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Molecular Analysis and Heterologous Expression of an Inducible Cytochrome P-450 Protein from Periwinkle (*Catharanthus roseus* L.)[1]

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ABSTRACT

We screened cDNA libraries from periwinkle (*Catharanthus roseus*) cell cultures induced for indole alkaloid synthesis and selected clones for induced cytochrome P-450 (P-450) proteins by differential hybridization, size of the hybridizing mRNA, and presence of amino acid motifs conserved in many P-450 families. Four cDNAs satisfying these criteria were analyzed in detail. They were grouped in two classes (pCros1, pCros2) that represented two closely related genes of a new P-450 family designated CYP72. Antiserum against a cDNA fusion protein overexpressed in *Escherichia coli* recognized in *C. roseus* a protein band of 56 kD. Quantification of western blots showed that it represented 1.5 ± 0.5 and 6 ± 1 μg/mg of protein in the membranes from noninduced and induced cells, respectively, and analysis of the total P-450 content suggested that the cDNA-encoded protein was one of the dominant P-450 proteins. The pathway to indole alkaloids contains two known P-450 enzymes, geraniol-10-hydroxylase (GE10H) and nerol-10-hydroxylase (NE10H). The induction kinetics of the cloned P-450 protein and of GE10H activity were similar, but those of NE10H were different. Western blots with membranes from other plants suggested that P-450 CYP72 is specific for *C. roseus* and other plants with GE10H activity. A tentative assignment of CYP72 as GE10H is discussed. The cDNA was recloned for expression in *Saccharomyces cerevisiae*, and the presence of the protein was demonstrated by western blots. Assays for GE10H failed to detect enzyme activity, and the same negative result was obtained for NE10H and other P-450 enzymes that are present in *C. roseus*.

P-450[2] are the terminal oxidases of a large number of biotransformations. The enzymic reactions include metabolism of steroids, fatty acids, prostaglandins, leukotrienes, biogenic amines, pheromones, drugs, plant metabolites, and numerous other substances, including mutagens (19). The importance of these enzymes led in the last 10 years to a dramatic increase of molecular research in animal systems (20).

The importance of P-450 enzymes in plants has been recognized (5), but the molecular analysis is lagging. The approach from purified protein to molecular biology has been difficult. In plants, the concentrations of these proteins are usually lower than in animals, the activities are unstable, the reconstitution of solubilized components to enzymically active complexes is difficult, and antisera against purified plant proteins or against P-450 enzymes from other sources most often recognize several polypeptides.

We investigated a molecular strategy to approach the analysis of P-450 enzymes. The system used in our experiments is a cell suspension culture of periwinkle (*Catharanthus roseus*), which is inducible for indole alkaloid biosynthesis (12, 23). This plant is a well-known source for pharmaceutically important indole alkaloids (see ref. 25 for review). Many but not all of these are also synthesized in cell cultures (26), and much effort has been directed toward the selection of high producers.

Alkaloid production is influenced by many factors, and the induction by changes of the medium composition has been well investigated (see for example refs. 12, 23, 25). The biosynthetic pathway to indole alkaloids contains several hydroxylation steps (25), and at least two are catalyzed by P-450 enzymes (GE10H and NE10H) (15). These proteins have been partially purified (14–17) and the induction kinetic of GE10H in a medium designed for indole alkaloid production has been investigated (23). Taken together, the available results suggested that these cell cultures are a suitable system for the molecular analysis of P-450 enzymes in a specific pathway.

We describe the isolation and molecular characterization of cDNAs for a new plant P-450 family (CYP72), the regulation of protein expression by medium changes, the correlation with P-450 enzyme activities in *C. roseus*, and the expression of the protein in the yeast *Saccharomyces cerevisiae*.

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[2] Abbreviations: P-450, cytochrome(s) P-450; CA4H, cinnamic-acid-4-hydroxylase; 14DM, lanosterol 14α-demethylase; FL3'H, flavonoid-3'-hydroxylase; FL3'5'H, flavonoid-3',5'-hydroxylase; GE10H, geraniol-10-hydroxylase; LAH, lauric-acid-hydroxylase (in chain); NE10H, nerol-10-hydroxylase; EROD, 7-ethoxyresorufin-*O*-deethylase; MX medium, growth medium; IM2 medium, alkaloid induction medium.

MATERIALS AND METHODS

Plant Material, Growth, and Induction Conditions

The cell suspension culture of periwinkle (*Catharanthus roseus* L. G. Don, line CP3a) had been selected for high indole alkaloid production (12, 23). Its maintenance on MX growth medium (18) and the induction of indole alkaloid biosynthesis by transfer into an 8% sucrose solution (12) or by growth in production medium IM2 (23) have been described. IM2 differs from MX medium in the concentrations of the following components: higher sucrose (8 versus 3%), higher potassium nitrate (0.63 versus 0.19 g/L), lower potassium phosphate (80 versus 170 mg/L), and the absence of 2,4-D (0.4 mg/L in MX medium).

The soybean (*Glycine max* L. Merr. cv Harosoy 63), peanut (*Arachis hypogaea* L.), *Ammi visnarga*, *Ammi majus*, and parsley (*Petroselinum crispum* L.) cell cultures were from the laboratory collection. For some experiments, the parsley cell cultures were induced by irradiation with UV-A (7). The Jerusalem artichoke (*Helianthus tuberosus* L.) system has been described (21). Parsley leaves were purchased at a local market. Leaves from *C. roseus*, *Allamanda neriifolia*, *Thevetia peruviana*, and *Nerium oleander* were from the Botanical Garden of the University of Freiburg. Like *C. roseus*, the latter three plants belong to the Apocynaceae family, which contains many members synthesizing indole alkaloids.

Isolation of Membranes and Measurements of Total P-450 Content

This was carried out as published for *C. roseus* (23), except that $K_2S_2O_5$ was omitted from the homogenization buffer. Microsomes from *Petunia hybrida* (cv Red Titan), *Ammi majus*, and *Ammi visnarga* were gifts from L. Britsch and D. Hamerski (University of Freiburg). Total P-450 content was determined according to published procedures (9).

Enzyme Assays

GE10H and NE10H Activity with Nonradioactive Substrate

These were based on the procedure of Schiel et al. (23), but the conditions were optimized with respect to the linearity of product formation with time and protein concentration. The assays contained in a final volume of 0.1 mL: 25 mM potassium phosphate (pH 7.5), 3 mM DTT, 1 mM geraniol or nerol, and 20 to 60 μg of protein from the membrane preparations. After preincubation for 5 min at 25°C, the enzyme reactions were initiated by addition of 1 mM NADPH (final concentration). They were stopped after 25 min by addition of 15 μL of 4 N KOH. After addition of 5 μg of 1,10-decanediol (internal standard for the quantification) (23), the assays were extracted twice with 0.25 mL of ethylacetate and the extracts were dried in a light stream of nitrogen. The residues were further dried over P_2O_5, dissolved in 5 μL of dimethylformamide, and silylated with 15 μL of *N*-trimethylsilyl-*N*-methyltrifluoro-acetamide for 45 min at 70°C. The hydroxylated products were quantified by capillary GC (WCOT fused Silica, CP Sil5, 25 m, with nitrogen as carrier gas; temperature program: 120 to 200°C with 6°C/min, then with 30°C/min to 300°C; Packard Instruments model 439). The products were identified in routine assays by their retention values. The identity of 10-hydroxygeraniol was also confirmed by mass spectrometric analysis (column, 15 m OV 1; carrier gas, helium, 2 mL/min; temperature program, 1 min at 80°C, then with 10°C/min to 300°C; mass spectrum recording, full scan mode electron impact, 70 eV).

GE10H Incubations with Radioactive Substrate

[1-^{14}C]Geranyl pyrophosphate (55 μCi/μmol) was prepared enzymically from dimethylallyl pyrophosphate and [1-^{14}C]-isopentenyl pyrophosphate (Amersham Corp.) (8), and the pyrophosphate was removed by treatment with acid phosphatase (Sigma). The free geraniol was extracted into pentane and the solvent was removed by evaporation. The enzyme incubations were as outlined above for the unlabeled substrate, except that the assays contained 20 μM [1-^{14}C]geraniol (2×10^5 cpm). This is in the range of the K_m values for geraniol (15), and therefore, the results are not directly comparable with those from the assay with unlabeled substrate that contained 1 mM geraniol. The separation (TLC in benzene-acetone-ethylacetate [2:1:1, v/v/v]) and quantification of substrate and product have been described (15).

Other Enzymes

The measurements of CA4H (7), LAH (21), and NADPH Cyt *c* reductase (6) were as published. FL3'H and FL3'5'H activities were analyzed with ^{14}C-labeled naringenin as substrate (3). These two enzymes operate in sequence, i.e. FL3'H synthesizes from naringenin the substrate for the FL3'5'H reaction. Therefore, FL3'H activity was calculated from the sum of both products.

RNA Isolation, cDNA Libraries, and Screening Procedures

Poly(A)$^+$ RNA was isolated (24) from *C. roseus* cell cultures induced for 7.5 h by a change from MX medium to 8% sucrose. The cDNA library was constructed using 5 μg of poly(A)$^+$ RNA and cDNA synthesis kits from Amersham Corp. (cDNA Synthesis System Plus, No. RPN1256Y) and Pharmacia LKB Biotechnology, Inc. (You-Prime cDNA Synthesis Kit No. 27–9273–01). After addition of *Eco*RI or *Eco*RI/*Not*I adaptors, the cDNAs were ligated to *Eco*RI digested phage lambda NM1149 (24) and packaged with a kit from Amersham Corp. (Lambda in vitro Packaging Kit No. N334L).

The library was sequentially screened in two steps to obtain a subset of clones corresponding to induced mRNAs of the sizes expected for P-450 proteins: (a) selection of clones representing induced mRNAs (differential hybridization with labeled single-stranded cDNAs from noninduced and induced cultures), and (b) selection of a second subset of cDNAs that hybridize in northern blots to induced mRNAs of the size expected for P-450 transcripts (1.3–1.9 kb for proteins of 45 to 65 kD; the known eukaryotic P-450 enzymes are in this size group [19]). The techniques have been described (22, 24).

Antiserum, Western Blots, and Quantification of the Immunoreactive Protein

The antiserum against the cDNA-encoded protein was obtained against an overexpressed bacterial fusion protein. The open reading frame in cDNA pCros1 was fused in frame with the amino terminal part of MS2 polymerase in expression vector pEX31c. This vector, expression of the fusion protein, its purification, and the raising of antiserum in rabbits have been described (27).

The separation of the proteins was routinely carried out in gels containing 0.1% SDS and 12.5% polyacrylamide. In some cases, the polyacrylamide concentration was reduced to 9% to obtain a better separation of proteins in the size range from 40 to 70 kD. Standard western blots used a secondary antibody coupled with alkaline phosphatase (Sigma Biochemicals). The quantification of the immunoreactive protein was performed with western blots incubated with biotin-coupled secondary antibodies and ^{125}I-labeled streptavidin (0.74–1.5 MBq/μg, Amersham Corp.) as described by the manufacturer. The radioactive bands were excised from the nitrocellulose sheets and quantified in a scintillation counter. The purified pCros1 cDNA fusion protein in various concentrations served as standard for the quantification. The immunoglobulin fraction of the anti-P-450 antiserum was purified by chromatography on Protein-A sepharose (Pharmacia LKB Biotechnology, Inc.) for these experiments.

DNA Sequence Analysis

The DNA fragments were sequenced by the dideoxy nucleotide chain termination technique using vectors and phages as described in ref. 13. The pTZ18R and pTZ19R system, helper phage M13K07, *Escherichia coli* strain JM109 (Pharmacia LKB Biotechnology, Inc.), and the reverse sequencing primer (Boehringer Mannheim) were used routinely on subcloned cDNA fragments. DNA polymerization reactions were performed with [^{35}S]adenosine 5′-*O*-(1-thiotriphosphate) (37 TBq/mmol, Amersham Corp.) and modified T7 DNA polymerase (Sequenase, Biochemical Corp.). The alignment of the sequences was carried out with the ALIGN2 program of M. Trippel (Infomed GmbH, Freiburg) and the PCGENE software package (IntelliGenetics Inc., Mountain View, CA). The FSTPSCN program of PCGENE was employed for homology searches in the SWISSPROT database (release 20).

Expression of the Plant P-450 Protein in *S. cerevisiae*

Strain JL745 (MAT α *ade* 2 *his* 4–419 *trp ura* 3–52 *cir*$^+$) (11) and plasmid pSCDM111 have been described (29). The plasmid contains a *SalI*/*Hin*dIII fragment with the yeast Pho5 promoter region (acid phosphatase, promoter repressed in the presence of phosphate in the medium), the coding region for the 14DM protein, and the transcription terminator of the 14DM gene (Fig. 1).

The *SalI*/*Hin*dIII fragment containing the yeast 14DM gene with the regulated Pho5 promoter was cloned into *E. coli* vector pTZ19R that had been modified before by deletion of the *Eco*RI site in the polylinker (pTZ19R▲E in Fig. 1). Most of the 14DM coding region was then removed by deletion of

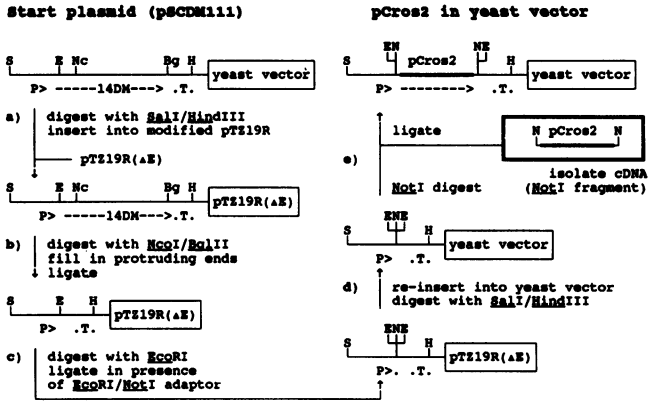

Figure 1. Cloning strategy for expression of the P-450 protein encoded in pCros2a and pCros2b in *S. cerevisiae*. The sizes are not drawn to scale. The *SalI*/*Hin*dIII fragment of the yeast plasmid pSCDM111 contains the Pho5 promoter region (P>), the coding region for the 14DM protein (–14DM–>), and the terminator region (.T.) (29). The principle of the strategy was: (a) cloning of the *SalI*/*Hin*dIII fragment in vector pTZ19R▲E (▲E indicates that the vector had been modified by deletion of the *Eco*RI site of the polylinker); (b) deletion of the 14DM protein coding region; (c) insertion of an *Eco*RI/*Not*I adaptor into the *Eco*RI site; (d) recloning of the modified *SalI*/*Hin*dIII fragment into the yeast vector; (e) insertion of pCros2a or pCros2b as *Not*I fragment. Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; Nc, *Nco*I; S, *Sal*I.

a *NcoI*/*Bgl*II fragment. This plasmid was opened at the single *Eco*RI site, and an *Eco*RI/*Not*I adaptor was inserted to provide a *Not*I site for introduction of the cDNAs pCros2a or pCros2b. The final constructs contained the coding region of the plant P-450 protein in the correct orientation positioned between the regulated Pho5 promoter and the transcription terminator sequences of the 14DM gene. Plasmids with the cDNAs oriented in the antisense direction served as controls. Plasmids were transformed into yeast strain JL745 (11) for analysis of the protein expression. For the induction experiments, the cells were grown to A_{600} = 1 in medium containing ammonium phosphate, concentrated by centrifugation, resuspended to the same density in phosphate-free medium, and harvested after incubation for an additional 7 to 8 h (29).

Membranes were isolated from *S. cerevisiae* as in ref. 4. Briefly, the cells were harvested, washed, treated with lyticase (Sigma; 10 mg/g fresh weight of cells) for 1 h at 30°C, and then broken in a French pressure cell (300–400 kg/cm^2). After a 10-min centrifugation at 15,000*g*, the membranes were pelleted at 110,000*g* for 60 min. The pellet was washed once, resuspended in 50 mM potassium phosphate, pH 7.5, 2 mM DTT, 25% glycerol, and stored at −70°C.

RESULTS

cDNA Clones for an Induced P-450 Protein

The first cDNA clone isolated with the sequential screening steps was designated pCros1. It contained an *Eco*RI fragment of 1 kb and an open reading frame throughout the DNA sequence. A search in the SWISSPROT library showed that the deduced protein possessed significant similarity with the

blocks A, B, and C, which are conserved in many P-450 families (10). The alignment with these proteins also predicted that pCros1 represented an *Eco*RI subfragment of a larger mRNA, and that the 5′ and the 3′ end of the mRNA were missing from this cDNA. Because it was possible that the *Eco*RI sites at the ends of cDNA pCros1 represented true restriction sites instead of adaptor sequences, we established a new library with *Eco*RI/*Not*I adaptors attached to the cDNA (this allows excision of the cDNA with *Not*I), and rescreened the first library with cDNA pCros1. Three new clones 1.8 kb in length, identified in this second screening, are aligned with pCros1 in Figure 2.

The sequence analysis (Fig. 3) showed that the three larger cDNAs, designated pCros2a, pCros2b, and pCros2c, differed in the extent of sequences at the 5′ and the 3′ ends, but were otherwise identical. The pCros2 clones contained the *Eco*RI sites of pCros1, and therefore, these did not represent adaptor sequences in the first cDNA. pCros1 differed from the pCros2 clones in 63 of 1033 positions. Because most of the exchanges were silent, only eight amino acid differences occurred with five of these considered as conservative changes. The results indicated that pCros1 and the pCros2 cDNAs represented two genes that code for very similar proteins (93.8 and 97.7% identity in the compared DNA and protein sequences, respectively).

The pCros2 cDNAs contained an open reading frame of 525 amino acids. With the first methionine as start (position 1 in Fig. 3), the protein would have 524 amino acids, a calculated size of 60,557 D, and a predicted membrane associated α-helix close to the amino terminal end (Fig. 3). The amino acid sequence had overall similarities with other P-450 proteins. The best values were 25% with animal LAH (CYP4A group) and 23% with several members of the CYP3A group (20). The similarity with other P-450 proteins was most pronounced in the four blocks of amino acids that are conserved in eight P-450 families (10) (Fig. 4). Block D contains the cysteine binding the heme group, and some of the amino acids to the right and to the left of this cysteine are strictly conserved (1). This motif was also present in the amino acid sequence derived from the pCros2 cDNA (■ in Fig. 4).

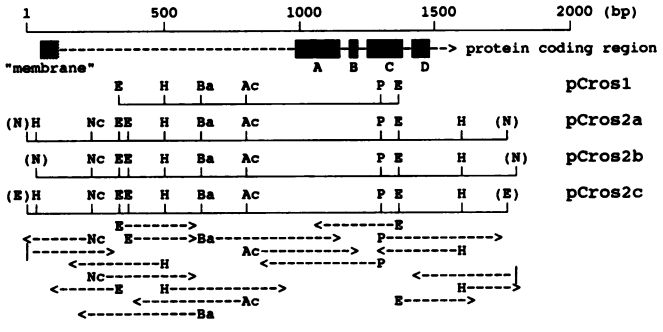

Figure 2. Alignment of the cDNA clones from *C. roseus* and position of the blocks A to D that are conserved in eight P-450 families (10). Membrane, Predicted membrane-associated α-helix at the amino terminal end. Bottom, Sequence strategy for the clones. Ac, *Acc*I; E, *Eco*RI; Ba, *Bam*HI; H, *Hin*dIII; N, *Not*I; Nc, *Nco*I; P, *Pst*I; in brackets, sites from the cloning of the cDNAs with *Eco*RI or *Eco*RI/*Not*I adaptors.

RNA and Protein in *C. roseus*

The northern blot analysis of RNA isolated from cultured *C. roseus* cells with the cloned cDNAs revealed a transcript of 1.8 kb (Fig. 5A), indicating that the pCros2 clones represented full-length or nearly complete copies of the mRNA. The RNA was present in noninduced cells and was induced 3- to 5-fold by change of the medium (Fig. 5A, lane 1). The probe did not discriminate between pCros1 and pCros2, and therefore, the relative contributions of the transcripts are not known.

The antiserum raised against the pCros1 cDNA fusion protein overexpressed in *E. coli* reacted with a single protein band of 56 ± 1 kD in *C. roseus* cells (Fig. 5C). The polyclonal rabbit antiserum did not discriminate between the proteins encoded in pCros1 and pCros2, and the presence of two cDNA classes suggested that the band contained at least two polypeptides. The size was smaller than predicted from the pCros2 DNA sequence, but this is typical for these hydrophobic polypeptides (19). The protein was present in noninduced cells and was induced 3- to 5-fold by a change from MX to IM2 indole alkaloid production medium (see Fig. 5C for example of a western blot). In noninduced as well as in induced cells it was detected only in the membrane fraction (110,000*g* pellet; Fig. 5B).

The quantification of western blots (see "Materials and Methods") indicated that the immunoreactive protein represented 1.5 ± 0.5 and 6 ± 1 μg/mg of the total protein in the membranes from noninduced and induced cells, respectively. This corresponds to 27 and 110 pmol/mg for the 56-kD cDNA-encoded protein. Determinations of the total P-450 content in membrane preparations from noninduced and induced cells yielded values of 200 to 250 pmol/mg of protein. The cDNA-encoded protein, therefore, appeared to represent a major fraction of the total P-450 proteins in *C. roseus* cell cultures induced for indole alkaloid biosynthesis.

Correlation of the Protein with P-450 Enzymes in *C. roseus*

In these experiments, we investigated whether the induction kinetics of the cDNA-encoded protein correlated with changes in the activities of P-450 enzymes.

A first set of experiments analyzed a change from MX medium to indole alkaloid production medium IM2. GE10H and NE10H were investigated in detail because the enzymes were known to be induced under these conditions (23). CA4H had not been measured before in *C. roseus*, but was included because it was induced in many other cell cultures after a switch of the medium. The results are summarized in Figure 6. Western blots quantifying the protein reacting with the antiserum showed a 2- to 3-fold increase within 24 h after change of the medium, and large amounts were present until the end of the experiment (Fig. 6A). The induction curves for immunoreactive protein and GE10H activity were similar throughout most of the time course, but significant deviations occurred at day 6 and 7 (Fig. 6A). NE10H was induced with lower activities than GE10H (181 versus 345 pkat/mg) (Fig. 6B). The two enzyme kinetics were similar in the first 8 d, but diverged afterwards. NE10H revealed only a few percent

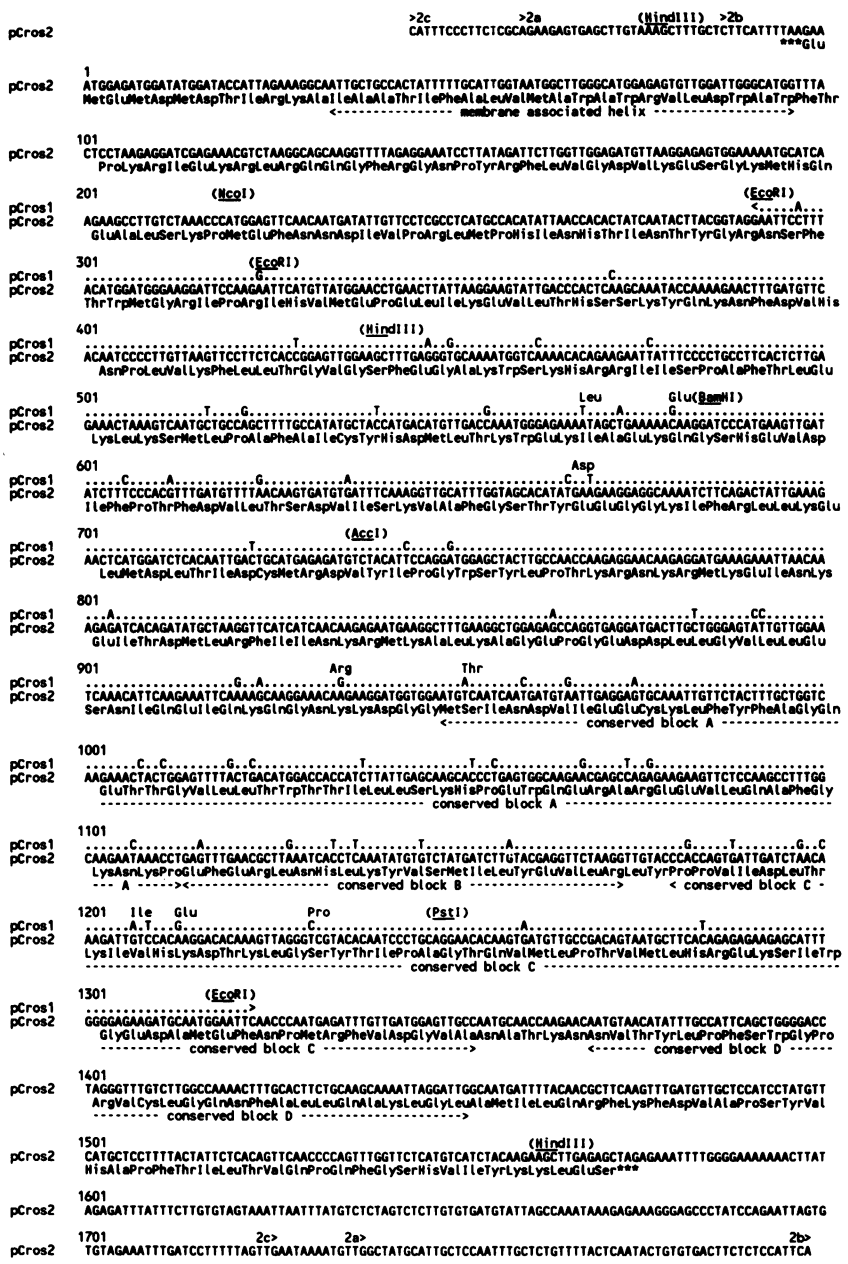

Figure 3. Nucleotide and deduced protein sequences of the *C. roseus* cDNA clones pCros2a, pCros2b, pCros2c, and pCros1. Only the differences to the pCros2 cDNAs are indicated in pCros1, and the amino acids are given if the base exchanges result in amino acid differences. Position 1 is the first base of the first methionine codon of the open reading frame in pCros2, >2a, >2b, and >2c mark the start and end of the pCros2 cDNAs. ***, Stop codons defining the start and end of the open reading frame. Below the protein sequence of pCros2 are blocks conserved in many P-450 enzymes (10). Block D contains the cysteine that binds the heme group (1).

of the maximum values at day 11 and later, whereas 50 to 60% of the cDNA-encoded protein was still present. This difference suggested that the cloned protein is not NE10H. The different kinetics of GE10H and NE10H also suggested that the hydroxylations of geraniol and nerol are performed by different proteins. This is of interest because previously it had not been possible to separate the two activities during enzyme purification (14, 15). The medium change induced a rapid, transient increase of CA4H activity with a peak at day 1 and very low activities after day 10 (Fig. 6B). This induction did not correlate with that of the cDNA-encoded protein, suggesting that the cDNAs do not code for CA4H.

A second set of experiments investigated the amount of the cDNA-encoded protein during growth of the culture in MX medium and after induction by transfer of cells from MX medium into a solution of 8% sucrose. In addition to GE10H, NE10H, and CA4H, the analysis included measurements of NADPH-dependent Cyt *c* reductase and of the P-450 enzymes LAH, FL3'H, and FL3'5'H, because other experiments had indicated that these activities were present in the *C. roseus* cell culture.

Experiment A of Table I summarizes the data from dilution of a 7-d-old culture into fresh MX growth medium. The highest values for the cDNA-encoded protein, the tested P-450 enzymes, and NADPH-dependent Cyt *c* reductase were measured at the earliest time point taken (3 d after the start

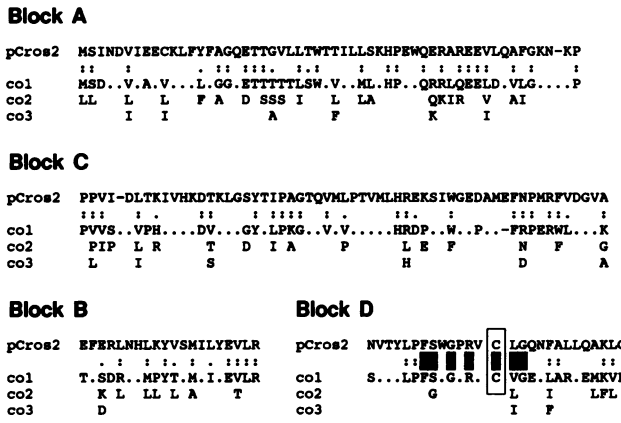

Figure 4. Alignment of sequences in the pCros2 protein from *C. roseus* with the amino acid sequence blocks A to D that are conserved in eight P-450 families (10). ■ in block D, The amino acids of the strictly conserved P-450 signature (1); the cysteine binding the prosthetic group is framed. :, Identity with the extended consensus (co1, co2, or co3); ., conservative exchanges.

of the experiment), indicating that the dilution of the cells into fresh growth medium was sufficient for induction. In most cases, the values decreased during further growth of the culture. The only exception was LAH, which revealed increased activity again at day 14. The amount of cDNA-encoded protein was very low at this point, and this difference suggests that the cDNAs do not code for LAH.

Experiment B of Table I presents the results obtained after resuspension of the cells from a 7-d-old culture into a solution of 8% sucrose. The cDNA-encoded protein increased more than 2-fold within 16 h, and similar kinetics were found for the activities of GE10H and CA4H. Cyt *c* reductase and LAH activity increased until the end of the experiment, and the two flavonoid hydroxylases showed a peak after 8 h. The differences between the kinetics of the cDNA-encoded protein and the P-450 enzymes were not large enough to exclude any one of them as a candidate for the function of the cDNA-encoded protein.

Related Proteins and GE10H Activities in Other Plants

The western blots in Figure 7 summarize experiments to test whether other plants contained proteins that cross-reacted with the antiserum against the cDNA-encoded protein. Membranes from parsley cell cultures revealed a weak immunological cross-reaction with a protein slightly smaller than in *C. roseus* (Fig. 7A, lanes 2 and 3; compare with lane 1), and very low GE10H was detected (close to detection limit with the nonradioactive assay). This band was also present in parsley leaves (not shown), and long exposures of the western blots revealed an additional band at 47 to 50 kD. A staining of all proteins in the gels with the red dye Ponceau-S showed a dominant protein at this position. Its size and abundance suggested that it represented the large subunit of ribulose bisphosphate carboxylase in the leaf membrane preparation. This interpretation is consistent with the finding that the band was absent in membranes from cell cultures (Fig. 7B, lanes 6 and 7). Experiments with a partially purified preparation of ribulose bisphosphate carboxylase from spinach confirmed that this protein, when present in high concentrations, bound small amounts of immunoglobulins that led to nonspecific bands in western blots (not shown). Such bands in blots with leaf preparations were, therefore, considered as nonspecific (marked by dots in lanes 1–4 of Fig. 7B).

C. roseus leaves revealed a protein of the same size as in the cell cultures (Fig. 7B, compare lanes 4 and 5), and GE10H activity was also detected (0.24 ± 0.05 pkat/mg in the assay with radioactive geraniol). Immunoreactive proteins of similar size were present in membranes from leaves of *Allamanda neriifolia* (lane 1) and *T. peruviana* (lane 2), and these preparations had GE10H activity (1.74 ± 0.1 and 0.27 ± 0.04 pkat/mg, respectively, assay with radioactive geraniol). Similar results were obtained with *H. tuberosus* (not shown). Leaves

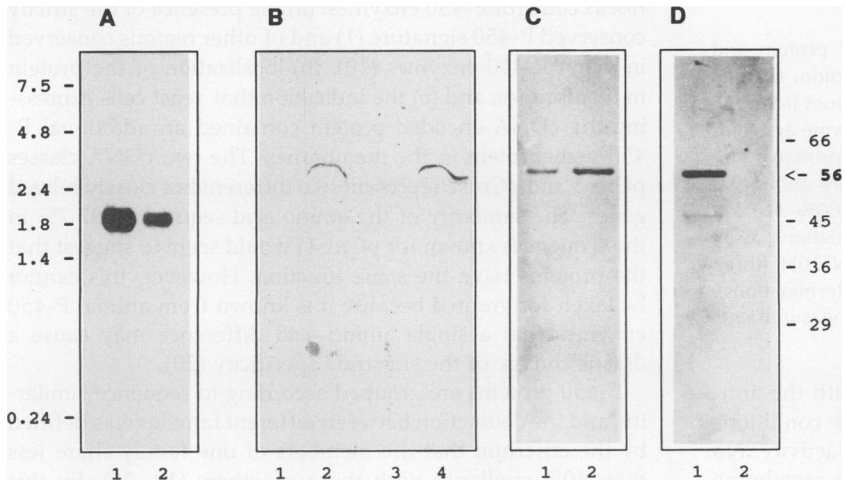

Figure 5. RNA and protein in *C. roseus*. Numbers at the left are size markers for the RNA (kb); at the right, size markers for the proteins (kD); <–56 marks the position of the cDNA-encoded protein. A, Northern blot of poly(A)$^+$ RNA (5 μg) probed with cDNA pCros1. Lane 1, Induced (7.5 h in a solution of 8% sucrose) (12); lane 2, noninduced (MX medium). B, Western blots with supernatants (lanes 1 and 3) and pellets (lanes 2 and 4) from a centrifugation for 60 min at 110,000g. Lanes 1 and 2, Noninduced cells; lanes 3 and 4, induced cells (8% sucrose for 24 h). Each lane contained 50 μg of protein. C, Western blots with membranes (12 μg of protein) from noninduced (lane 1, MX medium) and from induced cells (lane 2; 5 d in indole alkaloid production medium IM2). D, Western blots with membrane proteins from yeast (12 μg protein). Lane 1, Construct with sense insertion of cDNA pCros2b; lane 2, construct with antisense orientation of the cDNA.

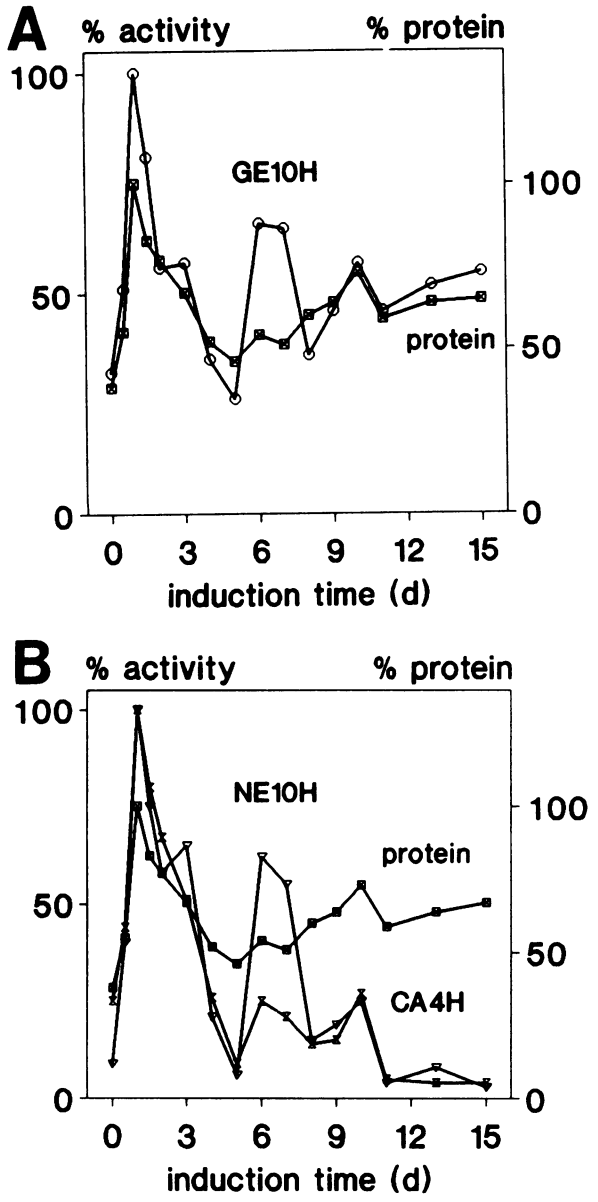

Figure 6. Correlation between the cDNA-encoded protein and induced P-450 enzyme activities in *C. roseus*. Induction, Change from MX medium to indole alkaloid production medium IM2 (23). Note that the scales are different for protein and enzyme activities to facilitate the comparison. Protein, Immunoreactive protein quantified in western blots with biotin-coupled secondary antibodies and radioactive streptavidin (four determinations), 100% = 5 μg/mg of total membrane protein; GE10H (three determinations; assay with unlabeled geraniol), 100% = 345 pkat/mg; NE10H (three determinations), 100% = 181 pkat/mg; CA4H (two determinations), 100% = 31 pkat/mg. Experimental variation in the measurements ± 8%.

from *N. oleander* contained a protein reacting with the antiserum (lane 3), but assays under the standard conditions developed for *C. roseus* failed to detect GE10H activity. No immunological cross-reaction was observed with membrane preparations from soybean (lane 6) and peanut (lane 7) cell cultures, and GE10H activity was not detectable even in the highly sensitive assay with radioactive geraniol. The same negative results were obtained with membranes from *Petunia hybrida* or spinach (*Spinacia oleracea*) leaves, and from *Ammi visnarga* or *Ammi majus* cell cultures (not shown).

Heterologous Expression in *S. cerevisiae*

These experiments were performed both with pCros2a and pCros2b in the sense and antisense orientation with respect to the vector promoter. Because it was not known whether the protein would be deleterious to yeast cells, the cloning strategy (Fig. 1) was designed to obtain a regulated expression of the polypeptide.

No immunoreactive protein was detected with the antisense control (Fig. 5D, lane 2). The construct with pCros2b in the sense orientation generated a polypeptide of the same size as in the plant cells (Fig. 5D, lane 1) and localized in the membrane. The sense pCros2a construct did not synthesize significant amounts of the protein, possibly because the length of the 5′ noncoding region is critical for protein expression (28). This is a possible explanation because pCros2a contains a longer 5′ noncoding region than pCros2b (Fig. 3).

The total P-450 content (9) of the yeast membrane preparations containing the cloned protein was about two times higher than in the controls (81 ± 8 versus 37 ± 2 pmol/mg of protein). A calculation based on the size of the cDNA-encoded protein and the assumption that each polypeptide contained a heme group suggested that the additional protein corresponded to approximately 2 to 4 μg/mg of the total protein in the yeast membranes. This was in the range of the concentrations found in *C. roseus*, and a direct comparison of the yeast and plant membrane preparations by quantification of the 56-kD protein reacting with the antiserum in western blots confirmed the estimate. Enzyme assays, however, failed to detect any of the P-450 enzyme activities found to be present in the plant cells.

DISCUSSION

The available evidence indicates that the cDNAs from *C. roseus* code for P-450 enzymes: (a) the presence of the strictly conserved P-450 signature (1) and of other regions conserved in many P-450 enzymes (10); (b) localization of the protein in membranes; and (c) the indication that yeast cells expressing the cDNA-encoded protein contained an additional P-450 hemeprotein in the membranes. The two cDNA classes pCros1 and pCros2 represent two different but closely related genes. The similarity of the amino acid sequences (97.7% in the sequences known for pCros1) would seem to suggest that the proteins have the same function. However, this cannot be taken for granted because it is known from animal P-450 enzymes that a single amino acid difference may cause a drastic change of the substrate specificity (20).

P-450 proteins are grouped according to sequence similarity, and the distinction between different families was defined by the criterium that the members of one family share less than 40% similarity with those of others (19, 20). By this criterium, the proteins encoded in pCros1 and pCros2 represented a new P-450 family (CYP72). There was less than

Table I. *Immunoreactive Protein, P-450 Enzyme Activities, and Cyt c Reductase in C. roseus*

Both experiments started with a 7-d-old cell culture grown in MX medium. The data are the average of three determinations (experimental variation ± 15%).

Experiment	Time	CYP72[a] Protein	P-450 Enzyme Activities					Reductase[c]
			GE10H[b]	CA4H	LAH	FL3'H	FL3'5'H	
	d	*μg/mg*			*pkat/mg*			*nkat/mg*
A. Dilution into fresh MX growth medium	3	4.6	3.0	14.2	0.7	>2.0[d]	>2.0[d]	0.80
	7	2.4	2.6	14.8	0.5	1.7	0.6	0.56
	10	0.6	0.1	6.2	0.2	0.3	nd[e]	0.25
	14	0.1	nd[e]	0.6	0.4	nd[e]	nd[e]	0.16
B. Induction by transfer into 8% sucrose	0	2.4	2.6	14.8	0.5	1.7	0.6	0.56
	8	3.4	5.8	23.2	0.7	>2.0[d]	1.1	0.90
	16	5.5	8.2	30.6	0.8	1.8	0.9	0.93
	24	5.1	8.1	33.8	1.0	1.5	0.8	1.25

[a] 56 kD protein band recognized by the antiserum in quantified western blots. [b] Assay with radioactive geraniol. [c] NADPH-dependent Cyt c reductase. [d] Out of linear range under the standard assay conditions. [e] Not detectable.

22% similarity with the only other published plant P-450 sequence (CYP71; the ripening-related P-450 from *Persea americana*) (2), indicating that this protein represents a different family.

The *C. roseus* system was chosen because conditions for the induction of a specific pathway with at least two well-known P-450 enzymes (GE10H and NE10H) were well established, and the previous selection of the cell culture and the optimization of the induction for indole alkaloid production suggested at least some specificity. However, our results showed that the induction was by no means specific for this pathway. The activation of the ubiquitous CA4H might have been suspected, but LAH, FL3'H, and FL3'5'H were new discoveries in these cultures. In particular, the two flavonoid-hydroxylases were unexpected because the growth or induction conditions did not include irradiation of the cells. Very recent experiments (not shown) indicated that the cells also contained 7-ethoxycoumarin-O-deethylase and EROD activity. EROD activity had been detected so far in only a few plants (30).

In view of this complexity, the identification of the protein function requires the functional expression in a heterologous system. Yeast cells appeared to be a suitable system, although they may present some problems even with well-investigated animal P-450 enzymes (28). Our results indicated that the plant protein was expressed, but it was not possible to demonstrate an enzyme activity for any of the eight reactions found in the plant cell cultures (including EROD and 7-ethoxycoumarin-*O*-deethylase). It is possible that the correct substrate was not yet tested, but it is also possible that the expression system or the assay conditions must be modified for functional studies of plant P-450 enzymes. The use of the yeast system requires further investigation, especially because it is unclear if the yeast reductase will interact with plant P-450. Supplementation of the yeast membranes with P-450 reductase purified from Jerusalem artichoke had no effect, but the enzyme also did not stimulate GE10H, NE10H, or CA4H activity with the preparations from *C. roseus*. One potentially serious complication is that some plant P-450 enzymes including GE10H and NE10H are localized in the (pro)vacuolar membranes (14, 16). In these cases, the targeting and/or an unsuitable environment may present basic problems for the function of the enzyme in the heterologous system.

The failure to detect enzyme activity in the yeast cells does

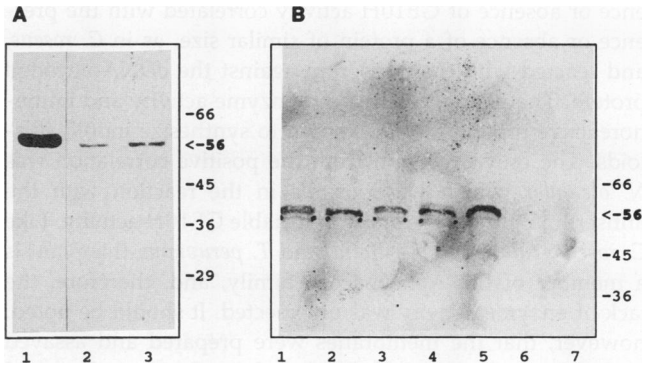

Figure 7. Western blots of membrane preparations from other plants. The numbers at the right indicate the protein size markers (kD); <−56 marks the position of the cDNA-encoded protein. A, Comparison of membranes from noninduced *C. roseus* cell cultures (lane 1) and parsley cell cultures (lane 2, noninduced; lane 3, induced by irradiation with UV-A for 20 h [7]). Each lane contained 120 μg of protein; this high amount was necessary to visualize the immunoreactive protein in parsley. The proteins were separated in 12.5% polyacrylamide gels. B, Membranes from other plants. The proteins were separated in 9% polyacrylamide gels. *Allamanda neriifolia* leaves (lane 1); *T. peruviana* leaves (lane 2); *N. oleander* leaves (lane 3); *C. roseus* leaves (lane 4); *C. roseus* cell culture (lane 5); *G. max* cell culture (lane 6); *Arachis hypogaea* cell culture (lane 7). Each lane contained 50 μg of protein. The dots in lanes 1 to 4 mark the position of ribulose bisphosphate carboxylase in the leaf preparations (see text).

not allow at present the unambiguous identification of the function encoded in the cloned cDNAs. Nevertheless, some conclusions are possible. The high abundance of the P-450 protein indicates that it is part of a major pathway. This would be expected for enzymes in the biosynthesis of secondary plant metabolites that are important in terms of quantity, but not for enzymes in the biosynthesis of hormones (e.g. gibberellins). Because the cell culture line was originally selected for high inducible production of indole alkaloids, the P-450 enzymes GE10H and NE10H are prime candidates. The kinetics of GE10H activation in indole alkaloid production medium IM2 were indeed similar to those of the cDNA-encoded protein, with the exception of day 6 and 7 (Fig. 6). P-450 reactions require a complex interaction of several components, and one of the most important is the reductase that transfers electrons to the P-450 protein. The discrepancies at specific time points of the kinetics could reflect the availability of the reductase in the competition with other P-450 enzymes rather than differences in the amount of GE10H protein. This seems to be a possible explanation because the available results indicated that the tested P-450 enzymes did not follow identical kinetics (Fig. 6, Table I).

It seems unlikely that the pCros cDNAs code for NE10H or CA4H because the kinetics of the enzyme activities clearly diverged from that of the immunoreactive protein (Fig. 6). The conclusion for CA4H is supported by experiments with an antiserum against CA4H from Jerusalem artichoke (6). This antiserum reacted with a protein in membranes from *C. roseus*, but not with the cDNA-encoded protein expressed in yeast or in *E. coli* (not shown).

The experiments with other plants suggested that the presence or absence of GE10H activity correlated with the presence or absence of a protein of similar size, as in *C. roseus*, and reacted with the antiserum against the cDNA-encoded protein. The plants lacking both enzyme activity and immunoreactive protein are not known to synthesize indole alkaloids. The only exception from the positive correlation was *N. oleander*, which was positive in the reaction with the antiserum but failed to show detectable GE10H activity. Like *C. roseus*, *Allamanda neriifolia*, and *T. peruviana*, this plant is a member of the Apocynaceae family, and, therefore, the lack of enzyme activity was unexpected. It should be noted, however, that the membranes were prepared and assayed according to the protocol developed for *C. roseus*. It would seem necessary to test modifications of the procedures before a definite conclusion on the absence of GE10H activity in the leaves of this plant can be reached.

The sum of these results is consistent with a working hypothesis that the cDNA-encoded protein is part of a major pathway that appears to be confined to *C. roseus*, relatives from the same family, and other plants that possess GE10H activity. Therefore, we suggest that GE10H is a prime candidate for the function of the cloned protein. This is supported by the finding that the cDNA-encoded protein has the same size as GE10H purified from *C. roseus* (56 kD) (17) and by the finding that this protein strongly reacts in western blots with the antibodies against the cloned protein through all purification steps (A.H. Meijer, R. Verpoorte, J.H.C. Hoge, personal communication). Future experiments will test the tentative assignment by functional expression of the protein in transgenic plants.

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