

Isolation and characterization of a cDNA clone from *Catharanthus roseus* encoding NADPH:cytochrome P-450 reductase, an enzyme essential for reactions catalysed by cytochrome P-450 mono-oxygenases in plants

Annemarie H. Meijer^{1*}, M. Inês Lopes Cardoso², John Th. Voskuilen², Anthony de Waal^{1,†}, Robert Verpoorte¹ and J. Harry C. Hoge²

¹Center for Bio-Pharmaceutical Sciences, Division of Pharmacognosy, Leiden University, Gorlaeus Laboratories, PO Box 9502, 2300 RA Leiden, The Netherlands, and

²Institute of Molecular Plant Sciences, Leiden University, Clusius Laboratory, PO Box 9505, 2300 RA Leiden, The Netherlands

Summary

The membrane-bound flavoprotein NADPH:cytochrome P-450 (cytochrome c) reductase, that functions in electron transfer to cytochrome P-450 mono-oxygenases, was purified from a cell suspension culture of the higher plant *Catharanthus roseus*. Antiserum raised against the purified protein was found to inhibit NADPH:cytochrome c reductase activity as well as the activities of the cytochrome P-450 enzymes geraniol 10-hydroxylase and *trans*-cinnamate 4-hydroxylase, which are involved in alkaloid biosynthesis and phenylpropanoid biosynthesis, respectively. Immunoscreening of a *C. roseus* cDNA expression library resulted in the isolation of a partial NADPH:cytochrome P-450 reductase cDNA clone, which was identified on the basis of sequence homology with NADPH:cytochrome P-450 reductases from yeast and animal species. The identity of the cDNA was confirmed by expression in *Escherichia coli* as a functional protein capable of NADPH-dependent reduction of cytochrome c and neotetrazolium, two *in vitro* substrates for the reductase. The N-terminal sequence of the reductase, which was not present in the cDNA clone, was determined from a genomic NADPH:cytochrome P-450 reductase clone. It was demonstrated that the reductase probably is encoded by a

single copy gene. A sequence comparison of this plant NADPH:cytochrome P-450 reductase with the corresponding enzymes from yeast and animal species showed that functional domains involved in binding of the cofactors FMN, FAD and NADPH are highly conserved between all kingdoms. In *C. roseus* cell cultures a rapid increase of the reductase steady state mRNA level was observed after the addition of fungal elicitor preparations that are known to induce cytochrome P-450-dependent biosynthetic pathways.

Introduction

NADPH:cytochrome P-450 reductase (NADPH:cytochrome c reductase; EC 1.6.2.4) is a membrane-bound flavoprotein of eukaryotes that is essential for reactions catalysed by cytochrome P-450 mono-oxygenases, since it functions in electron transfer from NADPH to the cytochrome P-450 proteins (Lu *et al.*, 1969). The primary structures of NADPH:cytochrome P-450 reductases from the animal species rat (Porter and Kasper, 1985), rabbit (Katagiri *et al.*, 1986), pig (Haniu *et al.*, 1986), trout (Urenjak *et al.*, 1987) and human (Yamano *et al.*, 1989) as well as from the yeasts *Saccharomyces cerevisiae* (Yabusaki *et al.*, 1988) and *Candida tropicalis* (Sutter *et al.*, 1990) have been determined. Furthermore, genes encoding enzymes that comprise a cytochrome P-450 domain and an NADPH:cytochrome P-450 reductase domain in a single, soluble protein have been cloned from *Bacillus megaterium* (Ruettinger *et al.*, 1989) and rat (Bredt *et al.*, 1991; Lyons *et al.*, 1992; White and Marletta, 1992). Domains involved in binding of the cofactors flavin mononucleotide (FMN), FAD and NADPH are highly conserved among all known NADPH:cytochrome P-450 reductase sequences.

Cytochrome P-450 mono-oxygenases in plants are implicated in the biosynthesis of a wide range of compounds, including lignins, terpenoids, sterols, fatty acids, plant hormones, pigments and defence-related phytoalexins (reviewed by Donaldson and Luster, 1991). In addition, certain plant cytochrome P-450 enzymes are involved in detoxification of herbicides (reviewed by O'Keefe *et al.*, 1987). Cytochrome P-450 enzymes from animals and micro-organisms have been studied extensively, but knowledge about these enzymes in plants is still limited.

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*For correspondence at Institute of Molecular Plant Sciences, Leiden University, Clusius Laboratory, PO Box 9505, 2300 RA Leiden, The Netherlands (fax +31 71 274999).

†Present address: Netherlands Cancer Institute, Department of Molecular Biology, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands.

Purification of plant cytochrome P-450 enzymes has been achieved in very few cases and only recently was the first plant cytochrome P-450 gene cloned (Bozak *et al.*, 1990). Like in yeast and animal species, the expression of cytochrome P-450 enzymes in plants appears to be highly regulated. Different external factors such as wounding and elicitor treatment induce the activities of specific plant cytochrome P-450 enzymes and in some cases a concomitant induction of NADPH:cytochrome P-450 reductase has been observed (Benveniste *et al.*, 1977; Reichhart *et al.*, 1980).

NADPH:cytochrome P-450 reductase was purified from the higher plant *Catharanthus roseus* by Madyastha and Coscia (1979). The protein was shown to resemble the mammalian enzyme with respect to flavoprotein nature, molecular weight, isoelectric point, substrate specificity and sensitivity to reductase inhibitors. Moreover, it was demonstrated that the purified reductase was capable of reconstituting the activity of the *C. roseus* cytochrome P-450 enzyme geraniol 10-hydroxylase, when added to a partially purified cytochrome P-450 preparation and a lipid fraction. Geraniol 10-hydroxylase catalyses the conversion of geraniol to 10-hydroxygeraniol in the biosynthesis of secologanin, which is a precursor of terpenoid indole alkaloids, a group of secondary plant metabolites with important pharmaceutical applications. Geraniol 10-hydroxylase represents a possible site for regulatory control in the biosynthesis of terpenoid indole alkaloids (McFarlane *et al.*, 1975; Schiel *et al.*, 1987). Recently we have described the further purification of this cytochrome P-450 enzyme from cell cultures of *C. roseus* (Meijer *et al.*, 1990). To gain insight into the molecular mechanisms governing terpenoid indole alkaloid biosynthesis and the

activity of cytochrome P-450 systems in general, we set out to obtain cDNA clones of both the geraniol 10-hydroxylase and the NADPH:cytochrome P-450 reductase from *C. roseus*.

Here we report on cloning of a *C. roseus* NADPH:cytochrome P-450 reductase cDNA and show the functional expression of the encoded protein in *Escherichia coli*. Furthermore, we demonstrate that the reductase is probably encoded by a single copy gene in the *C. roseus* genome. Some properties of antiserum against the reductase are described and a sequence comparison with NADPH:cytochrome P-450 reductases from other species is discussed. Finally, we demonstrate that the steady state mRNA level of the reductase is regulated in a tissue-specific manner and induced by fungal elicitor preparations.

Results

Purification of NADPH:cytochrome P-450 reductase and properties of reductase antiserum

NADPH:cytochrome P-450 reductase from *C. roseus* was characterized by Madyastha and Coscia (1979), who purified the enzyme from etiolated seedlings. We used a cell suspension culture of *C. roseus* as the enzyme source. The artificial substrates cytochrome c and neotetrazolium were used to follow the purification of the reductase. The purification strategy comprised cholate-solubilization from a 1000 to 20 000 g membrane fraction, ion-exchange chromatography on DEAE-Sephacel and affinity chromatography on 2'5'ADP-Sepharose 4B. DEAE-Sephacel chromatography resulted in an almost complete separation of cytochrome P-450 proteins from NADPH:cytochrome c reductase activity. Following this separation, enzyme activities of cytochrome P-450 enzymes can only be measured in a reconstituted system consisting of a cytochrome P-450-containing fraction, the reductase and a lipid extract. In agreement with the results of Madyastha *et al.* (1976), we found that the partially purified reductase preparation from DEAE-Sephacel was capable of reconstituting the activity of the cytochrome P-450 enzyme geraniol 10-hydroxylase, that is involved in terpenoid indole alkaloid biosynthesis in *C. roseus*. The dependence of cytochrome P-450 activity on reconstitution with NADPH:cytochrome P-450 reductase is shown in Figure 1. The reductase preparation was also capable of reconstituting the activity of the cytochrome P-450 enzyme *trans*-cinnamate 4-hydroxylase that is instrumental in phenylpropanoid biosynthesis (data not shown).

The pooled active fractions from DEAE-Sephacel were further purified by affinity chromatography on 2'5'ADP-Sepharose 4B. The NADPH:cytochrome c reductase activity eluted in two peaks, the first of which contained

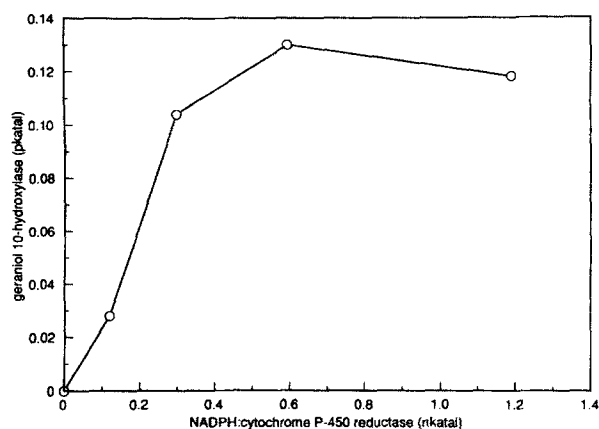


Figure 1. Dependence of the activity of the cytochrome P-450 enzyme geraniol 10-hydroxylase on reconstitution with NADPH:cytochrome P-450 reductase.

Different amounts of partially purified NADPH:cytochrome P-450 reductase and an extract of *C. roseus* lipids were added to a partially purified cytochrome P-450 preparation. After detergent removal geraniol 10-hydroxylase activity was determined.

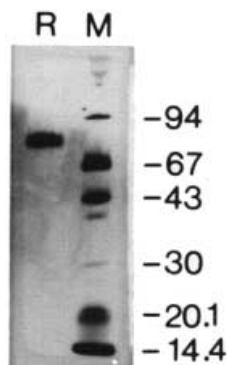


Figure 2. SDS-PAGE of affinity-purified NADPH:cytochrome P-450 reductase.

Lane R, affinity-purified NADPH:cytochrome P-450 reductase (200 ng); lane M, low molecular weight markers (Pharmacia-LKB Biotechnology; approximately 50 ng of each protein; M_r values are indicated on the right hand side of the figure). Gel electrophoresis was performed with the PhastSystem (Pharmacia-LKB Biotechnology). A 10–15% PhastGel with SDS bufferstrips was used. Protein visualization was obtained by silver staining.

70–75% of the total activity. The pooled fractions of this peak showed a single protein species with a molecular mass of 74 000 on SDS-PAGE after silver staining (Figure 2). The purified protein was found to have a pI of 4.6 and a specific activity for cytochrome c of 1.2 $\mu\text{katal mg}^{-1}$. No cytochrome c reduction could be observed when NADH was used as electron donor instead of NADPH. All activity-containing fractions from the affinity column were also subjected to native PAGE and stained for reductase activity with NADPH and neotetrazolium. This revealed that the second peak of NADPH:cytochrome c reductase activity contained a reductase protein with a slightly lower M_r than the purified protein in the first peak. Madyastha and Coscia (1979) also observed two forms of the reductase in their

purification, of which the smaller one was assumed to represent a proteolytic fragment of the intact reductase. This assumption was based on the situation in mammalian systems, where a single reductase functions with all different cytochrome P-450 enzymes, but where a smaller form representing a purification artefact formed by protease action can be observed. It has been demonstrated that removal of the hydrophobic domain of the reductase by trypsin results in an enzyme that is still able to reduce cytochrome c and neotetrazolium, but is no longer functional in the reduction of cytochrome P-450 (Black *et al.*, 1979; Lu *et al.*, 1969).

Like the partially purified reductase pool from DEAE-Sephacel, the purified protein was capable of reconstituting geraniol 10-hydroxylase activity. However, the purified preparation was less efficient in reconstitution than the partially purified enzyme. One possible explanation for this difference was gradual loss of flavin groups as purification progressed, despite the presence of 1 μM FMN and 1 μM FAD in all chromatographic steps. When flavins were omitted from the enzyme assay, the NADPH:cytochrome c reductase activity of the purified enzyme was reduced by only 10%. This observation does not necessarily mean that little dissociation had occurred, since a decreased enzyme activity due to loss of FAD cannot be completely restored by the addition of this compound to the enzyme assay (Vermilion and Coon, 1976). Another explanation for the difference in capability of reconstitution between the partially purified and homogeneous reductase preparations became apparent when the characteristics of antiserum raised against the purified enzyme were studied. On Western blots (Figure 3) an immunoreactive band with an M_r of 74 000 was detected in the affinity-purified reductase preparation (lane 3), whereas in crude membrane fractions

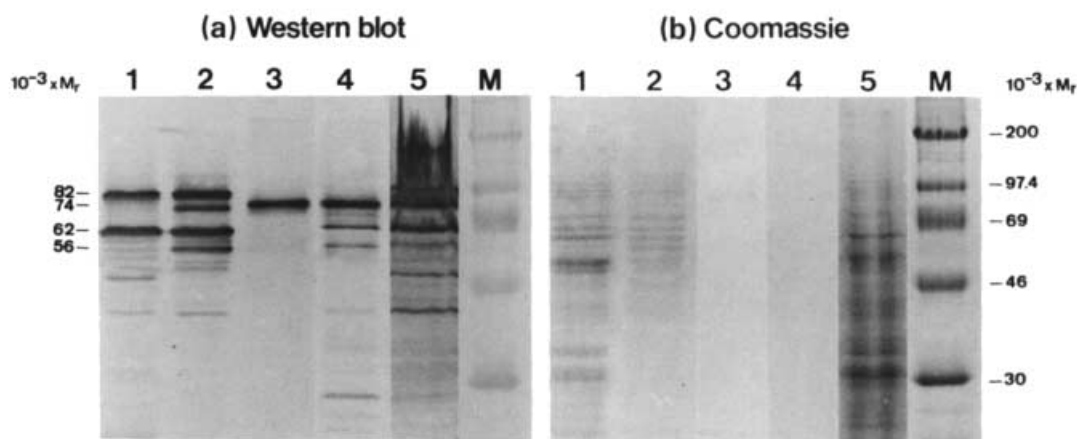


Figure 3. Western blot analysis of *C. roseus* protein samples at different stages of purification.

(a) Western blot and (b) Coomassie brilliant blue staining of *C. roseus* protein samples. Lane 1, 1000–20 000 g membrane fraction (35 μg); lane 2, NADPH:cytochrome P-450 reductase pool from DEAE-Sephacel (5 μg); lane 3, affinity-purified NADPH:cytochrome P-450 reductase (0.2 μg); lane 4, trypsin digestion of affinity-purified NADPH:cytochrome P-450 reductase (0.2 μg); lane 5, trypsin digestion of 1000–20 000 g membrane fraction (35 μg); lane M, Rainbow™ protein molecular weight markers (Amersham; approximately 7 μg of each protein). M_r values of immunoreactive bands are indicated at the left hand side of the figure and M_r values of molecular weight markers on the right hand side.

bands with M_r values of 82 000 and 62 000 were seen (lane 1). In the reductase pool from DEAE-Sephacel four bands with M_r values of 82 000, 74 000, 62 000 and 56 000 were visible (lane 2). Immunoreactive bands with M_r values of 62 000 and 56 000 were also observed after incubation of the affinity purified protein with trypsin (lane 4). Trypsin digestions of the crude membrane fraction resulted in disappearance of the band with an M_r of 82 000 and appearance of an immunoreactive protein of the same size as the affinity purified enzyme (lane 5). We therefore propose that the protein with an M_r of 82 000, observed in crude and partially purified enzyme preparations, represents the intact reductase and that all other immunoreactive proteins represent proteolytic degradation products of this protein. This would explain the less efficient reconstitution by the affinity-purified enzyme compared with the partially purified preparation.

We also examined the effects of antiserum against NADPH:cytochrome P-450 reductase on NADPH:cytochrome c reductase activity and on the activities of two cytochrome P-450 mono-oxygenases, geraniol 10-hydroxylase and *trans*-cinnamate 4-hydroxylase. Prior to measuring enzyme activities, protein extracts (1000–20 000 g *C. roseus* membrane fractions) were incubated for 20 min at room temperature with an equal volume of undiluted anti-reductase serum. In the control, the antiserum was replaced by 50 mM sodium phosphate buffer (pH 7.4), containing 150 mM NaCl. Under these experimental conditions the anti-reductase serum inhibited NADPH:cytochrome c reductase activity by nearly 100%, geraniol 10-hydroxylase activity by 87% and *trans*-cinnamate 4-hydroxylase activity by 94%. Pre-immune serum had no effect. These results support the assumption that the purified reductase functions in electron transfer from NADPH to cytochrome P-450 enzymes.

Isolation of a partial NADPH:cytochrome P-450 reductase cDNA clone and its functional expression in *E. coli*

By immunoscreening of a *C. roseus* cDNA expression library in λ ZAP II a cDNA clone, pSK-R9, containing an insert of 2.3 kb was isolated. Sequencing of parts of this cDNA clone revealed homology to domains involved in cofactor binding identified in NADPH:cytochrome P-450 reductase sequences from other species. At the 5' end an ORF was identified encompassing a region homologous to the binding site for the pyrophosphate group of FMN. This ORF was not preceded by an ATG start codon, indicating that the clone represented a partial cDNA clone. In yeast and animal NADPH:cytochrome P-450 reductases all other domains involved in cofactor binding are situated between the FMN-pyrophosphate binding site and the C-terminus of the protein. Hence it seemed likely that the protein specified by the putative *C. roseus* reductase

clone pSK-R9 comprised all functional domains of the enzyme and only lacked a small part of the N-terminal membrane anchoring region. Therefore we tried to confirm the identity of pSK-R9 by expressing the encoded protein as a β -galactosidase fusion protein in *E. coli* strain XL-1 and analysing the capacity of this protein to catalyse the reduction of cytochrome c and neotetrazolium, two *in vitro* substrates for the P-450 reductase. *E. coli* XL-1 containing pBluescript SK (pSK) without cDNA insert was used as a control. NADPH-dependent cytochrome c reductase activity in extracts from *E. coli* XL-1 containing pSK-R9 was 20-fold higher than that of control extracts. No difference in activity between the pSK-R9 and pSK extracts was observed when NADH instead of NADPH was used as electron donor (Table 1), which is in agreement with the fact that the purified cytochrome P-450 reductase from *C. roseus* is strictly dependent on NADPH. The protein extracts were subjected to native PAGE, and staining for reductase activity showed an NADPH/neotetrazolium-positive band in the pSK-R9 extracts that was not observed in the pSK extracts (Figure 4a). Western blotting of an identical gel showed that this band corresponded to the major immunoreactive protein with anti-reductase serum in the pSK-R9 extracts (Figure 4b). All results confirmed that pSK-R9 represents NADPH:cytochrome P-450 reductase.

Determination of the complete NADPH:cytochrome P-450 reductase sequence from a partial cDNA clone and a genomic clone

The partial NADPH:cytochrome P-450 reductase cDNA clone, pSK-R9, contained an insert of 2329 bp that harboured an ORF specifying a protein of 644 amino acids. RNA sequencing of the corresponding mRNA showed that cDNA clone pSK-R9 lacked approximately 370 nucleotides at the 5' end, but it was not possible to read the precise sequence of the missing part. After extensive rescreening of the cDNA library with a probe

Table 1. Cytochrome c reductase activity in protein extracts from *E. coli* expressing the NADPH:cytochrome P-450 reductase cDNA as a β -galactosidase fusion protein

Extract	NADPH specific activity ($A_{550} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$)		NADH specific activity ($A_{550} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$)	
	I	II	I	II
pSK	0.91	0.92	1.15	0.90
pSK-R9	18.63	18.00	1.06	1.10

The reduction of cytochrome c was measured at 550 nm with NADPH or NADH as electron donor.

pSK: extract from *E. coli* XL-1 containing pBluescript II SK without cDNA insert; pSK-R9: extract from *E. coli* XL-1 containing the putative NADPH:cytochrome P-450 reductase cDNA cloned in pBluescript II. I and II SK represent duplicate extracts.

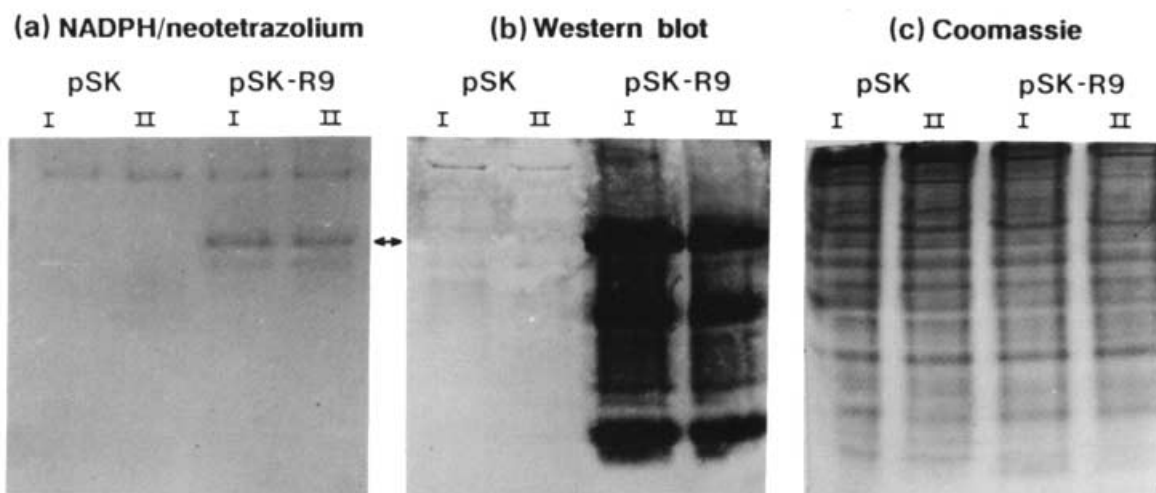


Figure 4. Functional expression of the *C. roseus* NADPH:cytochrome P-450 reductase cDNA clone as a β -galactosidase fusion protein in *E. coli*. (a) NADPH/neotetrazolium chloride staining, (b) Western blot analysis and (c) Coomassie brilliant blue staining of protein extracts from *E. coli* XL-1 containing pBluescript II SK without cDNA insert (pSK) and from *E. coli* XL-1 containing the putative NADPH:cytochrome P-450 reductase cDNA (pSK-R9) cloned in pBluescript II SK. The arrows indicate the position of the NADPH:cytochrome P-450 reductase protein.

corresponding to the 5' end of pSK-R9, we concluded that a full length cDNA clone was not present in our library. Since we had evidence that the reductase is encoded by a single copy gene (see below) it was possible to determine the missing part of the sequence by isolation of the corresponding gene. A genomic DNA library of *C. roseus* in λ Gem11 was screened and a 2 kb *Hind*III fragment, hybridizing with the 5' end of pSK-R9, was isolated from a positive genomic clone. Sequence analysis of part of this fragment revealed that the sequence of pSK-R9 is preceded by an ORF of 214 nucleotides, containing five in frame putative translation initiation codons. The isolated fragment also contained the sequence information for 74 bases upstream of the first ATG. Nine nucleotides upstream of this ATG a stop codon was present. The sequence derived from the genomic clone that represented the missing part of cDNA pSK-R9 is shown in Figure 5 together with the sequence of pSK-R9. It is difficult to predict which of the putative translation initiation sites is the true start site. The first (GAGATGGA) and third (GTTATGGC) putative sites show a closer resemblance to the initiation start consensus for dicot plants than the other three sites (Cavener and Ray, 1991). RNA sequencing had revealed that the length of the mRNA is sufficient to comprise all putative start codons. If the first ATG is assigned as the start codon, the encoded protein consists of 714 amino acids and has an M_r of 78 958 and a pI of 5.06. Including the FMN and FAD groups the molecular weight of the protein is 80 221. The calculated M_r corresponds well to the value of 82 000, estimated on SDS-PAGE for the protein that was assumed to represent the intact form of the reductase. In Figure 6 the *C. roseus* protein and yeast NADPH:cytochrome P-450 reductase are both aligned

with the reductase of rat. The alignment was obtained by the program BESTFIT (Devereux *et al.*, 1984), with the gap weight set at 3.0 and the gap length weight at 0.1. Other animal cytochrome P-450 reductase sequences are between 79 and 92% homologous with the rat reductase and are not shown in the alignment. Also, the cytochrome P-450 reductase from the yeast *C. tropicalis*, which is 51% homologous with *S. cerevisiae* is omitted from the alignment. On the amino acid level, the *C. roseus* protein shows 39% identity to the rat protein. The percentage identity between the yeast and rat proteins is 36%. An optimal alignment of the *C. roseus* and *S. cerevisiae* proteins revealed an identity of 31%. Functional domains involved in cofactor binding, identified previously in animal and yeast reductases (Porter and Kasper, 1985, 1986; Sutter *et al.*, 1990; Yabusaki *et al.*, 1988), are well conserved in the *C. roseus* protein. Hydropathy profiles (not shown) of the *C. roseus*, *S. cerevisiae* and rat reductases, calculated according to the method of Kyte and Doolittle (1982), revealed that all three proteins possess a hydrophobic domain close to the N-terminus, which could serve as a membrane anchor (Black *et al.*, 1979). A more hydrophilic region, preceding the hydrophobic domain, varies in length between different NADPH:cytochrome P-450 reductases, and is probably not involved in membrane attachment.

Estimation of NADPH:cytochrome P-450 reductase gene copy number

A Southern blot of *C. roseus* total DNA digested with different restriction enzymes, was hybridized with the pSK-R9 insert (Figure 7). The number of bands that were

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1  AAGCTTCTCTGTAGCATTCTTCCGGCGACGGAGTTGGGATTTATAATCTTTCTTGA
1321 CAGATCTCTTAAATACTCCTAAGAAGTCGCTTTGTTAGCTCTAGCAGCTTATGCATCTG
   D L L N T P K K S A L L A L A Y A S D
61  GTTAATATAGTTAACGGGAGATGGATTCTAGCTCGGAGAAGTTGTCGCCGTTCGAATTGA
1381 ATCCAAATGAGCCGATCGCTTAAATATCTTGCTTCTCCAGCCGGAAGGATGAATATG
   M D S S S E K L S P F E L M
   P N E A D R L K Y L A S P A G K D E Y A
121 TSAGCCGATCTTGAAGGAGCTAAATAGATGGGTCTAACTCTCAGATCTCGCGTAG
1441 CTCAGTCACTAGTTGCAAACTCAGAGAAGCTCCCGAGGTCATGGCTGAATTTCCATCAG
   S A I L K G A K L D G S N S S D S G V A
   Q S L V A N Q R S L L E V M A E F P S A
181 CTGTGTCGCCGAGTTATGGCTATGTTGTTGGAGAATAAGGAGTTAGTGATGATTTTGA
1501 CAAAGCCTCCTCTTGAGTATCTTTGAGCAATTTGCTCCAGCCTCCAACCCAGATTCT
   V S P A V M A M L L E N K E L V M I L T
   K P P L G V P F F A A I A P R L Q P R F Y
241 CTACTTCAGTGGCGGTTTGTAGCGTTGCTGCTAGTTTGTATGCGCGGATCTCCG
1561 ATTTATATCGTCTTCTCCAAGGATGGCACCATCAGAAATTCATGCTACTTGTGCATCTG
   T S V A V L I G C V V V L I W R R S S G
   S I S S S P R M A P S R I H V T C A L V
301 GATCGGCTAAAAGTCTCGGAGCCTCCGAGCTCATAGTCCCTAAATCTGTTGTAGAAC
1621 TTTATGAAAAACACCTGGAGGACCAATCACAAGGGTGTGTTGTCGACATGGATGAAGA
   S G K K V V E F P K L I V P K S V V E P
   Y E K T P G G R I H K G V C S T W H K N
361 CGGAGGAAATGATGAAGGAGAAGAAATTTACCATATTTTGGAAACAACTGGAA
1681 ATGCCATTCATGGAGGAAGCCGTGAGTGCAGCTGGGCTCTATCTTGTGACGGCAGT
   E E I D E G K K K F T I F F G T Q T G T
   A I P L E E S R D C S W A P I F V R Q S
421 CAGCTGAAGCTTCGCTAAGGCTCTAGCTGAGGACCAAGCTCGATGAAAAGGACG
1741 CTAACCTCAAACCTCCCTGCCGATCTCAAAGTCCCTTATAATGATCGGCCCTGGTACG
   A E G F A K A L A E E A K A R Y E K A V
   N F K L P A D P K V P V I M I G P G T G
481 TTATCAAAGTATGATATAGATGATATCGCCGCTGATGTAAGAATAACGAGGAGAAT
1801 GACTACTCCCTCAGAGGATTCCTTCCAGAAAGATTAGCTCTGAAGGAAGAAGGAGCTG
   I K V I D I D D Y A A D D E E Y E E K F
   L A P F R G F L Q E R L A L K E E G A E
541 TCAGAAAAGAGACCTTGGCATTTTTCATCTGGCCACGTAAGGAGTGGTGAACACCG
1861 AACTGTGACTGACGATTTTCTTTTGGATGAGGAAACCGCAAAATGATACATCTATG
   R K E T L A F F I L A T Y G D G E P T D
   L G T A V F F F G C R N R K M D Y I Y E
601 ACAATGCTCAAGGTTCTCAAAATGTTGTAGAGGAAATGATAGAGGGGACTGGCTAA
1921 AAGATGAGCTAAACATTTCTTGAATTTGGTGCATTTCCGAGCTACTTGTGCTTTCT
   N A A R F Y K W F V E G N D R G D W L K
   D E L N H P L E I G A L S E L L V A F S
661 AGAATCTGCAATATGAGCTTTTGGCTTGTGTAACAGCAATATGAGCATTTCAACAAGA
1981 CACGTGAGGGACCCACTAAGCAGTATGTCAACACAAGATGGCAGAAAAGGCTTCTGATA
   N L Q Y G V F G L G N R Q Y E H F N K I
   R E G P T K Q Y V Q H K M A E K A S D I
721 TTGCTAAAGTGGTGGATGAGAAAGTTGCTGAACAGGGTGTGTAAGCGGATGTTCCATTGG
2041 TTTGAGGATGATTTCTGATGGAGCATATGTTTACGCTCGCGTGTGCAAGGATGG
   A K V V D E K V A E Q G G K R I V P L V
   W R M I S D G A Y V Y V C G D A K G M A
781 TTCTGGAGAGCATGACCAGTGCATTGAAGATGACTTTGCTGATGGCTGAGAAATGAT
2101 CCAGGGATGCCACAGAACTCCACACCATTTGCTCAAGAGCAGGATGATGGATAGCA
   L G D D D G Q C I E D D F A A W R E N V W
   R D V H R T L H T I A Q E Q G S M D S T
841 GGCCTGATGATAACTTCTCCGGGATGAGGATGATCAACTTTTCTACAACCTACA
2161 CACAGGCTGAGGTTTGTGAAGAATCTCAAAATGACCGCAAGGATCTCCGAGATGCT
   P E L D N L L R D E D D T T V S T T Y T
   Q A E G F V K N L Q M T G R Y L R D V W
901 CTGCTGCTATCCAGAAATCGTGTGTGTTCCCTGACAATCAGATTCACATTTTCAG
2221 GGTGATATAAGTATTGCCCTCCGCTGTTCCAACCTCTCCCTAGAAATGGAGACTCCGAAAT
   A I P E Y R V V F P D K S D S L I S E
   *
961 AAGCAATGGCCATGCCAAATGGTTATGCTAATGGCAACCGTATATGATGCCAGCATC
2281 TAAGTATACCCCACTTTTATATATATGATAGATATAACAAGCATTTTAGGAAGGCCA
   A N G H A N G Y A N G N T V Y D A Q H P
   TGTGCAAAATTTACCAGCCTTCCCTCCACCTCCAGATTTTCAAGTGTGGTGGTT
2341
2401 CTTAAGATATCTTTTTTTTTTTTTTTGGTGGATTTTGGTTCGATGTAATTCATATAAAT
2461 GTAATATTTATAGTAAATATATATACCAATAATTTGGTGGTAGCAACCGCAACTATTAGGG
2521 GAAACTATGCTCAAGTCTGTATTATTTAATCAGTAATGAGAGACAGCAAAATGTTCTGTA
2581 CCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 2618
1261 GGAGCTCATGCTCCTCCTTTCCACCTTGTACTCTAAGAACCCTCCACTCGTTATG
   S S L P P P P P P C T L R T A L T R Y A

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Figure 5. Sequence of NADPH:cytochrome P-450 reductase from *C. roseus*.

The nucleotide sequence derived from a genomic NADPH:cytochrome P-450 reductase clone and from a partial cDNA clone (pSK-R9) as well as the deduced amino acid sequence of the reductase protein are shown. The cDNA clone pSK-R9 starts at the position of the arrow. The ATG codon starting at position 81 is presumed to be the translation initiation start codon. The asterisk indicates a stop codon and a putative polyadenylation signal is underlined.

observed following digestion with *Bgl*II, *Sac*I and *Xba*I was consistent with the presence of two *Bgl*II restriction sites and one site for the latter two enzymes in the cDNA sequence, assuming that two of the weaker hybridizing bands in the *Bgl*II digest are due to partial digestion. Multiple bands observed in the *Hind*III digest were due to a restriction site in an intron of the reductase gene (unpublished results). The results strongly suggest that

NADPH:cytochrome P-450 reductase is encoded by a single copy gene.

NADPH:cytochrome P-450 reductase expression in C. roseus plants and elicitor-treated cell cultures

The expression levels of NADPH:cytochrome P-450 reductase mRNA were determined in different organs of

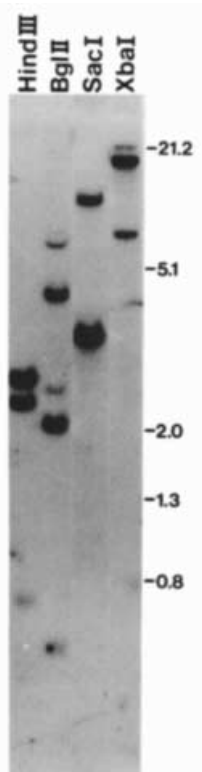


Figure 7. Estimation of NADPH:cytochrome P-450 reductase gene copy number. A Southern blot of *C. roseus* genomic DNA was probed with the NADPH:cytochrome P-450 reductase cDNA. Ten micrograms of DNA were digested with *Hind*III, *Bgl*II, *Sac*I and *Xba*I. Sizes of marker fragments are indicated in kbp.

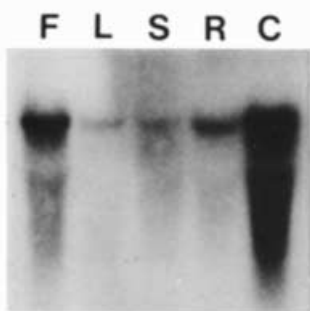


Figure 8. NADPH:cytochrome P-450 reductase steady state mRNA levels in *C. roseus* plants and cell cultures. A Northern blot of total RNA (25 µg) from flowers (F), leaves (L), stems (S) and roots (R) of 3-month-old *C. roseus* greenhouse-grown plants and from a *C. roseus* cell suspension culture (C) was probed with the NADPH:cytochrome P-450 reductase cDNA (pSK-R9).

flowering *C. roseus* plants grown in a greenhouse, and in a *C. roseus* cell suspension culture grown on LS medium (Linsmaier and Skoog, 1965) containing 3% sucrose, 2 mg l⁻² 1-naphthaleneacetic acid and 0.2 mg l⁻¹ kinetin. Total RNA was extracted from these tissues and Northern blot analysis was performed with cDNA pSK-R9 as a probe (Figure 8). The highest steady state levels of the

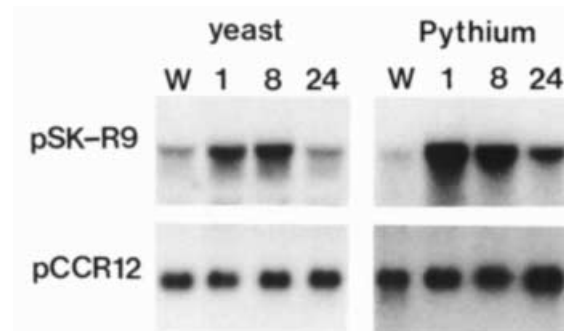


Figure 9. Effects of fungal elicitor preparations on the NADPH:cytochrome P-450 reductase mRNA level. *C. roseus* suspension-cultured cells were exposed for 1, 8 and 24 h to yeast extract or *P. aphanidermatum* culture filtrate. Control cultures were incubated for 24 h with water (W). Northern blots containing total RNA (10 µg per lane) were probed with the NADPH:cytochrome P-450 reductase cDNA (pSK-R9) or pCCR12 control clone.

reductase mRNA of around 2700 nucleotides were observed in the cell suspension culture and in flowers. In leaves and stems much lower expression levels were detected. An intermediate mRNA level was observed in roots, the source of cDNA clone pSK-R9.

In cell suspension cultures a rapid induction of the NADPH:cytochrome P-450 reductase steady state mRNA level was observed after addition of elicitor preparations from *Pythium aphanidermatum* or yeast (Figure 9). As a control the blots were probed with cDNA clone pCCR12, which corresponds to an abundant, unidentified mRNA of around 700 nucleotides. Elicitor treatment had no effect on the steady state mRNA level of the pCCR12 control.

Discussion

Characterization of an NADPH:cytochrome P-450 reductase cDNA from *C. roseus*: conservation of functional domains involved in cofactor and substrate binding

We have determined the complete nucleotide sequence of NADPH:cytochrome P-450 reductase from *C. roseus* from a partial cDNA clone and a genomic DNA clone. The cDNA could be identified by its functional expression in *E. coli* and on the basis of a high degree of homology with previously published NADPH:cytochrome P-450 reductase sequences from yeast and animal species. To the best of our knowledge this is the first report of an NADPH:cytochrome P-450 reductase clone from a higher plant. Sequence comparisons of the NADPH:cytochrome P-450 reductases from yeasts and rat (Sutter *et al.*, 1990; Yabusaki *et al.*, 1988) had already revealed a high degree of conservation in regions involved in cofactor binding. The same is true for the *C. roseus* reductase, as can be seen in Figure 6 where the *C. roseus* sequence is aligned with the sequences of the reductases of *S. cerevisiae* and rat.

A detailed comparison of the assumed cofactor binding sites in the plant, yeast and mammalian proteins, as discussed below, may yield additional information about the amino acid residues essential for cofactor binding and the structure of the cofactor binding sites.

By comparison with bacterial FMN-containing flavodoxins (Porter and Kasper, 1985) the regions from position 79 to 101 and from 138 to 202 in the reductase of rat were identified as FMN binding sites. In the first region residues Ser-86, Thr-88 and Thr-90 form hydrogen bonds with the pyrophosphate of FMN. The second region binds the FMN-isoalloxazine ring and may also be involved in binding the FMN-ribose through a hydrogen bond with Thr-139. Two tyrosine residues (positions 140 and 178) are supposed to shield and stabilize the isoalloxazine ring through stacking interactions between the aromatic residues. Their functional importance was confirmed by site-directed mutagenesis studies (Shen *et al.*, 1989). In the reductases from both *S. cerevisiae* and *C. roseus* all residues mentioned above are identical to the rat sequence or show a conservative substitution.

Regions in the rat reductase homologous to other FAD-containing proteins have also been discussed (Karplus *et al.*, 1984; Ostrowski *et al.*, 1989; Porter and Kasper, 1985, 1986). The region from position 292 to 326 in rat reductase may be involved in both ribose and pyrophosphate binding. Asp-318 of rat reductase could bind the ribose OH-groups and is conserved in the *S. cerevisiae* and *C. roseus* proteins. The region from Gln-452 to Glu-477 of rat reductase, which is also highly conserved in the *S. cerevisiae* and *C. roseus* proteins, is in the correct position to interact with the FAD isoalloxazine ring and is homologous to *E. coli* fumarate reductase (Porter and Kasper, 1985), which has an FAD covalently attached to a His residue. This His corresponds to His-470 of rat reductase and is conserved in *S. cerevisiae* and *C. roseus*, even though the FAD is non-covalently bound in the cytochrome P-450 reductases.

The positions of the binding sites for the pyrophosphate and ribose groups of NADPH remain unclear. The assignment of Porter and Kasper (1985, 1986) of residues 483–519 in rat reductase as the pyrophosphate binding site is not supported by the *C. roseus* sequence, since the region around Gly-504 of rat reductase is not conserved in the *C. roseus* protein. An alternative binding region for pyrophosphate could be position 532–536 (Karplus *et al.*, 1984; Ostrowski *et al.*, 1989). This region is also conserved in the *C. roseus* reductase. Porter and Kasper (1986) proposed residues 520–553 of rat reductase to be involved in both ribose and pyrophosphate binding. The relevant amino acid residues are consistent with the *C. roseus* sequence. The region around Lys-602 of rat reductase is another possibility for binding the NADPH-ribose and is highly homologous between rat, *S. cerevisiae* and *C.*

roseus. The region from position 624 to 640 of rat reductase is a possible site for contact with the NADPH-nicotinamide ring (Hackett *et al.*, 1986; Ruettinger *et al.*, 1989) and is almost completely conserved in the *C. roseus* and *S. cerevisiae* proteins.

Sutter *et al.* (1990) identified 25 invariant residues in all previously isolated NADPH:cytochrome P-450 reductase sequences and the FMN, FAD and NADPH binding domains of several other flavoproteins. Of these residues 22 (indicated with dots in Figure 6) are also conserved in the *C. roseus* sequence, which further supports their supposed essential role. The other three amino acids are two Leu residues (positions 562 and 574 of rat reductase) located outside the discussed binding sites, and Ser-86 in the FMN binding domain of rat reductase, where the *C. roseus* sequence shows a conserved mutation to Thr.

Putative binding domains for the substrates cytochrome P-450 (residues 109–130; Nadler and Strobel, 1991) and cytochrome c (residues 207–215; Nisimoto, 1986) were also identified in the rat reductase. Characteristic of these binding sites is the presence of several acidic residues and these are also found in the corresponding regions of *S. cerevisiae* and *C. roseus* reductase.

Like the yeast and animal reductases the *C. roseus* protein contains a hydrophobic domain close to the N-terminus, which could serve as a membrane anchor. Assuming we assigned the correct ATG codon as start position, the *C. roseus* reductase has a calculated M_r of 78 958 and contains 36 amino acids more than the rat reductase, with an M_r of 76 962. Benveniste *et al.* (1986, 1991) purified three isoforms of NADPH:cytochrome P-450 reductase from *Helianthus tuberosus*, with M_r values between 80 000 and 84 000. Cross-reactivity experiments with antiserum raised against the *H. tuberosus* reductases indicated that plant cytochrome P-450 reductases in general have slightly higher M_r values than the mammalian reductases (Benveniste *et al.*, 1989). The immunological studies of these authors also revealed that animal cytochrome P-450 reductases were not recognized by the antiserum against the plant enzyme. On the basis of this result they concluded that important structural differences would exist between higher plant and animal reductases. Our characterization of the *C. roseus* cytochrome P-450 reductase shows that the structure of the functional domains as well as the positioning of these domains in the protein are highly conserved between the plant and animal reductases, even though the overall amino acid identity is only 39%.

NADPH:cytochrome P-450 reductase probably is encoded by a single copy gene

In yeast and animal species all different cytochrome P-450 mono-oxygenases are dependent on a single form of the

reductase enzyme. It remains unclear whether the same situation applies to all higher plants. In *H. tuberosus* three isoforms of NADPH:cytochrome P-450 reductase were found (Benveniste *et al.*, 1991), but it has not been established whether these forms originate from different genes or represent modifications of a single gene product that occur post-translationally or during isolation. For *C. roseus* the results of Southern blot analysis strongly suggest that the reductase gene occurs as a single copy in the plant genome. Moreover, approximately 0.35 kb of the ORF specified by a genomic reductase clone was determined and no differences with the cDNA sequence were found. On Western blots the antiserum recognized more than one band in crude and partially purified *C. roseus* protein preparations. However, trypsin digestions performed on different protein fractions indicated that the immunoreactive bands with lower molecular weights are purification artefacts that are formed from the immunoreactive protein with the highest M_r value. The sensitivity of animal NADPH:cytochrome P-450 reductase to protease action is well known (Black *et al.*, 1979; Lu *et al.*, 1969), and in purifications of this enzyme from other plant species proteolytic degradation products have also been observed (Benveniste *et al.*, 1986; Fujita and Asahi, 1985). When the NADPH:cytochrome P-450 cDNA (pSK-R9) was expressed as a β -galactosidase fusion protein in *E. coli*, staining for reductase activity revealed more than one positive band (Figure 8), which is further evidence for the presumed sensitivity of the reductase to protease action.

NADPH:cytochrome P-450 reductase mRNA levels in C. roseus plants and elicitor-treated cell cultures

Since a wide range of metabolic pathways are dependent on cytochrome P-450 mono-oxygenases it is likely that several different cytochrome P-450 species are found in all plant organs. The activities of all these enzymes depend on NADPH:cytochrome P-450 reductase, therefore it is not surprising that the reductase mRNA could be detected in all tissues. The level of reductase mRNA expression appeared to be regulated in a tissue-specific manner. Possibly the level of expression shows a positive correlation with the metabolic activity of cytochrome P-450 enzymes. In flowers, where higher levels of reductase mRNA were found than in the other plant organs, several cytochrome P-450 enzymes that are involved in producing pigments, aromatics and defence-related compounds are likely to be expressed. Our research project is focused on the regulatory mechanisms that control the biosynthesis of terpenoid indole alkaloids in *C. roseus*. In mature *C. roseus* plants the activity of the cytochrome P-450 enzyme geraniol 10-hydroxylase is highest in roots (Meijer, unpublished results). Steady state mRNA levels of tryptophan decarboxylase and strictosidine synthase, two other key

enzymes in terpenoid indole alkaloid biosynthesis previously cloned in our group, were also found to be highest in roots (Pasquali *et al.*, 1992). In flowering *C. roseus* plants the reductase gene appeared not to be co-ordinately expressed with these two genes. At present the importance of the expression levels of either the reductase or geraniol 10-hydroxylase for the overall production of terpenoid indole alkaloids cannot be evaluated. The isolation of the reductase cDNA clone enables us to investigate this aspect of the regulation of this biosynthetic pathway. The reductase seems to be encoded by a single copy gene, which facilitates studies on its regulation. Gaining insight in the regulation of NADPH:cytochrome P-450 reductase is especially interesting since this enzyme is an essential component of a variety of different biosynthetic pathways in plants. As a first step in this direction we studied the effects of fungal elicitor preparations on expression of the reductase gene in cell cultures of *C. roseus*. Addition of yeast extract or *P. aphanidermatum* culture filtrate was found rapidly to enhance the reductase steady state mRNA level. It is likely that these extracts also induce the expression of a number of cytochrome P-450 genes, since these are involved in various defence-related biosynthetic pathways that are known to be elicitor-inducible (reviewed by Donaldson and Luster, 1991). We recently obtained evidence for this assumption, since we isolated two elicitor-inducible cytochrome P-450 cDNA clones from *C. roseus* (unpublished results). Our results demonstrate that steady state mRNA levels of the reductase are strictly regulated and induced under conditions that also induce the expression of cytochrome P-450 genes. Hence, the reductase may be of key importance in regulation of plant defence metabolism.

Potential applications of the NADPH:cytochrome P-450 reductase cDNA in genetic modification of cytochrome P-450-dependent biosynthetic pathways

The *C. roseus* NADPH:cytochrome P-450 reductase cDNA clone may be a useful tool for research projects concerning plant cytochrome P-450 enzymes. One application can be its use in experiments designed to identify a particular plant cytochrome P-450 cDNA clone by demonstrating its enzyme activity in a heterologous system. Heterologous expression studies of mammalian cytochrome P-450 cDNAs in *E. coli*, yeast and viral systems, have shown that co-expression of NADPH:cytochrome P-450 reductase is usually essential for obtaining high levels of cytochrome P-450 activity (Murakami *et al.*, 1986; Porter *et al.*, 1987; Tamura *et al.*, 1992; Yamano *et al.*, 1989). Heterologous expression of plant cytochrome P-450 enzymes can possibly be achieved with yeast or animal cytochrome P-450 reductase. Until now most studies have indicated that the NADPH:cytochrome P-450

reductases are rather species-unspecific, for example, an animal cytochrome P-450 has been shown to be active in tobacco (Saito *et al.*, 1991). Still, it is a safer approach to use a plant reductase, preferably from the same species, in attempts to demonstrate cytochrome P-450 enzyme activity in a heterologous system. For this type of experiment it will be necessary to use a full length reductase cDNA clone, which in the case of the *C. roseus* reductase can be constructed from the genomic clone and the partial cDNA clone. Although the partial cDNA clone was shown to be active in *E. coli* with the *in vitro* substrates cytochrome c and neotetrazolium, the missing N-terminal amino acid sequence is essential for membrane anchoring and interaction with cytochrome P-450 (Black *et al.*, 1979; Lu *et al.*, 1969).

A second application of the reductase cDNA can be its use in genetic modification of biosynthetic pathways that comprise reactions catalysed by cytochrome P-450 enzymes. The pathways leading to several secondary plant products with important pharmaceutical applications are dependent on cytochrome P-450 enzymes. Some examples are the terpenoid indole alkaloids formed by *C. roseus* and related plant species, and cardiac glucosides in *Digitalis lanata* (Petersen and Seitz, 1988). Overexpression of the appropriate cytochrome P-450 species may improve product yield of these compounds. In rat liver microsomes the molar ratio of NADPH:cytochrome P-450 reductase to cytochrome P-450 is about 1 to 15 (Shephard *et al.*, 1983). One might envisage that, when a P-450 is overexpressed, the reductase may become limiting, or may already be a limiting factor in the natural situation. Overexpression of the full length *C. roseus* reductase cDNA, alone and in combination with overexpression of cytochrome P-450, may answer these questions.

Experimental procedures

Plant material

Cell suspension cultures of *C. roseus* (L.) G. Don were grown in the light at 25°C on gyratory shakers at 120 r.p.m. The cultures were grown in LS medium (Linsmaier and Skoog, 1965) containing 3% sucrose, 2 mg l⁻¹ 1-naphthaleneacetic acid and 0.2 mg l⁻¹ kinetin, and subcultured every 7 days by a fourfold dilution. For enzyme purification 7-day-old *C. roseus* cultures were diluted fivefold in 500 ml alkaloid production medium according to Berlin *et al.* (1983) and grown in 2 l Erlenmeyer flasks for 5–6 days.

C. roseus seeds (*Vinca rosea*, variety Morning mist) were obtained from Kieft (Blokker, The Netherlands), and plants were grown in a greenhouse at 23°C.

Purification of NADPH:cytochrome P-450 (cytochrome c) reductase

Cell suspensions of *C. roseus* were harvested by filtration under suction, washed once with water, and frozen in liquid nitrogen.

The frozen cells were homogenized in a Waring blender equipped with a stainless steel beaker at top speed for 1 min, rapidly thawed in 2 volumes of 50 mM potassium phosphate buffer (pH 7.6) containing 0.3 M sucrose, 1 mM EDTA, 1 mM DTT and 5 µg ml⁻¹ leupeptin, and additionally homogenized with an Ultra Turrax at medium speed for 2 min. Membranes sedimenting between 20 min at 1000 g and 60 min at 20 000 g were suspended in an approximately equal volume of 50 mM Tris/HCl buffer (pH 7.8) containing 20% (v/v) glycerol, 1 mM EDTA, 1 mM DTT and 5 µg ml⁻¹ leupeptin. The membrane fraction (5–10 mg protein per ml) was solubilized on ice by the dropwise addition of a 10% (w/v) sodium cholate solution to a final concentration of 1%. After 30 min of gentle stirring the suspension was centrifuged at 100 000 g for 90 min. The supernatant was applied on a DEAE-Sephacel column (2.6 × 34 cm), equilibrated in 50 mM Tris/HCl buffer (pH 7.8) containing 15% (v/v) glycerol, 0.1% (v/v) Renex 690, 0.1 mM DTT, 1 µM FMN and 1 µM FAD. The column was washed with 400 ml equilibration buffer and protein was eluted with a KCl gradient in the same buffer, at a flow rate of 0.5 ml min⁻¹. After elution with a linear gradient of 0–100 mM KCl in 1000 ml, followed by 400 ml 100 mM KCl, the NADPH:cytochrome P-450 (cytochrome c) reductase-containing fractions were eluted with 600 ml 300 mM KCl. The pooled reductase-containing fractions from DEAE-Sephacel were fractionated on a 2'5'ADP-Sephacel 4B column (1 × 19 cm) equilibrated in 10 mM potassium phosphate buffer (pH 7.7) containing 20% (v/v) glycerol, 0.005% (w/v) sodium cholate, 0.02 mM EDTA, 0.2 mM DTT, 1 µM FAD and 1 µM FMN. The column was washed with 60 ml equilibration buffer and 75 ml 200 mM potassium phosphate buffer (pH 7.7) containing 20% (v/v) glycerol, 0.005% (w/v) sodium cholate, 0.4 mM EDTA, 0.2 mM DTT, 1 µM FAD and 1 µM FMN. Elution was with a linear gradient of 0–5 mM 2'AMP, followed by 10 ml 5 mM 2'AMP in equilibration buffer. The flow rate was 0.1 ml min⁻¹. All chromatographic steps were performed at 4–7°C. Purified enzyme preparations were stored at –80°C.

Determination of enzyme activities

The assay for NADPH:cytochrome c (P-450) reductase activity was modified from Madyastha *et al.* (1976). The incubation mixture (total volume 1.0 ml) consisted of 20 µl of enzyme solution and 50 nmol cytochrome c (type III, from horse heart), 0.15 µmol NADPH, 0.5 µmol KCN, 5 nmol FMN and 5 nmol FAD, in 0.5 M Tris/HCl buffer (pH 7.5). The reactions were started by the addition of enzyme and the reduction of cytochrome c was monitored at 20°C for 3–4 min at a wavelength of 550 nm ($\epsilon = 21 \text{ mM}^{-1} \text{ cm}^{-1}$).

Geraniol 10-hydroxylase activity was measured as the formation of [1-³H]10-hydroxygeraniol from [1-³H]geraniol in the presence of an NADPH-regenerating system according to Madyastha *et al.* (1976), with modifications. To determine the dependence of geraniol 10-hydroxylase activity on reconstitution with NADPH:cytochrome P-450 reductase, different amounts of a partially purified reductase preparation (DEAE-Sephacel pool) and 100 µg of *C. roseus* lipids, extracted from a crude membrane preparation with CHCl₃/MeOH (2:1, v/v), were added to a partially purified cytochrome P-450 preparation (4.9 pmol cytochrome P-450 determined from a CO difference spectrum according to Omura and Sato (1964)). Before the addition of substrates, the incubation mixture was passed through Extracti-gel D (Pierce) for detergent removal.

Trans-cinnamate 4-hydroxylase activity was determined by an HPLC-based method according to M. Petersen (University of Düsseldorf, Germany; personal communication). Reconstitution of a partially purified cytochrome P-450 preparation with NADPH:cytochrome P-450 reductase (360 pkat) and lipids was performed

as described for geraniol 10-hydroxylase. The incubation mixture (total volume of 500 μ l) consisted of 0.5 μ mol *trans*-cinnamate in 20 μ l 50% ethanol, 0.5 μ mol NADPH, and the reconstituted proteins in 0.1 M Tris/HCl buffer (pH 7.5), containing 20 mM DTT. After 60 min incubation at 30°C, the reaction was stopped by the addition of 100 μ l 6 M HCl, and the mixture was extracted twice with 1 ml ethyl acetate. The organic phase was dried under a stream of nitrogen and the residue was dissolved in 150 μ l methanol/water (50:50, by vol.), containing 100 μ l H₃PO₄ (85%) per litre. HPLC analysis was carried out at room temperature on a 4.6 mm (internal diameter) \times 250 mm Shandon Hypersil ODS column with a particle size of 5 μ m, at a flow rate of 1.5 ml min⁻¹. The analytical column was used in combination with a 2 mm (internal diameter) \times 20 mm pre-column (Upchurch) hand-packed with Perisorb RP-8 (Merck) with a particle size of 30–40 μ m. The mobile phase consisted of methanol/water, containing 100 μ l H₃PO₄ (85%) per litre and elution was with a linear gradient of 40–60% (v/v) methanol in 10 min, followed by 60% methanol for 3 min. The injection volume was 20 μ l and detection was at 309 nm.

Antiserum against NADPH:cytochrome P-450 reductase

Purified NADPH:cytochrome P-450 reductase was denatured by the addition of SDS to a concentration of 0.5% (w/v) and heating at 80°C for 15 min. New Zealand white rabbits were injected subcutaneously with 100 μ g of the denatured protein in complete Freund's adjuvant. Subsequently, two subcutaneous booster injections of 100 μ g denatured reductase in incomplete Freund's adjuvant were given at 2-week intervals. Blood was drawn from the ear vein 1–2 weeks after each booster injection, and serum was prepared. The presence of antibodies against the reductase was determined by enzyme-linked immunosorbent assays and Western blot analysis.

Gel electrophoresis, isoelectric focusing and Western blot analysis

SDS-PAGE and native gel electrophoresis were performed according to Laemmli (1970). Proteins were visualized with Coomassie brilliant blue or by silver staining. Native gels were stained for enzyme activity at room temperature in 25 ml 0.5 M Tris/HCl buffer (pH 7.6) containing 5 mg NADPH and 4 mg neotetrazolium chloride for at least 60 min in the dark (Fan and Masters, 1974). Trypsin digestions of protein samples for electrophoresis were performed on ice for 2 min in 50 mM sodium phosphate buffer (pH 7.4) containing 25 μ g ml⁻¹ trypsin (from bovine pancreas, Sigma) and 150 mM NaCl. The reactions were stopped by the addition of soybean trypsin inhibitor (Sigma) to a concentration of 100 μ g ml⁻¹. Electrophoresed proteins were electroblotted on to nitrocellulose filters with a Multiphor II Novablot (LKB) apparatus for 1 h at 0.8 mA cm⁻², with blot buffer consisting of 39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS and 20% (v/v) methanol. Immunodetection with antiserum against NADPH:cytochrome P-450 reductase was performed as described for cDNA library screening. Isoelectric focusing was carried out in PhastGel IEF 3-9 with Phastsystem (Pharmacia LKB Biotechnology).

Construction and screening of cDNA and genomic DNA libraries

A cDNA library consisting of 1.7×10^6 primary recombinants was constructed according to the λ ZAP II-cDNA Synthesis protocol (Stratagene) from 5 μ g of poly(A) RNA isolated from roots of 3-

month-old *C. roseus* plants. The library was amplified and around 400 000 plaques were screened on nitrocellulose filters (Schleicher and Schuell BA85) with antiserum against NADPH:cytochrome P-450 reductase, according to the Protoblot Immunoscreening System Manual (Promega). The antiserum was used in 1000-fold dilution and pre-incubated with 0.5 mg ml⁻¹ *E. coli* strain XL-1 protein extract. Goat anti-rabbit IgG conjugated to alkaline phosphatase (Promega) was used as second antibody.

In order to isolate a full length clone the cDNA library was rescreened with a DNA fragment derived from the 5' end of the incomplete reductase cDNA clone pSK-R9 resulting from immunoscreening as described in this paper. Plaques were lifted with nylon filters (Hybond-N, Amsterdam) and hybridization and washing were performed as described for Southern blot analysis. The same conditions were employed for screening of a λ GEM11 (Promega) genomic DNA library that was constructed from *C. roseus* total DNA, partially digested with *Sau*3a, as described (Goddijn, 1992).

Nucleic acid sequencing

Sequence analysis of (subcloned) cDNA inserts in pBluescript II SK (Stratagene) was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) with Sequenase version 2.0 (USB). The entire NADPH:cytochrome P-450 reductase sequence was determined on both strands. Sequence data were analysed with the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package (Devereux *et al.*, 1984). RNA sequencing was performed according to Geliebter (1987) with 10 μ g poly(A) RNA and the oligonucleotide 5'-GGCACTATGAGCTTCGG-3'.

Plant nucleic acid isolation

Total RNA was isolated from tissue ground in liquid nitrogen, by phenol/chloroform extraction and precipitation with LiCl at a final concentration of 2 M (Van Slogteren *et al.*, 1983). Total DNA was purified from the supernatant by isopropanol precipitation and CsCl-EtBr centrifugation. Poly(A) RNA was isolated with Poly(A) Quick columns (Stratagene).

Southern and Northern blot analysis

Digested DNAs were electrophoresed in 1% agarose gels in Tris-borate/EDTA buffer and transferred to GeneScreen (NEN) membranes by capillary blotting with 0.5 M NaOH. Total RNA was electrophoresed in 1.5% agarose/formaldehyde gels as described by Ausubel *et al.* (1987) and transferred to Duralon UV or Genescreen (NEN) membranes by capillary blotting with 50 mM sodium phosphate, 5 mM EDTA pH 6.5. Baked blots were (pre)-hybridized in 50% deionized formamide, $5 \times$ SSPE ($1 \times$ SSPE = 180 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate pH 6.5), 5% SDS at 42°C, washed with $0.1 \times$ SSPE, 0.5% SDS at 65°C, and exposed at -80°C to Fuji-RX films mounted on Kyokko intensifying screens. The hybridization probe was ³²P-labelled by the random primer labelling method (Feinberg and Vogelstein, 1984).

Preparation of *E. coli* protein extracts

E. coli (XL-1) cultures (50 ml), transformed with pSK or pSK-R9, were grown to an OD of 0.2. IPTG was then added to a concentration of 2 mM and the cells were grown for an additional 4 h. After centrifugation the cells were resuspended in 2 ml 50 mM Tris/HCl buffer (pH 7.8) containing 1 mM EDTA, 5 mM DTT and 20% (v/v)

glycerol, and sonicated on ice with a Branson microtip sonifier for five periods of 30 sec with 30 sec intervals. The sonicated extracts were used directly to assay cytochrome c reduction. Before native gel electrophoresis the extracts were incubated with 25 $\mu\text{g ml}^{-1}$ DNase I and RNase A for 30 min at 37°C, and then clarified by centrifugation.

Elicitor preparation and induction experiments

Pythium elicitor was prepared by autoclaving a filtered (0.2 μm) *P. aphanidermatum* culture grown in 100 ml of LS medium for 6 days at 27°C after inoculation with 1 cm² of fresh mycelium. Yeast extract (Difco) was dissolved in water and autoclaved. For elicitation experiments *C. roseus* cultures (10–12 days old) were subcultured by an eightfold dilution, and 10 ml of *Pythium* culture filtrate or 20 mg of yeast extract in 0.5 ml water were added 1, 8 or 24 h before harvesting at day 3 after subculturing. Control cultures were incubated for 24 h with water.

Protein determination

During enzyme purification, protein was determined according to Peterson (1977). For protein determinations of *E. coli* extracts the method of Bradford (1976) was used. Bovine serum albumin was used as standard.

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