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# Cloning, Yeast Expression, and Characterization of the Coupling of Two Distantly Related *Arabidopsis thaliana* NADPH-Cytochrome P450 Reductases with P450 CYP73A5*

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**Two NADPH-cytochrome P450 reductase-encoding cDNAs were isolated from an *Arabidopsis* cDNA library by metabolic interference in a *Saccharomyces cerevisiae* mutant disrupted for its endogenous *cpr1* gene. *ATR1* encodes a protein of 692 amino acids, while *ATR2* encodes either a 712-residue protein (ATR2-1), or a 702-residue protein (ATR2-2) depending on the choice of the initiation codon. Comparative analysis of ATR1 and ATR2-1 indicates 64% amino acid sequence identity and the absence of conservation in the third base of conserved amino acid codons. The two *Arabidopsis* reductases are encoded by distinct genes whose divergence is expected an early event in angiosperms evolution. A poly(Ser/Thr) stretch reminiscent of a plant chloroplastic targeting signal is present at the ATR2-1 N-terminal end but absent in ATR1. The cDNA open reading frames were expressed in yeast. The recombinant polypeptides were found present in the yeast endoplasmic reticulum membrane and exhibited a high specific NADPH-cytochrome *c* reductase activity. To gain more insight into the respective functions of the two reductases, the *Arabidopsis* cDNA encoding cinnamate 4-hydroxylase (CYP73A5) was cloned and co-expressed with ATR1 or ATR2 in yeast. Biochemical characterization of the *Arabidopsis* ATR1/CYP73A5 and ATR2-1/CYP73A5 systems demonstrates that the two distantly related *Arabidopsis* reductases similarly support the first oxidative step of the phenylpropanoid general pathway.**

P450 monooxygenases are involved in various biosynthesis pathways and in degradation of a large range of exogenous compounds (1, 2). In plants, besides their roles in herbicide and pesticide detoxication (3, 4) and in promutagen oxidative activations (5), P450s are widely involved in several secondary metabolisms such as the phenylpropanoid pathway (6, 7). This pathway, which leads to important molecules such as lignins, pigments, coumarins, flavonoids, and phytoalexins (6), involves a common oxidative step: the *para*-hydroxylation of cinnamate catalyzed by a P450 of the CYP73 family.

In animals, the microsomal P450 system associates in the same organism multiple P450 isoenzymes but a single ubiquitous NADPH-P450 reductase (CPR).[1] Conclusive evidence for the presence of a single CPR-encoding gene was provided with Chinese hamster cells, mouse spleen cells (8), and rat hepatocytes (9). Unlike animals, higher plants express multiple forms of CPRs as inferred from Western blot analysis of purified material (10, 11). Durst *et al.* (12) have recently isolated two distinct partial length cDNAs (*HTR1* and *HTR3*) from *Helianthus tuberosus*, corresponding to distinct (2.4- and 2.6-kb) mRNA species.

This observation questioned the physiological significance of the multiplicity of plant CPRs. Among hypotheses, one could consider the requirement of different CPRs to support the activities of specific plant P450s or the differential CPR expressions in plant tissues or at various development stages. The possibility of multiple subcellular locations for CPRs in plant-specific organelles in addition to the regular endoplasmic reticulum (ER) location is also of particular interest. To address these questions, full-length *Arabidopsis* CPR-encoding cDNAs were cloned by a function-based approach involving complementation in yeast of an endogenous CPR defect. The selection relies on the reduced growth rate and the strong ketoconazole hypersensitivity of a yeast strain, which has been disrupted for the *CPR1* gene, which encodes yeast microsomal CPR. The addition of ketoconazole, a potent inhibitor of yeast P450 lanosterol demethylase, is required for selection due to the presence of alternate electron donors in yeast, making *CPR1* deletion non-lethal. In the *cpr1* strain, the CPR defect and ketoconazole inhibition contribute together to cause a full depletion of ergosterol biosynthesis, leading to cell growth arrest (13–15). Screening of an *Arabidopsis* cDNA library in a multicopy plasmid pFL61 under the transcriptional control of the phosphoglycerate kinase (*PGK*) promoter (16) was carried out in the W(R) strain. This strain is engineered to overexpress the yeast CPR in galactose- but not in glucose-containing medium (17). The *PGK* promoter is active on both carbon sources, allowing complementation by heterologous CPRs of the conditional mutant phenotype induced by culture of transformed W(R) cells on glucose. The isolated *Arabidopsis* CPRs were further characterized for a physiologic coupling with *Arabidop-*

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X66016 (for ATR1), X66017 (for ATR2), and U37235 (for CYP73A5).*

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[1] The abbreviations used are: CPR, NADPH-cytochrome P450 reductase; kb, kilobase pair; bp, base pair; *CPR1*, yeast gene encoding microsomal CPR; HTR1, *H. tuberosus* CPR1; SCR1, *S. cerevisiae* CPR; HSR1, *Homo sapiens* CPR; ER, endoplasmic reticulum; P450, hemethiolate protein P450; PGK, phosphoglycerate kinase; PCR, polymerase chain reaction; ETBI, experimental third base identity; RTBI, reference third base identity.

TABLE I
*Sequence of primers used in the PCR amplifications*

Lowercase and uppercase letters indicate sequences without and with homology to the template DNA, respectively. The translation initiation codon or the sequence complementary to the stop codon is underlined.

| Primer name | Sequence |
|---|---|
| ATC4H-5 | ggatccATGACCTCCTCTTGC |
| ATC4H-3 | ggagatcTTAACAGTTCCTTGG |
| ORS21 | gcggatccTTACCATACATCTCTAAGATATCT |
| ORS22 | ccggatccATGTCCTCTTCTTCTTCTTCGTCAACC |
| ORS23 | ccggatccATGATCGATCTCATGGCAGC |
| ORS24 | gcgagatctATGACTTCTGCTTTGTATGCTTCC |
| ORS25 | gggaattcTCACCAGACATCTCTGAGGTATC |
| ORS31 | cgggaattccagctggcggccgcGTATGTTGTCTTTGAAGATGCACCCGTGGG |
| ORS32 | gcggaattcTAATCAGCCCACTGATCAACGTTCGG |
| ORS33 | ccagatctgcggccgcCAGTAAATTCACTTGGAATCACACCG |
| ORS34 | gcgaagcttGCGACAGTAGATCACTTGAAAACCG |

*sis* cinnamate 4-hydroxylase P450 (CYP73A5) by coexpression in yeast. For that purpose, the *Arabidopsis* cDNA encoding CYP73A5 was cloned from the same library using a hybridization probe derived from the corresponding *H. tuberosus CYP73A1* gene (18).

## EXPERIMENTAL PROCEDURES

*Materials*—Restriction and DNA modification enzymes were obtained from New England Biolabs. Thermostable *Taq* and *Pfu* DNA polymerases were from Boehringer Mannheim and Stratagene, respectively. The Sequenase™ (version 2.0) DNA sequencing kit was purchased from U.S. Biochemical Corp. The pCRScript™ cloning kit was from Stratagene. NADPH, horse heart cytochrome *c*, and cinnamic and coumaric acids were from Sigma, and 2-naphthoic acid was from Aldrich.

*Yeast Strains and Vectors*—The *Saccharomyces cerevisiae* strain W303–1B (*MAT***a**; *ade2*-1; *his3*-11,-15; *leu2*-3,-112; *ura3*-1; *trp1*-1) is designated as W(N). Yeast strain W(R) was constructed by substitution of the natural promoter of the *CPR1* gene (encoding microsomal CPR) by the galactose-inducible and glucose-repressed promoter *GAL10-CYC1*. WRΔ derives from W(N) by disruption of the *CPR1* gene with a *TRP1* selection marker (17).

The pFL61 vector has been previously described (16). The yeast expression vector pYeDP60 (V60) contains both *URA3* and *ADE2* as selection markers, and an expression cassette constituted by *GAL10-CYC1* promoter and *PGK* terminator sequences surrounding a cDNA insertion polylinker (19). The pGP1 vector contains a 5500-bp-long fragment that encompasses the full *CPR1* gene at the *Hin*dIII site of pUC19. The pYeDP51 yeast expression vector is identical to pYeDP1/8-2 (20), except that the *Pvu*II site close to the yeast origin of replication is replaced by a *Bgl*II site.

Using ORS31 and ORS32 as primers (Table I) and pGP1 as a template, the 428-bp-long *CPR1* gene terminator sequence was PCR-amplified and cloned into the *Sma*I site of pUC19. The 420-bp-long *Eco*RI-*Pvu*II fragment of the resulting vector was ligated to the *Eco*RI-*Pvu*II fragment of pYeDP51 encompassing *URA3*. The 6900-bp-long resulting vector, named pYeDP100, places the inserted cDNA under the transcriptional control of *GAL10-CYC1*-inducible promoter and *CPR1* terminator. By PCR amplification using ORS33 and ORS34 primers and pGP1 as a template, a 631-bp-long *Hin*dIII-*Bgl*II fragment of the upstream part of the *CPR1* promoter has been obtained and cloned in pUC19. The *Hin*dIII-*Bgl*II fragment of the *CPR1* promoter was ligated with the larger *Hin*dIII-*Bgl*II fragment of pYeDP100, which encompasses *URA3*, yielding the 5210-bp-long pYeDP110 integrative vector. The pYeDP110 vector bears *GAL10-CYC1* promoter and *CPR1* terminator sandwiching a cDNA insertion polylinker, and the *CPR1* 5′-noncoding region placed upstream of the *URA3* selection marker. The entire integrative fragment can be removed by a *Not*I digestion. The respective orientation of the two *CPR1* noncoding regions allows targeted integration of the expression cassette at the *CPR1* locus by homologous recombination.

*Cell Culture*—Transformation of yeast strains was performed by a modified lithium acetate procedure as described (21), except for the transformation of W(R) cells with the pFL61 library (see below). Transformed cells were selected onto glucose-containing SGI plates. Culture media, cell cultures, and galactose induction procedures of individual clones in both SLI and YPGE were as described previously (17, 22).

*Screening of the Arabidopsis cDNA Expression Library in Yeast*—A sized (inserts >2 kb) cDNA library from *Arabidopsis thaliana* seedlings including roots (two-leaf stage) in pFL61 yeast expression vector was kindly provided by Dr. M. Minet. In pFL61, cDNAs were inserted as *Not*I cassettes between the yeast *PGK* promoter and terminator (16). Yeast W(R) cells were grown on galactose, thus overexpressing yeast CPR, up to a cell density of $2 \times 10^7$ cells/ml. Cells are harvested and resuspended at a cell density of $10^6$ cells/ml in a glucose-containing YPGA medium, thus turning off *CPR1* gene expression. W(R) cells were further grown on glucose for 5 h until the microsomal CPR activity decreases to the value found in wild-type W(N) cells. About $10^8$ cells were lithium-treated and used for transformation by 10 μg of the *Arabidopsis* cDNA library, 0.2 mg of heat-denatured herring sperm DNA in 2 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 0.1 M LiCl. After a 10-min incubation at room temperature, 5 ml of the same buffer containing 50% of poly(ethylene)glycol-4000 was added and gently mixed to the previous 2 ml, and the incubation was continued for 20 min at 37 °C and then 5 min at 42 °C. The cells were sprayed in 0.1-ml aliquots onto 20 plates of SGAI synthetic medium for uracil prototrophy selection.

Two pools of $Ura^+$ transformants were constituted, and cells from each pool were sprayed at $10^4$ cells/plate onto a series of SGAI plates containing increasing concentrations of ketoconazole (0, 5, 10, 20, and 50 μg/ml ketoconazole) and incubated for 36 h at 28 °C. The 52 clones found to be resistant to 10 and 20 μg/ml ketoconazole were striped on SGAI medium containing 10 μg/ml ketoconazole. The plasmidic DNA of 24 of the resistant clones was rescued in *Escherichia coli*. The *Bam*HI and *Hin*dIII digestion patterns revealed five different restriction profiles (profiles A–E).

*Isolation of Arabidopsis CYP73A5-encoding cDNA*—The *Arabidopsis* cDNA library in pFL61 was transformed in *E. coli* by electroporation. About $10^5$ independent clones were recovered and transferred on nitrocellulose. The blots were screened by hybridization in SSC × 6 buffer containing 0.1% SDS and 0.05% milk overnight at 55 °C with the $^{32}$P-labeled 1800-bp-long cDNA encoding *Helianthus* CYP73A1 (18). Of 12 independent clones selected, two strongly hybridize with the same probe after subcloning. The plasmidic DNA of one of the two clones, named pCYP73A5, was isolated and the *Not*I fragment encompassing the full-length cDNA sequenced.

*DNA Sequence Determination*—The *Not*I cDNA inserts encoding ATR1 and ATR2 were extracted from pFL61 and subcloned into the unique *Not*I site of a derivative of pUC9. For *ATR1* and *ATR2* cDNAs, sequencing was carried out with M13 universal oligonucleotides and a series of specific oligonucleotides belonging to the sequences. For *CYP73A5* cDNA, the sequencing using M13 universal oligonucleotides was performed by using nested deletions with exonuclease III of pCYP73A5 digested by *Kpn*I and *Hin*dIII. Nucleotide sequences were determined on both strands by the dideoxynucleotide chain termination procedure.

*Southern Blot Analysis*—The *Arabidopsis* genomic DNA (kindly provided by Dr. Clint S. Chapple) was digested with *Bam*HI, *Eco*RI, or *Hin*dIII. The digested aliquots (10 μg) were separated by 0.8% agarose electrophoresis and then transferred to a Nylon membrane (Schleicher and Schüll). The blotted membrane was probed in stringent conditions with $^{32}$P-labeled *ATR1* or *ATR2* open reading frame.

*Tailoring of the Selected cDNAs*—Oligonucleotides were synthesized and purified as described previously (19). The 2079-bp-long *ATR1* coding sequence was PCR-amplified using ORS24 and ORS25 primers with 10 ng of pFL61/ATR1 as template. These primers introduce a *Bgl*II site immediately upstream of the ATG codon and a *Eco*RI site downstream of the stop codon of the PCR-amplified fragment. The 2139-bp-long

ATR2-1 coding sequence was PCR-amplified using the combination of ORS21 and ORS22 primers, and the 2109-bp-long ATR2-2 coding sequence was PCR-amplified using ORS21 and ORS23 primers and 10 ng of pFL61/ATR2 as template in both cases. The three *ATR2* primers introduce a *Bam*HI site immediately upstream of the ATG initiation codon and downstream of the stop codon of the PCR-amplified fragments. The expected PCR-amplified products were cloned at the *Sma*I site of pUC19, giving, respectively, pUC19/ATR1, pUC19/ATR2-1, and pUC19/ATR2-2. The ATR1 coding sequence was then excised by *Bgl*II-*Eco*RI digestion and inserted into pYeDP60 linearized at *Bam*HI and *Eco*RI unique sites, resulting in plasmid pATR1/V60. The ATR2-1 and ATR2-2 coding sequences were excised by *Bam*HI digestion and inserted into pYeDP60 linearized at *Bam*HI site, a clone with the proper orientation was chosen for each insert giving, respectively, pATR2-1/V60 and pATR2-2/V60. Amplification of the 1518-bp-long CYP73A5 coding sequence was performed by PCR using ATC4H-5 and ATC4H-3 primers, and the product was cloned at the *Sfr*I site of pCRScript vector, giving pCRScript/ATC4H. The primers introduce a *Bam*HI site immediately upstream of the ATG codon and a *Bgl*II site downstream of the stop codon. The coding sequence was excised by *Bam*HI-*Bgl*II digestion and inserted at the *Bam*HI site of pYeDP60, and a clone with the correct orientation was selected and named pATC4H/V60.

*Engineering of WAT11, WAT21, and Derived Yeast Strains*—The ATR1 and ATR2-1 open reading frames were isolated from pUC19/ATR1 and pUC19/ATR2-1 as *Bam*HI-*Eco*RI and *Bam*HI-*Bam*HI fragment, respectively. The *ATR1* fragment was inserted at the *Bam*HI-*Eco*RI sites of pYeDP110, yielding pATR1/DP110. Similarly, the ATR2-1 fragment was inserted at the unique *Bam*HI site of pYeDP110, and a clone with the proper orientation was selected and named pATR2-1/DP110. The *Not*I fragment of each plasmid encompassing *URA3* and the ATR coding sequence was isolated and used to transform WRΔ cells. Since in WRΔ the *CPR1* locus is disrupted with the *TRP1* gene, integration events result in a phenotype shift from $Ura^-$ $Trp^+$ to $Ura^+$ $Trp^-$. This procedure yielded two new yeast strains, WAT11 (for ATR1) and WAT21 (for ATR2-1). They express the *Arabidopsis* CPR instead of the yeast enzyme when grown on galactose. On glucose, WAT cells express no CPR activity. The *URA3* selection marker in both WAT strains was mutated to *ura3* by selecting 5-fluoroorotate-resistant clones (23), giving WAT11U and WAT21U strains.

*Subcellular Fractionation and P450 Quantitation*—W(R) and WAT cells were harvested after a 12-h galactose induction and microsomal fractions prepared as described in Ref. 17. P450 content in yeast microsomes was calculated from the reduced carbon monoxide difference using a differential absorption coefficient (450 *versus* 490 nm) of 91 $mM^{-1}\cdot cm^{-1}$ (24). Protein concentrations were determined by the Pierce BCA (bicinchoninic acid) protein microassay using bovine serum albumin as a standard.

*Enzymatic Activities*—Cinnamate 4-hydroxylase activity was assayed by high pressure liquid chromatography-monitoring 4-hydroxycinnamate production as described previously (22). For naphthoate hydroxylation reactions, activity was determined using a 1-ml assay mixture containing 120 $\mu$M NADPH, various concentrations of 2-naphthoate, and the microsomal aliquot in 50 mM Tris/HCl buffer, 1 mM EDTA, pH 7.4. The time dependence of the fluorescence emission was monitored at 440 nm, the excitation wavelength being set at 290 nm. The rate of the 2-naphthoate 6-hydroxylation reaction was calculated from the slope of the linear graph obtained. Transformed yeast was grown and assayed for cinnamate bioconversion essentially as described in Refs. 17 and 22.

CPR activity was measured on microsomal fractions as described previously (22). The rate of cytochrome *c* reduction was calculated using a differential absorption coefficient of 21 $mM^{-1}\cdot cm^{-1}$ at 550 nm. The rates of dichlorophenolindophenol and ferricyanide reductions were monitored, respectively, at 600 nm (22 $mM^{-1}\cdot cm^{-1}$) and 420 nm (1 $mM^{-1}\cdot cm^{-1}$).

## RESULTS

*Expression Cloning of Arabidopsis CPRs*—Yeast cells W(R) that carry the *CPR1* gene under the transcriptional control of the galactose-inducible *GAL10-CYC1* promoter were transformed with the *Arabidopsis* cDNA expression library in pFL61 (16). Extinction of the modified *CPR1* gene, when glucose is used as a carbon source for culture, is not lethal (14, 17). However, yeast CPR deficiency caused a dramatic lowering in transformation efficiency due to changes in the cell wall structure. This led us to the design of an "on the fly" transformation strategy (see "Experimental Procedures"). The ketoconazole resistance criterion was used for the selection of clones expressing constitutively an alternate CPR in the pFL61-based cDNA expression library.

Approximately $10^5$ $Ura^+$ colonies were selected, pooled, and sprayed on a series of SGAI plates containing increasing ketoconazole concentrations. Fifty-two colonies were sorted out as resistant to 10 and 20 $\mu$g/ml ketoconazole. Twenty-four of them, selected at random, were further analyzed by restriction digestion. Five types of restriction profile were evidenced: A (7 clones), B (6 clones), C (5 clones), D (1 clone), and E (5 clones). The plasmidic inheritance of the ketoconazole resistance associated with each type of sequence was tested by transforming WRΔ, a yeast strain carrying a permanent *CPR1* disruption. Four (A, B, C, and E) of the five plasmid classes were shown to confer ketoconazole resistance at 40 $\mu$g/ml to WRΔ. Partial cDNA sequencing was used for further identification and showed that classes A, C, and E (~2300-bp-long cDNA *Not*I fragment) contain related inserts differing only by their orientation and their 5′- and 3′-noncoding extremities when class B (2199-bp-long *Not*I insert) appeared containing a different open reading frame. The relative amounts of both *ATR* mRNAs in the plant were estimated by transforming *E. coli* with the *Arabidopsis* cDNA library in pFL61. Out of $10^4$ independent clones, two hybridize *ATR1* probe and seven hybridize *ATR2* probe (not shown). This result demonstrates that in the cDNA library used, *ATR1* cDNA is 3 times less represented than *ATR2* cDNA.

*Sequence Analyses of Arabidopsis CPRs*—The cDNA inserts of class B and C plasmids were sequenced on both strands. The *ATR1* cDNA is 2199 bp long and encompasses 69 bp of the 5′-noncoding region followed by a 2079-bp-long open reading frame and 51 bp of the 3′-noncoding region (Fig. 1). A termination TGA codon (from −13 to −15) immediately precedes in frame the first putative initiation codon. The first methionine codon of *ATR1* cDNA closely matches the dicot plant initiator codon consensus sequence (25). The *ATR1* open reading frame encodes a protein of 692 amino acid residues with an estimated molecular mass of 76,720 Da, similar to that of rat liver CPR (77 kDa) (26).

The 2290-bp-long *ATR2* cDNA, also obtained as a full-length clone, consists of a 2139-bp-long open reading frame sandwiched by a 5′-noncoding region of 50 bp and a 3′-flanking region of 101 bp (Fig. 2). The *ATR2* open reading frame encodes a protein of 712 amino acid residues, which is 64% identical to ATR1, with an estimated molecular mass of 79,077 Da, more similar to the $M_r$ observed for higher plant CPRs (11, 27, 28). The assignment of the starting codon was not straightforward, since the flanking sequence preceding the first ATG of *ATR2* cDNA does not contain any stop codon in frame and since two other ATG codons were found in the 15 first codons. Comparison with the consensus sequences around the initiation codon in dicot plant mRNAs (25) indicates that both the first and the second ATG of *ATR2* cDNA could be a suitable translation initiation site; it is therefore difficult to predict which of these is the true starting site. The ATR2 protein encoded by the first ATG was designated ATR2-1 and is 712 residues long; the shorter protein, starting at the second ATG, was named ATR2-2 and is 702 residues long (Fig. 3).

Fig. 4 shows, based on sequence comparison with flavodoxin (26, 29) and $NADP^+$-ferredoxin reductase (30, 31), that each ATR presents the conserved segments typical of all known CPRs for the binding of FMN, FAD, and NADPH. In this respect, ATR1 and ATR2 are typical NADPH-P450 reductases. In contrast, no clear sequence similarity is found between the 85 first residues of ATR1 and the 106 first residues of ATR2.

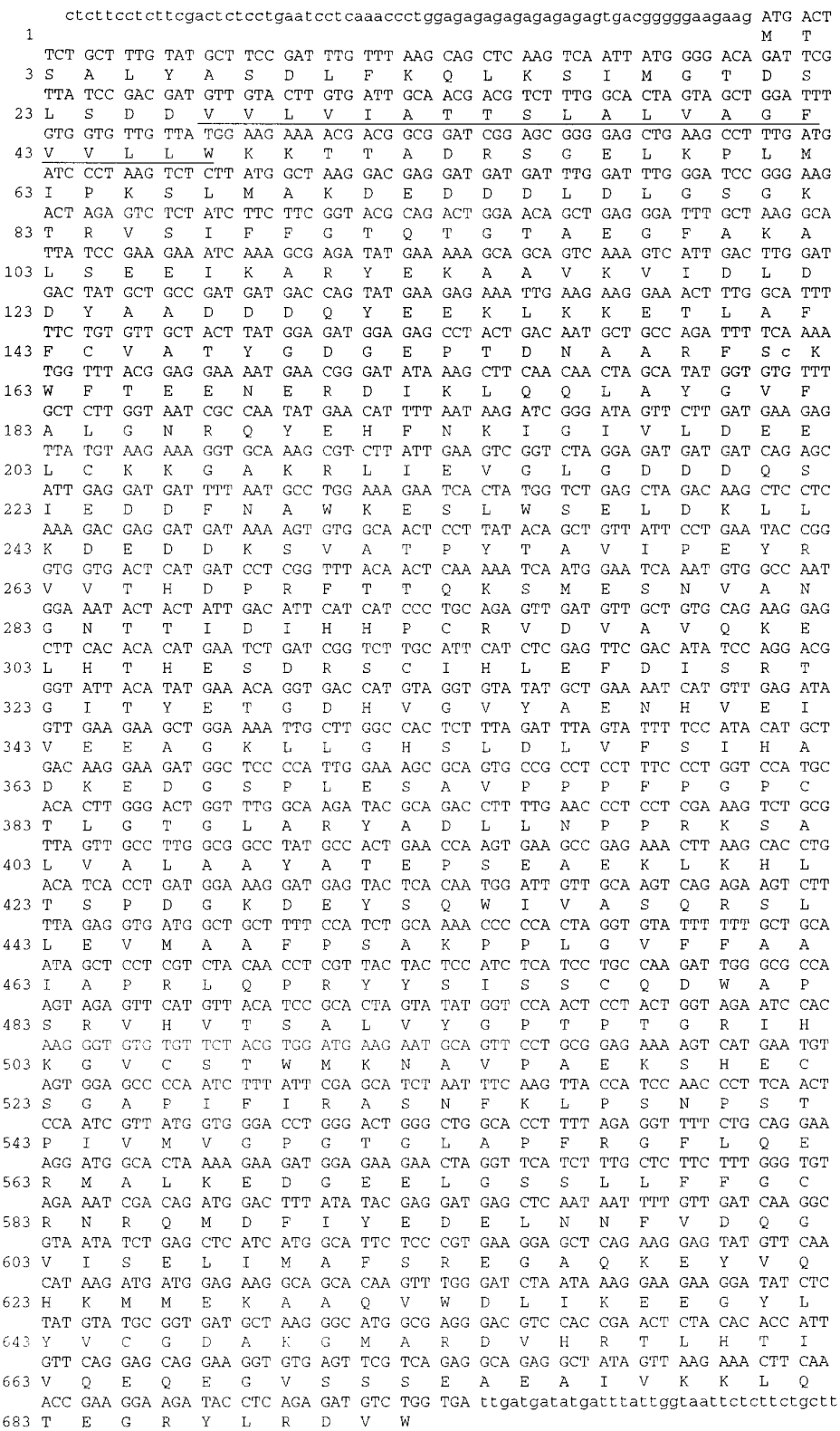


FIG. 1. **Nucleotide sequence and deduced protein sequence of *Arabidopsis ATR1*.** The deduced gene product is indicated in one-letter code *below* the DNA sequence. The 2199-bp-long cDNA encompasses a single open reading frame beginning at positions 70–72 and encoding a protein of 692 amino acids. A proposed motif for anchoring to the endoplasmic reticulum membrane is *underlined*. *Numbers* on the *left* indicate amino acid positions.

However, despite the absence of similarity in their N-terminal part, both ATR1 and ATR2 present in this part an hydrophobic segment that could act as a membrane anchor, as observed in other microsomal CPRs. Another distinct difference between the two ATRs is the net charge of the protein; ATR1 is predicted to be more acidic than ATR2 at neutral pH, exhibiting a calculated charge *versus* pH profile very different from those of ATR2 and human and yeast CPRs (Fig. 5). The differences in

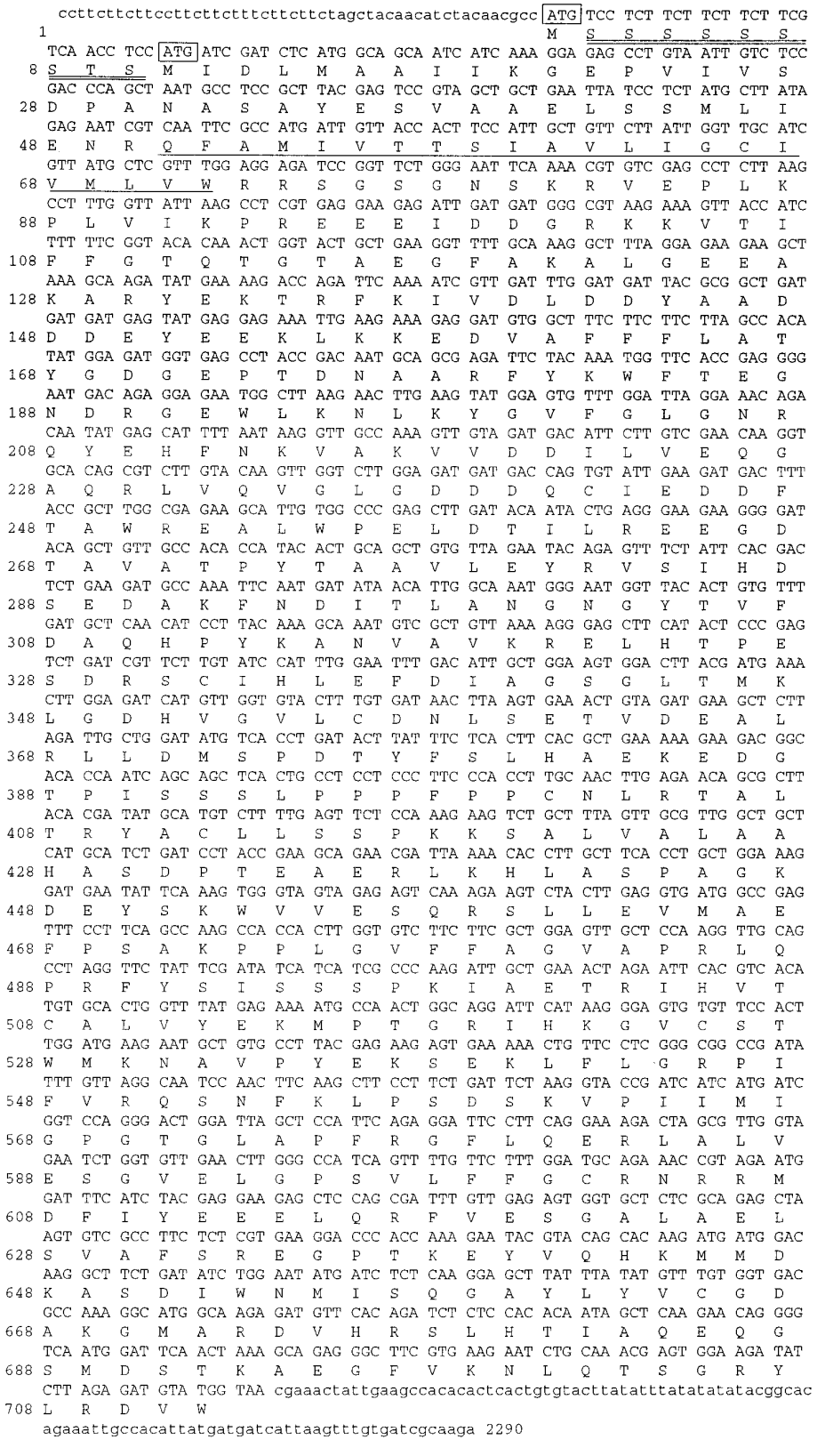


FIG. 2. **Nucleotide sequence and deduced protein sequence of *Arabidopsis ATR2*.** The deduced gene product is indicated in one-letter code *below* the DNA sequence. The 2290-bp-long cDNA encompasses a single open reading frame beginning at positions 51–53 or at positions 81–83 and encoding either a protein of 712 amino acids (ATR2-1) or a protein of 702 residues (ATR2-2). A proposed motif for anchoring to the endoplasmic reticulum membrane is *underlined*. The poly(Ser/Thr) stretch is *double-underlined*. The two putative ATG translational start codons are *boxed*. *Numbers* on the *left* indicate amino acid positions.

charge density appear to be mainly localized in the C-terminal moieties of the ATRs. More unusual is the presence at the N terminus of ATR2 of a poly(Ser/Thr) stretch that is not found in any other fungal, plant, or animal CPR. This particular segment could account for the specific glycosylations found in certain higher plant CPRs. The protein ATR2-2, encoded from

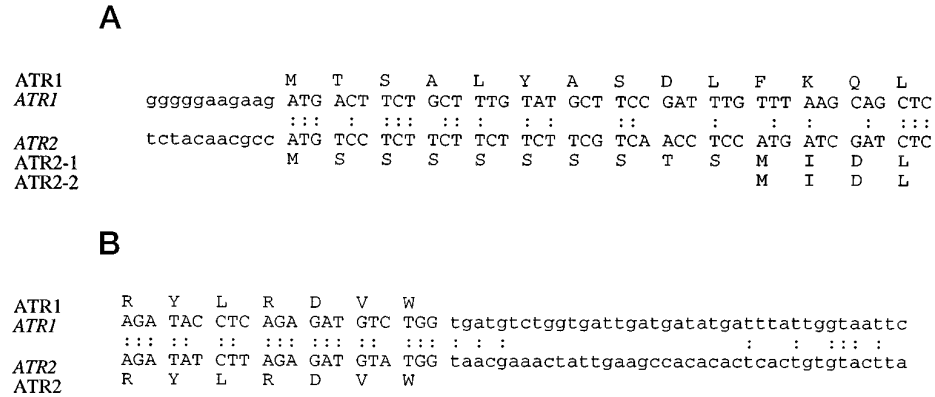


FIG. 3. **Comparison of *Arabidopsis* CPR sequences.** *A*, nucleotide and amino acid sequences of the N-terminal part of ATR1 and ATR2 with, in the latter case, the two ATR2 putative proteins. *B*, nucleotide sequence of the 3′-end of *ATR1* and *ATR2* cDNAs and the deduced amino acid sequences.

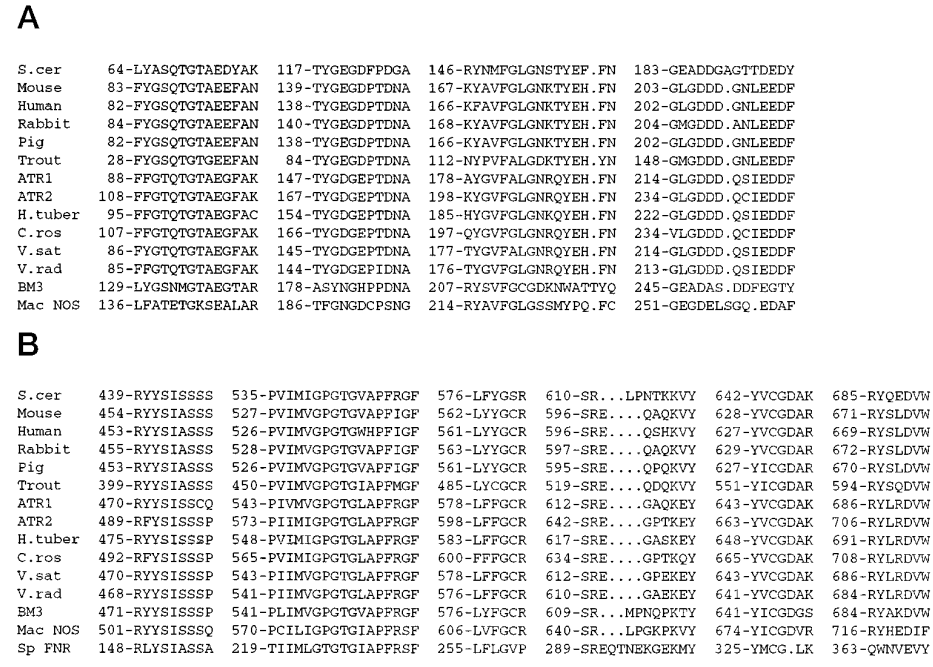


FIG. 4. **Conserved regions in the CPR family.** Shown are the eight peptide segments known to be involved in cofactors binding in comparison with other flavoenzymes of known three-dimensional structure. The first entry for each enzyme gives the residue number for the first amino acid in each segment. *A*, sequence alignments in the FMN-binding domain. *B*, sequence alignments in the FAD- and NADPH-binding domains. The compared sequences are, from *top* to *bottom*, *S. cerevisiae* (*S.cer*; GenBank™ D13788); three mammalian CPRs (mouse, human (GenBank™ S90469), and rabbit (GenBank™ X04610)); one insect CPR (fly (*Musca domestica*, GenBank™ L19897); one fish CPR (trout); six plant CPRs (the *Arabidopsis* ATR1 and ATR2, HTR1 from Jerusalem artichoke (*H. tuberosus*, GenBank™ Z26250), *Catharanthus roseus* from Madagascar periwinkle (*C.ros*, GenBank™ X69791), *Vicia sativa* from broad bean (*V.sat*, GenBank™ Z26252), and *Vigna radiata* from Mung bean (*V.rad*, GenBank™ L07843)); two flavoprotein domains (that of cytochrome P450 BM3, a soluble bacterial P450 involved in long chain hydrocarbon hydroxylation (BM3, GenBank™ J04832), and that of the murine macrophage nitric-oxide synthase (Mac NOS, GenBank™ M84373)); and spinach ferredoxin:NADP$^+$ reductase (*Sp FNR*, GenBank™ X07981).

the second ATG, corresponds to ATR2-1 depleted of this stretch of hydroxylated amino acid residues. Moreover, a second hydrophobic stretch that could serve as a membrane anchor is found at the N-terminal side of the canonic ER membrane-anchoring element in both ATR2-1 and ATR2-2.

*Cloning and Sequencing of Arabidopsis CYP73A5*—Using as a probe the coding sequence of *Helianthus CYP73A1* (18), one pFL61 clone of the *Arabidopsis* library was selected by hybridization. The 1735-bp-long full-length cDNA contains an open reading frame of 1518 bp. The translation initiation site was assigned to the first ATG, since it is preceded in frame by a stop codon (from −37 to −39-positions). The 5′- and 3′-noncoding regions are 48 and 169 bp long, respectively (Fig. 6). This *Arabidopsis* cDNA encodes a protein of 505 amino acid residues with a calculated molecular mass of 57,751 Da, a value typical of eukaryotic P450s. The high sequence identity found between *Arabidopsis* and *Helianthus* CYP73s (82%) confirms that cinnamate 4-hydroxylase enzymes share highly conserved sequence.

*Characterizations and Evolution of ATR Genes*—A Southern blot of *Arabidopsis* genomic DNA digested with different restriction enzymes was probed with either *ATR1* or *ATR2* coding sequence (Fig. 7). With *ATR1* probe, a single band of 12.5 kb is observed with *Eco*RI digestion, a site absent in the cDNA, while two bands are observed with *Bam*HI digestion and three distinct bands with *Hin*dIII. On the other hand, with *ATR2* probe, two bands are observed with *Eco*RI and *Hin*dIII and only one with *Bam*HI digestion (absent from the cDNA). No cross-hybridization is observed. Therefore, the fact that a digestion gives in each case a single band strongly suggests that each *ATR* cDNA sequence originates from a single copy gene.

PCR amplification starting from *Arabidopsis* genomic DNA

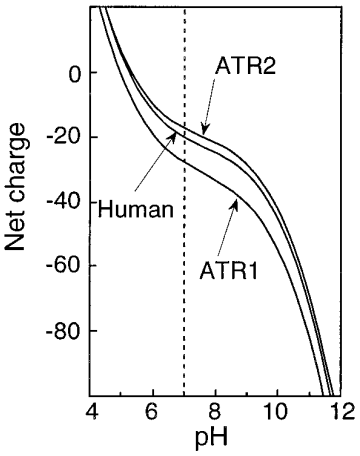


FIG. 5. **Computed net charge *versus* pH profiles of *Arabidopsis* CPRs.** Profiles shown are those of human CPR, ATR1, and ATR2 in the 4–12 pH range. The *broken line* highlights values at pH 7. The isoelectric program of the UWGCG software package was used.

and using the primers situated at both extremities of the open reading frames led to the amplification of a 3.9–4.0-kb fragment for *ATR1* and of a 3.5–3.6-kb fragment for *ATR2*. This result confirms the presence of a total of 1.8–1.9 kb and of 1.4–1.5 kb of intronic sequences in the *ATR1* and *ATR2* genes, respectively. The PCR amplification of *CYP73A5* DNA from *Arabidopsis* genomic DNA as a template using the two primers situated at both extremities of the *CYP73A5* coding sequence resulted in amplification of a 1.7–1.8-kb fragment, indicating the presence of a total of about 200 bp of intron sequence, which is a rather small amount.

Dating of the *ATR1/ATR2* gene divergence event was attempted by calculation of the level of identity (or ETBI value) between the third base of codons encoding conserved amino acid residues. A reference value (or RTBI value) was also calculated similarly by comparing all possible nucleotide sequences encoding ATR1/ATR2-1 conserved amino acid residues using the observed average codon usage as a ponderation factor. For ATR1/ATR2, ATR1/HSR1, and HSR1/SCR1 couples, Table II shows that ETBI and RTBI values are similar, as expected for distantly related genes (no memory of the third base usage). In contrast, ETBI and RTBI values still significantly differ for H1A1/H1A2 human P450s taken as a control. Such a difference is also detected for the comparison of *Helianthus* CPR cDNA (*HTR1*) (12) with *ATR2* but not with *ATR1*. The lower amino acid sequence identity observed between ATR1 and ATR2 (64%) than between ATR2 and HTR1 (73%) and the higher ETBI value for the HTR1/ATR2 couple therefore indicate that the ATR2 gene is probably more distant from the *ATR1* gene, although it belongs to the same species, than from the *Helianthus* HTR1-encoding gene (interspecies comparison).

To confirm these findings, a phylogenetic analysis was performed on CPR protein sequences using the CLUSTER algorithm and the BLOSUM62 sequence comparison matrix (32). The phylogenetic tree (not shown) revealed that plant CPRs clearly partition into two divergent subfamilies, one including *ATR1* and Mung bean CPR and the second clustering *ATR2* and all other plant CPR sequences available today. Interestingly, the two known *Helianthus* CPR sequences, the full-length *HTR1* and the partial length *HTR3* (which are 85% similar), cluster in the same subfamily together with *ATR2*. Thus, *Arabidopsis ATR1* and *ATR2* sequences constitute the first characterized presence of two distantly related CPR subfamilies in a single organism. These data highly suggest that the corresponding gene divergence probably occurred early during angiosperms evolution.

*High Level Expression of Arabidopsis CPRs in Yeast*—To optimize expression, the cDNAs encoding ATR1 and ATR2 were reformatted by deletion of their flanking regions and cloned in pYeDP60, placing the coding sequences under the transcriptional control of a *GAL10-CYC1* promoter. WRΔ cells were transformed by the resulting vectors, namely pATR1/V60, pATR2-1/V60, and pATR2-2/V60. Upon expression in strain WRΔ following galactose induction, the three *Arabidopsis* enzymes confer a resistance to ketoconazole at least equal to that observed with the original pFL61 clones. This result demonstrates that each ATR is fully competent to substitute for endogenous yeast CPR and particularly that both ATR2-1 and ATR2-2 are functional in coupling with yeast P450s. Thus, deletion of the poly(Ser/Thr) stretch in ATR2-2 has no detectable consequence on the subcellular location or function in yeast.

Fig. 8 shows that microsomes prepared from ATR-expressing yeasts present a dramatically reduced endogenous P450 content as compared with the WRΔ control. The high level of P450s found in the ER of WRΔ cells was shown previously to be induced by CPR deficiency and decreased upon yeast cytochrome $b_5$ overexpression (15). The expression of ATR1 or ATR2-1 in WRΔ thus reestablishes a wild type transcriptional regulation of endogenous P450s in CPR-deficient cells, indicating that both *Arabidopsis* CPRs efficiently substitute physiologically for the endogenous yeast enzyme.

Microsomal fractions prepared from transformed WRΔ cells were prepared and assayed for several NADH- and NADPH-dependent acceptor reduction reactions typical of the CPR enzymes (Table III). As a control, microsomes prepared from WRΔ cells transformed by a cDNA-free pYeDP60 exhibited very low NADPH-cytochrome *c* reductase activity. NADH does not support any ATR-dependent reductase activity. In contrast, a strong microsomal NADPH-cytochrome *c* reductase activity is observed for the three ATRs, with ATR2-1 exhibiting an unusually high activity (2840 nmol/min/mg) as compared with ATR1 and ATR2-2 (100 and 280 nmol/min/mg, respectively). These values are to be compared with a microsomal specific activity of 100 nmol/min/mg for human CPR overexpressed in WRΔ cells using the same vector system. This result indicates that the N-terminal poly(Ser/Thr) stretch of ATR2 is not essential for its CPR activity or ER targeting in yeast but significantly enhances the expressed ATR2-1 reductase activity as compared with ATR2-2. The $K_m$ value of ATR1 and ATR2-1 are, respectively, 12 ± 2 and 8 ± 1 μM for NADPH and 3 ± 1 and 3 ± 1 μM for cytochrome *c*. These values compare well with those reported for other plant CPRs (10, 27, 33, 34).

Analysis of the coupling between any ATR and a plant P450 requires coexpression in yeast. Genomic integration of the ATR1 and ATR2-1 expression cassettes at the *CPR1* locus was considered. For this purpose, ATR1 and ATR2-1 coding sequences were cloned into pYeDP110 integration vector, yielding pATR1/V110 and pATR2-1/V110. The pYeDP110 vector places the heterologous coding sequence under the transcriptional control of *GAL10-CYC1* promoter and *CPR1* terminator and provides sequences from both flanking regions of the *CPR1* gene that direct the integration at this locus, resulting in the deletion of most of the *CPR1* gene. The ATR1 and ATR2-1 coding sequences were integrated at the *CPR1* locus, resulting in two new yeast strains, WAT11 and WAT21, respectively. PCR analysis and Southern blotting confirmed that the full ATR expression cassette has been integrated within the *CPR1* locus of each selected clone (not shown).

For each WAT strain, a uracil auxotrophic mutant was selected on 5-fluoroorotate (23) and named WAT11U and WAT21U. When cultivated on glucose, WAT cells exhibit no

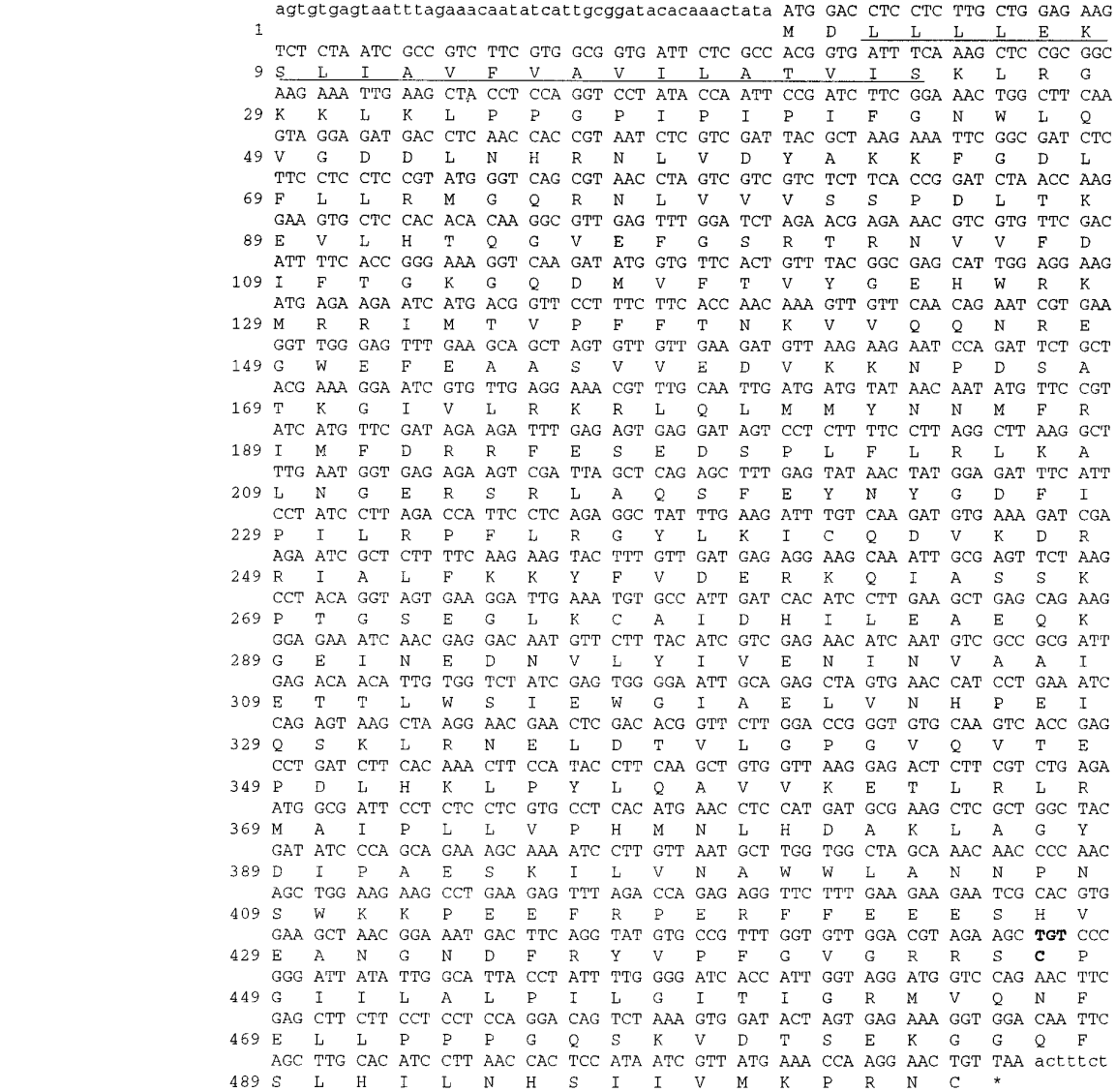


FIG. 6. **Nucleotide sequence and deduced amino acid sequence of *Arabidopsis* cinnamate 4-hydroxylase.** The deduced gene product is indicated in one-letter code *below* the DNA sequence. The 1735-bp-long cDNA encompasses a single open reading frame beginning at positions 49–51 and encoding a protein of 505 amino acids. A proposed motif for anchoring to the endoplasmic reticulum membrane is *underlined*. Numbers on the *left* indicate amino acid positions.

microsomal CPR activity and are phenotypically equivalent to WRΔ cells. But when grown on galactose, WAT11U and WAT21U cells express, respectively, microsomal ATR1 and ATR2-1 from the integrated expression cassette. Whether on glucose or in galactose, WAT cells express no yeast CPR. The generation time on galactose of the engineered strains was compared with that of the W(R) strain, which overexpresses yeast CPR, and no difference was observed. On the other hand, WAT11U and W(R) cells grown on glucose have identical generation times, significantly longer than upon growth on galactose.

WAT11U and WAT21U cells were grown on galactose to induce ATR expressions. The microsomal NADPH-cytochrome *c* reductase activity was measured (Table IV). A single integrated copy of the ATR1 expression cassette in WAT11 allows a CPR expression level similar to the value observed with a multicopy plasmid. In contrast, the reductase activity is reduced about 10-fold in WAT21, as compared with the plasmid-based expression, reaching a figure similar to WAT11. This suggests that the observed difference between ATR2-1 and ATR2-2 expression levels on multicopy plasmid might be related to different plasmid copy numbers and not to intrinsic properties of these two CPRs. All of these strains are fully isogenic, with the exception of the modified *CPR1* locus. They can thus be used for comparing the effect of the CPR nature on any P450 catalytic properties.

*Coupling Arabidopsis CPR and Cinnamate 4-Hydroxylase Activities in Yeast*—The CYP73A5 coding sequence was PCR-amplified and cloned in pYeDP60. The resulting expression vector, pCYP73A5/V60, was used to transform W(R), WAT11U, and WAT21U. Coexpression of cinnamate 4-hydroxylase and *Arabidopsis* or yeast CPRs was initiated by galactose induction, and bioconversion was assayed by adding cinnamic acid directly into the culture medium. Rapid accumulation of *p*-coumarate was observed (see Table IV). In contrast, no detectable product formation was observed with the same strains transformed by a cDNA-free pYeDP60 vector. The rate of bioconversion was found to be roughly identical in the three tested

strains, suggesting that the three CPRs similarly support CYP73A5 *in vivo* activity in yeast.

Microsomal fractions of pCYP73A5/V60-transformed W(R), WAT11U, and WAT21U cells were collected, and carbon monoxide-induced difference spectra were recorded on dithionite-reduced yeast microsomes suspensions. Spectrally detectable P450 content was found to be approximately 200 pmol of CYP73A5/mg of microsomal protein in W(R), 100 pmol/mg for WAT11 and WAT21 strains, and undetectable (<10 pmol/mg) in control. These values compare well with those usually observed in microsomes of yeast expressing mammalian P450s. The addition of cinnamate to microsomes from CYP73A5-expressing W(R), WAT11, and WAT21 cells causes a spectral shift with a differential absorption peak centered at 389 nm and a trough at 423 nm (not shown), typical of a ligand-induced low spin to high spin transition of the P450 heme ferric iron.

Microsomes from CYP73A5-expressing yeast cells were assayed for NADPH-dependent CYP73A5-catalyzed cinnamate and naphthoate hydroxylations. These reactions were found to be strictly NADPH-dependent and were not supported by NADH. Table IV lists the apparent kinetic parameters at steady state for the two substrates of the recombinant CYP73A5 in each of the three microsomal redox environments. Whatever the nature of the CPR, yeast or *Arabidopsis*, the turnover of the recombinant CYP73A5 was very high, reaching 470 $min^{-1}$ in W(R). Although the level of NADPH cytochrome *c* reductase activity was 15-fold lower in WAT11 as compared with W(R), the cinnamate hydroxylase turnover number was only reduced 2.3-fold. Intermediate results between these two cases were obtained with WAT21. $K_m$ values of the P450 for the two substrates tested (cinnamate and naphthoate) were found to be identical whatever the strain (W(R), WAT11, or WAT21). These results suggest that ATR2-1 and ATR1 are similarly efficient in supporting *Arabidopsis* CYP73A5 activity when coexpressed in yeast.

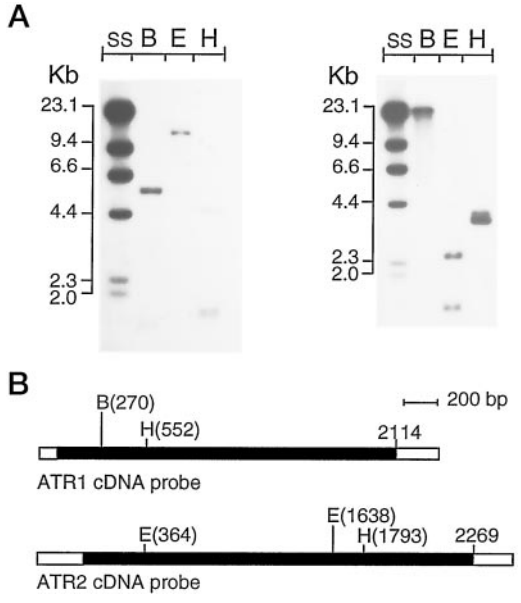


FIG. 7. **Southern analysis of *Arabidopsis* genomic DNA.** *A*, 10 μg of DNA were digested with *Bam*HI (*B*), *Eco*RI (*E*), and *Hin*dIII (*H*), transferred onto a nylon filter, and probed in stringent conditions with 150 ng of $^{32}$P-labeled ATR coding sequence obtained by *Not*I digestions from pUC19/ATR1 (*left*) or pUC19/ATR2 (*right*). Sizes of marker fragments (*lane ss*) are indicated in kb. *B*, restriction map of both ATR cDNAs for *Bam*HI, *Eco*RI, and *Hin*dIII. The *numbering* refers to the first bp of the cDNA. *Open rectangles* refer to noncoding regions, and *solid rectangles* refer to coding sequence.

## DISCUSSION

In this work, two *Arabidopsis* cDNAs, whose products increase the resistance of CPR-deficient yeast cells to the antifungal drug ketoconazole and reestablish a physiological expression level of yeast endogenous P450s, have been isolated. These cDNAs encode two distantly related (64% amino acid residue identity) NADPH-P450 reductases, ATR1 and ATR2, which are similarly found in the ER membrane when expressed in yeast. The two ATRs efficiently support the activity of P450 cinnamate 4-hydroxylase from the same plant when coexpressed in yeast. The use of a cloning procedure by metabolic interference allowed us to overcome the strict requirement of sequence similarity, which is critical for cDNA cloning by standard hybridization strategies. Southern blot analysis and sequence comparison in flanking regions of the cDNAs demonstrated that *ATR1* and *ATR2* correspond to two distinct genes, each existing as a single copy in the *Arabidopsis* genome. These CPRs are not close allelic variants but are encoded by distantly related genes as shown by the amino acid sequence divergence and the third base randomization in conserved codons. Similar cases have been found in several other *Arabidopsis* gene families and in particular with phenylalanine ammonia lyases,

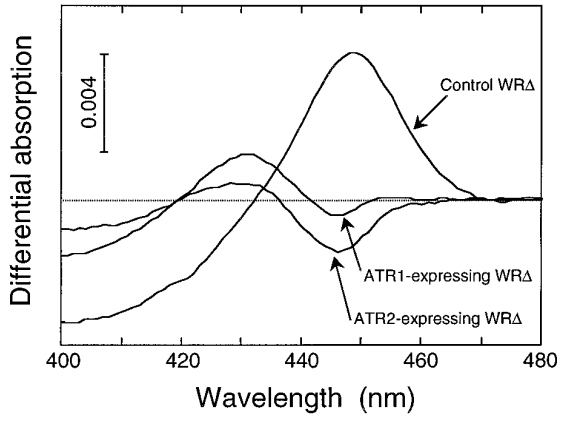


FIG. 8. **Carbon monoxide-induced difference spectra exhibited by dithionite-reduced microsomal fractions of transformed yeast cells.** WRΔ cells were transformed by a cDNA-free pYeDP60 vector (control yeast), by pATR1/V60 (ATR1-expressing), or by pATR2-1/V60 (ATR2-expressing). Cell culture was performed in SLI up to a density of 3 OD at 600 nm. Microsomal fractions were isolated, and the difference spectra were recorded using 1 mg of microsomal proteins/ml of 0.1 M Tris-HCl buffer, pH 7, containing an excess of sodium dithionite.

TABLE II
*Sequence comparisons*

The comparison was carried out on the amino acid sequences and on the open reading frames. The difference value is measured by subtracting the RTBI value from the ETBI. The higher this value is, the more recent is the divergence of the two compared genes. H1A1 and H1A2 are human CYP1A1 and CYP1A2.

| Compared sequences | ATR1 ATR2 | ATR1 HTR1 | ATR2 HTR1 | HSR1 SCR1 | HSR1 ATR1 or ATR2 | SCR1 ATR1 or ATR2 | H1A1 H1A2 |
|---|---|---|---|---|---|---|---|
| Amino acid identity (%) | 64 | 69 | 73 | 33 | 36 | 30 | 72 |
| ETBI | 46 | 47 | 54 | 44 | 34 | 38 | 77 |
| RTBI | 42 | 43 | 44 | 41 | 32 | 37 | 52 |
| Difference | 4 | 4 | 10 | 3 | 2 | 1 | 25 |

TABLE III

*Reductase activities catalyzed by Arabidopsis CPRs in microsomal fractions from transformed WRΔ cells*

Control, ATR1, ATR2–1, and ATR2–2, respectively, stand for cDNA-free pYeDP60-, pATR1/V60-, pATR2–1/V60-, and pATR2–2/V60-transformed WRΔ cultured in YPGE/Gal medium. The reductase activities were measured spectrophotometrically in 50 mM Tris HCl buffer, pH 7.4, containing 1 mM EDTA at room temperature. Data represent the average of values measured on microsomal fractions from five independent transformed clones.

| Donor | Acceptor | CPR activity | | | |
|---|---|---|---|---|---|
| | | Control | ATR1 | ATR2–1 | ATR2–2 |
| | | *nmol/min/mg microsomal protein* | | | |
| NADPH | Cytochrome *c* | 4 | 100 | 2840 | 280 |
| | DCPIP[a] | 20 | ND[b] | 1800 | 75 |
| | Ferricyanide | 100 | ND[b] | 3940 | 220 |
| NADH | Cytochrome *c* | 350 | 60 | 270 | 290 |
| | DCPIP | 320 | 330 | 220 | 310 |
| | Ferricyanide | 600 | ND[b] | 900 | 700 |

[a] DCPIP, dichlorophenolindophenol.
[b] ND, not determined.

TABLE IV

*Reconstitution of Arabidopsis cinnamate 4-hydroxylase activity in yeast*

The yeast strains were constructed as described under "Experimental Procedures." For bioconversions, the rate is expressed in molecules of the substrate transformed per min per cell. The microsomal fractions used for the *in vitro* assays were isolated from cells grown at high (YPGE/Gal) or low (SLI) cell density. Experiments were carried out in 50 mM Tris-HCl buffer, pH 7.4, and 37 °C. Protein concentration of pATCA4HV60-transformed yeast microsomes was 15–30 μg/ml depending on the assay. Data represent mean calculated from three independent clones. Turnover numbers for CYP73A5-catalyzed reactions are expressed in nmol/min/nmol P450.

| Strain | Type of CPR | Culture medium | CPR specific activity | Bioconversion rates of cinnamate | Kinetic parameters | | |
|---|---|---|---|---|---|---|---|
| | | | | | 2-Naphtoate ($K_m$) | Cinnamate | |
| | | | | | | $k_m$ | $k_{cat}$ |
| | | | | | $\mu M$ | $\mu M$ | $min^{-1}$ |
| W(R) | Yeast | SLI | 3000 | 3700 | 1.5 ± 0.3 | 8 ± 2 | 470 ± 30 |
| | Yeast | YPGE/Gal | 2500 | 3100 | ND[a] | ND | 420 ± 30 |
| WAT11 | ATR1 | SLI | 200 | 2400 | 1.1 ± 0.2 | 8 ± 2 | 200 ± 20 |
| | ATR1 | YPGE/Gal | 150 | 3100 | ND[a] | ND | 160 ± 30 |
| WAT21 | ATR2–1 | SLI | 250 | 3300 | 1.1 ± 0.3 | 10 ± 3 | 250 ± 20 |
| | ATR2–1 | YPGE/Gal | 190 | 4100 | ND[a] | ND | 150 ± 30 |

[a] ND, not determined.

phytochromes, and chalcone synthases (35–39).

The presence of multiple CPR genes in plants was also suggested by the multiplicity of bands detected in Western blots of CPR purified to homogeneity from Helianthus (11), sweet potato (10), and mung bean (27) and by the multiplicity of CPR peaks eluting from the 2′,5′-ADP-Sepharose column when purifying CPRs from periwinkle (28), tulip bulbs (33), and petunia flowers (34) with, in the latter case, an absence of antigenic cross-reaction between the proteins eluted in two peaks. In *Helianthus*, two partial length cDNAs probably encoding distinct CPRs have recently been cloned (12), but the phylogenetic comparison carried out in this work demonstrates that they cluster in the same plant CPR subfamily together with ATR2. In contrast, ATR1 belongs to the other plant CPR subfamily, clustering with the unique mung bean CPR sequence characterized to date. The fact that ATR2 appears to be more similar to *Helianthus* HTR1 and HTR3 than to ATR1 suggests that a parent common to both *ATR* genes would have existed before the separation of *Arabidopsis* (*Brassicaceae*) and *Helianthus* (*Asteraceae*). *Arabidopsis* is thus the first organism in which the cloning and function of CPR genes of two distinct subfamilies are achieved. In contrast, a single CPR-encoding gene has been systematically reported in lower eukaryotes, like fungi, and in mammals. This raises the question of the physiological role for two distant CPRs in higher plants. A hypothesis would be a differential expression of each ATR either during various stages of plant development or in different plant tissues. There are several known cases of multiple gene families that encode protein isoforms with different tissue localizations in *Arabidopsis* such as potassium channels isoforms (40), zinc finger proteins (41), and the myosin gene family (42). There are also examples of plant protein isoforms encoded by multiple gene families, which present differential expressions during development of the plant seedling such as maize manganese superoxide dismutases (43) and, of course, the *Arabidopsis* floral homeotic MADS-box regulatory gene family (44). In other cases, the members of a gene family exhibit both differential temporal and tissue-specific regulations, such as the *Arabidopsis* gibberellin aldehyde-induced transcripts (45).

Alignments reveal the presence in the two ATRs of all expected conserved FMN-, FAD- and NADPH-binding domains and thus are new members of the ferredoxin-$NADP^+$ reductase family of proteins (30, 31, 46). A very efficient cinnamate bioconversion is observed by co-expression in yeast of *ATR1* or *ATR2* and *Arabidopsis CYP73A5*. ATR1, ATR2, and yeast CPR each support rather similar CYP73A5 turnover numbers for cinnamate. Based on these data, the two ATRs would have virtually exchangeable roles. Nevertheless, the relative molar ratio between CPR and P450 expressed in yeast is 10-fold lower in the case of ATR expressions than in the case of yeast CPR in W(R) cells. The apparent equivalence between ATR1 and ATR2 to support cinnamate hydroxylase activity in yeast microsomes might thus be related to a saturation of CYP73A5 activity by high level of CPR. The W(R) or WAT engineered strains permit us to obtain turnover numbers for *Arabidopsis* and *Helianthus* CYP73s about 1000-fold higher than the value reported for *Medicago* CYP73 expressed in wild-type yeast (47). Similarly, expression in *E. coli* of a functional fusion protein consisting of the *Catharanthus* CYP73 fused at the N terminus of *Catharanthus* CPR, was recently reported (48). However, the CYP73 specific activity in this case was found 40-fold lower than in the present work. Functional analysis of the large number of plant P450-encoding cDNAs reported to date is thus expected to take advantage of the engineered WAT strains.

Almost no amino acid sequence similarity is found between the 100 first residues of ATR1 and ATR2-1, except for the presence of the hydrophobic stretch that is critical for the binding of CPRs to microsomal membrane (49). In ATR2-1, this stretch is preceded by a poly(Ser/Thr) N-terminal extension and an unusually long amphipatic sequence segment. This motif, which has no equivalent in any other fungal, plant, or animal CPRs, is highly reminiscent of a chloroplastic targeting sequence (50, 51), thus questioning the authentic subcellular location of ATR2-1 in plants. The observed addressing in yeast could thus be artifactual due to the absence of the plant-specific organites. The presence of P450s in chloroplast still remains debatable, but very recently it has been shown that *Linum* allene oxide synthase, CYP74, presents a typical N-terminal chloroplastic transit peptide (52). Moreover, it is highly suggested that the gibberellin biosynthesis pathway, which produces phytohormones, is localized at least partially in the chloroplast (Ref. 53 and references therein). *ent*-kaurene synthesis, from which all gibberellins derive, has been clearly established to take place in the chloroplast (54, 55). Nevertheless, the subcellular location in plants of the P450-catalyzed steps of *ent*-kaurene and gibberellin oxidations is still debated (53, 56, 57). This leaves open the hypothesis that in *Arabidopsis* chloroplasts could house some P450-catalyzed reactions with ATR2-1 as a chloroplastic CPR. In this hypothesis, ATR1 would be the *Arabidopsis* microsomal counterpart. A similar, but clearly established situation, has been found with *Arabidopsis* signal recognition particles. Distinct genes have been shown to encode two SRP54p isoforms, one cytosolic (58) and the other chloroplastic (59). This raises the question of the occurrence in plants of chloroplast-specific CPR and P450s whose physiological roles remain to be fully investigated.

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