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Regulation and Functional Expression of Cinnamate 4-Hydroxylase from Parsley

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A previously isolated parsley (*Petroselinum crispum*) cDNA with high sequence similarity to cinnamate 4-hydroxylase (C4H) cDNAs from several plant sources was expressed in yeast (*Saccharomyces cerevisiae*) containing a plant NADPH:cytochrome P450 oxidoreductase and verified as encoding a functional C4H (CYP73A10). Low genomic complexity and the occurrence of a single type of cDNA suggest the existence of only one C4H gene in parsley. The encoded mRNA and protein, in contrast to those of a functionally related NADPH:cytochrome P450 oxidoreductase, were strictly coregulated with phenylalanine ammonia-lyase mRNA and protein, respectively, as demonstrated by coinduction under various conditions and colocalization in situ in cross-sections from several different parsley tissues. These results support the hypothesis that the genes encoding the core reactions of phenylpropanoid metabolism form a tight regulatory unit.

C4H (EC 1.14.13.11) constitutes the CYP73 family of the large group of Cyt P450 monooxygenases (Teutsch et al., 1993). It catalyzes the 4-hydroxylation of *trans*-cinnamate, the central step in the generation of Phe-derived substrates for the many branches of phenylpropanoid metabolism (Russell, 1971). The first and the last enzymes of this short sequence of closely related reactions, termed the general phenylpropanoid metabolism, are PAL (EC 4.3.1.5) and 4CL (EC 6.2.1.12), respectively (Hahlbrock and Scheel, 1989). A second metabolic link couples C4H to the membrane-localized CPR (EC 1.6.2.4; Durst and O'Keefe, 1995; Schuler, 1996). The resulting pivotal role of C4H at the interface between cytosolic phenylpropanoid pathways and membrane-localized electron-transfer reactions is schematically illustrated in Figure 1.

The expression patterns of all three C4H-linked enzymes, PAL, 4CL, and CPR, and of the corresponding mRNAs have recently been analyzed in cell-suspension cultures and various intact tissues of parsley (*Petroselinum crispum* L.; Logemann et al., 1995; Reinold and Hahlbrock, 1996, 1997; Koopmann and Hahlbrock, 1997; Batz et al., 1998) and Arabidopsis (Bell-Lelong et al., 1997; Mizutani et al., 1997; Mizutani and Ohta, 1998). In our studies with parsley C4H was included to the extent that was possible, using a tentatively identified cDNA probe (Logemann et al., 1995; Koopmann and Hahlbrock, 1997; Batz et al., 1998). However, a thorough comparison required more detailed information concerning the genomic complexity of C4H in this plant, as well as the unequivocal verification of its biochemical function.

All previous results, although preliminary with respect to C4H, indicated a remarkable degree of coordination in the regulation of PAL, C4H, and 4CL at both the mRNA and protein levels (Logemann et al., 1995; Reinold and Hahlbrock, 1996, 1997). By contrast, CPR and C4H appeared to be less tightly coregulated (Koopmann and Hahlbrock, 1997), despite the essential requirement of C4H for CPR activity. Thus, the question arose as to whether C4H formed a closer regulatory unit with PAL and 4CL than with CPR, and whether it was as strictly coregulated with PAL and 4CL, as previously demonstrated for the latter two under a large variety of conditions. Here we show that this is the case, although each of the three coregulated enzymes appears to be encoded by a different number of genes.

MATERIALS AND METHODS

C4H Expression in Yeast

Primers for amplification of the C4H-coding sequence and introduction of the *Eco*RI and *Bam*HI restriction sites were purchased from MWG Biotech (Ebersberg, Germany). PCR was performed using 100 ng of the template and 60 pmol of the primer in the following cycles: one reaction cycle for 5 min at 95°C and 30 cycles for 1 min at 95°C, 1 min at 50°C, and 2.5 min at 72°C. The product was sequenced and the *Eco*RI/*Bam*HI fragment was cloned into the expression vector pYeDP60. The yeast (*Saccharomyces cerevisiae*) strains W(R) and W(At11) were transformed, the yeast cultures grown, and the microsomes prepared as described by Urban et al. (1994).

C4H Antiserum and Immunotitration

Primers for amplification of the C4H-coding sequence and introduction of the *Nco*I and *Bam*HI restriction sites were from MWG Biotech and PCR was performed as described above. The PCR product was cloned into the expression vector pQE60 and the construct was used for transformation of *Escherichia coli* strain SG13009 containing

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Abbreviations: C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CPR, NADPH:Cyt P450 oxidoreductase; PAL, Phe ammonia-lyase.

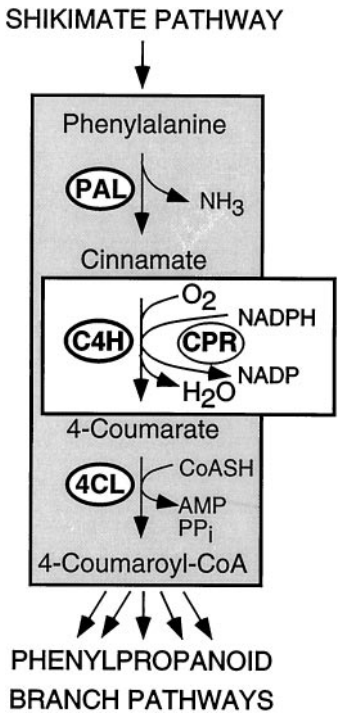

Figure 1. Schematic diagram of the pivotal role of C4H as a functional link between the cytosolic enzymes of general phenylpropanoid metabolism, PAL and 4CL, and the membrane-associated electron-transfer reactions catalyzed by CPR.

the plasmid pUBS520. For a 100-mL culture grown at 37°C, an overnight culture of transformed cells was taken as inoculum (1:50). At an A_{600} of 0.8, 1 mM isopropylthiogalactopyranoside was added and the culture was grown for another 2 h.

Cells were harvested by centrifugation (2,500*g* for 10 min at 4°C). The pellet containing the recombinant protein in inclusion bodies was resuspended in 1 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 35% [w/v] Suc, and 1 mM EDTA), 200 μL of lysozyme (10 mg/mL lysis buffer) was added, and the solution was incubated for 30 min on ice. After 52 μL of DNase I (1 mg/mL lysis buffer), 20 mM $MgCl_2$, and 2 mM $MnCl_2$ were added, the solution was again incubated for 30 min on ice and then vigorously mixed with 4 to 6 mL of detergent buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% [w/v] desoxycholate, 1% [w/v] Nonidet P40, 2 mM EDTA, and 10 mM mercaptoethanol) and centrifuged (15,000*g* for 10 min at 4°C). The pellet was washed several times with Triton buffer (20 mM Tris-HCl, pH 7.5, 0.5% [v/v] Triton X-100, 1 mM EDTA, and 10 mM mercaptoethanol) and centrifuged as above until the pellet appeared white.

To remove the detergent, the pellet was washed in Tris buffer (50 mM Tris-HCl, pH 7.5, and 10 mM mercaptoethanol), centrifuged, dissolved again in Tris buffer, frozen in liquid nitrogen, and stored at −80°C until use. This preparation of purified inclusion bodies was dissolved in buffer (8 M urea, 0.1 M sodium phosphate, and 10 mM Tris-HCl, pH 8.0), and the protein was purified further on a Ni-nitrilotriacetic acid column (Qiagen, Düsseldorf, Germany) according to the manufacturer's protocol. The mixture was then injected into a rabbit (4 × 60 μg) following the immunization procedure of Eurogentec (Seraing, Belgium). The final blood collection after 12 weeks was used as the C4H antiserum.

For immunoinhibition of C4H activity, 11 μg of microsomal protein from cultured parsley cells that had been treated for 15 h with fungal elicitor (Ayers et al., 1976; Kombrink and Hahlbrock, 1986) was incubated with increasing amounts of purified IgGs from the C4H antiserum or from the preimmune serum.

C4H Activity Assay

Microsomes from cultured parsley cells were prepared and C4H activity was determined as described by Vetter et al. (1992). The assay mixture contained 0.5 to 15 μg of microsomal protein from parsley or yeast cells, 50 mM potassium phosphate, pH 7.0, 2 mM DTT, 10 mM Glc-6-P, 0.5 unit of Glc-6-P dehydrogenase, and 20.8 μM [3-^{14}C]cinnamate (53.6 mCi/mmol, Isotopchim, Ganagobie, France) in a total volume of 50 μL. The reaction was started by adding 0.5 mM NADPH and stopped with 5 μL of 4 N HCl after incubation for 7.5 min at 30°C. Tracer (1 μL of an ethanolic solution containing 5 mg/mL each of cinnamate and 4-coumarate) was added, and the mixture was extracted twice with 100 μL of ethylacetate. The organic phase was evaporated, the residue dissolved in 10 μL of ethylacetate and spotted on a silica F60 TLC plate (Merck, Darmstadt, Germany), and the chromatogram developed in diethylether:petrol ether (30°C–50°C):formic acid (70:30:1, v/v). Radioactivity was visualized and quantified using a phosphor imager (Molecular Dynamics, Krefeld, Germany).

In Situ mRNA and Protein Localization

All methods used for plant propagation, including inoculation of leaf buds with zoospores of *Phytophthora sojae*, tissue sectioning and fixation, probe generation, in situ RNA/RNA hybridization, immunolocalization of proteins, and the PAL probes used, have recently been described (Reinold and Hahlbrock, 1996, 1997). Sense and antisense C4H riboprobes were generated using the *Sna*BI/*Spe*I cDNA fragment cloned into the pBluescript vector.

Analytical Methods

Proteins were separated by SDS-PAGE (Laemmli, 1970) and transferred onto PVDF membranes (Millipore) according to the method of Towbin et al. (1979). C4H and PAL antisera were used at a dilution of 1:1000 in 5% (w/v) dry milk powder in TBS.

RESULTS

Functional Identification

Several characteristic features, including >80% sequence identity with putative or functionally identified C4H pro-

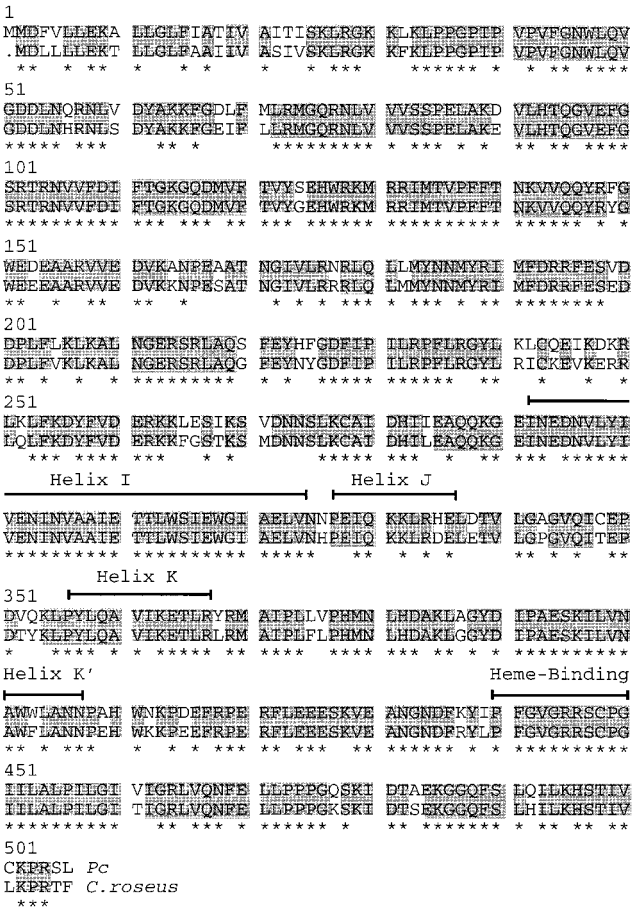

Figure 2. Comparison of deduced amino acid sequences for C4H from parsley (*Pc*) and a previously reported C4H from periwinkle (Hotze et al., 1995). Identical amino acids are marked in gray and putative helical and heme-binding domains (Durst and Nelson, 1995) are indicated by brackets. Asterisks highlight sequence identity among all functionally established C4H. The GAP program from the Genetics Computer Group package (Madison, WI) was used with the gap-weight penalty set at 3.0 and the gap-length penalty set at 0.1.

teins from other plants, were previously used as a basis for the tentative assignment of function to a parsley cDNA obtained by heterologous screening (Logemann et al., 1995). Among the most striking features (Fig. 2) is the combined occurrence of a putative ER membrane anchor at the N terminus (Nelson and Strobel, 1988), a Pro-rich region that may be responsible for correctly orienting the protein in the membrane (Szczesna-Skorupa et al., 1993; Yamazaki et al., 1993), the helical domains I, J, K, and K′, and the heme-binding motif PFGXGRRXCXG near the C terminus (Durst and Nelson, 1995).

To verify the deduced function of the encoded protein, we expressed the cDNA in two different yeast strains. One strain, W(R), contained the endogenous *CPR* gene under the control of a Gal-inducible promotor. The other strain, W(At11), used the same promotor, but the endogenous *CPR* gene was replaced by the *CPR* gene *ATR1* from Arabidopsis (Urban et al., 1997). Both strains, when transformed with the expression vector pYeDP60 containing the parsley cDNA, exhibited high C4H activity upon CPR induction by Gal. The specific C4H activity in microsomal fractions from these strains (1 μkat/mg protein) was 50-fold higher than that obtained with analogous preparations from elicitor-treated parsley cells (20 nkat/mg protein), probably at least in part because of the high induced level of CPR activity (approximately 5-fold higher in Gal-stimulated yeast cells than in elicitor-stimulated parsley cells; Koopmann and Hahlbrock, 1997). Yeast cells transformed with the vector alone showed no detectable C4H activity.

HPLC analysis confirmed the exclusive formation of 4-coumarate from cinnamate using microsomal fractions from the transformed yeast cells. Neither 2- nor 3-coumarate was detectable. An apparent K_m value of 5 μM for cinnamate was in agreement with similar values reported for C4H from other plant sources (Gabriac et al., 1991; Mizutani et al., 1993) or from transformed yeast (Urban et al., 1994). The pH optimum was found to be slightly lower (7.0) than usual (7.5). The preference of parsley C4H for the *trans*-isomer of cinnamic acid has previously been demonstrated (Pfändler et al., 1977). An antiserum raised against the *E. coli*-expressed and purified C4H protein (Koopmann and Hahlbrock, 1997) inhibited the C4H activity of parsley microsomes by 70%, in contrast to the completely ineffective preimmune serum (data not shown).

Genomic Complexity and Definition of Probes

Similar to the results reported for Arabidopsis (Bell-Lelong et al., 1997; Mizutani et al., 1997) and pea (Frank et al., 1996), gel-blot analysis with genomic parsley DNA and 3′ or 5′ fragments from the C4H cDNA for hybridization gave very simple patterns. Because complete genomic clones were difficult to obtain and were thus not available for direct comparison of these patterns with defined fragments, we analyzed further the cDNA library from which the original clone had been isolated. The 18 additional cDNA clones hybridizing under moderately stringent conditions gave the same restriction fragmentation patterns, except for differences in length, and 12 were at least partially sequenced and found to be identical to the original cDNA by this criterion.

Although these results do not exclude with certainty the presence of an additional C4H gene(s) in parsley, they strongly suggest the existence of only one gene, in contrast to the existence and expression of four PAL genes (Logemann et al., 1995), two 4CL genes (Douglas et al., 1987), and at least two CPR genes (Koopmann and Hahlbrock, 1997). For the following comparative studies, a PAL-1 cDNA (Logemann et al., 1995) and a PAL-1 antiserum (Appert et al., 1994) were used, both of which detected all four PAL isoforms. For C4H mRNA analysis, a hybridization probe lacking the putative N-terminal membrane anchor and the highly conserved heme-binding domain was used to prevent cross-hybridization with mRNAs encoding other P450 proteins. The C4H antiserum has been described elsewhere (Koopmann and Hahlbrock, 1997).

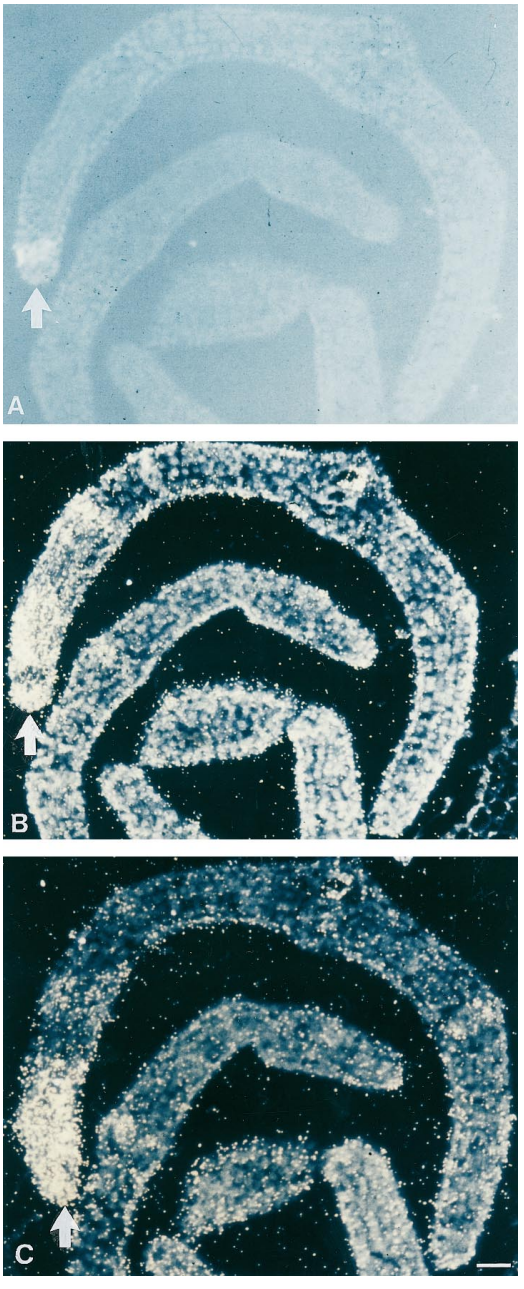

Figure 3. In situ localization of C4H and PAL mRNAs in parsley leaf buds 6 h postinoculation with *P. sojae*. The fungal infection site (A, arrow) was visualized by autofluorescence of the developing necrotic spot under UV/blue light of 365 nm. The same and an adjacent cross-section were hybridized with ^{35}S-labeled C4H (B) and PAL (C) antisense riboprobes, respectively. Magnification bar = 100 μm.

mRNA Accumulation upon Fungal Infection

Rapid and transient accumulation of C4H mRNA in response to treatments of leaves or cultured cells of parsley with various external stimuli, including fungal elicitor, UV-containing white light, or wounding, was previously reported (Logemann et al., 1995). However, the cDNA probe used in those studies proved unsuitable for in situ mRNA localization under the stringency conditions required for hybridization in fixed tissue slices. By using the probe that lacked frequently occurring domain structures, we were able to apply this technique and used it first to test the induction of C4H mRNA by fungal attack in cross-sections of parsley leaf buds. Figure 3, A and B, demonstrates the strong accumulation of C4H mRNA around a fungal infection site, with spatial distribution patterns identical to those of PAL mRNA (Fig. 3C).

Attempts to immunolocalize the encoded C4H protein in parallel tissue sections were unsuccessful, probably because of the high preexisting level in uninfected tissue. Similar, although surmountable, difficulties had previously been encountered with PAL and 4CL protein accumulation around infection sites (Reinold and Hahlbrock, 1996).

Cell-Type-Specific Expression Patterns

When protein extracts from three selected organs of parsley plants (flower, leaf, and pedicel) at various developmental stages were used, immunoblot analysis indicated similar overall expression patterns for C4H and PAL (Fig. 4). Both were strongly expressed in the pedicel and at all flower stages analyzed, whereas young and old leaves contained relatively small amounts of PAL protein, and C4H protein was not detectable under these conditions. Based on these and recently reported results of the tissue localization of PAL and 4CL (Reinold and Hahlbrock, 1997), we decided to use the pedicel and two different flower stages for in situ C4H mRNA and protein localization. Again, PAL was used as a reference.

The results revealed three major features of C4H expression (Fig. 5). First, C4H mRNA and protein occurred fairly ubiquitously throughout the tissue areas analyzed, although with distinct patterns. Second, among the most

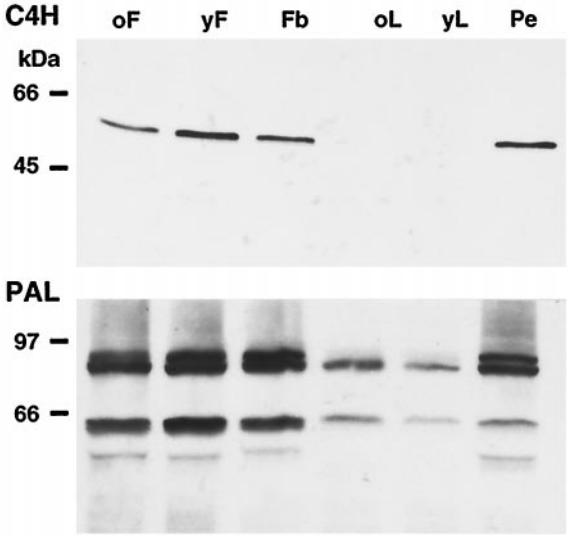

Figure 4. Comparison of total extractable C4H and PAL levels in various aerial parts of parsley plants. Protein blots on PVDF membranes (Millipore) after SDS-PAGE (10 μg of protein each, 10% polyacrylamide) were treated with C4H or PAL antiserum at a dilution of 1:1000. oF, Old flower; yF, young flower; Fb, flower bud; oL, old leaf; yL, young leaf; and Pe, pedicel.

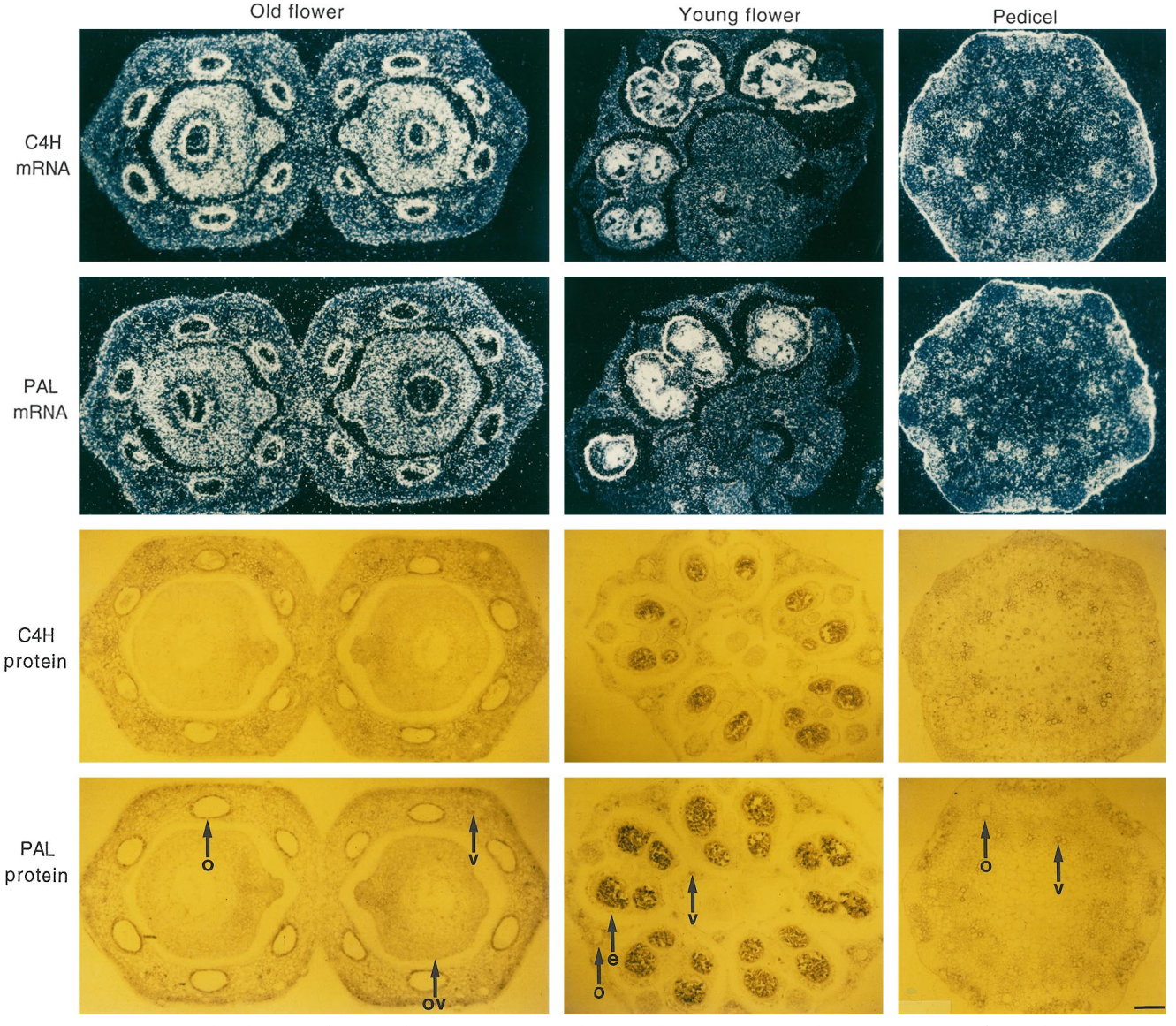

Figure 5. In situ localization of C4H and PAL mRNA (top) and protein (bottom), respectively, in cross-sections from selected parsley tissues as indicated. ^{35}S-Labeled antisense riboprobes and antisera were the same as described in Figures 3 and 4, respectively. e, Epidermis; o, oil-duct epithelial cells; ov, ovary; and v, vascular tissue. Magnification bar = 100 μm.

prominent accumulation sites were those known for high phenylpropanoid biosynthetic activity: epidermal and oil-duct epithelial cells, vascular tissue, and major parts of the ovule (Reinold and Hahlbrock, 1997). Third, the mRNA and protein-distribution patterns were identical within experimental error for C4H and PAL. Control experiments with sense RNA or preimmunserum as the probes gave low background levels in all cases, similar to those observed for PAL and 4CL under the same conditions as used here (Reinold and Hahlbrock, 1997).

In analogous experiments, C4H was also compared with CPR1, the CPR isoform with an apparent close metabolic relationship to C4H (Koopmann and Hahlbrock, 1997). However, CPR1 mRNA and protein showed much lower cell-type specificity than C4H in various tissues from adult parsley plants (data not shown), in contrast to the previously reported, massive accumulation of both C4H and CPR1 mRNAs in the same tissue area at fungal infection sites in primary leaf buds (Koopmann and Hahlbrock, 1997).

DISCUSSION

These results have clarified three related points: (a) the previous, tentative identification of the parsley C4H cDNA by sequence comparison was confirmed by functional data in vitro; (b) in contrast to all three associated enzymes (PAL, 4CL, and CPR), C4H appears to be encoded by a single gene; and (c) expression of this gene was regulated in tight coordination with the PAL gene family, strongly supporting its function in vivo as the predicted metabolic link between PAL and 4CL (Fig. 1).

Sequence identity of 80% or more with a functionally identified ortholog is often taken as a sufficiently strong criterion for the assignment of function to a newly isolated gene, cDNA, or protein. Although this has frequently proven to be a reliable approximation, several cases of considerable uncertainty exist. For instance, many of the numerous, structurally closely related proteins within the Cyt P450 family have widely differing functional properties, and even the exchange of only three amino acid residues can lead to a drastic change in substrate specificity (Lindberg and Negishi, 1989). At the opposite extreme, P450 proteins with only 26% sequence identity may catalyze the same biochemical reaction (Ma et al., 1994). Therefore, an important step toward the functional identification of the putative parsley C4H cDNA is the demonstration in vitro of its exclusive hydroxylation of cinnamate in the *para* position of the aromatic ring to give 4-coumarate.

A prerequisite for the second line of evidence supporting a specific role in phenylpropanoid metabolism was an analysis of the genomic complexity. Although an unequivocal determination of the number of genes encoding a particular enzyme in a given organism is very difficult to achieve, our results are taken as a strong indication that a single *C4H* gene encoded the mRNA and protein analyzed, in contrast, for example, to corn, in which at least three *C4H* genes seem to exist (Potter et al., 1995). This result enabled us to determine their cell-type-specific distribution patterns in vivo without interference from structurally similar gene products, the functional relatedness of which would have remained uncertain. However, we cannot definitely exclude the existence of functionally related but structurally dissimilar proteins. This latter possibility could be tested using only defined mutants. The fact that no null mutant for C4H has been reported may be taken as a hint of the functional uniqueness of this enzyme.

Both the C4H mRNA and the C4H protein occurred under all tested conditions in various parsley tissues in a highly coordinated fashion with PAL mRNA and protein. This high degree of coordination was observed not only here for the spatial distribution patterns (Figs. 4 and 5), but also in a previous study for the temporal patterns of accumulation (Logemann et al., 1995). Since analogous results have been obtained previously for PAL and 4CL (Logemann et al., 1995; Reinold and Hahlbrock, 1996, 1997), we conclude that all genes or gene families encoding the small set of three closely related enzymes of general phenylpropanoid metabolism (Fig. 1) form a tight regulatory unit. Surprisingly, this unit does not include the likewise closely related CPR1, whose mRNA accumulation patterns in parsley differ from those of C4H (Koopmann and Hahlbrock, 1997). CPR mRNA and protein were uniformly distributed throughout the tissue and appear to be more generally involved in various metabolic activities than is observed for C4H, with its apparent specific involvement in general phenylpropanoid metabolism.

These results strengthen further the hypothesis (Batz et al., 1998) that the genes or gene families encoding PAL, C4H, and 4CL, despite their different sizes, constitute an exceptional case of tightly linked regulation, possibly mediated through an unusually large structural and functional similarity of their promotors (Hahlbrock et al., 1995; Logemann et al., 1995; Bell-Lelong et al., 1997; Mizutani et al., 1997). This tight regulatory link at the gene expression level, coupled with large similarities in the apparent mRNA and protein turnover rates (Hahlbrock et al., 1976, 1981; Logemann et al., 1995), may indicate a likewise tight association at the enzyme activity level, perhaps including the formation of a true multienzyme complex. Experimental results that point in this direction come not only from previous studies (Czichi and Kindl, 1977; Stafford, 1981; Hrazdina and Wagner, 1985) but also from recent experiments in our own laboratory (E. Koopmann, unpublished results). In such a complex, C4H could serve as both a structural and metabolic anchor of cytosolic activities to the ER.

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