Cytochrome P450 CYP79A2 from *Arabidopsis thaliana* L. Catalyzes the Conversion of L-Phenylalanine to Phenylacetaldoxime in the Biosynthesis of Benzylglucosinolate*

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Glucosinolates are natural plant products gaining increasing interest as cancer-preventing agents and crop protectants. Similar to cyanogenic glucosides, glucosinolates are derived from amino acids and have aldoximes as intermediates. We report cloning and characterization of cytochrome P450 CYP79A2 involved in aldoxime formation in the glucosinolate-producing *Arabidopsis thaliana* L. The CYP79A2 cDNA was cloned by polymerase chain reaction, and CYP79A2 was functionally expressed in *Escherichia coli*. Characterization of the recombinant protein shows that CYP79A2 is an N-hydroxylase converting L-phenylalanine into phenylacetaldoxime, the precursor of benzylglucosinolate. Transgenic *A. thaliana* constitutively expressing CYP79A2 accumulate high levels of benzylglucosinolate. CYP79A2 expressed in *E. coli* has a $K_{m}$ of 6.7 µmol liter$^{-1}$ for L-phenylalanine. Neither L-tyrosine, L-tryptophan, L-methionine, nor DL-homophenylalanine are metabolized by CYP79A2, indicating that the enzyme has a narrow substrate specificity. CYP79A2 is the first enzyme shown to catalyze the conversion of an amino acid to the aldoxime in the biosynthesis of glucosinolates. Our data provide the first conclusive evidence that evolutionarily conserved cytochromes P450 catalyze this step common for the biosynthetic pathways of glucosinolates and cyanogenic glucosides. This strongly indicates that the biosynthesis of glucosinolates has evolved based on a cyanogenic predisposition.
cytochrome P450-independent and suggested to be catalyzed by flavin-containing monooxygenases (16–19). The formation of indole-3-acetaldoxime from tryptophan in the biosynthesis of indoleglucosinolates has been shown to be catalyzed by a plasma membrane-bound peroxidase in microsomes from Chinese cabbage (Brassica campestris ssp. pekinensis) (20).

In the biosynthesis of cyanogenic glucosides, cytochromes P450 of the CYP79 family catalyze the formation of aldoximes from amino acids (21–24). Several CYP79 homologues have previously been identified in glucosinolate-producing plants (25), but their function has never been determined. In the present paper, we report cloning and functional expression of the cytochrome P450 CYP79A2 from A. thaliana. We show that CYP79A2 catalyzes the conversion of L-phenylalanine to phenylacetaldoxime and that transgenic A. thaliana expressing CYP79A2 under control of CaMV35S1 promoter accumulate high levels of benzylglucosinolate. Our data are consistent with the involvement of CYP79A2 in the biosynthesis of benzylglucosinolate in A. thaliana.

**EXPERIMENTAL PROCEDURES**

**PCR Primers**—The following PCR primers (Fig. 1) were designed from the genomic CYP79A2 sequence (GenBank accession no. AB010692; Arabidopsis thaliana L. cv. Columbia) (add restriction sites are underlined, sequence encoding CYP71A7 is indicated by italics): A2F1 (5′-GTCGATACATGTTGACCTACCCGACG-3′), A2R1 (5′-ATGAATCTTCTAGAATATGTCG-3′), A2F2 (5′-CGCTCGATGCTTGCCTGATCTTACCTCTGATGACCGCAGATGTTTATATAGGTTTTCTCCTCCTGATCAATGACTG-3′), A2R2 (5′-CGAGCAAGTTAATGGTTTGATACACATGTA-3′), A2R3 (5′-GTCGATGCTCTGGATGATACACATGTA-3′), A2F3 (5′-GACTAAGTTGGGCCGCAATGTTG-3′), A2F1X (5′-CGCGAATTCCGTCGATCAGTTTACCTCTGATGACCGCAGATGTTTATATAGGTTTTCTCCTCCTGATCAATGACTG-3′), A2R4 (5′-CATCTGCGTCTTCCTAGCTGCCTTACCTCTGATGACCGCAGATGTTTATATAGGTTTTCTCCTCCTGATCAATGACTG-3′). In addition, the following primers were used: 17AF (5′-CGCGAATTCCGTCGATCAGTTTACCTCTGATGACCGCAGATGTTTATATAGGTTTTCTCCTCCTGATCAATGACTG-3′), 17AR (5′-GGGCCACGGCAATATGTAAGTTGTTTTGTTATATAGGTTTTCTCCTCCTGATCAATGACTG-3′).

dDNA Cloning—PCR was performed on phage DNA representing 2.5 × 106 plaque-forming units of the A. thaliana L. (cv. Wassilewskija) silique cDNA library CD4–12 (kindly provided by Dr. Linda A. Castle and Dr. David W. Meinke, Department of Botany, Oklahoma State University, Stillwater, OK, and ABRC) using primers A2F1/A2R1. PCR reactions were set up in a total volume of 50 µl in Expand HI buffer with 1.5 mM MgCl2 (Roche Molecular Biochemicals) supplemented with 200 µM dNTPs, 50 pmol of each primer, and 5% (v/v) Me2SO. After incubation of the reactions at 97 °C for 3 min, 30 PCR cycles of 20 s at 94 °C, 10 s at 60 °C, and 30 s at 72 °C were run. After digestion of the PCR fragments with EcoRI (exon 1) and HindIII (exon 2), the blunt ends were ligated with primers A2F3 and A2F2, respectively, and phosphorylated with T4 polynucleotide kinase (New England Biolabs). The two clones were then ligated into EcoRI/HindIII-digested pX223. The cloned cDNA was sequenced to exclude incorporation of PCR errors.

Four expression constructs (Fig. 2) were made in the expression vector pBluescriptSK (Ref. 27; kindly provided by Dr. Henry Barnes, Synthetic Genetics/Immune Complex Inc., San Diego, CA); 79A2 (“native”), 17A1–879A2 (“modified”), and 17A1–879A1–75–79A2D1–40 (“chimeric”) (79A2: CYP79A2; 17A1–879A2, modified; 17A1–879A1–75–79A2D1–40) (chimeric) (79A2: CYP79A2; 17A1–879A2, modified; 17A1–879A1–75–79A2D1–40). The N termini were modified by introducing PCR fragments from the ATG start codon to the PstI site of the CYP79A2 cDNA (Fig. 1). These fragments were ligated with the PstI/HindIII fragment of the CYP79A2 cDNA (Fig. 1) and EcoRI/HindIII-digested pYX223. For the modified and the truncated modified CYP79A2, primers A2F3X1/A2R2 and A2F3X2/A2R4, respectively, were used. The fusion with the N terminus of CYP79A1 was made by blunt-end ligation of a PCR fragment containing the CYP79A1 (1–25) sequence (GenBank accession no. AB010692) with primers 17AF/17AR with a PCR fragment generated from the CYP79A2 cDNA with primers A2F3X3/A2R4. PCR products were cloned and sequenced to exclude incorporation of PCR errors. The different CYP79A2 cDNAs were excised from pYX223 by digestion with NdeI and HindIII and ligated into NdeI/HindIII-digested pSP19g10L.

**Expression in Escherichia coli—**E. coli cells of strain JM109 transformed with the expression constructs were grown overnight in LB medium supplemented with 100 µg ml−1 ampicillin and used to inoculate 100 ml of modified TB medium containing 50 µg ml−1 ampicillin, 1 ml thiamine, 75 µg ml−1 3-aminolevulinic acid, and 1 ml isopropl-β-D-thioglucosidase (28). The cells were grown at 28 °C for 65 h at 125 rpm. Cells from 75 ml of culture were pelleted and resuspended in buffer composed of 0.1 M Tris-HCl, pH 7.5, 0.5 mM EDTA, 250 mM sucrose, and 250 µM phenylmethylsulfonyl fluoride. Lysozyme was added to a final concentration of 100 µg ml−1. After incubation for 30 min at 4 °C, magnesium acetate was added to a final concentration of 10 mM. Spheroplasts were pelleted, resuspended in 5 ml of buffer composed of 10 mM Tris-HCl, pH 7.5, 14 mM magnesium acetate, and 60 mM potassium phosphate, pH 7.4, and homogenized in a Potter-Evah姆 homogenizer. After DNAse and RNase treatment, glycerol was added to a final concentration of 29%. Temperature-induced Triton X-114 phase partitioning was performed as described previously (29). The Triton X-114-rich phase was analyzed by SDS-polyacrylamide gel electrophoresis.

**Co Difference Spectroscopy—**Fe2+–CO versus Fe2+–O2 difference spectroscopy (30) was performed on 100 µl of E. coli spheroplasts resuspended in 900 µl of buffer containing 50 mM KPi, pH 7.5, 1 mM Mg2+, 20% (v/v) glycerol, 0.2% (v/v) Triton X-100, and a few grains of sodium dithionite. The solubilize was distributed between two cuvettes, and a base line was recorded between 400 and 500 nm on a SLM Aminco DW-2000 TM spectrophotometer (SLM Instruments, Urbana, IL). The sample cuvette was flushed with CO for 1 min, and the difference spectrum was recorded. The amount of functional cytochrome P450 was estimated, based on an absorption coefficient of 91.1 mmol−1 cm−1.

**Measurements of Enzyme Activities—**The activity of CYP79A2 was measured in E. coli spheroplasts reconstituted with NADPH:cytochrome P450 oxidoreductase purified from Sorghum bicolor (L.) Moench as described previously (21). In a typical enzyme assay, 5 µl of spheroplasts and 4 µl of NADPH:cytochrome P450 reductase (equivalent to 0.4 units defined as 1 µmol of cytochrome c min−1) were incubated with 3.3 µl [U-14C]phenylalanine (453 mCi mmol−1) in buffer containing 30 mM KP, pH 7.5, 4 mM NADPH, 3 mM reduced glutathione, 0.042% (v/v) Tween 80, and 1 mg ml−1 1,6-dialkyroxy phosphatidylcholine in a total volume of 30 µl. To study substrate specificity, 3.7 µl [U-14C]tyrosine (449 mCi mmol−1), 0.1 µl [methyl-14C]methionine (56 mCi mmol−1), and 1 µl [U-3H]thiopropionate (33 Ci m−1) were used instead of [U-14C]phenylalanine. After incubation at 26 °C for 4 h, half of the reaction mixture was analyzed by thin layer chromatography on Silica Gel 60 F254 sheets (Merck) using toluene:ethyl acetate (5:1, v/v) as eluent. Radioactive bands (14C) were visualized and quantified by STORM 840 Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA). Radioactive bands (1H) were visualized by autoradiography. Product formation from L-[U-
using 0.005% (v/v) Silwet L-77 and 5% (w/v) sucrose in 10 mM MgCl2. With this construct was used for plant transformation by floral dip (34) Agrobacterium tumefaciens pPZP111 (32).

HindII, EcoRI, and Sacl fragmentations with 200 °C. The retention times of the (Z)-isomers of phenylacetolide were 12:43 and 13.06 min. The two isomers had identical fragmentation patterns with m/z 135, 117, and 91 as the most prominent peaks. Radio-labeled amino acids were purchased from Amersham Pharmacia Biotech. Homophenylalanine and 3-phenylpropanaldehyde were kindly provided by Prof. John A. Pickett (IACR Rothamsted, Rothamsted, United Kingdom), and phenylacetolide (mixture of the (E)- and (Z)-isomers) was a gift from Dr. Jens Lykkjesfeldt (KVL, Copenhagen, Denmark).

Generation of Transgenic Plants—A. thaliana L. cv. Columbia was used for all experiments. Plants were grown in a controlled-environment Arabidopsis Chamber (Perivac AR-60 I, Boone, IA) at a photoperiod of 12 h for plants used for transformation and 8 h for plants used for biochemical analysis.

For expression of CYP79A2 under control of the CalMV35S promoter in A. thaliana, the native full-length CYP79A2 cDNA was introduced into EcoRI/KpnI-digested pRT101 (31) via several subcloning steps. The expression cassette was excised by HindIII digestion and transferred to pPZP111 (32). Agrobacterium tumefaciens strain C58 (33) transformed with this construct was used for plant transformation by floral dip (34) using 0.005% (v/v) Silwet L-77 and 5% (w/v) sucrose in 10 mM MgCl2. Seeds were germinated on Murashige and Skoog medium supplemented with 50 µg ml−1 kanamycin, 2% (w/v) sucrose, and 0.9% (w/v) agar. Transforms were selected after 2 weeks and transferred to soil.

HPLC Analysis of Plant Extracts—Rosette leaves (five to eight leaves of different age from each plant) were harvested from 6-week-old plants (nine transgenic plants and three wild-type plants), immediately frozen in liquid nitrogen and freeze-dried for 48 h. Desulfoglucosinolates were isolated as described in Ref. 35. Briefly, freeze-dried material (50–200 mg) was homogenized in 3.5 ml of boiling 70% (v/v) methanol by a Polytron homogenizer. Plant material was pelleted, washed in liquid nitrogen and freeze-dried for 48 h. Desulfoglucosinolates were extracted and subjected to HPLC analysis as described above.

For expression of CYP79A2 in E. coli—CYP79A2 was expressed in E. coli strain JM109 by use of the expression vector pSP19g10L and cDNAs encoding the native CYP79A2 or three N-terminally modified CYP79A2 (Fig. 2). The expression vector pSP19g10L contains the lacZ promoter fused with the short leader sequence (g10L) of gene 10 from T7 bacteriophage. The g10L sequence was derived previously from the expression of various heterologous proteins (40). N-terminal modifications of CYP79A2 were designed to apply previously obtained expression results for high-level expression of eukaryotic cytochromes P450 in E. coli (28, 29). Two constructs were made to introduce the eight N-terminal amino acids of the bovine cytochrome P450 CYP17A in front of the N-terminus of CYP79A2 (yielding modified CYP79A2) and a truncation of CYP79A2 from A. thaliana L. 14661

CYP79A2 and CYP79A1 cDNAs were amplified by RT-PCR from total RNA following poly(A) selection using a Superscript III First-Strand Synthesis Kit (GIBCO BRL). The PCR products were cloned into a Bluescript SK+ vector. The N-terminal protein sequence for the expression of various heterologous proteins (40) was used as an excellent leader sequence for the expression of heterologous proteins (40). N-terminal modifications of CYP79A2 were designed to apply previously obtained expression results for high-level expression of eukaryotic cytochromes P450 in E. coli (28, 29). Two constructs were made to introduce the eight N-terminal amino acids of the bovine cytochrome P450 CYP17A in front of the N-terminus of CYP79A2 (yielding modified CYP79A2) and a truncation of CYP79A2 from A. thaliana L. 14661

14C phenylalanine was linear with time within the first 2 h of incubation as determined by using time points 30 min, 1 h, 2 h, and 6 h. For estimation of Km and Vmax values, reaction mixtures were incubated for 2 h at 26 °C. For GC-MS analysis, 450 µl of reaction mixture containing 33 µM l-phenylalanine (Sigma) or 33 µM homophenylalanine were incubated at 30 °C for 60 min and extracted with 5% (v/v) chloroform (Normapur, May and Baker). The organic phases were combined and evaporated to dryness. The residue was dissolved in 15 µl of chloroform and analyzed by GC-MS. GC-MS analysis was performed on an HP5980 Series II gas chromatograph directly coupled to a Jeol JMS-AX503W mass spectrometer. An 85E column (BPX5, 25 m × 0.25 mm, 0.25 µm- thick (film thickness was used (head pressure, 17 psig, 170 °C splitless injection). The oven temperature program was as follows: 80 °C for 3 min, 80 °C to 180 °C at 5 °C min−1, 180 °C to 300 °C at 20 °C min−1, 300 °C for 10 min. The ion source was run in EI mode (70 eV) at 200 °C. The retention times of the (E)- and (Z)-isomers of phenylacetolide were 12:43 and 13.06 min. The two isomers had identical fragmentation patterns with m/z 135, 117, and 91 as the most prominent peaks. Radio-labeled amino acids were purchased from Amersham Pharmacia Biotech. Homophenylalanine and 3-phenylpropanaldehyde were kindly provided by Prof. John A. Pickett (IACR Rothamsted, Rothamsted, United Kingdom), and phenylacetolide (mixture of the (E)- and (Z)-isomers) was a gift from Dr. Jens Lykkjesfeldt (KVL, Copenhagen, Denmark).

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cated CYP79A2 (yielding truncated-modified CYP79A2), respectively. Previous experiments with CYP79A1 from S. bicolor had shown that expression levels as high as 900 nmol of cytochrome P450 (liter of culture) were obtained with a protein, in which the first 24 amino acids of CYP79A1 were replaced by the eight N-terminal amino acids of bovine CYP17A (CYP79A1Δ(1–24)_bob; Ref. 29). Thus the N terminus of this cytochrome P450 seems to be especially suitable for expression in E. coli. We have therefore generated a fourth construct (chimeric CYP79A2; Fig. 2) in which we fused the cDNA encoding the N terminus of CYP79A1Δ(1–24)_bob (50 amino acids) with the cDNA encoding the catalytic domain of CYP79A2 (amino acids 41–523).

Protein bands migrating with an apparent molecular mass of about 60 kDa on SDS-polyacrylamide gels were detected in the detergent-rich phase obtained by temperature-induced Triton X-114 phase partitioning of E. coli spheroplasts harboring expression constructs for the native, the truncated-modified, and the chimeric CYP79A2. The band was not present in the detergent-rich phase from cells harboring the modified CYP79A2 expression construct or the empty vector. GC-MS analysis showed that two compounds with identical retention times and the fragmentation pattern identified these compounds as the (E)- and (Z)-isomers of phenylacetaldoxime (Fig. 5). Administration of L-[14C]phenylalanine as the substrate. As no CO spectrum was obtained with native CYP79A2, we were not able to estimate the amount of functional native CYP79A2. However, based on the expression level of functional chimeric CYP79A2, we can estimate a turnover number of 0.24 min⁻¹ for native CYP79A2.

The activity of recombinant CYP79A2 was strongly dependent on the pH of the reaction mixture and, to a lesser extent, on several other factors. Compared with the activity at pH 7.5, the activity of chimeric CYP79A2 was 25% at pH 6.5, 50% at pH 6.5, 80% at pH 7.0, and 70% at pH 7.9. Addition of Tween 80 to a final concentration of 0.083% (v/v) resulted in a 1.5-fold increase in aldoxime production. Addition of reduced glutathione...
that cytochrome P450-dependent monooxygenases catalyze the

Constitutive Expression of CYP79A2 in Transgenic A. thaliana—We have expressed CYP79A2 under control of the CaMV35S promoter in A. thaliana cv. Columbia. The appearance of the transgenic plants was comparable to wild-type plants. The glucosinolate content of rosette leaves of the T1 generation was investigated by HPLC of the desulfoglucosinolates. All the transgenic plants analyzed accumulated benzylglucosinolate, whereas benzylisoglucosinolate was not detected in simultaneously grown wild-type plants (Fig. 6). The content of benzylglucosinolate varied between different transgenic plants. In the three plants with highest accumulation, benzylglucosinolate accounted for 38% (plant 10), 5% (plant 14), and 2% (plant 13), respectively, of the total glucosinolate content of the leaves. HPLC analysis of seeds of these plants showed that benzylglucosinolate accounted for 35% (plant 10), 12% (plant 14), and 3% (plant 13) of the total glucosinolate content of the seeds. In seeds of wild-type plants (cv. Columbia and Wassilewskija), minute amounts of benzylglucosinolate were detected (in cv. Columbia, 0.034 μmol (g fresh weight)-1 corresponding to 0.05% of the total glucosinolate content). The content of homophenylalanine-derived 2-phenylethylglucosinolate was unaffected in leaves and seeds of the transgenic plants compared with wild-type plants.

DISCUSSION

In the present study, we report cloning and characterization of CYP79A2, a cytochrome P450-dependent monooxygenase, which catalyzes the conversion of L-phenylalanine to phenylacetaldoxime, N-hydroxylation of aromatic amino acids to aldoximes in glucosinolate biosynthesis.

A. thaliana represents an ideal model plant for the study of glucosinolate biosynthesis, as the knowledge rapidly emerging from the Arabidopsis Genome Sequencing Initiative can directly be used to identify candidate genes involved in glucosinolate metabolism. Glucosinolates are related to cyanogenic glucosides as the biosynthesis of both classes of secondary plant products proceeds from amino acids via aldoximes. Cyanogenic glucosides are widely distributed in the plant kingdom. They are present in angiosperms, gymnosperms, and ferns, indicating that cyanogenesis has developed early in evolution (42). The occurrence of glucosinolates is restricted to the order Caparales and the genus Drypetes (Euphorbiales) (43). C. papaya is the only known example of a plant containing both glucosinolates and cyanogenic glucosides (15). It has been suggested that glucosinolate biosynthesis might have evolved based on a "cyanogenic predisposition" (43), implicating that homologous enzymes catalyze related reactions in the biosynthesis of both groups of compounds. CYP79A2 is one of several CYP79 homologues in A. thaliana. We have cloned the corresponding cDNA and characterized the recombinant protein expressed in E. coli. Our data show that CYP79A2 is an N-hydroxylase, which catalyzes the conversion of L-phenylalanine to phenylacetaldoxime. This provides the first evidence that CYP79 homologues are involved in the biosynthesis of glucosinolates. The substrate specificity of CYP79A2 seems to be rather narrow, as neither L-tyrosine, Dl-homophenylalanine, L-tryptophan, nor L-methionine are metabolized by the enzyme, even though the former two amino acids are structurally similar to phenylalanine and function as precursors of glucosinolates. The high substrate specificity is in agreement with results obtained with CYP79 homologues involved in the biosynthesis of cyanogenic glucosides (23, 29, 44). The kinetic data for recombinant

A
B
C
D
E

Fig. 4. Metabolism of L-phenylalanine by E. coli spheroplasts reconstituted with NADPH:cytochrome P450 reductase from S. bicolor. L-[14C]Phenylalanine was administered to spheroplasts of E. coli transformed with expression constructs for native CYP79A2 (A), modified CYP79A2 (B), truncated-modified CYP79A2 (C), chimeric CYP79A2 (D), or empty vector (E). The samples were incubated at 30 °C for 120 min and analyzed by thin layer chromatography.
CYP79A2 show that CYP79A2 has high affinity to l-phenylalanine, which is metabolized at low rate (turnover number 0.24 min\(^{-1}\)) compared with the conversion of l-tyrosine by CYP79A1 (turnover numbers of 200 min\(^{-1}\) and 350 min\(^{-1}\) for CYP79A1 purified from S. bicolor (Ref. 21) and recombinant CYP79A1 analyzed in E. coli membranes (Ref. 29), respectively).

E. coli does not contain endogenous cytochromes P450 or NADPH:cytochrome P450 reductase. However, two soluble E. coli flavoproteins, flavodoxin and NADPH-flavodoxin reductase, have been shown to support the catalytic activity by donating reducing equivalents to heterologously expressed cytochromes P450 (28, 45). Our experiments with CYP79A2 showed that the E. coli flavoproteins do not support the catalytic activity of recombinant CYP79A2. Detectable amounts of phenylacetaldoxime were only obtained in the presence of exogenously supplied NADPH:cytochrome P450 reductase. The stimulation of the enzyme activity upon addition of glutathione or Tween 80 to the reaction mixture might reflect an improved interaction of CYP79A2 with NADPH:cytochrome P450 reductase. Glutathione has been reported previously to stimulate cytochrome P450 activity in reconstitution experiments with purified CYP3A4 (46) and has been suggested to affect the reductase-cytochrome P450 interaction (29). Detergents, e.g. Tween 20 and Triton X-100, have been demonstrated to cause conformational changes of cytochrome P450 CYP1A2, leading to increased catalytic activity (47).

Additional evidence for the involvement of CYP79A2 in glucosinolate biosynthesis is provided by accumulation of the phenylalanine-derived glucosinolate benzylglucosinolate (also referred to as glucosepropaenol) in transgenic A. thaliana expressing CYP79A2 under the control of the CaMV35S promoter. All transgenic plants analyzed in the present study contained benzyl glucosinolate in the rosette leaves, whereas rosette leaves of wild-type plants did not contain detectable amounts of this glucosinolate. Although seeds of A. thaliana cv. Columbia are known to contain the homophenylalanine-derived 2-phenylethylglucosinolate, the occurrence of benzylglucosinolate has never been reported for A. thaliana. However, we have detected minute amounts of benzylglucosinolate in seeds of A. thaliana cv. Columbia and cv. Wassilewskija. Seeds of transgenic plants accumulated high levels of benzylglucosinolate. The content of 2-phenylethylglucosinolate was unchanged in seeds of transgenic plants compared with seeds of wild-type plants. This supports the data obtained with CYP79A2 expressed in E. coli and shows that CYP79A2 converts specifically phenylalanine, but not homophenylalanine to the corresponding aldoxime. As indicated by the accumulation of high levels of benzylglucosinolate in several transgenic plants, the formation of phenylacetaldoxime is the rate-limiting step in the biosynthesis of benzylglucosinolate in A. thaliana.

Benzylglucosinolate is only sporadically observed in roots and cauline leaves of wild-type A. thaliana cv. Columbia and may be induced by environmental conditions.\(^2\) The sporadic occurrence of benzylglucosinolate corresponds well with our observation that the CYP79A2 mRNA is a low abundant transcript. We were not able to detect the CYP79A2 mRNA in seedlings, rosette leaves of different developmental stages, and cauline leaves of A. thaliana cv. Columbia by Northern blotting and RT-PCR (data not shown). Initial experiments with wild-type A. thaliana cv. Columbia seedlings in liquid culture indicate that no induction of benzylglucosinolate biosynthesis occurs after treatments with salicylic acid, methyl jasmonate, or the ethylene precursor 1-aminoacyclopentane-1-carboxylic acid. The elucidation of environmental factors which lead to induction of benzylglucosinolates in A. thaliana must await further studies.

The nature of the enzymes involved in the conversion of amino acids to aldoximes in the biosynthesis of glucosinolates has been studied in different plant species (for review, see Ref. 12). In microsomal enzyme systems isolated from S. alba, T. majus, and C. papaya, tyrosine or phenylalanine are converted to the corresponding aldoximes by cytochrome P450-dependent monoxygenases, as evidenced by photoreversible carbon monoxide inhibition and other inhibitor studies (13–16). Biochemical studies with microsomal preparations from Brassica napus L. have indicated that the conversion of homophenylalanine, dihomo-, trihomo-, and tetrahomomethionine to the corresponding aldoximes is not affected by carbon monoxide and

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\(^2\) P. Brown and J. Gershenzon, personal communication.
other cytochrome P450 inhibitors (17, 18). Instead, the enzymes showed characteristics of flavin-containing monooxygenases, which have subsequently been suggested to catalyze these reactions as well as the N-hydroxylation of phenylalanine in other members of the Brassicaceae family (16, 19). A third enzyme system, a plasma membrane-bound peroxidase first characterized in Chinese cabbage (B. campestris ssp. pekinensis), has been shown to convert tryptophan to indole-3-acetaldoxime in the biosynthesis of indoleglucosinolates (20). It has been proposed that the involvement of cytochrome P450-dependent monooxygenase may be restricted to species that do not belong to the Brassicaceae family, implicating that the cytochrome P450-dependent formation of p-hydroxyphenylacetaldoxime in S. alba has to be regarded as a unique exception from the rule or an experimental artifact (16). Our data strongly suggest that aldoxime formation from aromatic amino acids is dependent on cytochrome P450 enzymes in members of the Brassicaceae as well as in other families.

In conclusion, we have identified a cytochrome P450-dependent monooxygenase, CYP79A2 from A. thaliana, which catalyzes the conversion of L-phenylalanine to phenylacetaldoxime, the first step in the biosynthesis of benzylglucosinolate. CYP79A2 is the first enzyme shown to catalyze aldoxime formation in glucosinolate biosynthesis. Our data provide the first evidence for the involvement of CYP79 homologues in this step, confirming that the aldoxime formation is catalyzed by evolutionarily conserved cytochromes P450 in the biosynthesis of glucosinolates and cyanogenic glucosides. This indicates that the biosynthesis of glucosinolates has evolved based on a cyanogenic predisposition. Several glucosinolates, including benzylglucosinolate (48–50), have been shown to be degraded to biologically active compounds with anticarcinogenic, fungitoxic, and herbicidal activity. Considering the increasing interest in glucosinolate-containing vegetables as foods possessing cancer-preventing properties (51, 52) and in the use of glucosinolates as biopesticides and herbicides (53), the identification of enzymes involved in the biosynthesis of glucosinolates has great potential for biotechnological uses and the development
of crops with improved pest resistance and increased nutriti-

tonal value.

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Note Added in Proof—After acceptance of this article, an article was
published on indole-3-acetaldoxime forming cytochrome P450s
(CYP79B2 and CYP79B3) by Hull et al. (Hull, A. K., Vj, R., and
Celenza, J. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2379–2384).

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