product and authentic RA (purchased from Funakoshi, Japan) were measured using a JASCO J-725 Spectropolarimeter in ethanol at 25°C.

2-O-Caffeoyl-3-(4-hydroxyphenyl)lactic acid: FAB-MS; *m/z*: 367 [M+Na]. ^{1}H-NMR (abbreviations: s, singlet; d, doublet; dd, doublet of doublets; m, multiplet); (CD_3OD) δ: 3.12–3.13 (1H, m, CH_aH_b-Ph), 3.16 (1H, d-like, J=7.3 Hz, CH_aH_b-Ph), 5.01 (1H, m, OCH<), 6.25 (1H, d, J=15.9 Hz, PhC*H*=CH), 6.68 (2H, d, J=8.3 Hz, Ar–H), 6.75 (1H, d, J=8.3 Hz, Ar–H), 6.91 (1H, dd, J=2.0, 8.3 Hz, Ar–H), 7.01 (1H, d, J=2.0 Hz, Ar–H), 7.12 (2H, d, J=8.3 Hz, Ar–H), 7.49 (1H, d, J=15.9 Hz, CO–C*H*=CH).

2.6. Quantitative determination of RA

The frozen cells (about 0.2 g) were extracted with 2 ml methanol at 65°C for 60 min with vigorous shaking. The slurry was centrifuged at 12 000×*g* for 5 min and the supernatant was subjected to HPLC. The conditions for HPLC analysis have been described previously [10].

3. Results

3.1. CYP98A6 cDNA was cloned as a YE-inducible gene by differential display

By a simplified differential display approach three YE-inducible cDNA clones (F86A, F86B and A07A) were obtained and sequenced. Characterization of the F86A and F86B cDNA fragments will be reported elsewhere. A BLAST search using about 700 bp of the A07A sequence showed that the nucleotide sequence is similar to those of various plant cytochrome P450s. The A07A fragment was digoxigenin-labeled and used as a probe to screen about 250 000 plaques from a cDNA library constructed from the YE-treated *L. erythrorhizon* cells. After three rounds of plaque hybridization, a cDNA clone covering the entire protein coding region was isolated and sequenced. The cDNA clone was 1702 bp long with an open reading frame corresponding to 506 amino acids, a 41-bp 5′ non-coding sequence and a 140-bp 3′ non-coding region. The deduced amino acid sequence displayed the presence of evolutionarily conserved regions typical of all cytochrome P450s, including a proline-rich region immediately after the N-terminal hydrophobic sequence, a threonine-containing binding pocket for the oxygen molecule and a heme-binding region [20]. A search of DDBJ/EMBL/GenBank databases revealed that the encoded protein shared 60–65% amino acid sequence identity with other cytochrome P450 proteins belonging to the CYP98A subfamily, and the protein was designated CYP98A6 by the Committee on Cytochrome P450 Nomenclature (D.R. Nelson, personal communication).

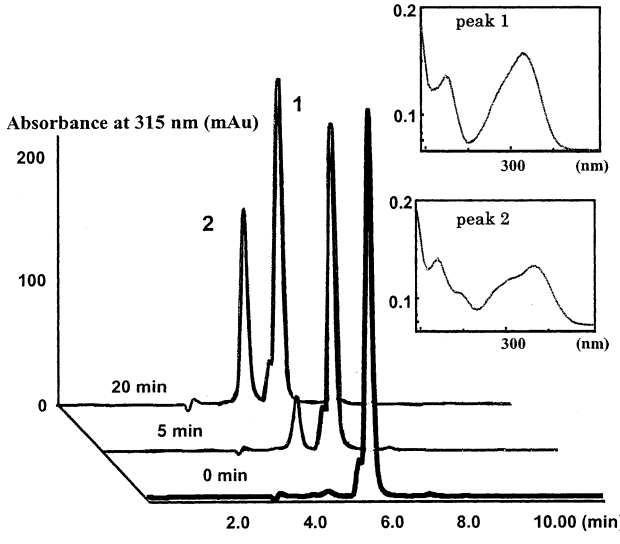

Fig. 2. HPLC analysis of the product formed from CHPL by incubation with recombinant CYP98A6. CHPL was incubated for 0, 5 and 20 min at 25°C with 0.56 mg microsomal protein from the induced yeast harboring pYES-CYP98A6. The insets show UV absorption spectra corresponding to peaks 1 and 2.

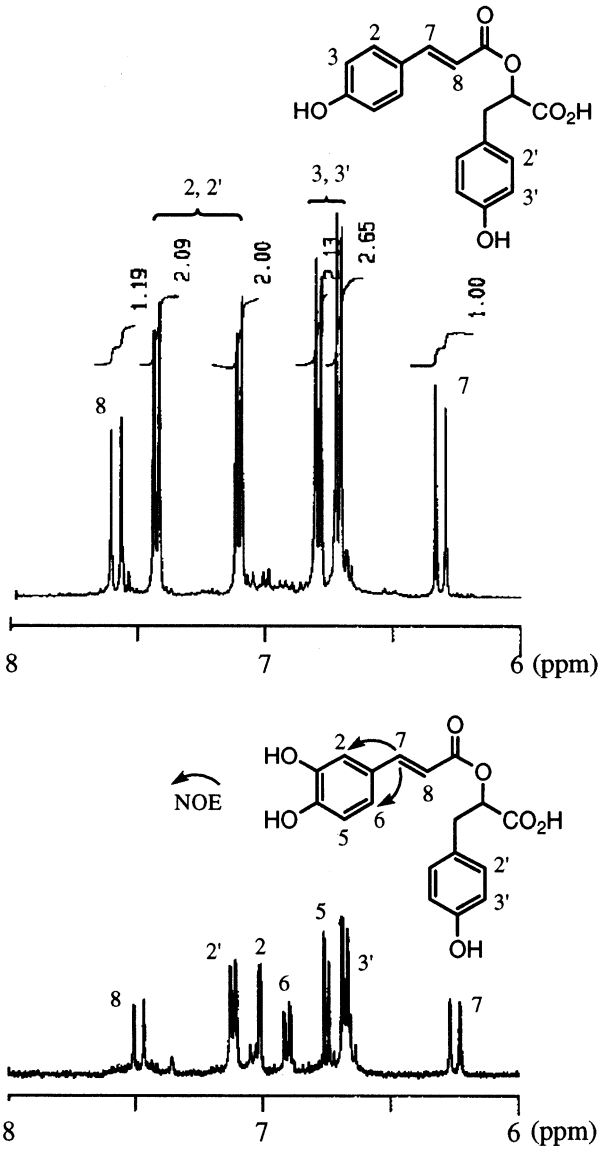

Fig. 3. ^{1}H-NMR spectra of CHPL (above) and the reaction product with the recombinant CYP98A6 (below). The numerals in the spectra indicate the assigned protons.

3.2. CYP98A6 specifically hydroxylates the coumaroyl moiety of CHPL

To examine the catalytic function of the gene product *CYP98A6* was heterologously expressed in yeast cells. Three cytochrome P450s are presumed to be involved in RA biosynthesis; one is C4H and the others would be required for two subsequent hydroxylation steps which convert CHPL to RA. Since C4H cDNAs have already been isolated from *L. eryth-*

rorhizon and identified as CYP73A30 and CYP73A31 [8], we used CHPL as a candidate substrate for assay of CYP98A6 enzyme activity. As shown in Fig. 2, recombinant CYP98A6 converted CHPL into a more hydrophilic compound. The conversion rate was linearly correlated with both time (up to 60 min) and microsome concentration (up to 1.3 mg microsomal protein). The reaction was also completely dependent on NADPH.

To determine the chemical structure of the product, the HPLC eluate containing the product was pooled, concentrated and subjected to NMR, MS and CD measurements. The FAB-MS spectrum of the product showed a pseudo molecular ion peak at *m/z* 367 [M+Na], 16 mass units larger than the substrate. The ^{1}H-NMR spectrum of the product indicated the presence of one *tri*-substituted phenyl group (δ 6.75, 6.91 and 7.01 ppm) and one *para*-substituted phenyl group (δ 6.68 and 7.12 ppm), a *trans*-olefin (δ 6.25 and 7.49 ppm) and a CH–CH_2 system (δ 5.01, 3.12–3.13 and 3.16 ppm) as shown in Fig. 3. Irradiation of the olefin signal at 6.25 ppm induced 5.1 and 5.9% nuclear Overhauser effects on the aromatic proton signals at 6.91 and 7.01 ppm, respectively. These results indicate that the product possesses a caffeoyl moiety rather than a 3,4-dihydroxyphenyllactic acid moiety. The substrate, CHPL, had therefore been hydroxylated by CYP98A6 at the 3-position of the coumaroyl moiety to produce caffeoyl-4′-hydroxyphenyllactic acid. Comparison of the CD spectra (data not shown) between the product and naturally occurring RA indicated that the stereochemistry of the product is same as that of RA, namely the *R*-form, although the substrate CHPL is a racemic mixture. Formation of RA could not be detected even when the reaction was continued for prolonged periods. Cinnamic acid, 4-coumaric acid and 4-coumaroyl-CoA were tested as substrates for hydroxylation, but none of these compounds was converted to the expected products, 4-coumaric acid, caffeic acid and caffeoyl CoA, respectively.

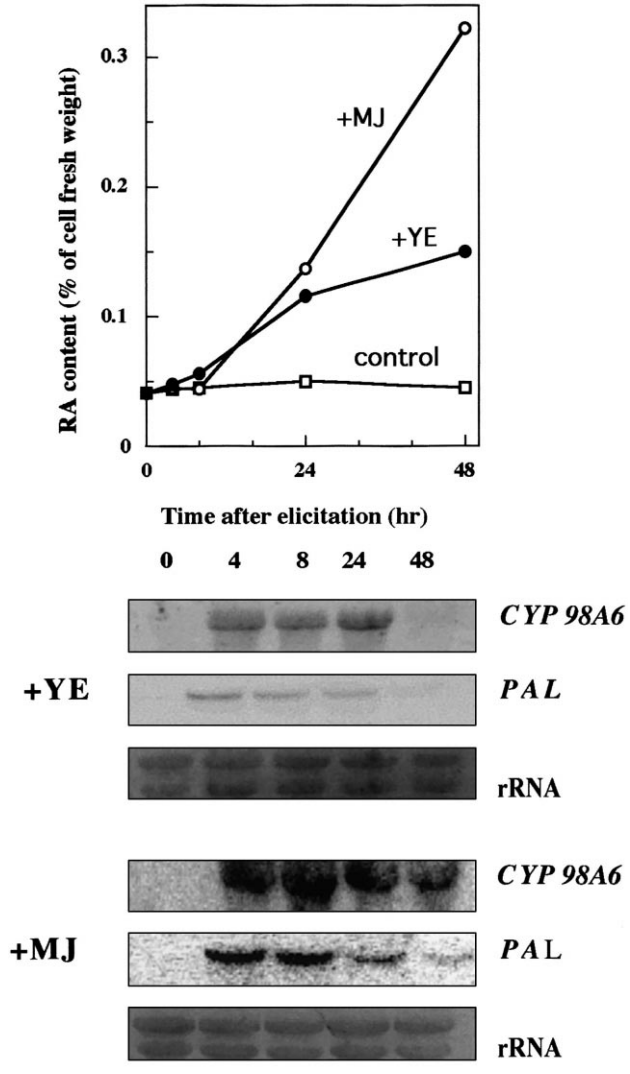

Fig. 4. Changes in RA accumulation and mRNA levels of CYP98A6 and of PAL in *L. erythrorhizon* suspension cultures after the addition of YE or MJ. The elicitors were added to the cultures 7 days after cell inoculation. The *L. erythrorhizon* PAL cDNA, which was generously provided by Dr. K. Yazaki of Kyoto University, was digoxigenin-labeled using the PCR DIG Probe Synthesis Kit and used as a probe. Dimethylsulfoxide was added to the control cultures.

3.3. *CYP98A6 expression is induced by various elicitors that also induce RA biosynthesis*

In order to characterize the relationship between *CYP98A6* expression and RA biosynthesis, we analyzed the changes in CYP98A6 mRNA level and in RA accumulation following different elicitor treatments (Fig. 4). RA accumulation in *L. erythrorhizon* cells started to increase gradually within 4–8 h after YE addition, followed by a rapid increase until 24 h. Expression of *CYP98A6* rose within 4 h after YE treatment, reached a peak at 24 h and thereafter decreased, consistent with the YE-induced changes in RA accumulation. MJ treatment, on the other hand, induced a larger increase in RA accumulation than did YE treatment, but the response of the cells to MJ was slower. RA production started to increase 8 h after MJ addition, and rose rapidly until 48 h after MJ treatment. A similar delay was observed in *CYP98A6* expression. Expression of *PAL* was also increased by YE or MJ treatment, but the extent of the increase was much smaller compared with *CYP98A6*.

4. Discussion

Characterization of plant cytochrome P450s by conventional biochemical approaches has been hampered by the low abundance and instability of these membrane-bound proteins. The relatively high degree of sequence conservation within the CYP superfamily has made it possible to use sequence-based PCR cloning to isolate plant cytochrome P450 genes [21], but this approach does not reveal the function of the encoded gene products unless the expressed protein can be assayed against appropriate substrates. T-DNA tagging provides an alternative method for unambiguously associating a given P450 gene with an in vivo metabolic function [22], but this is a time-consuming approach and applicable only within a limited number of species, such as *Arabidopsis*.

In this study we cloned a cytochrome P450 cDNA, *CYP98A6*, from elicited *L. erythrorhizon* suspension cultures using the differential display technique and established that CYP98A6 catalyzed the 3-hydroxylation of the hydroxycinnamoyl moiety of *R*-CHPL to form caffeoyl-4′-hydroxyphenyllactic acid, an immediate precursor of RA. This enzymatic activity had been detected in microsomes prepared from *C. blumei* suspension cells, based on the NADPH dependence,

inhibitor sensitivity and partially photoreversible carbon monoxide inhibition of the activity [9]. We have also observed that *L. erythrorhizon* microsome preparations supplemented with NADPH can efficiently convert CHPL to RA (data not shown). These observations point to the presence of two hydroxylating functions, but no further characterization of the hydroxylases involved has been reported.

Our results indicate that CYP98A6 represents the first of the two hydroxylases required to complete the RA biosynthetic pathway. Recombinant CYP98A6 converts CHPL exclusively to caffeoyl-4′-hydroxyphenyllactic acid rather than 4-coumaroyl-3′,4′-dihydroxyphenyllactic acid, and is unable to convert caffeoyl-4′-hydroxyphenyllactic acid to RA. This indicates that a second hydroxylase, responsible for 3′-hydroxylation of the 4′-hydroxyphenyllactic acid moiety, still remains to be discovered.

Despite the abundance of 3,4-dihydroxyphenylpropanoid metabolites found in plants, attempts to identify the enzyme(s) responsible for introduction of the 3-hydroxyl group to a 4-hydroxylated precursor have only recently met with notable success. 5-Hydroxylation of ferulic acid and its derivatives, such as coniferylaldehyde and coniferyl alcohol, has been shown to be catalyzed by ferulate-5-hydroxylase, a cytochrome P450 protein designated CYP84A1 [23]. In contrast, 3-hydroxylation of the 4-hydroxylated flavonoid B ring is catalyzed by a flavonoid 3′-hydroxylase belonging to a different P450 subfamily, CYP75B. Recently, Schoch et al. reported that CYP98A3 from *Arabidopsis thaliana* catalyzes 3-hydroxylation of shikimate and quinate esters of 4-coumaric acid to form corresponding caffeoyl esters [24]. This reaction is similar to the conversion of CHPL to caffeoyl 4-hydroxyphenyllactic acid, insofar as the primary substrate is a *p*-coumaroyl ester of a bulky hydrophilic acid.

A phylogenetic tree of various plant cytochrome P450s (Fig. 5) confirmed that among CYP98A members, CYP98A6 is most closely related to CYP98A3. CYP98A8 and CYP98A9, two other CYP98A members from *A. thaliana*, constitute a different cluster, and neither exhibits 3-hydroxylation activity against the phenolic esters [24]. Interestingly, CYP98A6 shares more sequence homology with C4H (CYP73A) than it does with F3′H (CYP75B), in spite of the fact that both CYP98A6 and F3′H catalyze 3-hydroxylation of a 4-hydroxylated aromatic ring. This suggests that the capacity to form *ortho*-dihydroxyphenylpropanoid metabolites may have evolved from an ancestral hydroxylase responsible for the original conversion of the unsubstituted cinnamic acid ring to its 4-hydroxy derivative.

The expression pattern of *CYP98A6* in cultured cells of *L. erythrorhizon* treated with elicitors is closely linked to elicitor-induced RA biosynthesis. RA production is stimulated more than 15-fold by addition of MJ, while the activities of PAL in the general phenylpropanoid pathway and HPR in tyrosine-derived pathway are induced only up to five-fold [9]. By contrast, *CYP98A6* expression was massively stimulated by MJ treatment. The striking increase in *CYP98A6* transcript levels after YE or MJ treatments, together with its demonstrated catalytic function, clearly places CYP98A6 in the RA biosynthetic pathway, and may indicate that *CYP98A6* plays a crucial role in regulation of the rate of RA formation.

While a key element in RA biogenesis has been discovered, unanswered questions remain. For example, CYP98A6 is clearly capable of introducing a 3-hydroxyl group to CHPL

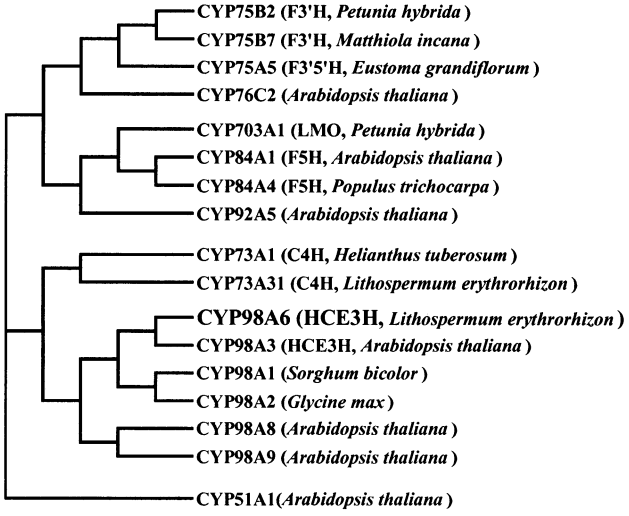

Fig. 5. Phylogenetic tree showing the relationship between the deduced amino acid sequences of CYP98A6 and some other plant P450s. The amino acid sequences were aligned using ClustalW and the tree was constructed by the neighbor-joining (NJ) method in PHYLIP software package (J. Felsenstein, Phylogeny Inference Package, version 3.573, University of Washington, Seattle, WA, USA). Applying other tree-constructing algorithms such as UPGMA and parsimony methods gave essentially the same topology. The tree was rooted using the CYP51A1 sequence as the outgroup. The branch length does not reflect genetic distance between two clusters. F3′H, flavonoid-3′-hydroxylase; F3′5′H, flavonoid-3′,5′-hydroxylase; F5H, ferulate-5-hydroxylase; LMO, lauric acid monooxygenase; HCE3H, hydroxycinnamoyl ester-3-hydroxylase.

to form the caffeoyl derivative which can then be converted to RA by a second 3′-hydroxylase acting on the 4′-hydroxyphenyllactic acid moiety. However, at this point we cannot exclude the possibility that CYP98A6 may normally catalyze 3-hydroxylation of 4-coumaroyl-3′,4′-dihydroxyphenyllactic acid that has been first formed from CHPL by the other 3′-hydroxylase (Fig. 1). Assay of recombinant CYP98A6 against 4-coumaroyl-3′,4′-dihydroxyphenyllactic acid will require preparation of this intermediate in the biosynthetic pathway, work which is now under way. Identification and characterization of the enzyme responsible for 3′-hydroxylation of the hydroxyphenyllactic moiety will also help to resolve this question. The relatively unreactive nature of the aromatic ring of 4′-hydroxyphenyllactic acid, compared to the more highly conjugated and reactive hydroxycinnamic acids, suggests that this hydroxylase may have significantly different properties from the enzymes characterized to date.

While *Lithospermum* CYP98A6 and *Arabidopsis* CYP98A3 are clearly evolutionarily related gene products, the specificity of CYP98A6 for RA biosynthesis, and of CYP98A3 for quinate/shikimate ester biosynthesis, remains uncertain. It is possible that CYP98A6 is not a specific enzyme for RA biosynthesis but represents a broader-specificity enzyme derived from CYP98A3, and that it still supports general phenylpropanoid biosynthesis in *Lithospermum*. To examine this question more closely, the ability of CYP98A6 to use shikimate and quinate esters of 4-coumaric acid as substrates, and of CYP98A3 to use CHPL, is also under investigation.

In summary, by using a differential display we have cloned from *L. erythrorhizon* suspension cultures a cDNA encoding a novel cytochrome P450-dependent monooxygenase (CYP98A6), and have demonstrated that the enzyme catalyzes

3-hydroxylation of a hydroxycinnamic acid ester involved in one of the final steps in RA biosynthesis. *CYP98A6* may prove to be useful for manipulation of RA productivity via genetic engineering.

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