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Molecular characterization and functional expression of dihydroxypterocarpan 6a-hydroxylase, an enzyme specific for pterocarpanoid phytoalexin biosynthesis in soybean (*Glycine max* L.)

Christel R. Schopfer[a], Georg Kochs[b], Friedrich Lottspeich[c], Jürgen Ebel[a,*]

[a] *Botanisches Institut, Ludwig-Maximilians-Universität München, Menzinger Str. 67, D-80638 Munich, Germany*
[b] *Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene, Universität Freiburg, D-79008 Freiburg, Germany*
[c] *Max-Planck-Institut für Biochemie, D-82152 Martinsried, Germany*

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Abstract Four cytochrome P450-dependent enzymes, among them dihydroxypterocarpan 6a-hydroxylase (D6aH), are specifically involved in the elicitor-inducible biosynthesis of glyceollins, the phytoalexins of soybean. Here we report that CYP93A1 cDNA, which we isolated previously from elicitor-induced soybean cells, codes for a protein with D6aH activity. Analysis of the catalytic properties of recombinant CYP93A1 expressed in yeast, its NADPH dependency, stereoselectivity and high substrate affinity confirmed that D6aH is the physiological function of CYP93A1. It thus represents the first isoflavonoid-specific CYP to be characterized at the molecular level. In elicitor-treated soybean cells producing phytoalexins, increases in D6aH activity were correlated with elevated transcript levels which indicates that expression of the enzyme is regulated at the level of transcription. Therefore, CYP93A1 cDNA can be used as a specific molecular marker for the inducible defense response against pathogen attack.

Key words: Cytochrome P450; Dihydroxypterocarpan 6a-hydroxylase; Glyceollin biosynthesis; Transcriptional control; Heterologous expression in yeast; *Glycine max* L. cell culture

1. Introduction

Soybean seedlings infected with *Phytophthora sojae* or soybean cell cultures treated with a glucan elicitor from this oomycete accumulate glyceollins, the phytoalexins of this plant [1]. Phytoalexin accumulation is preceded by increases of the enzyme activities involved in the biosynthetic pathway. For enzymes involved in early steps of glyceollin biosynthesis, such as phenylalanine ammonia lyase, 4-coumarate:CoA ligase and chalcone synthase, transcriptional activation has been shown ([1] and references therein). For later steps, however, knowledge about the mechanisms underlying the regulation of the activity levels of the enzymes is not well advanced.

One feature of glyceollin formation is that cytochrome P450-dependent enzymes (P450s) have a major role in later steps of the biosynthetic pathway. P450s are heme-containing proteins that function as terminal element in a membrane-bound electron transport chain and depend on electrons from the reaction catalyzed by NADPH-cytochrome P450 reductase (CPR). In plants, P450s constitute a superfamily that fulfils important functions both in secondary metabolism and in detoxification of xenobiotics [2,3]. Despite the recent cloning of more than 230 CYPs (cytochrome P450s) from plants, only in a few cases the physiological substrate of the encoded proteins could be identified (D. Nelson, personal communication; [3]). Of the five P450s involved in glyceollin biosynthesis [4], C4H catalyzes a reaction in the general phenylpropanoid pathway and has been thoroughly characterized at both the biochemical and the molecular level. The other four P450s catalyze reactions in the isoflavonoid/pterocarpanoid branch and are thus more specifically involved in the biosynthesis of glyceollins. The latter enzymes have been studied solely at the biochemical level. Among them is dihydroxypterocarpan 6a-hydroxylase (D6aH) (Fig. 1). Although the purification of D6aH protein to apparent homogeneity has been achieved previously, attempts to obtain amino acid sequence information have not been successful so far [5,6].

Molecular cloning of the four glyceollin-specific P450 cDNAs would allow us to study the regulation of reactions of the specific branch pathway and in addition, provide us with molecular markers for the phytoalexin defense response in soybean. We recently reported on the isolation of a soybean C4H cDNA and of seven other cytochrome P450 cDNAs that represented P450s whose mRNA levels were enhanced by elicitor treatment of soybean cells showing a time course similar to that of representative enzymes of the glyceollin pathway [7]. These seven CYPs with as yet unknown function were thus considered as candidates for the four glyceollin-specific P450s. We now present the functional identification of one of them. By heterologous expression CYP93A1 cDNA was demonstrated to encode a protein with D6aH activity equivalent to the enzyme biochemically characterized in elicitor-treated soybean tissue. This is the first report of the functional identification of a CYP cDNA specifically involved in the biosynthesis of isoflavonoids that are typical secondary metabolites of legumes.

2. Materials and methods

2.1. Materials

Sodium boro[^{3}H]hydride (13 GBq/mmol) was purchased from Amersham Buchler (Braunschweig, Germany); 2,4′,7′-trihydroxyisoflavon was provided by P.M. Dewick (Nottingham, England). Racemic (±)-3,9-dihydroxypterocarpan [6,11a-^{3}H]pterocarpan ((±)-DHP)

*Corresponding author. Fax: +49 (89) 1782274.
E-mail: j.ebel@botanik.biologie.uni-muenchen.de

Abbreviations: C4H, cinnamate 4-hydroxylase; CPR, NADPH-cytochrome P450 reductase; D6aH, 3,9-dihydroxypterocarpan 6a-hydroxylase; DHP, 3,9-dihydroxypterocarpan; DMSO, dimethylsulfoxide; HPLC, high pressure liquid chromatography; HPTLC, high performance thin layer chromatography; P450, cytochrome P450; THP, 3,6a,9-trihydroxypterocarpan; TLC, thin layer chromatography

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was obtained by reduction of 2,4′,7′-trihydroxyisoflavon with sodium boro[^{3}H]hydride [5,8]. The reaction product was purified by TLC on silica gel using solvent system 1, eluted from the gel with chloroform/methanol (8:2), dried, redissolved in methanol and stored at −20°C. The specific radioactivity was 4.51 Gbq/mmol and the radioactive yield 67%. The identity of the labeled compound was confirmed by cochromatography with a reference substance in solvent systems 1 and 2. The position of the label was confirmed in a control experiment with sodium boro[^{2}H]hydride and subsequent ^{1}H-NMR analysis. Unlabeled 3,9-dihydroxypterocarpan, 3,6a,9-trihydroxypterocarpan, and the radiolabeled (6a*S*, 11a*S*)-enantiomer of DHP were from our laboratory collection. Elicitor from *P. sojae* (60 μg of glucose equivalents/ml) was prepared by acid hydrolysis of purified mycelial cell walls [9].

2.2. Yeast strains and plasmids

The *Saccharomyces cerevisiae* W303-1B strain (*MAT*a, *leu*2, *his*3, *trp*1, *ura*3, *ade*2-1, can^R, cyr$^+$) designated W(N), had been engineered to overexpress either the yeast CPR upon galactose induction (strain W(R), [10]) or the *Arabidopsis thaliana* isoforms ATR1 and ATR2 (strains WAT11 and WAT21, [11]). The strains W(N) and W(R) were provided by Rhône-Poulenc Agro (Lyon). D. Pompon (Gif-sur-Yvette, France) provided the strains WAT11 and WAT21 as well as the yeast expression plasmid pYeDP60 [12].

2.3. Treatment of soybean cell cultures, pterocarpan extraction, RNA and microsome preparation

Cell suspension cultures of soybean (*Glycine max* L.) were propagated in 400 ml medium in the dark as described by Ebel et al. [13]. For induction experiments, 6-day-old cultures were transferred into fresh medium 12 h before addition of fungal elicitor to give a final concentration of 0.2 mg/ml medium. Suspension cells were harvested by filtration, frozen in liquid nitrogen and stored at −80°C. Pterocarpans were extracted from the culture filtrate twice with ethylacetate and analyzed by reverse phase HPLC on a LiChrosorb RP-18 (5 μm) column (250 mm long, 4 mm i.d.) with a flow rate of 1 ml min^{-1}. A linear gradient from 40 to 65% methanol in 16 min was used as eluent. Compounds were identified using reference substances. RNA was prepared according to Chang et al. [14] and analyzed by Northern blotting as described previously [7]. Microsomal fractions from soybean cells were isolated by a modified version of the protocol described by Diesperger et al. [15]. Frozen cells were homogenized by a mortar and pestle and resuspended in 0.2 M Tris/HCl, pH 7.5, 15% sucrose, 30 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol in the presence of Dowex1x2. After filtration through a nylon mesh and centrifugation at 12 000×*g* for 20 min, the microsomal fraction was collected from the supernatant by centrifugation for 30 min at 50 000×*g*, resuspended in 0.1 M KH_2PO_4/K_2HPO_4, pH 7.4, containing 30% glycerol, frozen in liquid nitrogen and stored at −80°C.

2.4. Enzyme assays

The standard assay for dihydroxypterocarpan 6a-hydroxylase contained in a total volume of 100 μl 50 mM KH_2PO_4/K_2HPO_4, pH 7.4, 5–30 μg microsomal protein from soybean cells or yeast transformants, 4.8 kBq (±)-dihydroxy[6,11a-^{3}H]pterocarpan (10 μM). After equilibration for 2 min at 30°C, the reaction was started with the addition of 1 mM NADPH and terminated after 20 min of incubation by the addition of 50 μl ethylacetate, 5 mg EDTA and 5 μl acetic acid. The reaction products were extracted twice with ethylacetate. The extract was applied to a silica gel plate, chromatographed with solvent system 2, and analyzed using a TLC scanner. The products were identified by cochromatography with reference substances. For determination of substrate affinity, the assay was scaled up 16 times. CPR activity was measured as described by Urban et al. [16].

2.5. Chromatography and product identification

TLC analysis was performed on silica gel plates (Merck F_{254}) with the solvent systems (ratios by volume) (1) chloroform/methanol (9:1) and (2) toluene/chloroform/acetone (45:25:35). The pterocarpans were detected under UV light or by spraying with a 0.1% solution of fast blue B salt (Merck).

2.6. Protein microsequencing

D6aH protein was purified to apparent homogeneity from elicitor-treated cell suspension cultures of soybean as described [6]. Approximately 4 μg protein were digested by proteinase LysC. Microsequencing of cleavage peptides was accomplished using a gas phase sequenator.

2.7. Construction of pK23

The coding region of CYP93A1 was inserted into the yeast expression vector according to Urban et al. [16] using PCR primers EF2 (5′-atatatagatctATGGCTTATCAAGTGTTGCTAATTTG) and ER2 (5′-tatatagaattcAATCAAATAGTAGGGAATGGGTTAATCC) in order to introduce a *Bgl*II restriction site upstream and an *Eco*RI restriction site downstream of the coding region. PCR was performed as described previously [7]. The resulting plasmid pK23 was analyzed by sequencing of one strand of the CYP93A1 coding region.

2.8. Yeast culture, transformation, and microsome preparation

Transformation of yeast and preparation of microsomal fractions was done as described previously [7,16]. CYP expression was induced according to the 'high-density procedure' described by Pompon et al. [17]. Cultures of 1–5×10^8 cells/ml were used for microsome preparation.

3. Results and discussion

3.1. Localization of a D6aH-derived decapeptide in the domain A of CYP93A1

D6aH protein was purified from elicitor-treated soybean cells and subjected to proteinase LysC digestion. Microsequencing of HPLC-purified cleavage peptides yielded a sequence of 10 amino acids (KARQEIDAVV). Out of the eight elicitor-inducible soybean CYP cDNAs that were isolated recently [7], only the deduced sequence of CYP93A1 contained a motif identical to the D6aH-derived peptide. It was located 20 amino acids C-terminal of the conserved motif ((A/G)Gx(D/E)T(T/S)) of the oxygen-binding domain of P450 proteins (Fig. 2). CYP93A3, the other member of the CYP93 family, which was also isolated in the screening for elicitor-inducible CYPs, contained a similar sequence, but the isoleucine was exchanged for a methionine. Generally, the pattern of hydrophobic and charged amino acids in the region corresponding to the D6aH-derived decapeptide was conserved in all plant CYPs available to date. The D6aH decapeptide, however, was not found in any other published plant P450 sequence, except CYP93A2, a jasmonate-induced soybean CYP of unknown function [18]. Because of the rare occurrence of the D6aH-derived decapeptide in plant CYPs, its presence in CYP93A1 strongly indicated that this CYP cDNA encoded D6aH.

3.2. Functional expression of CYP93A1 in yeast

In order to verify that CYP93A1 cDNA indeed encoded D6aH, the protein was expressed in a yeast strain that had been optimized for functional expression of CYPs [11,17]. Synthesis of the recombinant P450 as well as the electron transfer partner CPR can be induced in this expression system by the addition of galactose. The coding region of CYP93A1

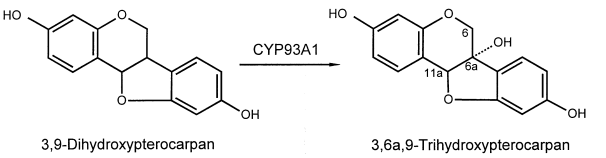

Fig. 1. Reaction catalyzed by dihydroxypterocarpan 6a-hydroxylase (CYP93A1).

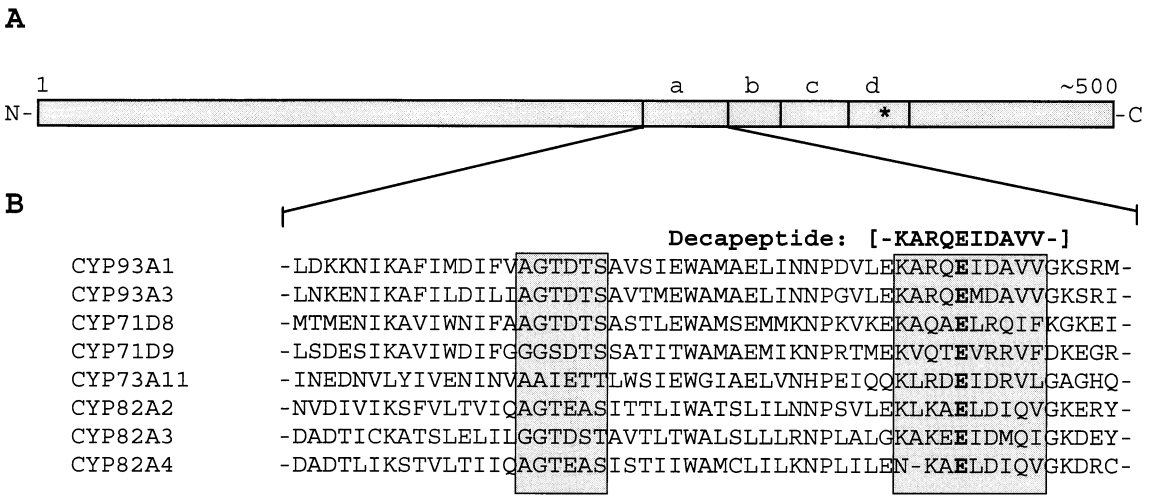

Fig. 2. Visualization of the D6aH-derived decapeptide in the CYP domain A. A: Overall structure of plant CYPs. a–d: Regions of high sequence conservation according to Kalb and Loper [19]. The heme-binding region is indicated by *. B: D6aH-derived decapeptide and comparison with the deduced amino acid sequences in domain A of the eight elicitor-inducible soybean CYPs isolated previously [7]. Boxes indicate conserved amino acids of the oxygen-binding site as well as amino acids corresponding to the D6aH-derived decapeptide. The glutamate residue conserved in all plant CYPs available to date is marked by boldface type.

cDNA was placed behind a galactose-inducible promoter in the yeast expression vector pYeDP60 [10]. The resulting plasmid pK23 was transformed into the yeast strain W(R) which expressed the yeast CPR upon induction by galactose. When a microsomal preparation from galactose-induced transformants was incubated with (±)-DHP in the presence of NADPH a new radioactive product was formed (Fig. 3). The identity of this product with THP was established by HPTLC on silica plates with solvent systems 1 and 2 (results not shown). The R_f value of the radioactive product coincided with that of authentic THP. The conversion of DHP to THP was dependent on the presence of NADPH. No reaction was observed using microsomes from yeast that was transformed with only the control vector pYeDP60 (Fig. 3). Therefore, D6aH activity resulted from the protein encoded by CYP93A1 cDNA (Fig. 1).

3.3. Catalytic properties of recombinant CYP93A1

The affinity of the CYP93A1 gene product for the substrate DHP was analyzed using a microsomal preparation of galactose-induced WAT11/CYP93A1 yeast cultures. The apparent K_m value for DHP of recombinant D6aH was found to be about 0.1 μM (Fig. 4A). This high affinity for the substrate implicated that DHP is the in vivo substrate of the CYP93A1 gene product. Similar experiments demonstrated that the K_m value for DHP of D6aH from elicitor-treated soybean cells was about 0.16 μM (Fig. 4B), closely matching the value found for the recombinant protein expressed in yeast. Both apparent K_m values were determined under the same experimental conditions, because the D6aH activities in yeast and soybean microsomes showed very similar optima for temperature (30°C), pH (7.4), and buffer concentration (50 mM K_2HPO_4/KH_2PO_4) (results not shown). Previous studies on D6aH of elicitor-induced soybean cells had yielded a value for the substrate concentration of half maximal specific activity ($[S]_{0.5}$) of 5 μM [5]. The apparent difference in the values for affinity towards DHP in the previous and present studies cannot be explained at present.

The reaction catalyzed by the recombinant D6aH was stereoselective. Maximally 50% of the racemic DHP was converted to THP by a microsomal preparation of CYP93A1-transformed yeast. With radioactively labeled (6a*S*, 11a*S*)-DHP no reaction was observed (results not shown). A similar

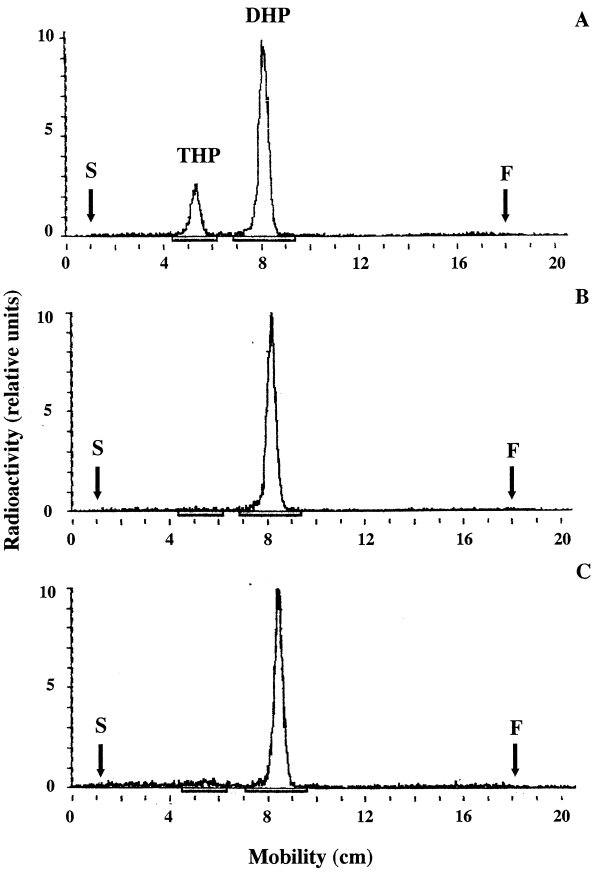

Fig. 3. TLC analysis of (±)-[^{3}H]DHP conversion using a microsomal fraction from galactose-induced W(R) cultures transformed with the CYP93A1-bearing plasmid pK23 (A, B) or with the control vector pYeDP60 (C). In B, NADPH was omitted. S, start; F, front.

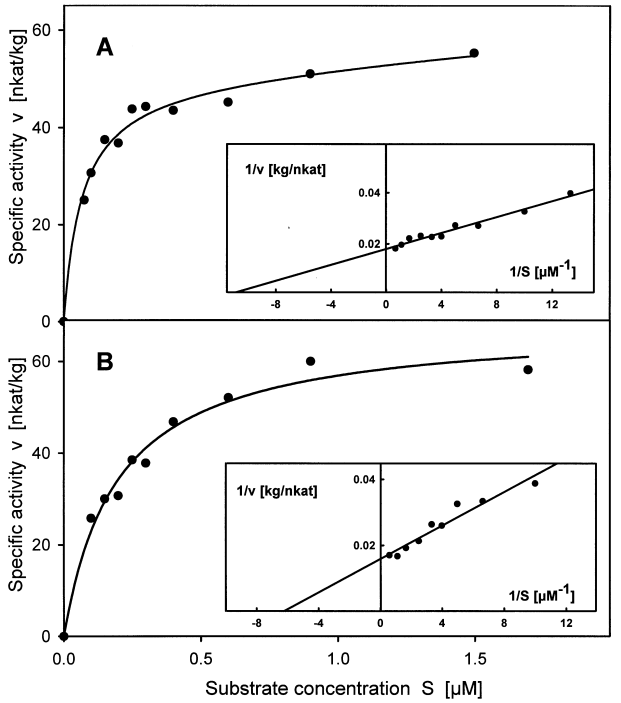

Fig. 4. Dependence of D6aH activity on (6a*R*, 11a*R*)-DHP concentration in microsomal fractions from galactose-induced WAT11/CYP93A1 cultures (A) or from elicitor-induced soybean cells (B). The inserts show double-reciprocal plots. The apparent K_m values were (A) 0.1 μM (repetition 0.1 μM) and (B) 0.1 μM (repetition 0.2 μM).

stereoselectivity had been previously observed for the D6aH activity of elicitor-induced soybean cells [5]. In addition to substrate affinity, also the demonstrated stereoselectivity for (6a*R*, 11a*R*)-DHP support the conclusion that the observed enzyme activity of the recombinant protein represents the in vivo function of this P450.

3.4. D6aH activity in different yeast strains

CYP93A1 cDNA was expressed in yeast strains differing in the origin and regulation of expression of CPR, the enzyme catalyzing the transfer of electrons from NADPH to the prosthetic heme group of the P450 protein. The different yeast strains used in the studies consisted of strain W(N) that was engineered to overexpress either yeast CPR upon induction with galactose (W(R)) or the *A. thaliana* CPR isoforms ATR1 and ATR2 (WAT11 and WAT21, respectively). Highest levels of apparent specific D6aH activity were measured in pK23-transformed WAT11 (Fig. 5). D6aH activities of the strains W(R), WAT21, and W(N) after transformation with pK23 were lower (26%, 19% and 12% of the value in WAT11, respectively). In contrast, highest specific activities for CPR were measured in strain W(R). These differences could be either due to variable levels of catalytically active D6aH protein in the different yeast strains, or it could result from unequal coupling efficiencies of recombinant D6aH protein with different CPRs. Because the expression levels of recombinant protein were too low for quantification of P450 content by CO difference spectroscopy, we could not differentiate between these possibilities. Interestingly, another CYP from soybean, CYP73A11 encoding C4H, showed highest catalytic activity when expressed in strain W(R) [7]. Further experiments

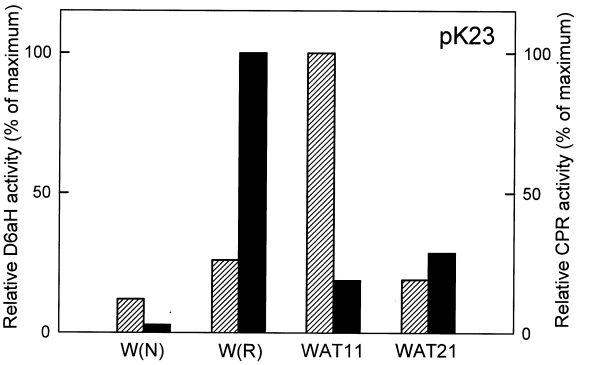

Fig. 5. Comparison of D6aH (hatched) and CPR (black) activities in microsomal extracts from yeast strains with different CPRs. Data represent mean values of relative activities in the four strains as determined in two independent experiments. Maximal values in the first experiment were 18 μkat/kg (D6aH) and 14 mkat/kg (CPR), in the second experiment 21 μkat/kg (D6aH) and 23 mkat/kg (CPR). W(N), *S. cerevisiae* strain W303-1B; W(R), strain overexpressing yeast CPR upon induction with galactose; WAT11, strain overexpressing CPR isoform 1 from *A. thaliana*; WAT21, strain overexpressing CPR isoform 2 from *A. thaliana*.

are required to establish whether particular P450s of soybean indeed display different coupling efficiencies with CPRs.

3.5. Elicitor-induced changes of D6aH transcript level, D6aH enzyme activity, and glyceollin amount

Soybean cells grown under standard conditions did not contain measurable amounts of either D6aH activity, D6aH transcripts, or of glyceollins, the phytoalexins of this plant. Addition of glucan elicitor resulted in a transient increase of

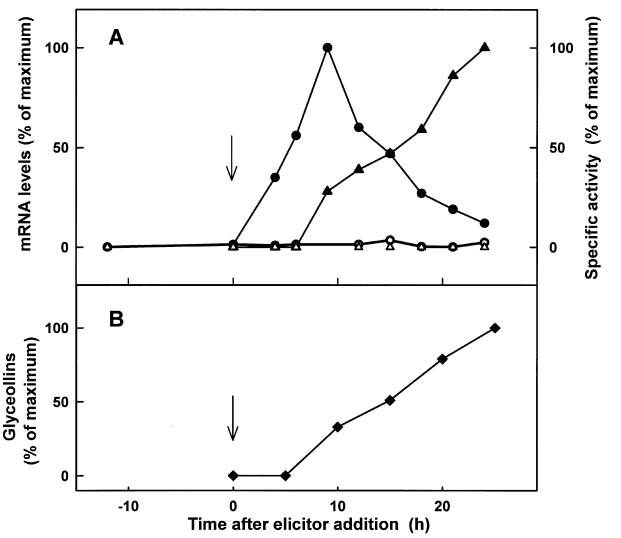

Fig. 6. Elicitor-induced increase of D6aH transcript level, D6aH activity and glyceollin level in soybean cells. A: At 12 h after transfer of the soybean cells into fresh medium, glucan elicitor (filled symbols) or water (open symbols) was added. Values for transcript level (circles), determined by Northern blot analysis, and enzyme activity (triangles), determined with microsomal protein, are given in percent of the maximum (100% D6aH activity represents 55 μkat/kg). Control hybridizations for Northern blot analysis were performed by using the *Hin*dIII fragment of SAc3 to monitor constitutively expressed actin transcripts [7]. B: Glyceollin accumultion in the culture filtrate of elicitor-treated soybean cells. Arrows indicate the time of addition of *P. sojae* elicitor.

CYP93A1 mRNA level after an apparent lag phase of less than 4 h (Fig. 6A). A maximal steady-state transcript level was observed at about 9 h after elicitor addition. In the same experiment, D6aH activity was first detectable at 6 h postelicitation and then steadily increased during the time analyzed. The period of high transcript levels correlated well with the time of increasing enzyme activity, indicating that D6aH activity might be controlled mainly at the transcriptional level under the conditions of the experiment. The final products of the biosynthetic pathway, the glyceollins, typically started to accumulate at about 10 h postelicitation (Fig. 6B). Consequently, elevated CYP93A1 transcript levels can be used to monitor activation of the defense-related pathway.

In summary, we have shown that CYP93A1 cDNA encodes D6aH that appears to be the first of the isoflavonoid-specific P450s to be characterized at the molecular level. The enzyme utilizes a pterocarpanoid intermediate as substrate that is specific for the final branch of the glyceollin biosynthetic pathway. Thus, experimental tools have been obtained for studies on the regulation at the molecular level of one of the late steps of phytoalexin biosynthesis. Furthermore, this CYP gene might be useful in transgenic approaches to extend plant disease resistance or in screening efforts for new herbicide targets.

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