Cinnamate 4-hydroxylase from *Catharanthus roseus*, and a strategy for the functional expression of plant cytochrome P_{450} proteins as translational fusions with P_{450} reductase in *Escherichia coli*

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Abstract A PCR-based approach was used to isolate cDNAs for cinnamate 4-hydroxylase (C4H) from *Catharanthus roseus* cell cultures. The protein shared 75.9% identity with C4H from other plants, and the transcription was induced under various stress conditions. The cloned protein was used to investigate the functional expression of plant P_{450}/P_{450}-reductase fusions in *E. coli*. Fusions containing a modified N-terminal membrane anchor were located in the membrane and possessed C4H activity without solubilization or addition of other factors. The results indicate that the fusion protein strategy provides a useful tool to analyze the activities encoded in the rapidly increasing number of plant P_{450} sequences of uncertain or unknown function. We also discuss critical elements of the strategy: the choice of the *E. coli* host strain, the N-terminal membrane anchor, and the conditions for protein expression.

Key words: *Catharanthus roseus*; Cinnamate 4-hydroxylase; Fusion protein; Heterologous expression; Membrane anchor; Cytochrome P_{450}; Cytochrome P_{450} reductase

1. Introduction

Cinnamate 4-hydroxylase (C4H), a cytochrome P_{450}-dependent enzyme (P_{450}) catalyzing the hydroxylation of cinnamate to 4-coumarate, is the second enzyme in the common branch of the phenylpropanoid pathway that leads to a large number of biologically important molecules, e.g. lignins, pigments and phytoalexins. The enzyme was completely purified some years ago [2], and cDNA clones were reported for a few plants (*Helianthus tuberosus* [3], *Medicago sativa* [4], *Phaseolus aureus* [5], and *Zinnia elegans* (AC U19922). In the first part of this work we describe the molecular characterization of C4H from Madagascar periwinkle (*Catharanthus roseus*) and studies on the regulation in cell cultures.

The second part addresses a problem of growing importance in view of the rapidly increasing number of sequences that are described as P_{450} on the basis of sequence similarities. Considering the difficulties with the purification of P_{450} from plants, the functional identification represents a challenging task. One of the methods of choice is the heterologous expression followed by functional assays. Plant cells are useful as hosts if mutants are available [6], but in many cases the distinction from resident activities may present a problem. Expression of functionally identified plant P_{450} in other cells has been reported (e.g. yeast [4,7] and animal cells [8,9]), but each of the available systems suffer from certain limitations including ease of use or expense.

E. coli had apparently not been used for plant P_{450}, although it represents one of the simplest systems for cloning and expression. One of the reasons may be that these bacteria lack P_{450} activities [1] and thus also the characteristic cytochrome P_{450} reductase (P_{450}-RED) that is necessary for P_{450} function. Results with some animal P_{450} suggested that it may be replaced by the combined action of two soluble *E. coli* flavoproteins [10,11], but a high efficiency required purified proteins [12], and the general applicability was not investigated. The obtaining of active P_{450} then requires the reconstitution with purified plant P_{450} reductase, but that is technically tedious and difficult. Experiments with a few animal steroid hydroxylases, however, suggested that the reductase problem could be avoided by fusing the P_{450} proteins translationally with the P_{450}-RED [13–15], an approach that imitated a naturally occurring protein in *Bacillus megaterium* [16]. Another problem could be that some plant P_{450} (including C4H) and P_{450}-RED have been shown to be glycoproteins [17–19], and that different glycosylation patterns in *E. coli* might result in inactive proteins.

The interest in a simple expression system for the functional identification and characterization of cloned plant P_{450} led us to investigate whether the fusion protein strategy can be applied to plant P_{450}. The results indicate that this strategy provides a useful tool, and we discuss some critical points of the approach.

2. Experimental

2.1. Plant material, induction, and isolation of membrane fractions

The cell suspension culture of Madagascar periwinkle (*Catharanthus roseus* L.G. Don, line CP3a), its maintenance on MX growth medium with subcultures every week, and the nutritional down-shift by transfer into a 8% sucrose solution have been described [20]. The PMG elicitor was a preparation from the cell walls of *Phytophthora megasperma* f. sp. *glycinea* [21]; it was used at a concentration of 1.2 mg/40 ml culture. The isolation of the membrane fractions has been published [20,22].

2.2. Vectors and E. coli strains

The vector pTZ19R [23] and the expression vector pQE-6 [24] as well as its modification by the introduction of a *Not*I site in the polylinker (pQE-6(*Not*I)) have been published [25]. The *E. coli* strains JM109, DH5α [26], M15 (recommended for pQE-6) [24], and the minicell producer DS410 and its use for selective expression of plasmid-encoded polypeptides [27] have been described. In the latter three strains, the pQE-6 promoter driving the expression of the cloned proteins was

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Abbreviations: AA, amino acids; C4H, cinnamate 4-hydroxylase (EC 1.14.13.11); P_{450}, cytochrome P_{450}-dependent enzyme(s) (recommended name heme-thiolate proteins [1]); P_{450}-RED, cytochrome P_{450} reductase(s) (EC 1.6.2.4); PCR, polymerase chain reaction.

The nucleotide sequence newly reported in this paper has been deposited in the GenBank/EMBL Data Banks with Accession no. Z32563.

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repressed under non-induced conditions by the *lacI*^{q} encoded in plasmid pREP4 [24] (DH5α[pREP4] and M15[pREP4]) or in plasmid pFDX500 [28] (DS410[pFDX500]).

2.3. cDNAs for C4H and P_{450}-RED

2.3.1. C4H. The cDNA synthesis with poly(A)-enriched RNA was carried out with a double-stranded primer (Fig. 1A) that incorporated a *Sal*I site and contained a single-stranded oligo(dT) extension for annealing with the poly(A)-stretch in mRNA. C4H-specific sequences were then amplified by PCR with a degenerated primer (by use of dITP) from the conserved sequences around the heme-binding cysteine in C4H and a primer from the sequences used for synthesis of the cDNA (Fig. 1A). The 0.35 kbp product was cloned into pTZ19R and used for screening cDNA libraries for full-length C4H clones.

2.3.2. P_{450}-RED. We used oligonucleotides from the published *C. roseus* sequence [29] to amplify the region from position 1360 to 2257 in a cDNA preparation. The resulting 0.9 kbp fragment was cloned in vector PCR^{TM}II (Invitrogen, San Diego, USA) and used to screen for full-length cDNA clones.

2.3.3. cDNA libraries. Poly(A)-rich RNA was isolated from *C. roseus* cell cultures induced for 7.5 h by a change from MX medium to 8% sucrose [20]. The construction of the libraries and the screening techniques have been described [30].

2.4. DNA sequence analysis

The cDNAs were sequenced by the dideoxy nucleotide chain termination technique. The vectors, phages, and methods were described in detail recently [30].

2.5. Antiserum against P_{450}-RED immunoblots

A cDNA fragment encoding the C-terminal 210 AA of the *C. roseus* P_{450}-RED was fused in frame with the sequence encoding the 42 kDa maltose-binding-protein in vector pMal-c2 [31]. The fusion protein (64.7 kDa) was purified by affinity chromatography on an amylose resin column and directly used for immunization of a rabbit. The cloning and purification procedures followed the protocol provided by the manufacturer (New England Biolabs). The separation of proteins for immunoblots was carried out in gels containing 0.1% sodium dodecyl sulfate and 8.5% polyacrylamide. The immunoreactions were performed with a secondary antibody coupled to alkaline phosphatase (Sigma Biochemicals) after protein transfer to nitrocellulose sheets [20].

2.6. C4H/P_{450}-RED fusion proteins

2.6.1. Modification of P_{450}-RED. A *Sal*I site was introduced at position 70 of the AA sequence by PCR using a custom-designed oligonucleotide (Fig. 1B) and the reverse sequencing primer from pTZ19R. A *Sal*I/*Asp*718 fragment (the latter site from the vector polylinker) containing the shortened protein coding region was then cloned into pTZ19R. This created a translational fusion between the N-terminal of the β-galactosidase in the vector and the P_{450}-RED. Enzyme assays indicated that the protein possessed P_{450}-RED activity (measured as cytochrome *c* reduction), and that at least 50% was present in the fraction of the soluble proteins. The cloning preserved the vector polylinker *Hin*dIII site that was useful for the later insertion of the 3′ end of C4H as *Sal*I/*Hin*dIII fragment.

2.6.2. Modification of C4H and fusion with P_{450}-RED. The C4H modifications and the fusion of the coding regions were carried out in several steps. The first eliminated by oligonucleotide-directed PCR mutagenesis the C4H stop codon and simultaneously introduced a *Sal*I site (Fig. 1B). The *Sal*I site and an internal *Hin*dIII site in the C4H cDNA were then used to insert a 0.37 kbp C4H fragment into the P_{450}-RED construct digested with *Sal*I and *Hin*dIII; this created a translational fusion with the P_{450}-RED. The addition of an internal 1 kbp *Hin*dIII fragment from the C4H cDNA added most of the C4H coding region, except for the N-terminal residues (32 AA). These were modified separately at different positions by PCR-directed mutagenesis to introduce *Nco*I sites and various AA exchanges (Fig. 1C, and section 3). The fragments were then combined with the previously constructed C4H/P_{450}-RED fusion. The completed constructs were recloned as *Nco*I/*Not*I fragments (the latter from the 3′ end of P_{450}-RED) into expression vector pQE-6(*Not*I). This positioned the protein-coding region via the *Nco*I site in optimal distance to the promoter-translation configuration of the vector.

All mutations were confirmed by sequence analysis, and sequences generated by PCR were exchanged against fragments from the original cDNAs as far as possible. The remaining PCR fragments were sequenced to establish the identity with the cDNA.

2.7. Protein expression in E. coli *DS410 and membrane preparation*

Strain DS410 was sequentially transformed with plasmid pFDX500 and the pQE-6 derivatives for expression of the fusion proteins. Precultures (2 ml) were grown overnight at 37°C in LB medium with ampicillin (0.1 mg/ml) and kanamycin (25 μg/ml). They were diluted 1:100 with fresh TB medium [26] and allowed to grow to $A_{600} = 0.6$. Protein expression was induced with 2 mM isopropyl-β-D-thiogalactopyranoside, and the cultures (100 ml) were shaken vigorously at 25°C for 24 h before harvest by centrifugation. The bacteria were washed twice with 50 ml of 10 mM potassium phosphate (pH 7.5) containing 0.15 M NaCl, and the pellets were stored at −20°C. All further steps were performed at 4°C. The cells were resuspended in 5–30 ml buffer A (75 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 0.25 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), sonicated for 10 s, and lysed in a French pressure cell. The lysate was centrifuged for 10 min at 10,000 × *g*, and the membrane fraction was obtained from the supernatant fluid by a 1 h centrifugation at 100.000 × *g*. The pellet was resuspended in 0.1–0.2

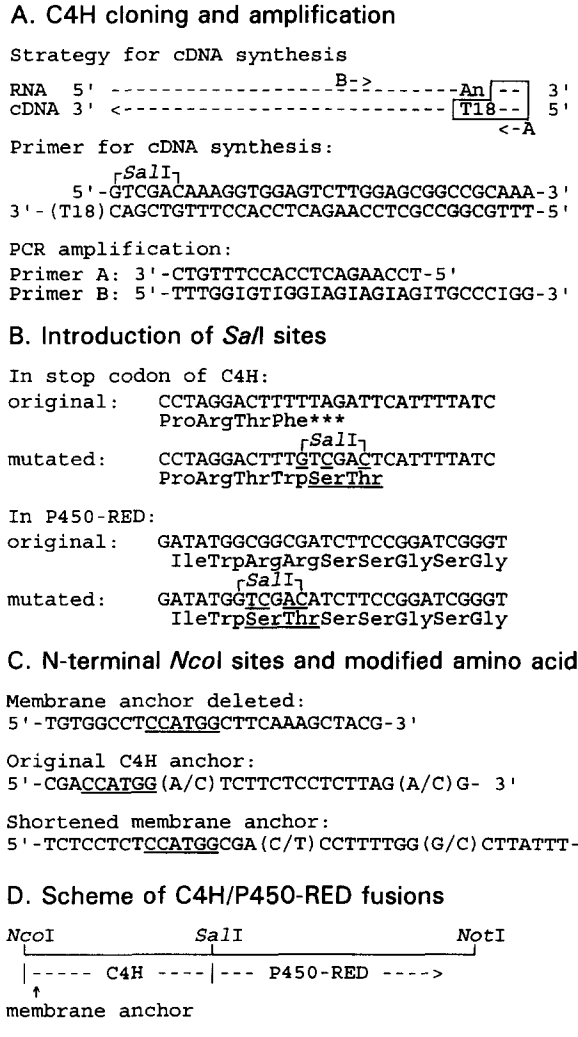

Fig. 1. Oligonucleotides for cDNA synthesis and amplification, for introduction of restriction sites and AA exchanges, and schematic drawing of the cassette used for protein expression. (B) *** = stop codon of C4H; underlined = mutated bases and Ser/Thr residues created by the *Sal*I site. (C) Underlined = introduced *Nco*I sites; (X/Y), bases incorporated at a 1:1 ratio during synthesis of the oligonucleotides.

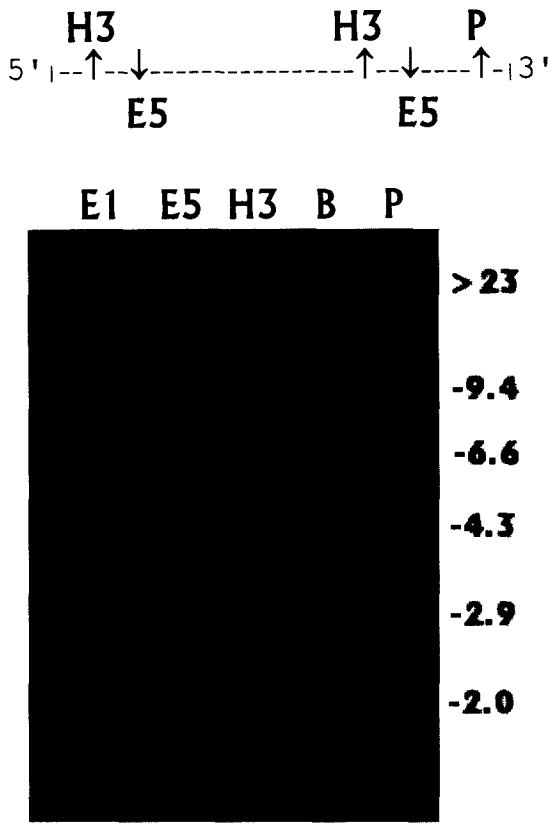

Fig. 2. Genomic Southern blot for C4H in *C. roseus*. (Top) Position of the sites in the cDNA (1713 bp), if present. Enzyme abbreviations (the positions of the sites are given in brackets): E1 = *Eco*RI; E5 = *Eco*RV (376,1361); H3 = *Hin*dIII (146,1202); B = *Bam*HI; P = *Pst*I (1626). The numbers at the right side of the blot indicate the size markers in kbp. Each lane contained 7.5 μg DNA.

ml buffer B (75 mM Tris-HCl, pH 7.5, 10% glycerol, 0.25 mM EDTA) and stored at −70°C until use for the enzyme assays.

2.8. Enzyme assays

The activities of C4H (with ^{14}C-labelled cinnamic acid) and of the P_{450}-RED (cytochrome *c* reduction) were determined as described [20].

2.9. Standard techniques

Standard cloning techniques, RNA blots with total RNA, and genomic blots were performed according to published procedures [26].

3. Results

3.1. C4H from C. roseus*: molecular characterization and expression in cell cultures*

We used a PCR strategy to obtain a partial sequence which was then used to isolate cDNAs from a cDNA library. The screening identified four clones of 1 kbp and one of 1.7 kbp, and the sequence analysis indicated that all were derived from the same gene. Hybridizations with genomic DNA were performed with the complete cDNA as probe, under hybridization and washing conditions which result in strong signals at more than 70% sequence identity [32]. The small number of hybridizing bands and the distribution of restriction sites in the cDNA (Fig. 2) suggested that the *C. roseus* culture contained either one gene with additional sites in introns or two closely related genes. The protein sequence translated from the cDNA revealed a close relationship with the four other C4H available from the databases (75.9% overall identity) (Fig. 3). There were also a number of positions in which all C4H contained conserved hydrophobic, acidic, or basic AA. The data suggested that the *C. roseus* clones encoded C4H, and that these enzymes are highly conserved proteins.

The expression in the cell cultures was investigated by Northern blots and enzyme activity determinations. Transcripts and C4H activity were detectable under all conditions. A nutritional downshift from MX medium to an aqueous solution of 8% sucrose had been shown to induce several P_{450} activities in *C. roseus* [20], and Fig. 4A shows a slow increase of the C4H transcripts during the 24 h of the experiments. The treatment with an elicitor preparation from *Phytophthora megasperma* induced a transient transcript increase (Fig. 4A). The changes correlated with the transcripts, taking into account the high basic enzyme activities prior to induction. The expression during the growth of the cell culture was also investigated with the P_{450}-RED, and Fig. 4B shows that both transcripts followed a similar time course.

3.2. Strategy for functional expression of plant P_{450} in E. coli

We investigated the expression of translational fusions of P_{450} with a cloned P_{450}-RED, because this promised to avoid the technically complex reconstitution of enzyme activity by addition of purified P_{450}-RED. All cloning steps were designed to utilize the cassette principle as much as possible. Full-length

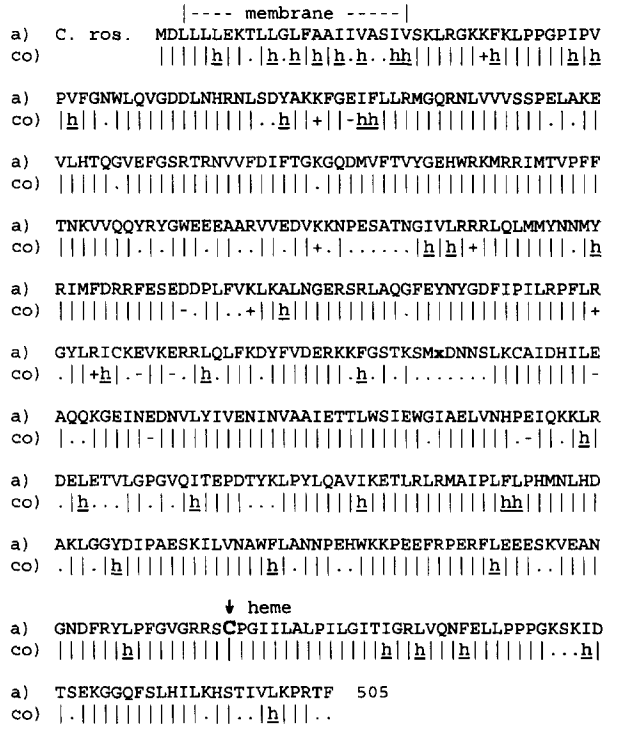

Fig. 3. C4H from *C. roseus* (a) and consensus (co) established from five different plants (the accession numbers are given in brackets): *C. roseus* (Z32563); *Helianthus tuberosus* (Z17369); *Phaseolus aureus* (L07634); *Medicago sativa* (L11046); *Zinnia elegans* (U19922). The predicted membrane spanning α-helix and the heme-binding Cys are marked. The X in the *C. roseus* sequence indicates a residue present only in *M. sativa*. In co (consensus): | = identical AA; − and + = acidic or basic residues, respectively; h = hydrophobic AA in all proteins. Dots indicate variable AA.

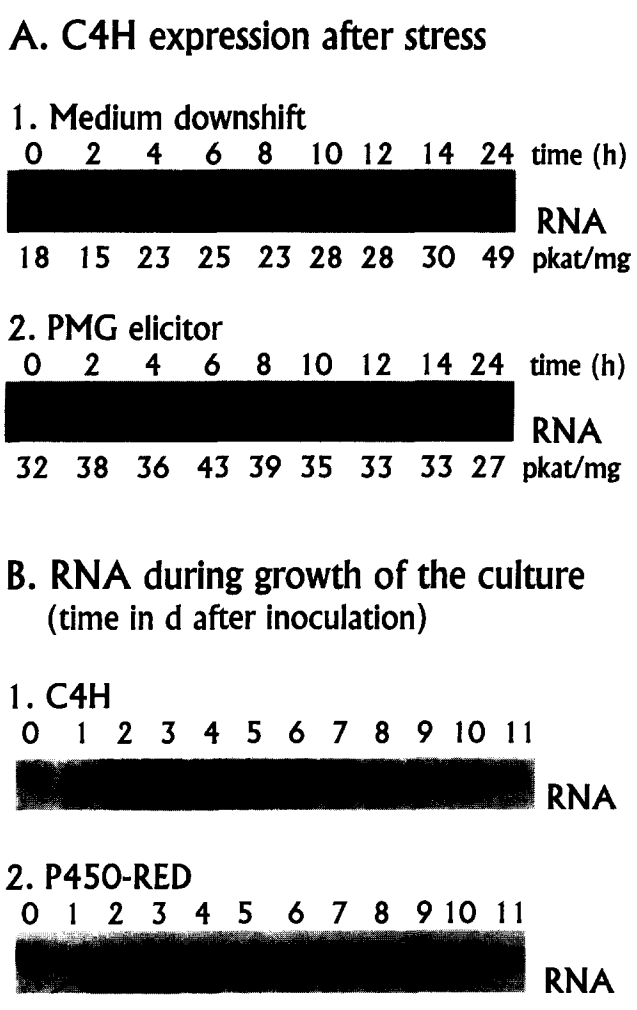

Fig. 4. Regulation of C4H. All experiments started with 7-day-old cell cultures. (A) C4H after exposure to stress (10 μg RNA per lane). 1 = medium downshift from MX medium to a solution of 8% aqueous sucrose; 2 = treatment with PMG elicitor. (B) Northern blots of of C4H and P_{450}-RED expression during growth of the cell culture (7.5 μg RNA per lane). The transcript sizes are 1.7 kb (C4H) and 2.8 kb (P_{450}-RED).

cDNAs for the reductase from *C. roseus* were obtained by a PCR-based approach (see section 2). The sequences were identical with those previously reported [29], except for a few differences.

The key elements of the strategy may be summarized as follows. The C4H N-terminal sequence was mutated at various positions to exchange certain AA and to introduce *Nco*I sites that incorporated protein start codons (CCATGG). This facilitated the insertion of the protein-coding regions in optimal distance to the promoter of the expression vector, and it allowed the precise definition of the critical N-terminal membrane anchor sequences of the fusions. The junction of C4H and P_{450}-RED was via a *Sal*I site introduced into the stop codon of C4H and at AA residue 70 of the reductase. A *Sal*I site encoding the dipeptide Ser-Thr was chosen because it created at the protein junction a Ser-Thr-Ser-Ser-Gly-Ser-Gly linker (Fig. 1B) that was unlikely to form extensive secondary structures possibly affecting the interaction of P_{450} and P_{450}-RED. The position 70 in P_{450}-RED was chosen because comparable experiments with animal reductases have shown that the N-terminal 50–70 residues could be deleted in such fusion proteins without loss of function [33,34]. Fig. 1D shows a schematic drawing of the completed cassette for expression of the fusion proteins.

3.3. *Functional expression of C4H/P_{450}-RED fusions in* E. coli: *importance of host strain and membrane anchor*

The first experiments were performed with a fusion in which the N-terminal P_{450} membrane anchor was completely deleted (Fig. 5A, construct 2). Immunoblots with the P_{450}-RED antiserum failed to detect the expected 129 kDa protein in any of the tested *E. coli* strains (JM109, DH5α, M15), even after extensive variations of the induction conditions. The results suggested that a hydrophobic membrane anchor was necessary for protein stability, and therefore we tested the N-terminal sequence of the original C4H, a shortened anchor, and its variations in certain residues (Fig. 5A). In the course of these experiments we also used an additional *E. coli* strain, DS410, because its property to produce minicells [27] provided a simple means to confirm the integrity of the protein coding regions in the fusion constructs. Surprisingly, the immunoblots revealed that this strain, when compared with the others, delivered the best protein yields. This was later confirmed with all constructs (not shown), and the experiments described below were carried out with this strain.

With the construct containing the original membrane anchor of C4H, the major product was a membrane-bound protein of 116 kDa, not the expected 129 kDa protein (Fig. 5, C4H), and C4H activity was not detected. Construct 6, containing a shorter N-terminal hydrophobic sequence (17 AA) than the original C4H, produced the 129 kDa protein, but the 116 kDa polypeptide was also present. The constructs 3 to 5 tested whether exchanges in AA representing potential breakers of the membrane-bound α-helix led to increased protein amounts and/or to a reduction of the smaller kDa protein, but no significant effect was observed (Fig. 5B, lanes 3–6). Fractionation

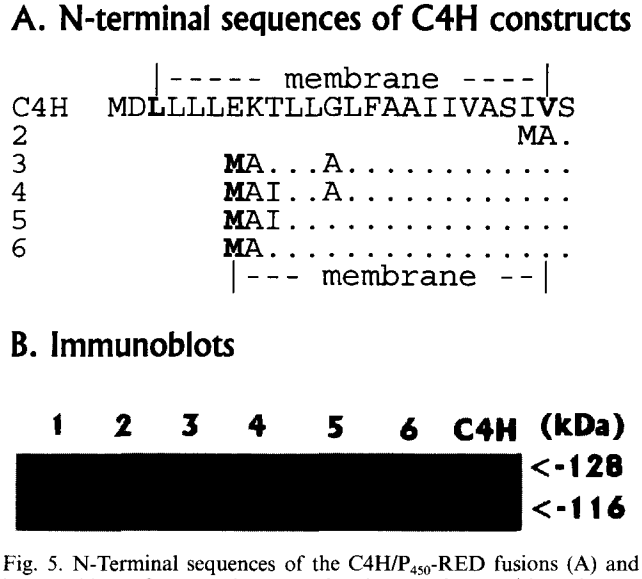

Fig. 5. N-Terminal sequences of the C4H/P_{450}-RED fusions (A) and immunoblots after protein expression in *E. coli* (B) with antiserum against P_{450}-RED. The lanes correspond to the constructs. C4H, original C4H membrane anchor. Lane 1 in B is the *E. coli* control (vector). Each slot contained 0.1 mg membrane protein.

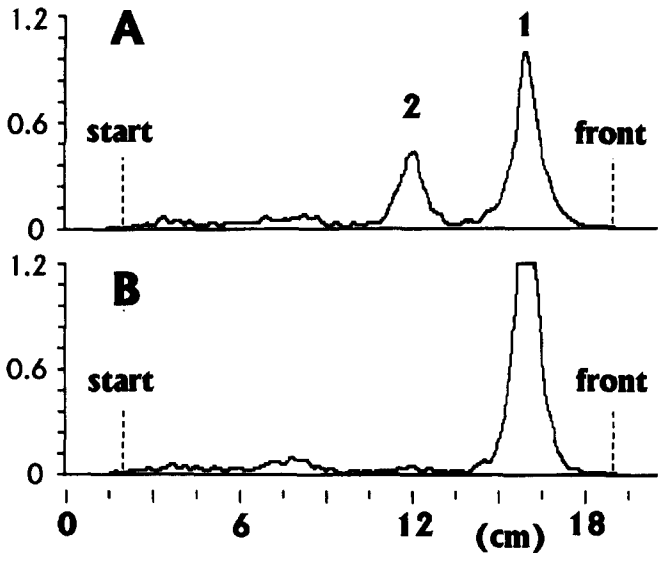

Fig. 6. C4H activity in *E. coli* membranes. (A) C4H/P_{450}-RED fusion (construct 3). (B) Membranes from control *E. coli* cells (vector). The figure shows the radioactivity scans obtained after thin-layer chromatographic separation of the substrate cinnamic acid (1) and the product 4-coumaric acid (2) in the solvent ethylether/petrol ether (b.p. 40–60°C)/formic acid (70:30:1, v/v/v). The incubations were performed for 20 min at 30°C with ^{14}C-labelled cinnamic acid and 50 μg protein. The numbers at the ordinate represent cpm × 10^{-3}.

experiments revealed that the majority of the 129 kDa protein was located in the pellet of centrifugations at 100,000 × *g*, suggesting that the protein had been inserted in the membranes. We also reinvestigated in DS410 the fusion lacking any membrane anchor, but never detected the expected protein (Fig. 5B, lane 2).

Enzyme assays showed that the membranes containing the 129 kDa protein possessed C4H activity (see Fig. 6 for an example with construct 3), and no solubilization step or addition of lipids was necessary. The enzyme activities of the four constructs ranged from 1.5 to 7 pkat/mg protein. The variability was most likely due to the fact that the fusion proteins were to some extent harmful to the *E. coli* cells, leading to reduced growth of the cultures and to a variable extent of cell lysis in the course of the protein induction (see also section 4). This could also explain the finding that the specific activity was about 4- to 7-fold lower than in microsomes from induced *C. roseus* cell cultures which contain very high C4H activities [20]. Antiserum against the C4H was not available, and therefore a direct comparison of the specific activities based on the amounts of immunoreactive protein was not possible.

4. Discussion

The extensive sequence similarity of the protein cloned from *C. roseus* with four other proteins suggested C4H as most likely candidate for the function. In particular with P_{450}, however, this could not be taken for granted because even a single AA difference can lead to drastic changes in the substrate specificity of P_{450} (reviewed in [1]). The heterologous expression was essential to confirm the tentative identification, and it also was an excellent possibility to investigate whether the fusion protein strategy can be used for the functional expression of plant P_{450} in *E. coli*. The positive results are to our knowledge the first demonstration for plant P_{450}, and the presence of C4H activity moreover demonstrated that plant-specific glycosylation steps are not necessary for C4H activity. It remains to be tested whether this applies to other glycosylated plant P_{450} as well.

The experiments with the C4H/P_{450}-RED fusions indicated a few parameters that may have to be tested for each fusion protein.

(a) E. coli *host strain.* We detected little or no protein in strain M15 which is recommended for pQE-6 derivatives, or in the strains DH5α and JM109 which have been used successfully for the expression of animal P_{450} alone or as P_{450}/P_{450}-RED fusions (see [11,13] for some examples). Those experiments revealed unexplained differences between DH5α and JM109 as well, and we have no simple explanation for the finding that the C4H/P_{450}-RED expression was successful in DS410, but not in the others.

(b) *N-Terminal of the fusion proteins.* Our experiments failed after complete deletion of the membrane binding domain, and the original sequence led to an inactive, truncated protein. They were successful with a shortened version of the original sequence, and a further modification by elimination of AA suspected as helix breakers (T > I, G > A; Fig. 5A) did not significantly improve the expression or stability. It is not excluded that other modifications may result in higher yields. The importance of the membrane anchor has been investigated to some extent with animal P_{450} (see [35] for a recent paper, and references therein). The original N-terminals were only rarely suitable, but otherwise no generalization seems to be possible. Successful expression was reported after deleting much or all of the N-terminal hydrophobic sequences (e.g. [10]), but other experiments succeeded only with modifications of the original anchor, and these followed the example of a once established successful sequence (M-A-L-L-L-A-V-F-L-F-C-L-V-F-W-V-L) [11] that bears no more than a superficial similarity to the shortened anchor used in our experiments (Fig. 5).

(c) *Regulation and conditions of protein expression.* The induction of the proteins led to a significant reduction in the growth of the cultures, suggesting that the fusion proteins were harmful to *E. coli*. It is therefore recommended to use a tightly regulated promoter in order to avoid the selection of mutants. We also observed that a variable percentage of the cells was lysed during the induction period, and this may well account for the observed variabilities of the enzyme activities in independent expression experiments. Extensive trials with the C4H/P_{450}-RED fusions in DS410 showed that expression for 24 h at low temperatures (25°C to 28°C) was a reasonable compromise to obtain active protein without extensive cell lysis. Under these conditions, the enzyme activity obtained in *E. coli* was more than sufficient to identify the C4H function. Experiments with other P_{450} fusions in the same expression vector suggested that the induction conditions may have to be optimized for each P_{450}/P_{450}-RED fusion (unpublished observations).

Our experiments tested a homologous P_{450}/P_{450}-RED combination, but it seems very likely that a once established P_{450}-RED cassette can also be used with P_{450} from other plants, and recent experiments with the flavonoid-3′,5′-hydroxylase from *Petunia*

hybrida support this suggestion (unpublished results). A low specificity in the interaction is suggested by the successful reconstitution of plant activities in vitro with heterologous plant combinations (e.g. [17]), and by the finding that a plant P_{450}-RED was able to support reactions of animal P_{450} [19]. It has also been shown that yeast reductase interacted successfully with plant P_{450} [4,7,36].

We conclude that the fusion protein strategy has the potential to provide a useful and simple technique to investigate cloned plant P_{450} functions in those cases where little or no other information is available. The cloning steps present no difficulties in laboratories that clone and analyze cDNA or genes, and no additional equipment is necessary.

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References

[1] Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Gonzalez, F.J., Coon, M.J., Gunsalus, I.C., Gotoh, O., Okuda, K. and Nebert, D.W. (1993) DNA Cell Biol. 12, 1–51.
[2] Gabriac, B., Werck-Reichhart, D., Teutsch, H. and Durst, F. (1991) Arch. Biochem. Biophys. 288, 302–309.
[3] Teutsch, H.G., Hasenfratz, M.P., Lesot, A., Stoltz, C., Garnier, J.-M., Jeltsch, J.-M., Durst, F. and Werck-Reichhart, D. (1993) Proc. Natl. Acad. Sci. USA 90, 4102–4106.
[4] Fahrendorf, T. and Dixon, R.A. (1993) Arch. Biochem. Biophys. 305, 509–515.
[5] Mizutani, M., Ward, E., DiMaio, J., Ohta, D., Ryals, J. and Sato, R. (1993) Biochem. Biophys. Res. Commun. 190, 875–880.
[6] Holton, T.A., Brugliera, F., Lester, D.R., Tanaka, Y., Hyland, C.D., Menting, J.G.T., Lu, C.-Y., Farcy, E., Stevenson, T.W. and Cornish, E.C. (1993) Nature 366, 276–279.
[7] Urban, P., Werck-Reichhart, D., Teutsch, H., Durst, F., Regnier, S., Kazmaier, M. and Pompon, D. (1994) Eur. J. Biochem. 222, 843–850.
[8] Song, W.-C., Funk, C.D. and Brash, A.R. (1993) Proc. Natl. Acad. Sci. USA 90, 8519–8523.
[9] Kraus, P.F.X. and Kutchan, T.M. (1995) Proc. Natl. Acad. Sci. USA 92, 2071–2075.
[10] Sagara, Y., Barnes, H.J. and Waterman, M.R. (1993) Arch. Biochem. Biophys. 304, 272–278.
[11] Barnes, H.J., Arlotto, M.P. and Waterman, M.R. (1991) Proc. Natl. Acad. Sci. USA 88, 5597–5601.
[12] Jenkins, C.M. and Waterman, M.R. (1994) J. Biol. Chem. 269, 27401–27408.
[13] Fisher, C.W., Shet, M.S., Caudle, D.L., Martin-Wixtrom, C.A. and Estabrook, R.W. (1992) Proc. Natl. Acad. Sci. USA 898, 10817–10821.
[14] Shet, M.S., Fisher, C.W., Holmans, P.L. and Estabrook, R.W. (1993) Proc. Natl. Acad. Sci. USA 90, 11748–11752.
[15] Shet, M.S., Fisher, C.W., Arlotto, M.A., Shackleton, C.H.L., Holmans, P.L., Martin-Wixtrom, C.A., Saeki, Y. and Estabrook, R.W. (1994) Arch. Biochem. Biophys. 311, 402–417.
[16] Ruettinger, R.T., Wen, L.-P. and Fulco, A.J. (1989) J. Biol. Chem. 264, 10987–10995.
[17] Kochs, G., Werck-Reichhart, D. and Grisebach, H. (1992) Arch. Biochem. Biophys. 293, 187–194.
[18] Benveniste, I., Lesot, A., Hasenfratz, M.P., Kochs, G. and Durst, F. (1991) Biochem. Biophys. Res. Commun. 177, 105–112.
[19] Shet, M.S., Sathasivan, K., Arlotto, M.A., Mehdy, M.C. and Estabrook, R.W. (1993) Proc. Natl. Acad. Sci. USA 90, 2890–2894.
[20] Vetter, H.-P., Mangold, U., Schröder, G., Marner, F.-J., Werck-Reichhart, D. and Schröder, J. (1992) Plant Physiol. 100, 998–1007.
[21] Habereder, H., Schröder, G. and Ebel, J. (1989) Planta 177, 58–65.
[22] Schröder, G., Beck, M., Eichel, J., Vetter, H.-P. and Schröder, J. (1993) Plant Mol. Biol. 23, 583–594.
[23] Zagursky, R.J. and Berman, M.L. (1984) Gene 27, 183–191.
[24] Crowe, J. and Henco, K. (1992) The QIAexpressionist. Qiagen Inc. Chatsworth, CA, USA.
[25] Tropf, S., Lanz, T., Rensing, S.A., Schröder, J. and Schröder, G. (1994) J. Mol. Evol. 38, 610–618.
[26] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[27] Dougan, G. and Kehoe, M. (1984) Methods Microbiol. 17, 233–258.
[28] Schnetz, K., Sutrina, S.L., Saier Jr. and Rak, B. (1990) J. Biol. Chem. 265, 13464–13471.
[29] Meijer, A.H., Cardoso, M.I.L., Voskuilen, J.T., De Waal, A., Verpoorte, R. and Hoge, J.H.C. (1993) Plant J. 4, 47–60.
[30] Schröder, G. and Schröder, J. (1995) Eur. J. Biochem. 228, 74–78.
[31] Maina, C.V., Riggs, P.D., Grandea, A.G.I., Slatko, B.E., Moran, L.S., Tagliamonte, J.A., McReynolds, L.A. and Guan, C. (1988) Gene 74, 365–373.
[32] Lanz, T., Schröder, G. and Schröder, J. (1990) Planta 181, 169–175.
[33] Sakaki, T., Shibata, M., Yabusaki, Y., Murakami, H. and Ohkawa, H. (1990) DNA Cell Biol. 9, 603–614.
[34] Shibata, M., Sakaki, T., Yabusaki, Y., Murakami, H. and Ohkawa, H. (1990) DNA Cell Biol. 9, 27–36.
[35] Gillam, E.M.J., Guo, Z., Marin, M.V., Jenkins, C.M. and Guengerich, F.P. (1995) Arch. Biochem. Biophys. 319, 540–550.
[36] Bozak, K.R., O'Keefe, D.P. and Christoffersen, R.E. (1992) Plant Physiol. 100, 1976–1981.