Functional Characterization and Subcellular Localization of Poplar (*Populus trichocarpa* × *Populus deltoides*) Cinnamate 4-Hydroxylase[1]

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Cinnamic acid 4-hydroxylase (C4H), a member of the cytochrome P450 monooxygenase superfamily, plays a central role in phenylpropanoid metabolism and lignin biosynthesis and possibly anchors a phenylpropanoid enzyme complex to the endoplasmic reticulum (ER). A full-length cDNA encoding C4H was isolated from a hybrid poplar (*Populus trichocarpa* × *P. deltoides*) young leaf cDNA library. RNA-blot analysis detected *C4H* transcripts in all organs tested, but the gene was most highly expressed in developing xylem. *C4H* expression was also strongly induced by elicitor-treatment in poplar cell cultures. To verify the catalytic activity of the putative C4H cDNA, two constructs, C4H and C4H fused to the FLAG epitope (C4H::FLAG), were expressed in yeast. Immunoblot analysis showed that C4H was present in the microsomal fraction and microsomal preparations from strains expressing both enzymes efficiently converted cinnamic acid to *p*-coumaric acid with high specific activities. To investigate the subcellular localization of C4H in vivo, a chimeric C4H-green fluorescent protein (GFP) gene was engineered and stably expressed in Arabidopsis. Confocal laser microscopy analysis clearly showed that in Arabidopsis the C4H::GFP chimeric enzyme was localized to the ER. When expressed in yeast, the C4H::GFP fusion enzyme was also active but displayed significantly lower specific activity than either C4H or C4H::FLAG in in vitro and in vivo enzyme assays. These data definitively show that C4H is localized to the ER in planta.

In plants, cytochrome P450 monooxygenases (P450s) are involved in the biosynthesis of extremely diverse metabolites (e.g. fatty acids, phenylpropanoids, alkaloids, and terpenoids) and in the processes of herbicide or pesticide detoxification (for review, see Chapple, 1998). More than 400 P450 genes from various plants are available in the genome databases, but the true complexity of this protein superfamily is better reflected by the presence of approximately 270 named P450 genes, falling into 45 distinct P450 families that have so far been identified in the Arabidopsis genome (http://drnelson. utmem. edu/CytochromeP450.html). An inter-kingdom P450 phylogenetic analysis derived from available sequence information indicates that all of the plant P450s are likely to have evolved from a common ancestral gene (Nelson, 1999). Thus, the present diversity of P450s in plants may reflect the rapid molecular evolution of P450s driven by biochemical demands for coevolution with herbivores and pathogens and for adaptation to environmental factors.

Among plant P450s, the CYP73A group, cinnamate 4-hydroxylase (C4H), has been most extensively studied. C4H catalyzes the first oxidative reaction in phenylpropanoid metabolism, the conversion of trans-cinnamic acid to *p*-coumaric acid. This reaction consumes molecular oxygen and a reducing equivalent from NADPH delivered via cytochrome P450 reductase (CPR). In conjunction with two other key enzymes of the core phenylpropanoid pathway, Phe ammonia-lyase (PAL) and 4-coumarate:coenzyme A ligase (4CL), C4H directs carbon flux to an array of important phenolic compounds in plants including lignin, suberin, flavonoids, and numerous other phenylpropanoids (Chapple, 1998). Due to the labile and membrane-bound nature of P450 proteins, cloning of *C4H* genes by protein purification followed by immuno-screening of cDNA libraries has been difficult. However, isolation of the first C4H cDNA from Jerusalem artichoke (Teutsch et al., 1993) enabled heterologous screening approaches, and, to date, at least 20 orthologues of *C4H* have been cloned from different plant sources. These generally share a high degree of identity in their deduced amino acid sequence (>85%) with the exception of two divergent isoforms from maize and French bean, which show only approximately 60% identity to other *C4H* genes (Potter et al., 1995; Nedelkina et al., 1999). The catalytic identity of several cloned C4H cDNAs has been confirmed by heterologous expression in yeast (Urban et al., 1994; Koopmann et al., 1999), in insect cells (Mizutani et al., 1997), or in *Escherichia coli* as a CPR-fusion protein (Hotze et al., 1995).

Regulation of *C4H* expression has been investigated in various plants and cell-culture systems.

[1] This work was supported by the Natural Science and Engineering Research Council of Canada (to C.J.D.) and by a University Graduate Fellowship from the University of British Columbia (to D.K.R.).

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Transcriptional regulation seems to be a major mechanism for control of *C4H* expression during development and in response to external stimuli as it is for *PAL* and *4CL*. Rapid up-regulation of *C4H* expression by light, wounding, elicitors, and pathogen infection has been documented in many plants (Chapple, 1998). Developmentally regulated *C4H* expression in parsley is correlated with lignification and other sites of active phenylpropanoid metabolism (Koopmann et al., 1999), and the Arabidopsis *C4H* promoter has been shown to specify a pattern of temporal and spatial gene expression correlated with lignification of bolting stems (Bell-Lelong et al., 1997). Since *C4H* promoter regions share common cis-elements with those of *PAL* and *4CL* (Logemann et al., 1995; Bell-Lelong et al., 1997; Mizutani et al., 1997), it is generally assumed that *C4H* is under similar regulatory control. This would be consistent with reports of tissue- and cell-type specific colocalization of PAL, C4H, and 4CL mRNA and protein (Koopmann et al., 1999). By extension of this concept, it has been hypothesized that these enzymes may be physically associated with each other in organized multienzyme complexes (MECs). Metabolite channeling from L-Phe to *p*-coumarate has been detected in microsomes from cucumber (Czichi and Kindl, 1977) and in cultured tobacco cells and tobacco stem tissue (Rasmussen and Dixon, 1999), as predicted by the MEC model. According to this model, C4H serves as a structural scaffold, anchoring the enzyme-complex on the endoplasmic reticulum (ER; Winkel-Shirley, 1999). Considering the potential importance of MECs for the compartmentalization and regulation of phenylpropanoid metabolism in plants, it is essential that the subcellular fate of C4H in plant cells be established.

While plant P450s, like most animal P450s, are generally considered to be localized to the ER, there are also reports that some plant P450s are localized to the plasmamembrane (Kjellbom et al., 1985) or the provacuole (Madyastha et al., 1977). As well, the N-terminal sequences from CYP74 and CYP79B2/3 most closely resemble chloroplast targeting transit peptides (Song et al., 1993; Hull et al., 2000). Thus, the subcellular locations of plant P450s appear to vary with the individual protein. In the case of C4H, subcellular fractionation of pea seedlings suggested that the enzyme was ER-localized (Benveniste et al., 1978), whereas subcellular fractionation of sweet potato roots found most of the C4H in unidentified organelles, perhaps provacuoles (Fujita and Asahi, 1985). In French bean, immunolocalization placed C4H in both the Golgi apparatus and the ER (Smith et al., 1994).

Invasive sample preparation, cross-reactivity of antibodies, and contamination between subcellular fractions may have confounded these studies and made it difficult to accurately determine the site of protein localization by these approaches. However, with the recent development of green fluorescent protein (GFP) tagging methods (for review, see Köhler, 1998), it is now possible to track the location of specific proteins within living cells.

In woody plants, C4H is particularly important for the biosynthesis of lignin, the second most abundant biopolymer after cellulose, and C4H is likely to play a key role in the ability of phenylpropanoid metabolism to channel carbon from primary metabolism into the biosynthesis of lignin and other polymers in trees. *Populus* species (poplars, cottonwoods, and aspens) provide models for molecular and genetic studies of tree biology because of their small genomes, ease of vegetative propagation, transformation systems, and genetic resources (Sterky et al., 1998, and references therein), and an expressed sequence tag (EST) genome project has been initiated in *Populus* (Sterky et al., 1998). Phenylpropanoid genes encoding *PAL* and *4CL* have been cloned and characterized from several *Populus* species (Subramaniam et al., 1993; Osakabe et al., 1995; Allina et al., 1998; Hu et al., 1998). As well, *C4H* sequences have been reported from two *Populus* species (Ge and Chiang, 1996; Kawai et al., 1996), and *C4H* is reported to be encoded by a small gene family in *Populus kitakamiensis* (Kawai et al., 1996). To better understand the catalytic and structural role of C4H in woody plants, we isolated a C4H cDNA from a *P. trichocarpa* × *P. deltoides* hybrid, profiled its expression relative to other phenylpropanoid genes, and demonstrated its catalytic activity by expression in yeast. Using a C4H::GFP fusion, we show for the first time that C4H is predominantly localized to ER in planta, consistent with its postulated role in anchoring phenylpropanoid enzyme MECs to the ER.

RESULTS

Isolation and Characterization of a Poplar C4H cDNA

Use of a heterologous C4H probe enabled us to retrieve a number of putative C4H cDNA clones from a hybrid poplar cDNA library. Sequence analysis showed that one cDNA, C4H-550 (GenBank accession no. AF302495), contained the complete coding sequence for a putative C4H protein. Comparison of this clone to two additional partially sequenced clones across their 5′ coding and 3′-untranslated regions revealed that each clone had a unique sequence. At the nucleotide level, the coding regions showed approximately 90% identity, whereas the 3′-untranslated regions showed approximately 80% to 90% identity to each other. Most of the differences occurred at the third nucleotide position of codons in the coding regions, and as a result, these clones were >96% identical in their deduced amino acid sequences (data not shown). This heterogeneity could be due either to allelic variation between *C4H* genes from two parental genomes in the H11 hybrid or to

the presence of multiple highly similar *C4H* gene family members.

The complete sequence of C4H-550 revealed an open reading frame encoding a 505-amino acid protein with a predicted molecular mass of 58 kD and a pI of 9.1. The predicted C4H-550 amino acid sequence is 85% to 91% identical to C4H sequences from other angiosperm species (Fig. 1) and is 98% and 99% identical to the predicted amino acid sequences of C4H from *P. tremuloides* (GenBank accession no. U47293; Ge and Chiang, 1996) and *P. kitakamiensis* (SWISS-PROT accession no. Q43054; Kawai et al., 1996), respectively. All diagnostic features of the primary structure for cytochrome P450 enzymes are present in the deduced protein sequence, including all four conserved domains (A to D domains in Fig. 1) identified in other eukaryotic P450s, and the heme-binding motif (FXXGXXXCXG) in the C-terminal region (Kalb and Loper, 1988). An N-terminal hydrophobic domain consisting of 21 amino acids is flanked by an acidic residue (Asp) and several basic residues. Similar primary structure has been shown to function as a signal-anchor sequence and to determine the correct orientation of P450s in the ER (Sakaguchi et al., 1992). Following the N-terminal anchoring sequence, a conserved PPGP tetrapeptide or Pro-rich region is present, which is essential for proper heme-incorporation and P450 protein stability (Szczesna-Skorupa et al., 1993).

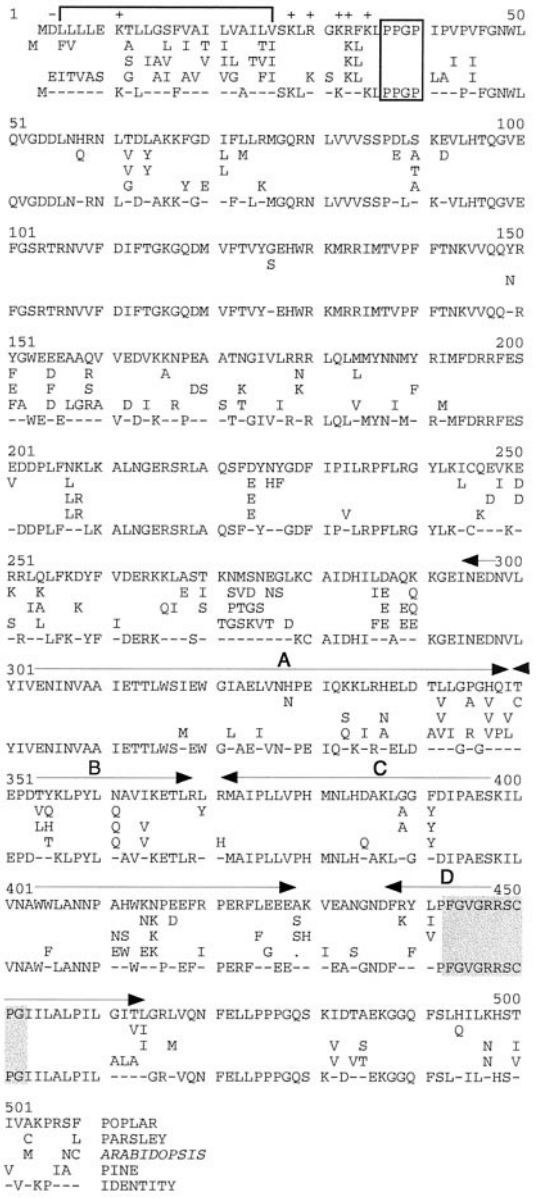

Figure 1. Deduced amino acid sequence of the poplar C4H-550 cDNA and comparison to other plant C4H amino acid sequences. The full amino acid sequence of poplar C4H-550 is given, and only the amino acids that differ from those in poplar are shown in the parsley (Q43033), Arabidopsis (AAB58355), and pine (AAD23378) sequences. The PILEUP and PRETTY options from Wisconsin Package (version 9.1, Genetics Computer Group, Madison, WI) were used to align and process the sequences. Charged amino acids conserved in the N terminus are indicated by + or − signs. The Pro-rich and heme-binding domains are highlighted by a black-lined box and a gray box, respectively. The membrane-anchoring region is indicated by black brackets. The four conserved domains typical of eukaryotic P450s (Kalb and Loper, 1988) are indicated by arrow lines labeled A to D.

C4H Expression in Poplar

Steady-state C4H mRNA accumulation patterns in various organs and in cell cultures were examined by northern-blot analysis, in parallel with the expression patterns of poplar *PAL* and *CHS* genes (Fig. 2), which are often expressed coordinately with *C4H*. *C4H* transcripts were detected in all organs examined, but their levels were highest in the xylem. C4H mRNA was accumulated more abundantly in young leaves than old leaves. Consistent with previous observations (Subramaniam et al., 1993; Gray-Mitsumune et al., 1999), H11 *PAL1/2* transcripts were barely detectable in xylem but were highly abundant in young leaf tissue. On the other hand, a probe specific to H11 *PAL3*, which is over 90% identical to the *P. kitakamiensis gpal2a* and *gpal2b* genes that are expressed in woody stems (Osakabe et al., 1995), detected *PAL* transcripts in all tissues, including secondary xylem. *CHS* transcripts were abundant only in young leaves and green stem. None of these genes was expressed in untreated poplar cell cultures, but treatment with a fungal elicitor induced high level of all transcripts except *CHS*. Thus, the poplar *C4H* expression profile overlaps with that of other poplar phenylpropanoid genes and is consistent with the central role of C4H in phenylpropanoid metabolism.

C4H Expression in Yeast

Although many cytochrome P450-encoding sequences have been found through genome and EST projects, it is often difficult to assign a catalytic func-

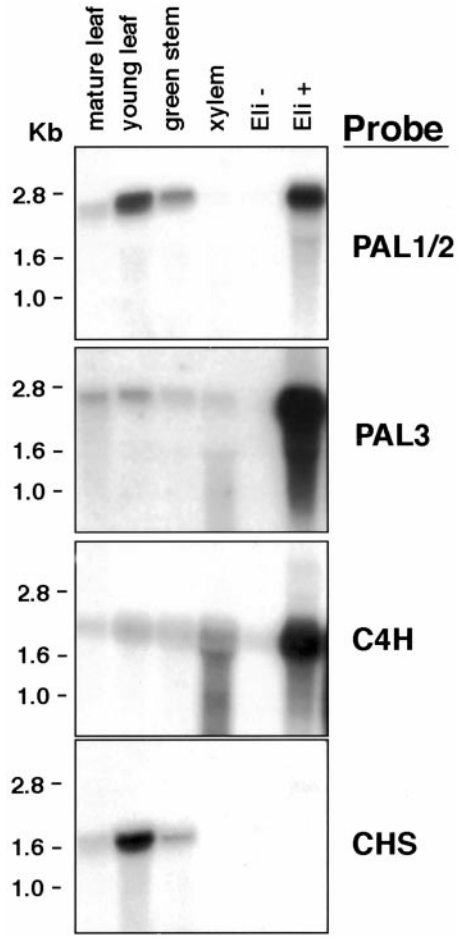

Figure 2. Northern-blot analysis of PAL, C4H, or CHS mRNA accumulation in organs and tissue culture cells. Total RNA (10 μg) was isolated from the organs indicated, or from cell cultures treated with (Eli+) or without (Eli−) an elicitor, separated on formaldehyde agarose gels, transferred to a nylon membrane, and hybridized to a poplar PAL, C4H, or CHS probes as described in "Materials and Methods."

tion to the cloned genes. Not only are the sequences generally closely related, but P450s are very plastic in their catalytic behavior, and their substrate use profiles can be changed by a single amino acid substitution (Lindberg and Negishi, 1989). Thus, expression in a heterologous host is an essential step in functionally identifying a newly cloned P450 gene. Our initial attempts to express the hybrid poplar C4H cDNA in insect cells using the baculovirus system resulted in >95% non-functional enzyme that was almost exclusively localized in the 10,000-g subcellular fraction. Only small amounts of weakly active enzyme were recovered from the microsomal fraction, although protein sequencing confirmed that the correct protein was being expressed (J. Norton, B. Ellis, unpublished data).

As an alternative, we expressed poplar C4H cDNA in a genetically modified *Saccharomyces cerevisiae* strain, WAT11, in which the yeast endogenous *CPR* gene was replaced by the Arabidopsis *CPR1* gene under the control of a GAL10-CYC1 hybrid promoter (Urban et al., 1997). Poplar *C4H* coding region was inserted behind the GAL10-CYC1 promoter in the pYeDP60 vector. To facilitate immunodetection of the expressed C4H protein, an epitope-tagged version of the poplar C4H was prepared by fusing the FLAG epitope to the C terminus of the C4H coding sequence. Coomassie Blue staining of SDS-PAGE fractionated microsomal proteins from yeast strains transformed with C4H or C4H::FLAG constructs revealed strong protein bands corresponding to the predicted positions, which were absent from microsomal preparations from vector-transformed yeast (data not shown). Immunoblot analysis using anti-FLAG antibodies confirmed the identity of the novel approximately 60-kD protein in the microsomal fraction of C4H::FLAG expressing strains as C4H (Fig. 3A).

Correct folding and heme-incorporation of the recombinant C4H and C4H::FLAG proteins was evaluated by use of reduced CO differential absorption spectroscopy (Omura and Sato, 1964). Microsomal proteins from yeast transformed with either the C4H

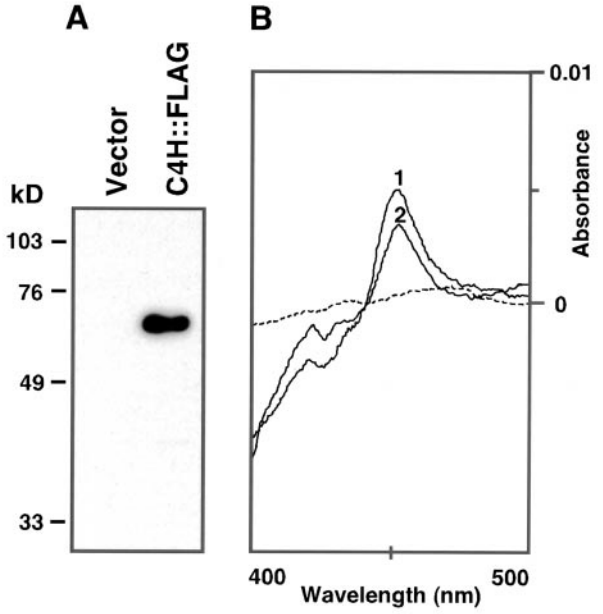

Figure 3. Immunoblot and carbon monoxide-induced differential absorption spectra analysis of microsomes from C4H::FLAG-transformed yeast. A, Immunoblot analysis of control (vector only) and experimental (C4H::FLAG-transformed) yeast strains. Microsomal protein preparations (2 μg) were separated by SDS-PAGE and transferred to a PVDF membrane. The blot was incubated with mouse monoclonal anti-FLAG antibody and signals detected by chemiluminescence. B, Carbon monoxide-induced differential absorption spectra recorded from reduced microsomes of C4H- or C4H::FLAG-transformed yeast. Microsomes from vector-transformed yeast yielded the spectrum shown by the dashed line, and spectrum 1 and 2 were recorded from C4H (0.46 mg/mL) and C4H::FLAG (0.30 mg/mL) containing microsomes, respectively.

or the C4H::FLAG construct showed characteristic absorption peaks at 450 nm after Gal induction (Fig. 3B). Based on the height of their 450 nm peaks, C4H and C4H::FLAG-expressing yeast routinely yielded from 100 to 400 pmol of P450 per milligram of microsomal protein.

The catalytic identity of the protein encoded by the C4H-550 cDNA was verified by enzyme assays using cinnamic acid as a substrate. Conversion of cinnamate to *p*-coumarate, detected by HPLC analysis, was efficiently catalyzed by microsomes from the yeast strains transformed with either C4H or C4H::FLAG construct (Table I). Vector-transformed control yeast strains produced no detectable *p*-coumarate. There was no significant difference between the specific activities of recombinant C4H and C4H::FLAG proteins. These activities are comparable with those previously reported for Arabidopsis C4H in the same yeast strain (Urban et al., 1997) and confirm that the poplar cDNA encodes an authentic, functional C4H enzyme.

Subcellular Localization of C4H

To explore the in planta subcellular location of the C4H encoded by the C4H-550 cDNA, we expressed a C4H::GFP fusion in Arabidopsis. An engineered red-shifted GFP (Davis and Vierstra, 1998), by itself or fused in frame to the C terminus of C4H, was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter and homozygous transgenic Arabidopsis lines were generated for each construct. Epidermal cells from living transgenic and control seedlings were examined by confocal laser scanning microscopy. Non-transformed Arabidopsis seedlings showed weak autofluorescence in hypocotyl epidermal cell walls, but no fluorescence was associated with other cell organelles (Fig. 4, A and D).

When GFP alone was expressed in transgenic Arabidopsis, fluorescence was observed throughout the cytoplasm, and cell organelles appeared as dark zones against this background (Fig. 4, B, E, and G). Seedlings transgenic for C4H::GFP, on the other hand, presented an entirely different pattern. In all six independent transgenic lines examined, epidermal cells displayed strong fluorescence that localized to a characteristic reticular network in both hypocotyl and cotyledon epidermal cells (Fig. 4, C and F).

Table I. *C4H activity in microsome preparations from C4H-, C4H::FLAG-, and C4H::GFP-expressing yeast strains*

Construct	Specific Activity[a]
	nmol p-coumarate min^{-1} nmol P450^{-1}
C4H	274 ± 56
C4H::FLAG	315 ± 22
C4H::GFP	148 ± 43

[a] Mean ± SD. Each value was calculated by three measurements each from two independent yeast transformants.

The fluorescent structures showed typical three-way junctions with approximately 120-degree angles between the branches, identical to the patterns generated by the ER marker DiOC6 in onion cells (Knebel et al., 1990). This pattern has also been observed in tobacco leaf epidermal cells expressing an ER-targeted chimeric GFP-KDEL fusion protein from a viral vector (Boevink et al., 1996). Optical sectioning through hypocotyl epidermal cells showed that most of the ER was peripherally compressed due to turgor pressure from the large central vacuole (data not shown). The congruence of the C4H::GFP fluorescence pattern with that expected for the ER suggests that C4H::GFP is in fact localized to the ER.

Dynamic unidirectional ER streaming was observed in cotyledon epidermal cells (Fig. 4F, arrow). No pattern of green fluorescence was detected that could correspond to other cell organelles such as mitochondria, the Golgi apparatus, or chloroplasts. Despite autofluorescence, fluorescence associated with the cell wall was negligible in comparison with that seen in the ER, and no fluorescence was observed in the apoplastic space. In addition to the reticulate ER pattern of fluorescence, a number of elongated fluorescent organelles were often observed in the hypocotyl epidermal cells and less frequently in the cotyledon epidermis (Fig. 4C, arrow heads). They were highly mobile when closely located to the streaming ER (data not shown) but otherwise static on the ER.

The uppermost optical sections of guard cells also presented typical ER-like patterns (Fig. 4H), but an unusual pattern of fluorescence was observed in inner sections (Fig. 4I). Here, fluorescence was detected around the nuclear envelope, which forms a continuous membrane system with the ER, and large uniformly fluorescent bodies were frequently observed. Absence of red fluorescence and 4′,6-diaminophenylindole staining clearly distinguished these bodies from chloroplasts and nuclei, respectively (data not shown), but their identity remains to be clarified.

C4H::GFP Fusion Gene Expression in Yeast

The C4H::GFP fusion provides a potentially powerful tool to investigate the physical organization of phenylpropanoid enzyme complexes in plant and yeast. As a prelude to such studies, we expressed the C4H::GFP construct in yeast to determine whether the fusion protein is folded correctly and therefore retains catalytic activity. In immunoblots of microsomal proteins from control or C4H::GFP expressing yeast strains probed with anti-GFP antibodies, a protein of the predicted size for C4H::GFP was expressed specifically in the C4H::GFP-expressing yeast strain (Fig. 5A). A microsomal protein preparation from a C4H::GFP-expressing yeast strain was used to record reduced CO differential spectra. Fig-

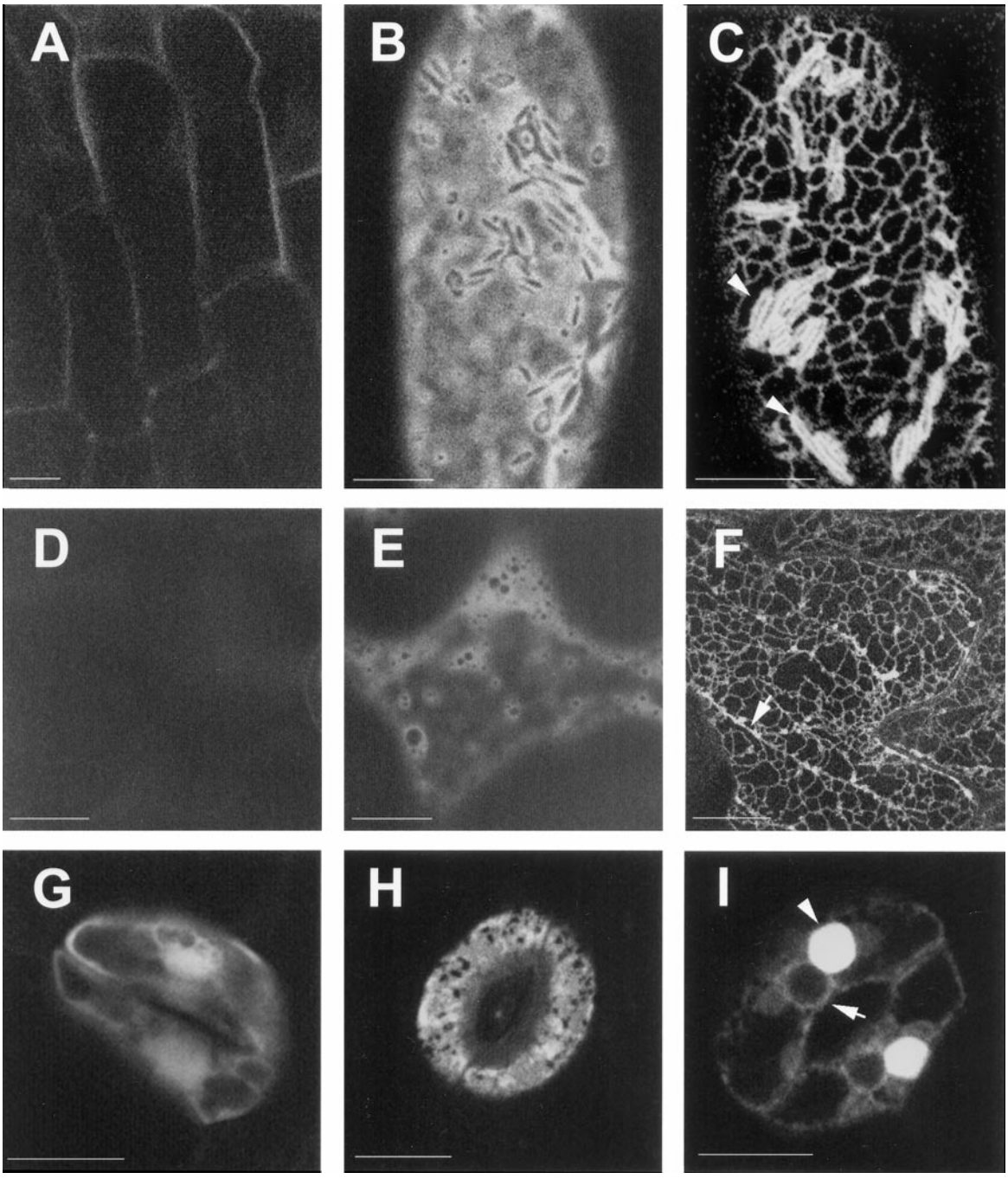

Figure 4. Subcellular localization of hybrid poplar C4H::GFP in transgenic Arabidopsis seedlings. A through C, Hypocotyl epidermal cells; D through F, cotyledon epidermal cells; G through I, guard cells from 5-d-old seedlings were examined by confocal microscopy. A and D, Non-transformed Arabidopsis; B, E, and G, 35S-GFP transformed Arabidopsis; C, F, and H, 35S-C4H::GFP transformed Arabidopsis. Arrowheads indicate dilated ER patterns in C, and a line of ER-streaming is shown by the arrow in F. I, Optical section of a guard cell from 35S-C4H::GFP transformed Arabidopsis showing fluorescence in an unidentified organelle; arrow indicates the perinuclear membrane, and the arrow-head indicates a strongly fluorescent organelle. Scale bar = 10 μm.

ure 5B shows that a typical P450 peak was observed after 20-h Gal induction but that it was accompanied by a peak at 420 nm, which has been attributed to denatured and inactivated forms of P450. By contrast, C4H or C4H::FLAG microsomal fractions prepared in parallel displayed only minor P420 peaks, indicating that the high P420 content in C4H::GFP was not caused by inappropriate microsomal preparation. Extended heterologous P450 expression has been reported to result in accumulation of P420 at the ex-

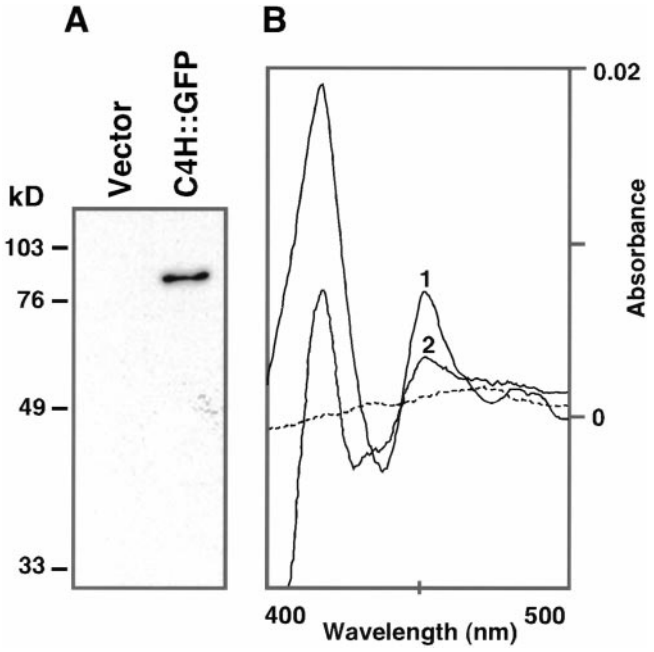

Figure 5. Immunoblot and carbon monoxide-induced differential absorption spectra analysis of microsomes from C4H::GFP-transformed yeast. A, Immunoblot analysis of control (vector only) and experimental (C4H::GFP-transformed) yeast strains. Microsomal protein preparations (2 μg) were separated by SDS-PAGE and transferred to a PVDF membrane. The blot was incubated with mouse monoclonal anti-GFP antibody, and signals detected by chemiluminescence. B, Carbon monoxide-induced differential absorption spectra recorded from reduced microsomes of C4H-GFP transformed yeast. Microsomes from 20 or 12 h Gal-induced C4H::GFP-transformed yeast, adjusted to a final assay concentration of 1.0 or 1.1 mg/mL, were used to record spectrum 1 or 2, respectively. The spectrum for microsomes from vector transformed yeast is shown by the dashed line.

pense of the P450 (Chen et al., 1996). We therefore examined the effect of using a shorter induction time for C4H::GFP-expression, but after a 12-h induction, a high P420 content was again observed in the microsomal preparation (Fig. 5B).

Most importantly, despite the dominance of the P420 fraction in C4H::GFP expressing yeast, sufficient amounts of the P450, ranging from 40 to 60 pmol per milligram of microsomal protein, were recovered from microsomal preparations to permit comparisons of enzyme activity. These microsomal fractions were able to efficiently convert cinnamic acid to *p*-coumaric acid in in vitro enzyme assays, but did so with approximately 2-fold lower specific activities than C4H or C4H::FLAG-expressing strains, when normalized on the basis of spectrally active P450 (Table I). However, the specific activity of C4H::GFP was much lower (approximately 8 times), when normalized to total microsomal protein amount (data not shown).

To exclude the possibility of preferential denaturation and inactivation of C4H::GFP during the process of microsome preparation, enzyme assays for C4H activity were performed in living yeast cells expressing C4H, C4H::FLAG, or C4H::GFP. Cinnamic acid fed to all three yeast strains was converted to *p*-coumarate in a linear time-dependant manner (Fig. 6). A vector-only control strain did not produce any *p*-coumarate. As observed in vitro, no significant difference was observed in the catalytic activities of the C4H and C4H::FLAG constructs, whereas the C4H::GFP construct converted cinnamate to *p*-coumarate approximately 2-fold less efficiently.

Taken together, these in vitro and in vivo data confirm that C4H::GFP proteins retain enzymatic activity and that the general protein-folding and heme-binding to the C4H::GFP apoprotein proceeds correctly in a significant fraction of the expressed proteins.

DISCUSSION

Although poplar *C4H* genomic clones and the sequence of a poplar C4H cDNA have been described (Ge and Chiang, 1996; Kawai et al., 1996), *C4H* genes and proteins have not been further characterized in this genus. In this paper, we present a functional characterization of poplar C4H and present data strongly suggesting that C4H is strictly localized to the ER.

Based on the data presented here and elsewhere (Kawai et al., 1996), C4H appears to be encoded by a small family of very similar genes in *Populus*. We identified three C4H cDNA clones, one of which (C4H-550) was completely sequenced. The other two clones were >90% identical to C4H-550 in the regions sequenced. This level of sequence divergence is consistent with the presence of three *C4H* genes with greater than 90% sequence identity in *P. kitakamiensis* (Kawai et al., 1996). Using the C4H-550 sequence, we searched a database of poplar xylem and cambium ESTs (Sterky et al., 1998) and identified sequences for

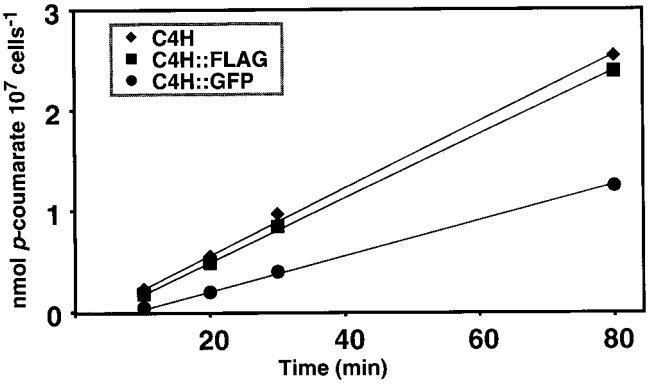

Figure 6. In vivo conversion of cinnamate to *p*-coumarate by transgenic yeast. The accumulation of *p*-coumarate in C4H-, C4H::FLAG-, and C4H::GFP-transformed yeast cultures was measured over the times given. Similar results were obtained when the experiment was repeated using independent yeast transformants.

six independent putative C4H clones, but these were all >95% identical to each other and to C4H-550 in regions where their sequences overlapped, suggesting that *C4H* genes with sequences significantly divergent from C4H-550 are not expressed in poplar xylem.

Taken together, this data suggests that there are multiple, but very similar, *C4H* genes in poplar, possibly having arisen from recent gene duplications in this genus. In maize and French bean, on the other hand, highly divergent C4H cDNAs have been identified (Potter et al., 1995; Nedelkina et al., 1999). Whereas these genes and their proteins have not yet been fully characterized, it has been proposed based on expression patterns that the divergent isoform could be involved in lignin biosynthesis. By contrast, C4H has been reported to be encoded by a single gene in Arabidopsis (Bell-Lelong et al., 1997; Mizutani et al., 1997), and our search of the complete Arabidopsis genome sequence revealed a single *C4H* sequence. Thus, in Arabidopsis, a single C4H form appears to participate in the biosynthesis of a wide array of phenylpropanoid natural products, including both lignin and flavonoids.

Poplar *C4H* transcripts detected by the C4H-550 probe accumulate in all organs and tissues tested, including both young leaves and green stems, the sites of flavonoid and lignin accumulation (Fig. 2). Thus, while we cannot rule out differential expression of very similar, cross-hybridizing *C4H* genes, the *C4H* expression pattern in poplar is consistent with a situation similar to Arabidopsis, where a single type of C4H enzyme (encoded by multiple, very similar genes in poplar) supports the biosynthesis of many or most phenylpropanoid natural products. In contrast, PAL and 4CL are encoded by multiple genes with divergent sequences and expression patterns in poplar (Subramaniam et al., 1993; Osakabe et al., 1995; Allina et al., 1998; Hu et al., 1998). For both enzymes, divergent genes whose expression is localized to either young leaves and stems or to developing secondary xylem have been described. As shown in Figure 2, poplar *C4H* expression overlaps with that of both *PAL1/2*, which is restricted to young leaves and stems, and a second divergent *PAL* gene (*PAL3*) that is more highly expressed in developing xylem. Both *C4H* and the two *PAL* genes are strongly induced by elicitor treatment, suggesting that all these genes may be involved in the biosynthesis of defense-related phenylpropanoid compounds.

The association of *C4H* expression with flavonoid biosynthesis in poplar is supported by the expression of both *C4H* and *CHS* in young leaves and stems (Fig. 2). It is interesting that *CHS* expression was not induced by elicitor, suggesting that flavonoids do not play roles as induced defense-related compounds in poplar. Although the flavonoid pinocembrin was proposed to be a potential poplar phytoalexin (Shain and Miller, 1982), our data do not support a role for the induced synthesis of this or other flavonoids in poplar defense against pathogens.

The functional identity of the protein encoded by the poplar C4H-550 cDNA as C4H was proven by expression in yeast. Microsomal fractions from yeast strains expressing C4H, C4H::FLAG, and C4H::GFP constructs all showed the typical peaks for cytochrome P450 enzymes by CO differential spectroscopy, and displayed high specific activities for cinnamic acid, which was efficiently converted to *p*-coumaric acid in a yeast strain engineered to express Arabidopsis CPR. The activities of all three versions of C4H were also confirmed by in vivo C4H assays in yeast. Although the C4H::GFP protein showed lower activities in both in vitro and in vivo, the fusion protein remained functional.

A likely explanation for the reduced specific activity of C4H::GFP in vitro (Table I) is reduced accessibility of the fusion enzyme to CPR brought about by interference of the C-terminal 26-kD GFP peptide with C4H-CPR interaction. In addition, we found a high content of a P420 species, which is considered to be an inactivated form of P450 enzymes in microsomes containing the fusion proteins. Destabilization of the C4H protein by the GFP fusion to its C-terminal is not unexpected, since the heme-binding region is very closely located to the C-terminal end (Fig. 1) and disruption of heme binding would lead to formation of the P420 species. The GFP domain could also potentially constrain the active site and make the fusion protein more susceptible to conformational change, thus leading to the high P420 content of C4H::GFP. The lower C4H activity in vitro and in vivo in general may be related to the instability of the fusion protein as indicated by the high P420 content in samples. In fact, the spectral shift from P450 to P420 provides a sensitive internal indicator of P450 denaturation (Yu et al., 1995).

Expression of the poplar C4H::GFP in transgenic Arabidopsis allowed its subcellular localization to be determined in vivo. To our knowledge, this is the first time that the subcellular localization of C4H, or any other phenylpropanoid enzyme, has been established using this technique. Optical sectioning of Arabidopsis cotyledon and hypocotyl epidermal cells by confocal microscopy demonstrated that C4H was strictly retained on the cortical ER, identified by its characteristic reticulate pattern. No fluorescence was detected in the Golgi apparatus, mitochondria, chloroplasts, or other cellular organelles, although these have been easily identified by GFP tags in other studies (Köhler, 1998). Although immunohistochemical studies in French bean suggested that C4H is abundant in the Golgi of that species (Smith et al., 1994), we found no evidence for fluorescent signals in the mobile, punctuate Golgi organelles visualized by GFP tagging in living tobacco cells (Boevink et al., 1998; Nebenführ et al., 1999), or numerous speck-like

Golgi detected by an immunolocalization method in fixed Arabidopsis cells (Wee et al., 1998).

Inappropriate retention of proteins in the ER can result from quality-control mechanisms that prevent defective proteins from leaving the ER (Lodish, 1988; Hurtley et al., 1989). The P420 species in yeast microsomes suggests the possible presence of defective C4H::GFP proteins in Arabidopsis. However, this explanation for C4H localization to the ER, and their apparent absence from the Golgi, can be excluded since a significant fraction of C4H::GFP in yeast microsomes retained normal catalytic and spectral properties. This suggests that functional C4H::GFP should be accessible to the machinery for potential trafficking outside the ER, and argues against the exclusive artifactual trapping of defective C4H::GFP fusion proteins in the ER.

Besides ER, elongated mobile organelles were frequently detected (Fig. 4C). In transformed Arabidopsis where the ER-targeted GFP was expressed, similar structures have been described as torpedo-shaped organelles (Cutler et al., 2000, supplemental data available at http://deepgreen.stanford.edu) or novel organelles (Köhler, 1998). However, these subcellular structures closely resemble the dilated ER cisternae characteristic of the Brassicaceae (Gunning, 1998, and references therein). We favor the idea that they are more likely to be subdomains of ER in Arabidopsis.

In addition to the ER localized fluorescent signals in epidermal cells, we frequently found strong fluorescent signals within unidentified organelles in the guard cells of cotyledons in Arabidopsis lines expressing C4H::GFP (Fig. 4I). These fluorescent round organelles, located next to non-fluorescent central vacuole, may be protein storage vacuoles (PSVs). Direct protein-movement from ER to the PSV, bypassing the Golgi apparatus, has been demonstrated (Hara-Nishimura et al., 1998; Jiang and Rogers, 1998). It is interesting that a series of optical sections of the organelles revealed that the fluorescence was uniformly labeled inside the organelle. This unique observation is consistent with the fact that integral membrane proteins are localized in the lumen of PSV in an internalized crystalloid membrane structure (Jiang et al., 2000). It is most likely that C4H localization to these unusual organelles, possibly PSVs, is an artifact of over expression of the C4H::GFP fusion protein, resulting in mislocalization of abundant C4H::GFP in metabolically active guard cells. How the C4H::GFP proteins are targeted to this organelle remains to be solved.

The C4H::GFP fusion proteins described here will provide a valuable tool for future studies on the mechanisms underlying C4H subcellular localization and trafficking in vivo. Expression of this fusion in planta and expression of the catalytically active C4H, C4H::FLAG, and C4H::GFP fusions in yeast together with other phenylpropanoid enzymes may also allow experimental reconstruction of potential phenylpropanoid MECs associated with ER-localized C4H.

MATERIALS AND METHODS

Isolation of cDNA Clones

A mixture of parsley (Logemann et al., 1995) and Arabidopsis C4H (Arabidopsis Biological Resource Center stock no. SCD12T7P) cDNAs was radiolabeled by random priming (Life Technologies/Gibco-BRL, Cleveland) and used as a probe to screen a Lambda ZAP (Stratagene, San Diego) *Populus trichocarpa* × *P. deltoides* hybrid H11-11 young leaf cDNA library (Moniz de Sá et al., 1992) according to standard methods (Sambrook et al., 1989). After washing filters at low stringency (65°C, 2× SSC), 20 positive plaques, identified from a total of 150,000 pfu screened, were partially purified and tested for insert length by PCR using T3 and T7 primers. The three longest clones were further purified and excised in vivo according to manufacturer's recommendations. One clone, designated C4H-550, had a 1.8-kb insert predicted to contain a full-length *C4H* gene, and was completely sequenced. DNA sequencing was carried out by automated Prism Cycle Sequencing (ABI, Sunnyvale, CA) at University of British Columbia Nucleic Acid-Protein Service Unit.

RNA Analysis

Total RNA was extracted using the methods of Hughes and Galau (1988). Total RNA (10 μg) was separated by electrophoresis in a 1.2% (w/v) formaldehyde-agarose gel and blotted onto a nylon membrane (Amersham, Buckinghamshire, UK). Radiolabeled probes were derived from the following sources: H11-11 *PAL1/2* from PAL7 (Subramaniam et al., 1993), H11-11 *C4H* from C4H-550 (this study), and *CHS* from a partial CHS cDNA clone obtained from the H11-11young leaf cDNA library (J. Jones, C. Douglas, unpublished data). The *PAL3* probe was generated from a *Populus triochocarpa* × *P. deltoides* hybrid 53-246 genomic restriction fragment containing a portion of *PAL* gene that is 90% identical to *P. kitakamiensis palg2a* and *palg2b* (Osakabe et al., 1995), and 70% identical to *PAL1*/2 (M. Gray-Mitsumune, C. Douglas, unpublished data). Final washing was carried out under high stringency condition (65°C, 0.2× SSC).

Fusion Gene Construction for Plant Expression

The CD3-327 clone, which contains a plant expression cassette for the red-shift soluble modified GFP (smRS-GFP; GenBank accession no. U70496) (Davis and Vierstra, 1998), was obtained from the Arabidopsis Biological Resource Center. To generate a C4H::GFP fusion construct for expression in plants, the coding sequence of GFP was PCR-amplified using Pfu polymerase (Stratagene; used in all PCR reactions hereafter) and the gene-specific oligonucleotide, 5′-CAGC*TCTAGA*ATGAGTAAAGGAGAAGAACT TT-3′ together with a T7 vector primer. The amplified GFP coding sequence was digested with *Xba*I and *Sac*I, and the

resulting fragment was inserted into the corresponding sites of the pSL1180 vector (Pharmacia Biotech, Piscataway, NJ). The inserted GFP coding region was sequenced to verify the accuracy of the PCR amplification. In parallel, the poplar C4H cDNA clone C4H-550 was amplified by PCR using a C4H-specific oligonucleotide, 5′-CAGC*TCT AGA*AAAGGACCTTGGCTTTGC-3′ and the M13 reverse primer, specific to pBluescript II SK. The resulting PCR product was digested with *Apa*I and *Xba*I, and the resulting fragment was inserted into the corresponding sites of pBluescript II KS. To avoid possible PCR errors, the *Apa*I and *Eco*RI fragment from this PCR-modified C4H clone was replaced by corresponding fragment from the original C4H cDNA, and the remaining 3′ end was sequenced. The entire C4H coding sequence, flanked by *Apa*I and *Xba*I restriction enzyme sites, was then inserted into the *Apa*I and *Xba*I sites of pSL1180, containing the GFP coding region between the *Xba*I and *Sac*I sites. This resulted in an in-frame fusion of C4H and GFP in the pSL1180 vector.

The pBin19 vector (Bevan, 1984) was modified for addition of a plant expression cassette as follows: A fragment of the pBin19 multiple cloning site was removed by digestion with *Eco*RI and *Sal*I. Both sites were made blunt by fill-in reaction with Klenow fragment, and the plasmid was religated. A *Hin*dIII fragment from the pRT101 vector (Töpfer et al., 1987), containing a plant expression cassette of CaMV 35S promoter and terminator flanking a multiple cloning site, was inserted into the *Hin*dIII site of the modified pBin19 vector to construct a Bin19/pRT101 binary vector. Finally, a *Sal*I and *Spe*I-digested 2.3-kb fragment containing the C4H::GFP fusion coding region was placed in the *Xho*I and *Xba*I site of Bin19/pRT101. Independently, a binary vector with a cassette expressing GFP alone was constructed by inserting the *Hin*dIII and *Eco*RI fragment from the CD3-327 clone, which includes the 35S CaMV promoter, GFP, and Nos terminator, into the same sites in the pBin19 vector (Bin19/GFP). Agrobacterium strain GV3101 was transformed either with the binary vector harboring the 35S-C4H::GFP fusion construct or with the 35S-GFP construct by electroporation. Transformants were selected on Luria-Bertani media containing 25 mg/L gentamycin, 25 mg/L rifampicin, and 100 mg/L kanamycin, and the presence of the binary vector was verified by PCR.

Plant Growth Conditions and Transformation

Arabidopsis ecotype Columbia was germinated and grown on Arabidopsis (AT) media (Somerville and Ogren, 1982) for a week at 20°C under constant light, and the seedlings were then transferred to soil (Redi-Earth, W.R. Grace and Company, Ontario, Canada) under the same growth conditions. Plants were grown until abundant immature floral clusters had formed and then transformed by the floral dip method (Clough and Bent, 1998) using 0.05% (v/v) Silwet L-77 (Lelhle Seeds, Round Rock, TX). Primary transformants (T_0) were selected by screening on AT media containing 40 mg/L kanamycin. T_0 lines were selfed, and the resulting seeds were screened by germination on kanamycin-AT media to select T_1 lines. Six C4H::GFP homozygous lines were identified by their T_2 segregation pattern of kanamycin resistance.

Confocal Laser Scanning Microscopy and Image Analysis

Confocal laser microscopy analysis was performed using six independent transgenic Arabidopsis lines. Arabidopsis plants 5 to 7 d old were mounted under a cover glass with a drop of distilled water. The intact seedlings were observed using a MRC-600 confocal laser scanning microscope (Bio-Rad Laboratories, Hercules) using the blue high sensitivity filter block. Excitation was provided by the 488-nm line of an argon laser, and the laser was reduced to 10% for optical sectioning at 0.2-μm intervals. Collected images were processed using NIH Image software, and selected sections were processed using Photoshop 5.0 (Adobe Systems, Mountain View, CA).

Yeast Expression Vector Constructions

The pYeDP60 expression vector and strain WAT11 (Urban et al., 1994, 1997) were used to express C4H and its fusion derivatives in yeast. To create the C4H constructs in pYeDP60, the complete C4H coding sequence from C4H-550 was amplified by PCR using two gene-specific primers, each with a *Bam*HI site: forward primer, 5′-ACA*GGATCC*-ATCATGGATCTCCTTCTCCTGGA-3′ and reverse primer 5′-ACA*GGATCC*TTAAAAGGACCTTGGCTTTGCAAC-3′. The PCR products were digested with *Bam*HI and ligated into the corresponding site of pYeDP60. The correct orientation and fidelity of this and all other constructs hereafter were confirmed by PCR and DNA sequencing, respectively. To create a C-terminal fusion of the FLAG epitope to C4H, the complete C4H coding sequence was PCR-amplified using a set of gene specific primers each containing a *Not*I site: forward primer 5′-ATAAGACT*GCGGCC-GC*ATCATGGATCTCCTTCTCCTG-3′ and reverse primer 5′-AAGTAGTA*GCGGCCGC*AAAGGACCTTGGCTTTGCA-ACAATAG-3′. The resulting PCR products were digested with *Not*I and cloned into the *Not*I site of pESC vector (Stratagene) to produce a C4H-FLAG in-frame fusion. The entire C4H::FLAG fusion construct was re-amplified by PCR using another set of specific primers containing a *Bam*HI site: the same forward C4H-specific primer and reverse primer, 5′-ACA*GGATCC*TCAGATCTTATCGTCA-TCATCCTT-3′. The PCR products were digested with *Bam*HI and cloned into the *Bam*HI site of pYeDP60. For the C4H::GFP fusion construct, the same forward C4H-specific primer was used together with the reverse primer, 5′-TATGAT*GGATCC*TTATTTGTATAGTTCATCCATGCCAT-GT-3′, to amplify the C4H-GFP fusion construct in the pSL1180 described above. The resulting PCR products were digested with *Bam*HI and cloned into the *Bam*HI site of pYeDP60. The yeast strain WAT11 was transformed by the polyethylene glycol-LiOAc method (Gietz et al., 1992) and transformants were selected as described (Urban et al., 1994).

Yeast Cell Culture and Microsomal Protein Preparation

Yeast cell culture and induction conditions, disruption of yeast cells using glass beads (425–600 μm; Sigma, St. Louis), and preparation of microsomal fractions were performed as described (Urban et al., 1994), except that ultracentrifugation at 100,000*g* was used for 60 min.

Differential Spectroscopy

Total cytochrome P450 content in protein preparations was determined by obtaining reduced carbon monoxide difference spectra (Omura and Sato, 1964). Microsomal protein was diluted in buffer containing 100 mM sodium phosphate (pH 7.4), 0.1 mM EDTA, and 30% (v/v) glycerol, and divided equally between two 1-mL cuvettes. A few grains of sodium dithionite were added to both cuvettes, and the baseline was recorded in a dual beam UV-visible spectrophotometer (Biospec-1601; Shimadzu). The contents of the sample cuvette were then gently bubbled with a carbon monoxide stream for 1 min, and the resulting differential spectrum was recorded between 400 and 500 nm.

SDS-PAGE and Immunoblots

Microsomal protein samples (2 μg) were separated on 10% (w/v) polyacrylamide gels and either stained with Coomassie Blue or transferred to PVDF membrane (Amersham) for immunoblot analysis. Immunodetection of target proteins was performed using the enhanced chemiluminescence system following the manufacturer's recommendations (Amersham). Protein-transferred membranes were blocked with 5% (w/v) non-fat milk for 16 h. Primary antibodies, either anti-FLAG monoclonal antibody (Stratagene) or anti-GFP monoclonal antibody (Sigma) at 1:2,500 dilution, were then reacted with the antigens on the membrane for 1 h at room temperature. Mouse horseradish peroxidase-conjugated anti-mouse antibody was used as the secondary antibody at 1:2,500 dilution for 1 h at room temperature and detected using the enhanced chemiluminescence system.

Enzyme Assays

The in vitro C4H enzyme assays were initiated by adding NADPH at a final concentration of 0.5 mM to a reaction mixture (600 μL total volume) containing 100 mM sodium phosphate buffer (pH 7.4), 0.1 mM cinnamic acid, and 30 to 50 μg microsomal protein. After incubation for 10 min at 30°C, the reaction was stopped by adding 40 μL 6 M HCl and the reaction mixture was extracted twice into 600 μL of ethylacetate, followed by evaporation of the organic phase in vacuum. Reaction products were analyzed and quantified by HPLC system equipped with a 996 photodiode array detector (Waters, Milford, MA). The residue was dissolved in 800 μL of HPLC-grade acetonitrile, and samples (40 μL) and standards were separated on 3.9 $\times$ 300 mm (10 μm) μBondapak C18 column (Waters). Solvents used were A (0.85% aqueous phosphoric acid [w/v]) and B (acetonitrile). A linear gradient of 80% solvent A, 20% solvent B to 48% solvent A, 52% solvent B was used at a flow-rate of 0.8 mL/min for 30 min. HPLC peaks specific to *p*-coumarate were identified by migration of standards and diagnostic UV absorption spectra, and peak area was used to quantify the products. For in vivo enzyme assays, yeast suspension culture (10 mL, approximately 3.8×10^8 cells mL^{-1}) induced for 12 h by 2% (w/v) Gal was pelleted by centrifugation at 4,000*g* for 5 min, and resuspended in 30 mL TE buffer (Tris-HCl, pH 7.4, 1 mM EDTA) supplemented with 0.2 mM cinnamic acid. The yeast cells were then incubated at 28°C with gentle shaking. A series of 1-mL samples was collected at different time points and pelleted by centrifugation at 14,000*g* for 1 min. The level of *p*-coumarate product in the supernatants were quantified by HPLC analysis as above.

ACKNOWLEDGMENTS

We thank Drs. Jürgen Ehlting and Lacey Samuels for critical reading of this manuscript, and Jason Marhue for making the GFP-Bin19 expression vector. We also thank Dr. Denis Pompon (Centre de Génétique Moléculaire du CNRS, Gif-sur-Yvette, France) for providing plasmid pYeDP60 and *S. cerevisiae* strain WAT11 and John C. Rogers (Washington State University, Pullman) for helpful discussions.

Received October 11, 2000; returned for revision January 25, 2001; accepted February 21, 2001.

LITERATURE CITED

Allina SM, Pri-Hadash A, Theilmann DA, Ellis BE, Douglas CJ (1998) 4-coumarate:coenzyme A ligase in hybrid poplar: properties of native enzymes, cDNA cloning, and analysis of recombinant enzymes. Plant Physiol **116:** 743–754

Bell-Lelong DA, Cusumano JC, Meyer K, Chapple C (1997) Cinnamate-4-hydroxylase expression in *Arabidopsis*: regulation in response to development and the environment. Plant Physiol **113:** 729–738

Benveniste I, Salaun JP, Durst F (1978) Phytochrome-mediated regulation of a monooxygenase hydroxylating cinnamic acid in etiolated pea seedlings. Phytochemistry **17:** 359–363

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res **12:** 8711–8721

Boevink P, Oparka K, Santa Cruz S, Martin B, Betteridge A, Hawes C (1998) Stacks on tracks: the plant Golgi apparatus traffics on an actin/ER network. Plant J **15:** 441–447

Boevink P, Santa Cruz S, Hawes C, Harris N, Oparka KJ (1996) Virus-mediated delivery of the green fluorescent protein to the endoplasmic reticulum of plant cells. Plant J **10:** 935–941

Chapple C (1998) Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. Annu Rev Plant Physiol Plant Mol Biol **49:** 311–343

Chen W, Peter RM, McArdle S, Thummel KE, Sigle RO, Nelson SD (1996) Baculovirus expression and purifica-

tion of human and rat cytochrome P450 2E1. Arch Biochem Biophys **335:** 123–130

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

Cutler SR, Ehrhardt DW, Griffitts JS, Somerville CR (2000) Random GFP::cDNA fusions enable visualization of subcellular structures in cells of *Arabidopsis* at a high frequency. Proc Natl Acad Sci USA **97:** 3718–3723

Czichi U, Kindl H (1977) Phenylalanine ammonia lyase and cinnamic acid hydroxylases as assembled consecutive enzymes on microsomal membranes of cucumber cotyledons: cooperation and subcellular distribution. Planta **134:** 133–143

Davis SJ, Vierstra RD (1998) Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. Plant Mol Biol **36:** 521–528

Fujita M, Asahi T (1985) Different intracellular localization of two cytochrome P-450 systems, ipomeamarone 15-hydroxylase and cinnamic acid 4-hydroxylase, in sweet potato root tissue infected with *Ceratocystis fimbriata*. Plant Cell Physiol **26:** 389–395

Ge L, Chiang VL (1996) A full length cDNA encoding *trans*-cinnamate 4-hydroxylase from developing xylem of *Populus tremuloides* (accession no. U47293) (PGR 96–075). Plant Physiol **112:** 861

Gietz D, St. Jean A, Woods RA, Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res **20:** 1425

Gray-Mitsumune M, Molitor EK, Cukovic D, Carlson JE, Douglas CJ (1999) Developmentally regulated patterns of expression directed by poplar PAL promoters in transgenic tobacco and poplar. Plant Mol Biol **39:** 657–669

Gunning BE (1998) The identity of mystery organelles in *Arabidopsis* plants expressing GFP. Trends Plant Sci **3:** 417

Hara-Nishimura I, Shimada T, Hatano K, Takeuchi Y, Nishimura M (1998) Transport of storage proteins to protein storage vacuoles is mediated by large precursor-accumulating vesicles. Plant Cell **10:** 825–836

Hotze M, Schroder G, Schroder J (1995) Cinnamate 4-hydroxylase from *Catharanthus roseus*, and a strategy for the functional expression of plant cytochrome P450 proteins as translational fusions with P450 reductase in *Escherichia coli*. FEBS Lett **374:** 345–350

Hu WJ, Kawaoka A, Tsai CJ, Lung J, Osakabe K, Ebinuma H, Chiang VL (1998) Compartmentalized expression of two structurally and functionally distinct 4-coumarate: CoA ligase genes in aspen (*Populus tremuloides*). Proc Natl Acad Sci USA **95:** 5407–5412

Hughes WD, Galau G (1988) Preparations of RNA from cotton leaves and pollen. Plant Mol Biol Rep **6:** 253–257

Hull AK, Vij R, Celenza JL (2000) *Arabidopsis* cytochrome P450s that catalyze the first step of tryptophan-dependent indole-3-acetic acid biosynthesis. Proc Natl Acad Sci USA **97:** 2379–2384

Hurtley SM, Bole DG, Hoover-Litty H, Helenius A, Copeland CS (1989) Interactions of misfolded influenza virus hemagglutinin with binding protein (BiP). J Cell Biol **108:** 2117–2126

Jiang L, Phillips TE, Rogers SW, Rogers JC (2000) Biogenesis of the protein storage vacuole crystalloid. J Cell Biol **150:** 755–770

Jiang L, Rogers JC (1998) Integral membrane protein sorting to vacuoles in plant cells: evidence for two pathways. J Cell Biol **143:** 1183–1199

Kalb VF, Loper JC (1988) Proteins from eight eukaryotic cytochrome P-450 families share a segmented region of sequence similarity. Proc Natl Acad Sci USA **85:** 7221–7225

Kawai S, Mori A, Shiokawa T, Kajita S, Katayama Y, Morohoshi N (1996) Isolation and analysis of cinnamic acid 4-hydroxylase homologous genes from a hybrid aspen, *Populus kitakamiensis*. Biosci Biotechnol Biochem **60:** 1586–1597

Kjellbom P, Larsson C, Askerlund P, Schelin C, Widell S (1985) Cytochrome P450/420 in plant plasma membranes: a possible component of the blue-light-reducible flavoprotein-cytochrome complex. Photochem Photobiol **42:** 779–783

Knebel W, Quader H, Schnepf E (1990) Mobile and immobile endoplasmic reticulum in onion bulb epidermis cells: short- and long-term observations with a confocal laser scanning microscope. Eur J Cell Biol **52:** 328–340

Köhler R (1998) GFP for *in vivo* imaging of subcellular structures in plant cells. Trends Plant Sci **3:** 317–319

Koopmann E, Logemann E, Hahlbrock K (1999) Regulation and functional expression of cinnamate 4-hydroxylase from parsley. Plant Physiol **119:** 49–56

Lindberg RL, Negishi M (1989) Alteration of mouse cytochrome P450coh substrate specificity by mutation of a single amino-acid residue. Nature **339:** 632–634

Lodish HF (1988) Transport of secretory and membrane glycoproteins from the rough endoplasmic reticulum to the Golgi: a rate-limiting step in protein maturation and secretion. J Biol Chem **263:** 2107–2110

Logemann E, Parniske M, Hahlbrock K (1995) Modes of expression and common structural features of the complete phenylalanine ammonia-lyase gene family in parsley. Proc Natl Acad Sci USA **92:** 5905–5909

Madyastha KM, Ridgway JE, Dwyer JG, Coscia CJ (1977) Subcellular localization of a cytochrome P-450-dependent monooxygenase in vesicles of the higher plant *Catharanthus roseus*. J Cell Biol **72:** 302–313

Mizutani M, Ohta D, Sato R (1997) Isolation of a cDNA and a genomic clone encoding cinnamate 4- hydroxylase from *Arabidopsis* and its expression manner *in planta*. Plant Physiol **113:** 755–763

Moniz de Sá M, Subramaniam R, Williams FE, Douglas CJ (1992) Rapid activation of phenylpropanoid metabolism in elicitor-treated hybrid poplar (*Populus tricorcarpa* Torr and Gray × *Pipulus deltoides* Marsh) suspension-cultured cells. Plant Physiol **98:** 728–737

Nebenführ A, Gallagher LA, Dunahay TG, Frohlick JA, Mazurkiewicz AM, Meehl JB, Staehelin LA (1999) Stop-and-go movements of plant Golgi stacks are mediated by the acto-myosin system. Plant Physiol **121:** 1127–1142

Nedelkina S, Jupe SC, Blee KA, Schalk M, Werck-Reichhart D, Bolwell GP (1999) Novel characteristics and regulation of a divergent cinnamate 4- hydroxylase

(CYP73A15) from French bean: engineering expression in yeast. Plant Mol Biol **39:** 1079–1090

Nelson DR (1999) Cytochrome P450 and the individuality of species. Arch Biochem Biophys **369:** 1–10

Omura T, Sato R (1964) The carbon monoxide-binding pigment of liver microsomes: I. Evidence for its hemoprotein nature. J Biol Chem **239:** 2370–2378

Osakabe Y, Osakabe K, Kawai S, Katayama Y, Morohoshi N (1995) Characterization of the structure and determination of mRNA levels of the phenylalanine ammonia-lyase gene family from *Populus kitakamiensis*. Plant Mol Biol **28:** 1133–1141

Potter S, Moreland DE, Kreuz K, Ward E (1995) Induction of cytochrome P450 genes by ethanol in maize. Drug Metabol Drug Interact **12:** 317–327

Rasmussen S, Dixon RA (1999) Transgene-mediated and elicitor-induced perturbation of metabolic channeling at the entry point into the phenylpropanoid pathway. Plant Cell **11:** 1537–1552

Sakaguchi M, Tomiyoshi R, Kuroiwa T, Mihara K, Omura T (1992) Functions of signal and signal-anchor sequences are determined by the balance between the hydrophobic segment and the N-terminal charge. Proc Natl Acad Sci USA **89:** 16–19

Sambrook J, Frisch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Shain L, Miller JB (1982) Pinocembrin: an antifungal compound secreted by leaf glands of eastern cottonwood. Phytopathology **72:** 877–880

Smith CG, Rodgers MW, Zimmerlin A, Ferdinando D, Bolwell P (1994) Tissue and subcellular immunolocalisation of enzymes of lignin synthesis in differentiating and wounded hypocotyl tissue of French bean (*Phaseolus vulgaris L.*). Planta **192:** 155–164

Somerville CR, Ogren WL (1982) Isolation of photorespiration mutants in *Arabidopsis thaliana. In* M Edelman, RB Hallick, NH Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier Biomedical Press, Amsterdam, pp 129–138

Song WC, Funk CD, Brash AR (1993) Molecular cloning of an allene oxide synthase: a cytochrome P450 specialized for the metabolism of fatty acid hydroperoxides. Proc Natl Acad Sci USA **90:** 8519–8523

Sterky F, Regan S, Karlsson J, Hertzberg M, Rohde A, Holmberg A, Amini B, Bhalerao R, Larsson M, Villarroel R et al. (1998) Gene discovery in the wood-forming tissues of poplar: analysis of 5,692 expressed sequence tags. Proc Natl Acad Sci USA **95:** 13330–13335

Subramaniam R, Reinold S, Molitor EK, Douglas CJ (1993) Structure, inheritance, and expression of hybrid poplar (*Populus trichocarpa* × *Populus deltoides*) phenylalanine ammonia-lyase genes. Plant Physiol **102:** 71–83

Szczesna-Skorupa E, Straub P, Kemper B (1993) Deletion of a conserved tetrapeptide, PPGP, in P450 2C2 results in loss of enzymatic activity without a change in its cellular location. Arch Biochem Biophys **304:** 170–175

Teutsch HG, Hasenfratz MP, Lesot A, Stoltz C, Garnier JM, Jeltsch JM, Durst F, Werck-Reichhart D (1993) Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. Proc Natl Acad Sci USA **90:** 4102–4106

Töpfer R, Matzeit V, Gronenborn B, Schell J, Steinbiss HH (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res **15:** 5890

Urban P, Mignotte C, Kazmaier M, Delorme F, Pompon D (1997) Cloning, yeast expression, and characterization of the coupling of two distantly related *Arabidopsis thaliana* NADPH-cytochrome P450 reductases with P450 CYP73A5. J Biol Chem **272:** 19176–19186

Urban P, Werck-Reichhart D, Teutsch HG, Durst F, Regnier S, Kazmaier M, Pompon D (1994) Characterization of recombinant plant cinnamate 4-hydroxylase produced in yeast: kinetic and spectral properties of the major plant P450 of the phenylpropanoid pathway. Eur J Biochem **222:** 843–850

Wee EG, Sherrier DJ, Prime TA, Dupree P (1998) Targeting of active sialyltransferase to the plant Golgi apparatus. Plant Cell **10:** 1759–1768

Winkel-Shirley B (1999) Evidence for enzyme complexes in the phenylpropanoid and flavonoid pathways. Physiol Plant **107:** 142–149

Yu XC, Shen S, Strobel HW (1995) Denaturation of cytochrome P450 2B1 by guanidine hydrochloride and urea: evidence for a metastable intermediate state of the active site. Biochemistry **34:** 5511–5517