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 monoxygenases 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). Conclusion 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 multi-copy 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-

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, hememolate protein P450; PGK, phosphoglycerate kinase; PCR, polymerase chain reaction; ETBI, experimental third base identity; RTBI, reference third base identity.

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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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sis cinnamate 4-hydroxyase 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 \textit{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\textsuperscript{TM} (version 2.0) DNA sequencing kit was purchased from U.S. Biochemical Corp. The pCRScript\textsuperscript{TM} 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 (MATa, ade2–1; his3–11, 15; leu2–3, –112; ura3–1; trpl–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 \textit{GAL10}-\textit{CYC1}. W(R) derives from W(N) by disruption of the CPR1 gene with a \textit{TRPI} selection marker (17).

The pFL61 vector has been previously described (16). The yeast expression vector pYeDP60 (V60) contains both \textit{URA3} and \textit{ADE2} as selection markers, and an expression cassette constituted by \textit{GAL10-CYC1} promoter and \textit{PGK} terminator sequences surrounding a \textit{cDNA} insertion polynucleotide (19). The \textit{pGp1} vector contains a 5500-bp-long fragment that encompasses the full \textit{CPR1} gene at the \textit{HindIII} site of the \textit{pUC19}. The \textit{pYeDP51} yeast expression vector is identical to pYeDP6/8-2 (20), except that the \textit{PvuII} site close to the yeast origin of replication is replaced by a \textit{BglII} site.

Using ORS1 and ORS22 as primers (Table I) and pGP1 as a template, the 428-bp-long \textit{CPR1} gene terminator sequence was PCR-amplified and cloned into the \textit{SmaI} site of \textit{pUC19}. The 420-bp-long EcorI-\textit{PvuII} fragment of the resulting vector was ligated to the EcoR1-\textit{PvuII} fragment of \textit{pYeDP51} encompassing \textit{URA3}. The 6900-bp-long resulting vector, named \textit{p YE DP100}, places the inserted \textit{cDNA} under the transcriptional control of \textit{GAL10-CYC1}-inducible promoter and \textit{CPR1} terminator. By PCR amplification using ORS33 and ORS34 primers and \textit{pGp1} as a template, a 631-bp-long \textit{HindIII-BglII} fragment of the upstream part of the \textit{CPR1} promoter has been obtained and cloned in \textit{pUC19}. The \textit{HindIII-BglII} fragment of the \textit{CPR1} promoter was ligated with the larger \textit{HindIII-BglII} fragment of \textit{pYeDP100}, which encompasses \textit{URA3}, yielding the 5210-bp-long \textit{pYeDP110} integrative vector. The \textit{pYeDP110} vector bears \textit{GAL10-CYC1} promoter and \textit{CPR1} terminator sandwiched a \textit{cDNA} insertion polynucleotide, and the \textit{CPR1} 5‘-noncoding region placed upstream of the \textit{URA3} selection marker. The entire integrative fragment can be removed by a \textit{NotI} digestion. The respective orientation of the two \textit{CPR1} noncoding regions allows targeted integration of the expression cassette at the \textit{CPR1} locus by homologous recombination.

**Cell Culture**—Transformation of yeast strains was performed by a modified lithium acetate procedure as described (21). E.coli cells were transformed by the lithium acetate method (22). The transformed cells were selected on glucose-containing SGI plates. Culture media, cell cultures, and galactose induction procedures of individual clones in both SLI and YPGF were as described previously (17, 22).

**Screening of the Arabidopsis cDNA Expression Library in Yeast**—A sized (inserts >2 kb) \textit{cDNA} library from \textit{Arabidopsis thaliana} seeds including roots (two-leaf stage) in \textit{pFL61} yeast expression vector was kindly provided by Dr. M. Minet. In \textit{pFL61}, cDNAs were inserted as \textit{NotI} cassettes between the yeast \textit{PGK} promoter and terminator (16). Yeast W(R) cells were grown on galactose, thus overexpressing yeast CPR, up to a cell density of 2 × 10\textsuperscript{7} cells/ml. Cells are harvested and resuspended at a cell density of 10\textsuperscript{8} cells/ml in a glucose-containing YPGA medium, thus turning off \textit{CPR1} gene expression. \textit{W(R)} cells were further grown on glucose for 5 h until the microsomal CPR activity decreases to the value found in wild-type \textit{W303–1B} cells. About 10\textsuperscript{6} cells were lithium-treated and used for transformation by 10 \mu g of the \textit{Arabidopsis} cDNA library, 0.2 mg of heat-denatured herring sperm DNA in 2 ml of 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 0.1% LiCl. After a 10-min incubation at room temperature, 5 ml of the same buffer containing 50% of polyethylene 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 SGA1 synthetic medium for uracil prototrophy selection.

Two pools of \textit{Ura}+ transformants were constituted, and cells from each pool were sprayed at 10\textsuperscript{4} cells/plate onto a series of SGA1 plates containing increasing concentrations of ketoconazole (0.5, 10, 20, and 50 \mu g/ml ketoconazole) and incubated for 36 h at 28 °C. The 52 clones found to be resistant to 10 and 20 \mu g/ml ketoconazole were striped on SGA1 medium containing 10 \mu g/ml ketoconazole. The plasmidic DNA of 24 of the resistant clones was rescued in \textit{Escherichia coli}. The \textit{Bar} and \textit{HindIII} digestion patterns revealed five different restriction profiles (profiles A–E).

**Isolation of Arabidopsis CYP73A5-encoding cDNA**—The \textit{Arabidopsis} cDNA library in \textit{pFL61} was transformed in \textit{E. coli} by electroporation. About 10\textsuperscript{5} independent clones were recovered and transferred on nitrocellulose filters. The blots were hybridized by denaturing \textit{SSC} × 6 2× \textit{Denatured DNA Sequence Determination—The NotI} cDNA inserts encoding \textit{ATR1} and \textit{ATR2} were extracted from \textit{pFL61} and subcloned into the unique \textit{NotI} site of a derivative of \textit{pUC9}. For \textit{ATR1} and \textit{ATR2} cDNAs, sequencing was carried out with the \textit{M13} universal and \textit{ATR1} or \textit{ATR2} as template. These primers introduce a \textit{BglII} site immediately upstream of the \textit{ATG} codon and a \textit{EcoRI} site downstream of the stop codon of the \textit{PCR}-amplified fragment. The 2139-bp-long

**Table I**

<table>
<thead>
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<th>Primer name</th>
<th>Sequence</th>
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<tr>
<td>ATCH1-5</td>
<td>ggacccATGACTCCCTCTTCTGGCC</td>
</tr>
<tr>
<td>ATCH1-3</td>
<td>ggccacctTTAAGCTTTCCTTCTGGCC</td>
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<tr>
<td>ORS21</td>
<td>ccgtaagccATGAACTTTCCTTCTGGCC</td>
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<td>ORSS2</td>
<td>cggattctcGACCAGACCTCCCTGGATCCAC</td>
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<td>ORS23</td>
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<tr>
<td>ORS25</td>
<td>cggattctcGACCAGACCTCCCTGGATCCAC</td>
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<tr>
<td>ORS31</td>
<td>cggaaacctccacctgcggccqcgATGATTGTCCTTCTGAGATGCAACCCGGTGG</td>
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<td>cggaaacctTAATCGAGCCCACTGACAGGTTCC</td>
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<td>ORS33</td>
<td>cggaaacctcGACCAGACCTCCCTGGATCCAC</td>
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<td>gcggaaacctcGACCAGACCTCCCTGGATCCAC</td>
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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 BamHI 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 SmaI site of pUC19, giving, respectively, pUC19/ATR1, pUC19/ATR2-1, and pUC19/ATR2-2. The ATR1 coding sequence was then excised by BglII-EcoRI digestion and inserted into pYeDP60 linearized at BamHI and EcoRI unique sites, resulting in plasmid pATR1/V60. The ATR2-1 and ATR2-2 coding sequences were excised by BamHI digestion and inserted into pYeDP60 linearized at BamHI/EcoRI, 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 SrfI site of PCRScript vector, giving pCRScript/ATC4H. The primers introduce a BamHI site immediately upstream of the ATG codon and a BglII site downstream of the stop codon. The coding sequence was excised by BamHI/BglII digestion and inserted at the BamHI site of pYeDP60 and a clone with the correct orientation was selected and named pATC4H/V60.

Expression 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 BamHI-EcoRI and BamHI-BglII fragments, respectively. The ATR1 fragment was inserted at the unique BamHI site of pYeDP110, yielding pATR1/DP110. Similarly, the ATR2-1 fragment was inserted at the unique BamHI site of pYeDP110, and a clone with the proper orientation was selected and named pATR2-1/DP110. The Norl fragment of each plasmid encompassing URA3 and the ATR coding sequence was isolated and used to transform W3A cells. Since in W3A the CPR1 locus is disrupted with the TRP1 gene, integration events result in a phenotype shift from Ura- to Ura+. 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 ura3-52 by selecting 5-fluoroorotate-resistant clones (23) giving WAT11U and WAT21U strains.

Subcellular Fractionation and P450 Quantitation—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 absorbance at 440 nm. The rate of cytochrome c oxidase reduction was measured on microsomal fractions prepared as described in Refs. 17 and 22.

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 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 SGA1 plates containing increasing ketoconazole concentrations. Fifty-two colonies were sorted out as resistant to 10 and 20 μg/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 WRA, 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 μg/ml to WRΔ. Partial cDNA sequencing was used for further identification and showed that classes A, C, and E (∼2300-bp-long cDNA Norl fragment) contain related inserts differing only by their orientation and their 5’- and 3’-noncoding exones when class B (2199-bp-long Norl 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_0 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.
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. 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.
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. 2

**Arabidopsis NADPH-P450 Reductase Isoforms**

![Figure 2](http://www.jbc.org/)

**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.
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 237 to 239-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 EcoRI digestion, a site absent in the cDNA, while two bands are observed with BamHI digestion and three distinct bands with HindIII. On the other hand, with ATR2 probe, two bands are observed with EcoRI and HindIII and only one with BamHI 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
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.

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. WRA cells were transformed by the resulting vectors, namely pATR1/V60, pATR2-1/V60, and pATR2-2/V60. Upon expression in strain WRA 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 WRA 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 bs overexpression (15). The expression of ATR1 or ATR2-1 in WRA 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 WRA 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 WRA 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 Km values 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-fluoro-orotate (23) and named WAT11U and WAT21U. When cultivated on glucose, WAT cells exhibit no...
microsomal CPR activity and are phenotypically equivalent to WR 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

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.
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 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 hydrolations. These reactions were found to be strictly NADPH-dependent and were not supported by NADH. Table IV lists the apparent kinetic parameters at 470 min 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. Kₚ 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.

**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.

**Figure 7.** Southern analysis of Arabidopsis genomic DNA. A. 10 μg of DNA were digested with BamHI (B), EcoRI (E), and HindIII (H), transferred onto a nylon filter, and probed in stringent conditions with 150 ng of 32P-labeled ATR coding sequence obtained by Not1 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 BamHI, EcoRI, and HindIII. The numbering refers to the first bp of the cDNA. Open rectangles refer to noncoding regions, and solid rectangles refer to coding sequence.

**Figure 8.** Carbon monoxide-induced difference spectra exhibited by dithionite-reduced microsomal fractions of transformed yeast cells. WR3 cells were transformed by a cDNA-free pYeDP60 vector (control yeast), by pATR1/V60 (ATR1-expressing), or by pATR2/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 the value is, the more recent is the divergence of the two compared genes. H1A1 and H1A2 are human CYPIA1 and CYPIA2.

<table>
<thead>
<tr>
<th>Compared sequences</th>
<th>ATR1</th>
<th>ATR2</th>
<th>HTR1</th>
<th>HTR2</th>
<th>HSRI</th>
<th>HSRI</th>
<th>CR1</th>
<th>CR1</th>
<th>CR1</th>
<th>H1A1</th>
<th>H1A2</th>
</tr>
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<tbody>
<tr>
<td>Amino acid identity (%)</td>
<td>64</td>
<td>69</td>
<td>73</td>
<td>33</td>
<td>36</td>
<td>30</td>
<td>72</td>
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<td>47</td>
<td>54</td>
<td>44</td>
<td>34</td>
<td>38</td>
<td>77</td>
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<td>43</td>
<td>44</td>
<td>41</td>
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<td>37</td>
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<td>4</td>
<td>10</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>25</td>
<td></td>
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</tbody>
</table>
transformed yeast microsomes was 15–30 nmol/min/mg microsomal protein. The numbers for CYP73A5-catalyzed reactions are expressed in nmol/min/nmol P450. 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/mmol P450.

### TABLE III

<table>
<thead>
<tr>
<th>Donor Acceptor</th>
<th>Control</th>
<th>ATR1</th>
<th>ATR2–1</th>
<th>ATR2–2</th>
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<tbody>
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<td>100</td>
<td>2840</td>
<td>280</td>
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<tr>
<td>NADPH DCPIP</td>
<td>20</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1800</td>
<td>75</td>
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<tr>
<td>NADH Ferricyanide</td>
<td>100</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>220</td>
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<td>280</td>
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<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>900</td>
<td>700</td>
</tr>
</tbody>
</table>

<sup>a</sup> DCPIP, dichlorophenolindophenol.  
<sup>b</sup> ND, not determined.

### TABLE IV

The yeast strains were constructed as described under “Experimental Procedures.” For bioconversions, the rate is expressed in molecules of the donor acceptor, 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 FMM-, FAD- and NADPH-binding domains and thus are new members of the ferredoxin-NADP<sup>+</sup> 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 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 organelles. 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 (53) and an unusually long amphipatic sequence segment. This stretch is preceded by a poly(Ser/Thr) N-terminal extension.

The 100 first residues of ATR1 and ATR2-1, except for the ent-kaurene and gibberellin oxidations is still debated (53, 56, 57). Nevertheless, the subcellular location in plants of the P450-catalyzed steps of phytohormones, is localized at least partially in the chloroplast (54, 55). Nevertheless, the subcellular location in plants of the P450-catalyzed steps of phytohormones, is localized at least partially in the chloroplast (Ref. 53 and references therein). The observed addressing in yeast could thus be artifactual due to the absence of the plant-specific organelles. 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 (53) and an unusually long amphipatic sequence segment. This stretch is preceded by a poly(Ser/Thr) N-terminal extension.

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Cloning, Yeast Expression, and Characterization of the Coupling of Two Distantly Related Arabidopsis thalianaNADPH-Cytochrome P450 Reductases with P450 CYP73A5

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