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Carsten Horslev Hansen
The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

Liangcheng Du
University of Nebraska - Lincoln, ldu3@unl.edu

Peter Naur
The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

Carl Erik Olsen
The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

Kristian B. Axelsen
The SwissProt Group, Swiss Institute of Bioinformatics, CH-1211 Geneva 4, Switzerland

See next page for additional authors

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Carsten Hørslev Hansen[a,c], Liangcheng Du[a,c], Peter Naur[a,c], Carl Erik Olsen[c,b], Kristian B. Axelsen[d], Alastair J. Hick[e], John A. Pickett[e], and Barbara Ann Halkier[a,c,*]

[a] *Plant Biochemistry Laboratory, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark*
[b] *Department of Chemistry, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark*
[c] *Center for Molecular Plant Physiology (PlaCe), The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark*
[d] *The SwissProt Group, Swiss Institute of Bioinformatics, CH-1211 Geneva 4, Switzerland*
[e] *IACR-Rothamsted, Harpenden, AL5 2JQ, United Kingdom*
[*] *Corresponding author. Email:* bah@kvl.dk.

Abstract: CYP83B1 from *Arabidopsis thaliana* has been identified as the oxime-metabolizing enzyme in the biosynthetic pathway of glucosinolates. Biosynthetically active microsomes isolated from *Sinapis alba* converted *p*-hydroxyphenylacetaldoxime and cysteine into *S*-alkylated *p*-hydroxyphenylacetothiohydroximate, *S*-(*p*-hydroxyphenylacetohydroximoyl)-L-cysteine, the next proposed intermediate in the glucosinolate pathway. The production was shown to be dependent on a cytochrome P450 monooxygenase. We searched the genome of *A. thaliana* for homologues of CYP71E1 (P450ox), the only known oxime-metabolizing enzyme in the biosynthetic pathway of the evolutionarily related cyanogenic glucosides. By a combined use of bioinformatics, published expression data, and knock-out phenotypes, we identified the cytochrome P450 CYP83B1 as the oxime-metabolizing enzyme in the glucosinolate pathway as evidenced by characterization of the recombinant protein expressed in *Escherichia coli*. The data are consistent with the hypothesis that the oxime-metabolizing enzyme in the cyanogenic pathway (P450ox) was mutated into a "P450*m*ox" that converted oximes into toxic compounds that the plant detoxified into glucosinolates.

Glucosinolates are naturally occurring amino acid-derived *S*-glucosides of thiohydroximate-*O*-sulfonates. They co-occur with endogenous thioglucosidases called myrosinases that upon tissue damage hydrolyze glucosinolates into a wide range of degradation products such as *e.g.* isothiocyanates, nitriles, and thiocyanates. Glucosinolates (or rather their degradation products) are involved in plant defense and constitute characteristic flavor compounds and cancer-preventive agents in *Brassica* vegetables.

The biosynthetic pathway from precursor amino acid to the core glucosinolate structure has been well studied, and many of the intermediates are known, including oximes, thiohydroximic acids, and desulfoglucosinolates (1, 2). Recently, it has been shown that cytochromes P450 belonging to the CYP79 family catalyze the conversion of amino acids to oximes (3–7). Little is known about the formation of thiohydroximic acids from oximes. The remaining part of the pathway for the core structure involves a UDP-glucose:thiohydroximic acid glucosyltransferase and a sulfotransferase (for review, see Ref. 2).

It has been proposed that *aci*-nitro compounds are intermediates in the conversion of oximes to thiohydroximic acids (8). This was supported by isolation of 1-nitro-2-phenylethane from *Tropaeolum majus* shoots and by *in vivo* conversion of phenylacetaldoxime into 1-nitro-2-phenylethane and of 1-nitro-2-[1,2-^{14}C]phenylethane into benzylglucosinolate (9). The *aci*-nitro is proposed to be conjugated with a sulfur donor to produce an *S*-alkyl thiohydroximate, possibly by a glutathione *S*-transferase (2). Biochemical studies indicate that the *S*-alkyl thiohydroximate is subsequently hydrolyzed to the thiohydroximic acid by a C-S lyase (10).

Glucosinolates are related to cyanogenic glucosides because both groups of natural plant products are derived from amino acids and have oximes as intermediates. This suggests that the oxime-metabolizing enzyme is the branching point between the cyanogenic glucoside and the glucosinolate pathway. In the biosynthetic pathway of the tyrosine-derived cyanogenic glucoside dhurrin from *Sorghum bicolor*, the oxime-metabolizing enzyme (designated P450ox or CYP71E1) catalyzes the conversion of oxime to α-hydroxynitrile by dehydrating the oxime to a nitrile, which is then C-hydroxylated to form the β-hydroxynitrile (11, 12).

We have previously used *Sinapis alba* as a model plant for biosynthetic studies of the glucosinolate pathway (13). The tyrosine-derived *p*-hydroxybenzylglucosinolate is the major glucosinolate in *S. alba.* In the present study, we characterize biochemically the oxime-metabolizing enzyme in microsomes from *S. alba* and show that the enzyme is dependent on a cytochrome P450 monooxygenase. In addition, we show that CYP83B1 is the oxime-metabolizing enzyme in glucosinolate biosynthesis in *Arabidopsis thaliana* as evidenced by biochemical characterization of the recombinant protein. The data substantiate the results recently obtained with the *rnt1-1* CYP83B1 knock-out mutant (29).

EXPERIMENTAL PROCEDURES

Chemical Synthesis

S-(Benzohydroximoyl)-L-cysteine—*N*-Chlorosuccinimide (280 g, 21 mmol) and HCl gas (40 ml) were added to benzaldoxime (2.42 g, 20.0 mmol) in dimethylformamide (40 ml) and stirred at room temperature for 4 h. Water (150 ml) was added, and the reaction mixture was extracted with diethyl ether (3 × 50 ml). The combined ether phases were washed with water (2 × 50 ml), dried, and concentrated *in vacuo*. The residue oil containing the benzohydroximic acid chloride was dissolved in ethanol (50 ml) and added to a solution of ice-cold L-*cys*-

teine (3.15 g, 20.0 mmol) in ethanol (100 ml). The mixture was treated with sodium methoxide (sodium 0.74 g, 32.2 mmol; methanol 50 ml) and left stirring for 3 h. The white precipitate was filtered, and the filtrate was concentrated *in vacuo*, washed in water, and dried (3.42 g, 71%). NMR in D_2O/NaOD: ^1H δ 7.4-7.5 (5H, m, Ph), 3.28 (1H, dd, *J* 8.3, 3.9 Hz, C*H*), 2.85 (1H, dd, *J* 13.2, 8.3 Hz, CH_2), 3.13 (1H, dd, *J* = 13.2, 3.9 Hz, CH_2); ^{13}C: δ 168.4 (*C*OOH), 150.9 (S-*C*=N), 133.7, 128.4, 128.4, 129.1 (Ph), 53.3 (*C*H), 52.7 (*C*H_2). MS[1]: $[M + Na]^+$ at *m*/*z* 263, $[M + H]^+$ at *m*/*z* 241, fragment ions at *m*/*z* 224, *m*/*z* 195, *m*/*z* 154, and *m*/*z* 120. After a few hours in methanol, the product underwent cyclization with concomitant release of hydroxylamine to produce (*R*)-2-phenyl-thiazoline-4-carboxylic acid. NMR (Me_2SO-d_6): ^1H δ 7.4-7.8 (5H, m, Ph), 5.17 (1H, t, *J* 9 Hz, C*H*), 3.62 (2H, d, *J* 9 Hz, CH_2); ^{13}C: δ 172.6 (S-*C*=N), 167.7 (*C*OOH), 133.0 131.7 128.3 128.9 (Ph), 80.2 (*C*H), 35.7 (*C*H_2). MS: $[M + H]^+$ at *m*/*z* 208, fragment ion at *m*/*z* 162.

*S-(Benzohydroximoyl)-N-acetyl-*L*-cysteine*—Benzohydroximic acid chloride (1.55 g, 10 mmol) diluted with tetrahydrofuran was added dropwise to ice-cold *N*-acetyl-L-cysteine (1.63 g, 10 mmol) in tetrahydrofuran (30 ml) with triethylamine (4.04 g, 0.04 mmol). After stirring for an additional 30 min, dilute sulfuric acid (5% v/v, 100 ml) was added, the tetrahydrofuran was removed *in vacuo*, and the aqueous layer was left at room temperature for 48 h. The white precipitate was filtered, and the filtrate was washed in diethyl ether and dried (2.06 g, 73%). NMR (D_2O, NaOD): ^1H: d 1.95 (^3H, s, CH_3CO), 2.98 (1H, dd, *J* 5.0, 14.0, C$H_a$$H_b$CH), 3.00 (1H, dd, *J* 5.0, 14.0, CH$_a$$H_b$CH), 4.17 (1H, t, *J* 5.0, CH$_a$H$_b$C*H*) 7.40-7.49 (5H, m, 5× Ar*H*), ^{13}C: d 24.8 (CO*C*H$_3$), 35.1 (*C*H$_2$), 57.6 (*C*H), 131.4 (2× *C*H), 131.5 (*C*H), 131.6 (2× *C*H), 137.0 (*C*$_q$), 155.3 (*C*=NOH), 175.8 (*C*O), 179.2 (*C*O). MS: $[M + Na]^+$ at *m*/*z* 305, $[M + H]^+$ at *m*/*z* 283, fragment ions at *m*/*z* 208, *m*/*z* 162, and *m*/*z* 130.

Preparation of Microsomal Enzyme System from S. alba

Seeds of *S. alba* were germinated in darkness and jasmonate-treated as previously described (13). The preparation of microsomes was done as previously described (13) except that the isolation buffer was modified to consist of 250 mM Tricine, 100 mM ascorbic acid, 2 mM EDTA, and 2 mM dithiothreitol, pH 8.2. The same procedure was used to prepare microsomes from seedlings of *T. majus*, *S. bicolor*, and *Zea mays*.

Measurements of Oxime-metabolizing Enzyme Activities in Microsomes from S. alba

[U-^{14}C]*p*-Hydroxyphenylacetaldoxime was produced enzymatically by CYP79A1 as previously described (14). In a typical enzyme assay, 1 mg of microsomal protein was incubated in buffer containing 30 mM KP_i, pH 7.9, 3 mM NADPH, 38 nCi (66 pmol) of [U-^{14}C]*p*-hydroxyphenylacetaldoxime, and 6 mM cysteine or *N*-acetylcysteine in a total volume of 200 µl. After incubation at 29 °C for 1 h, the reaction mixtures were extracted twice with 400 µl of ethyl acetate. If *N*-acetylcysteine was used as a sulfur donor, the remaining water phases were acidified by addition of 200 µl of 1% formic acid and extracted three times with 800 µl of ethyl acetate. The ethyl acetate phases were combined and dried *in vacuo*. The extracts were redissolved in 30 µl of 50% ethanol and analyzed by TLC. If cysteine was used as a sulfur donor 400 µl of 96% ethanol were added to the remaining water phases, and the solution was clarified by centrifugation for 15 min at 20,000 × *g*. The supernatants were concentrated *in vacuo*, redissolved in 30 µl of 50% ethanol, and analyzed by TLC. Boiled microsomes were used in control assays. For analysis of the effect of treatment with cytochrome P450 inhibitor, standard microsomal reaction mixtures were incubated in the presence of 0.1 mM tetcyclasis. The TLC was performed on Silica Gel 60 F_{254} sheets (Merck) using 2-propanol:ethyl acetate:water (7:1:2, v/v) as eluent. The TLC plates were eluted twice to improve separation. Radiolabeled bands were visualized and quantified on a STORM 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For LC-MS analysis, 5 × 200 µl of reaction mixtures were made as described above except that [U-^{14}C]*p*-hydroxyphenylacetaldoxime was exchanged for 3 mM unlabeled oxime. The compounds were extracted from the reaction mixtures as described above.

LC-MS Analysis

Electrospray ionization LC-MS analysis was done on a HP1100 LC coupled to a Bruker Esquire-LC ion trap mass spectrometer. The reversed-phase LC conditions were as follows: column: XTerra MS C18 3.5 µm, 2.1 × 100 mm (Waters Corp.); mobile phases A: 0.03% HCOOH, B: 80% MeCN, 0.1% HCOOH. The flow rate was 0.2 ml/min, and the gradient program was 0-2 min isocratic 5% B, 2-30 min linear gradient 5-100% B, and 30-35 min isocratic 100% B. The mass spectrometer was run in positive ion mode using a low sampling cone voltage to minimize fragmentation. Authentic standards of *S*-(benzohydroximoyl)-L-cysteine and *S*-(benzohydroximoyl)-*N*-acetyl-L-cysteine were synthesized and used to develop conditions for the recording of mass spectra of analogue compounds (see above).

Phylogenetic Trees

The protein sequences of all the members of the CYP71 family were corrected for prediction errors, and the full-length sequences were aligned including one member of each of the families CYP76, CYP98, and CYP84 using ClustalW (15). Phylogenetic analysis was performed with the Protdist and Fitch (Fitch-Margoliash and least squares method) programs of the Phylip package (16).

Generation of the Constructs for Escherichia coli Expression

The ESTs 226P8T7, 5G6, and 148G2T7 (Arabidopsis Biological Resource Center, Columbus, OH), which encode the full-length sequences of *CYP71B6*, *CYP71B7*, and *CYP83B1*, respectively, were expressed heterologously in *E. coli* using the pSP19 g10L expression vector (17). Silent mutations were introduced to enrich for A and T in the first 11 codons (17). The coding region of *CYP71B6* was amplified from the EST 226P8T7 by PCR with primer 1 (sense direction; 5'-GGAATTCCATATGTCACTTTTATCTTTCCCCATT-3') and primer 2 (antisense direction; 5'-GGCTGCAGGCATGCTTAAAGCTTGCGGTTGATGA-3'). The PCR was set up in a total volume of 100 µl in *Pwo* polymerase PCR buffer with 2 mM $MgSO_4$ using 4 units of *Pwo* polymerase (Roche Molecular Biochemicals), 1.3 µg of template DNA, 200 µM dNTPs, and 100 pmol of each primer. The PCR was incubated for 2 min at 94 °C, 21 cycles of 15 s at 94 °C, 20 s at 56 °C, and 70 s at 72 °C. The PCR fragment was digested with *Eco*RI and *Pst*I, ligated into pBluescript II SK (Stratagene), and transferred from pBluescript II SK to an *Nde*I/*Sph*I-digested pSP19 g10L vector. The *CYP71B7* gene was PCR-amplified from EST 5G6 using primer 3 (sense direction; 5'-GGAATTCATATGGCTATCTTGCTCTGTTTC-3') and primer 4 (antisense direction; 5'-CGGGATCCCATGATCGTCATCTTAATGATG-3'). The PCR was set up as described above. The PCR was incubated for 2 min at 94 °C, 23 cycles of 15 s at 94 °C, 45 s at 55 °C, and 2 min at 72 °C. The PCR product was digested with *Eco*RI and *Bam*HI, ligated into pBluescript II SK, and transferred from pBluescript II SK into an *Nde*I/*Bam*HI-digested pSP19 g10L. The *CYP83B1* coding region was amplified from the EST 148G2T7 by PCR with primer 5 (sense direction; 5'-GGAATTCCATATGAAACTCTTATTGATTATAGCTGGTTTAGTTGCGGCTGCAG-3') and primer 6 (antisense direction; 5'-CGGGATCCATTAGATGTGTTTCGTTGGTGC-3'). The PCR was set up as described above. The PCR was incubated for 2 min at 94 °C, 22 cycles of 15 s at 94 °C, 20 s at 57 °C, and 70 s at 72 °C. The PCR fragment was digested with *Eco*RI and *Bam*HI, ligated into pBluescript II SK, and transferred from pBluescript II SK to a *Nde*I/*Bam*HI-digested pSP19 g10L vector. All constructs were sequenced to exclude PCR errors.

Expression in E. coli

E. coli cells of strain C43(DE3) (18) were transformed with the expression constructs and grown overnight in LB medium supplemented with 100 µg ml^{-1} ampicillin. 2 ml of culture was used to inoculate 200 ml of modified TB medium containing 50 µg ml^{-1} ampicillin (14). The cultures were grown at 37 °C at 250 rpm until A_{600} = 0.5-0.7. Then 1 mM thiamine and 75 µg ml^{-1} δ-aminolevulinic acid were added, and after an additional 1 h of growth, the cultures were induced with 1 mM isopropyl-β-D-thiogalactoside and subsequently grown at 28 °C for 40 h at 125 rpm. Spheroplasts were prepared as previously described (14) except that glycerol was omitted in the final buffer. The amount of expressed functional cytochrome P450 was monitored by Fe^{2+}·CO *versus* Fe^{2+} difference spectroscopy and quantified using an extinction coefficient of 91 mM^{-1} cm^{-1}.

Characterization of Enzyme Activity of Recombinant CYP71B6, CYP71B7, and CYP83B1

The enzyme activity of CYP71B6, CYP71B7, and CYP83B1 was measured in reaction mixtures in which spheroplasts of *E. coli* transformed with the respective expressing construct were reconstituted with recombinant NADPH: cytochrome P450 reductase (ATR1) from *A. thaliana*. In a typical enzyme assay, 25 µl of spheroplasts and 0.06 units of NADPH:cytochrome P450 reductase were incubated in buffer containing 50 mM Tricine, pH 8.1, 3 mM NADPH, 38 nCi (66 pmol) of [U-^{14}C]*p*-hydroxyphenylacetaldoxime, and 6 mM cysteine or *N*-acetylcysteine in a total volume of 100 µl. Spheroplasts of *E. coli*

[1] Abbreviations: MS, mass spectrometry; LC, liquid chromatography; EST, expressed sequence tag; PCR, polymerase chain reaction.

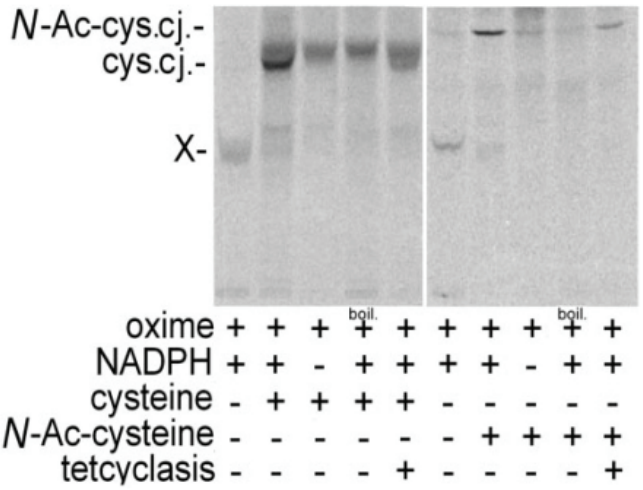

Figure 1. Production of the cysteine conjugate (*cys.cj.*), the *N*-acetylcysteine conjugate (*N-Ac-cys.cj.*), and an unidentified compound (*X*) by *S. alba* microsomes. [U-^{14}C]*p*-Hydroxyphenylacetaldoxime was incubated with microsomes from *S. alba* in the presence of cysteine (*columns 1-5*) or *N*-acetylcysteine (*columns 6-10*) as a sulfur donor. The reaction mixtures were extracted with ethyl acetate, and the water phases were analyzed by TLC. As a control boiled microsomes were used (*columns 4* and *9*). In the absence of a sulfur donor a compound (*X*) accumulated (*columns 1* and *6*). The production of the conjugates was inhibited by the cytochrome P450 inhibitor tetcyclasis (*columns 5* and *10*).

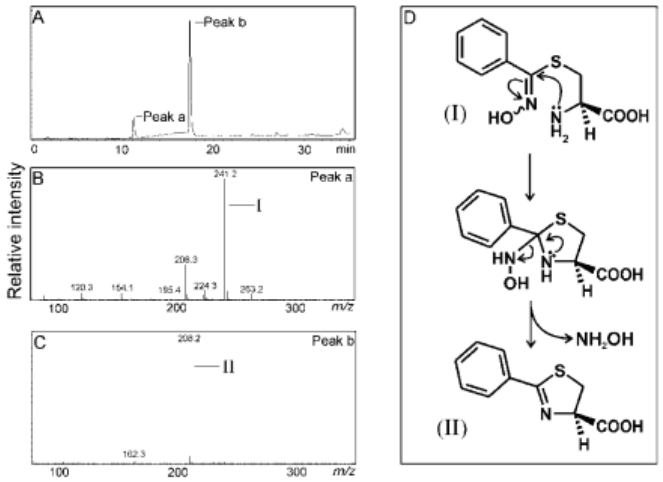

Figure 2. LC-MS analysis of *S*-(benzohydroximoyl)-L-cysteine. *A*, LC-MS analysis of *S*-(benzohydroximoyl)-L-cysteine (*I*) dissolved in methanol. The total ion chromatogram shows the starting material as *Peak a* and the pre-injection cyclized product, (*R*)-2-phenyl-thiazoline-4-carboxylic acid (*II*) as *Peak b*. The rising baseline between the two peaks stems from on-column cyclization. *B*, mass spectrum of *Peak a*: *I* gives rise to the ions $[M + Na]^+$ at *m*/*z* 263 and $[M + H]^+$ at *m*/*z* 241. C, mass spectrum of *Peak b*: *II* gives rise to the ion $[M + H]^+$ at *m*/*z* 208. *D*, scheme illustrating the cyclization process of *S*-(benzohydroximoyl)-L-cysteine to (*R*)-2-phenyl-thiazoline-4-carboxylic acid.

C43(DE3) transformed with empty vector were used as controls. After incubation at 29 °C for 1 h, the reaction mixtures were extracted and analyzed as described for the microsomal assays. Product formation from the oxime was linear with time within the first 10 min of incubation. For kinetic analysis, 5-50 μM *p*-hydroxyphenylacetaldoxime was added to the standard reaction mixture, which was incubated for 5 min and analyzed by TLC as described above. Radiolabeled bands were visualized and quantified on a STORM 840 PhosphorImager. For LC-MS analysis, 10 reactions of 100 μl were done as described above except that radiolabeled *p*-hydroxyphenylacetaldoxime was exchanged with 3 mM unlabeled oxime.

Substrate Binding Spectra

Substrate binding spectra were measured for recombinant CYP83B1 using partially purified enzyme. Purification was obtained by temperature-induced Triton X-114 phase partitioning of *E. coli* spheroplasts as previously described (14). The substrate binding spectra were performed on an SLM Aminco DW-2000 TM spectrophotometer (SLM Instruments, Urbana, IL) at 12 °C using 10 μl of the Triton X-114-rich phase in 990 μl of 50 mM KP_i, pH 7.5, and 0.2 mM of either *p*-hydroxyphenylacetaldoxime, *p*-hydroxyphenylacetonitrile, 1-nitro-2-(*p*-hydroxyphenyl)ethane, or *p*-hydroxyphenylacetamide.

RESULTS

Characterization of the Oxime-metabolizing Enzyme in Microsomes from S. alba—Biosynthetically active microsomes from *S. alba* seedlings were isolated under conditions where myrosinase was inhibited to prevent formation of inhibitory breakdown products from the glucosinolates in the tissue (13). When [U-^{14}C]*p*-hydroxyphenylacetaldoxime and cysteine were added to microsomes from *S. alba* in the presence of NADPH, a cysteine-dependent radiolabeled compound accumulated in the reaction mixtures as evidenced by TLC analysis (Figure 1, *column 2*). The compound was not detectable when boiled microsomes were used (Figure 1, *column 4*), indicating that the reaction required the presence of an active enzyme. The product formation was significantly reduced when NADPH was not added to the reaction mixture, further emphasizing that the reaction was enzyme-dependent and the electron source was NADPH (Figure 1, *column 3*). When the reaction mixtures were extracted with ethyl acetate, the cysteine-dependent compound remained in the water phase, whereas the oxime accumulated in the ethyl acetate phase. The water solubility indicated that the cysteine-dependent compound contained the polar groups of cysteine. The characteristics were consistent with the compound being the proposed intermediate *S*-(*p*-hydroxyphenylacetohydroximoyl)-L-cysteine, also referred to as cysteine conjugate. Such cysteine conjugates undergo cyclization with concomitant release of hydroxylamine to produce the corresponding 2-substituted thiazoline-4-carboxylic acids as evidenced by the chemically synthesized authentic standard *S*-(benzohydroximoyl)-L-cysteine (Figure 2). The cyclization product (*R*)-2-(*p*-hydroxybenzyl)-thiazoline-4-carboxylic acid formed from *S*-(*p*-hydroxyphenylacetohydroximoyl)-L-cysteine with $[M + H]^+$ at *m*/*z* 238 was identified by LC-MS (Figure 3, *A* and *B*). In the presence of tetcyclasis, an inhibitor of cytochrome P450, the production of cysteine conjugate was significantly reduced (Figure 1, *column 5*). This indicated that the oxime-metabolizing enzyme in the glucosinolate pathway is a cytochrome P450-dependent monooxygenase.

When *N*-acetylcysteine was used as a sulfur donor in the microsomal reaction mixtures containing [U-^{14}C]*p*-hydroxyphenylacetaldoxime and NADPH, an *N*-acetylcysteine-dependent compound was produced as evidenced on TLC (Figure 1, *column 7*). The *N*-acetylcysteine-dependent compound was not present when boiled microsomes were used (Figure 1, *column 9*), and the product formation was significantly reduced when NADPH was not added to the reaction mixture (Figure 1, *column 8*), showing that the reaction was enzyme-dependent and the electron source was NADPH. The production was inhibited by tetcyclasis as was seen for the cysteine conjugate compound (Figure 1, *column 10*). The *N*-acetylcysteine-dependent compound was identified by LC-MS analysis as *S*-(*p*-hydroxyphenylacetohydroximoyl)-*N*-acetyl-L-cysteine, also referred to as *N*-acetylcysteine conjugate (Figure 3, *C* and *D*). In contrast to the cysteine conjugate, no cyclization of the *N*-acetylcysteine conjugate took place, reflecting that the nucleophilic property of the amine (cysteine) is considerably greater than that of the amide (*N*-acetylcysteine). Rearrangement, as shown in Figure 2*D*, can only take place if

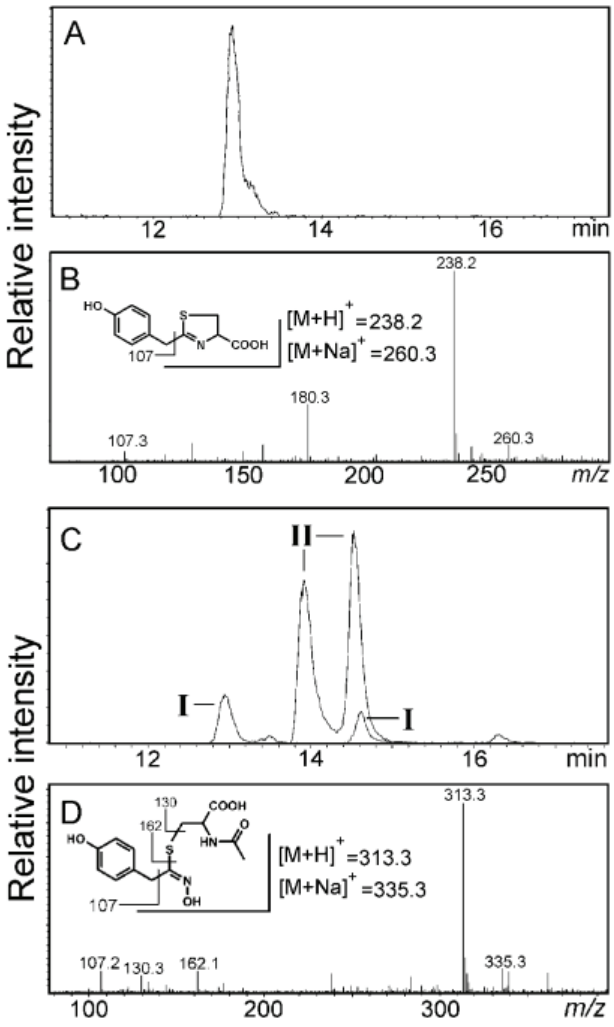

Figure 3. Identification by LC-MS of (*R*)-2-(*p*-hydroxybenzyl)-thiazoline-4-carboxylic acid and of *S*-(*p*-hydroxyphenylacetohydroximoyl)-*N*-acetyl-L-cysteine produced by *S. alba* microsomes. *A*, reconstructed ion chromatogram of *m*/*z* 238. *B*, mass spectrum of the cyclization product (*R*)-2-(*p*-hydroxybenzyl)-thiazoline-4-carboxylic acid of *S*-(*p*-hydroxyphenylacetohydroximoyl)-L-cysteine showing $[M + H]^+$ at *m*/*z* 238 and fragment ion at *m*/*z* 107. *C*, reconstructed ion chromatogram (*m*/*z* 313) showing the *E*- and *Z*-isomers of the product *S*-(*p*-hydroxyphenylacetohydroximoyl)-*N*-acetyl-L-cysteine (*I*) and reconstructed ion chromatogram (*m*/*z* 152) showing *E*- and *Z*-isomers of the substrate *p*-hydroxyphenylacetaldoxime (*II*). *D*, mass spectrum of *S*-(*p*-hydroxyphenylacetohydroximoyl)-*N*-acetyl-L-cysteine showing $[M + Na]^+$ at *m*/*z* 335, $[M + H]^+$ at *m*/*z* 313, and fragment ions at *m*/*z* 162, 130, and 107.

the *N*-acetyl group is eliminated; this was not seen over the time course of the reaction. It may take place, however, if the reaction is allowed to proceed for a longer period of time during which hydrolysis occurs. By the LC-MS analysis of the *N*-acetylcysteine conjugate two peaks were seen at retention times of 12.9 and 14.6 min corresponding to the *E*- and *Z*-isomers of the hydroximino group of the molecule, respectively. The two isomers had identical mass spectra with $[M + Na]^+$ at *m*/*z* 335, $[M + H]^+$ at *m*/*z* 313, and fragments at *m*/*z* 162, 130, and 107 (Figure 3*D*). Very low sampling cone voltage was required for detection of the molecular ion. The conditions for detecting the molecular ion were developed using the authentic standards *S*-(benzohydroximoyl)-L-cysteine and *S*-(benzohydroximoyl)-*N*-acetyl-L-cysteine.

When [U-^{14}C]*p*-hydroxyphenylacetaldoxime and NADPH were added to microsomes from *S. alba* in the absence of a sulfur donor, a radiolabeled compound (X) accumulated in the reaction mixtures as evidenced by TLC (Figure 1, *columns 1* and *6*). The accumulation of this product (X) was reduced when a sulfur donor was added (Figure 1, *columns 2* and *7*). Another compound accumulated in low amounts in ethyl acetate extracts of the reaction mixtures (data not shown). The compound co-migrated with authentic 1-nitro-2-(*p*-hydroxyphenyl)ethane. The identity of the compound was confirmed by gas chromatography-MS (data not shown). The specificity of the oxidation of oxime to nitro for the glucosinolate biosynthetic pathway was investigated by testing the ability of microsomes isolated from another glucosinolate-producing plant (*T. majus*) and a non-glucosinolate-producing plant (*Z. mays*) to produce the nitro compound. Both *T. majus* and maize were able to convert the *p*-hydroxyphenylacetaldoxime into 1-nitro-2-(*p*-hydroxyphenyl)ethane, showing that the reaction is not specific for glucosinolate-producing plants.

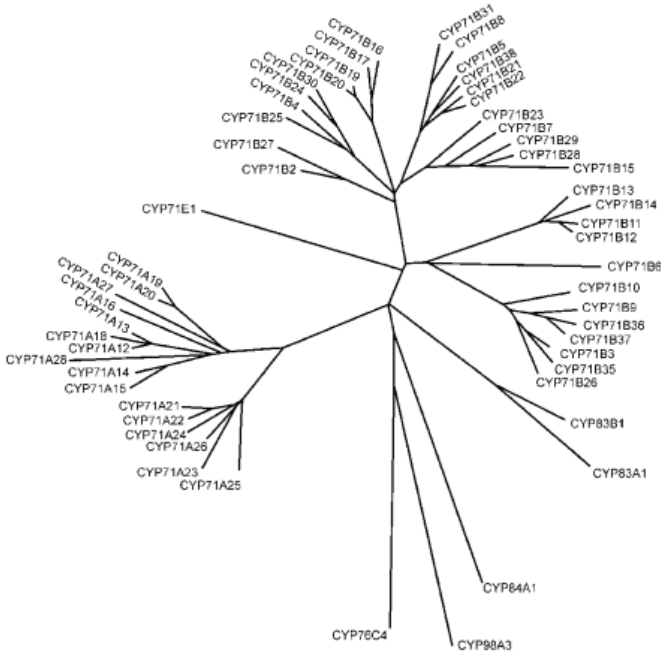

Figure 4. A phylogenetic tree of the CYP71 family in *A. thaliana* and CYP71E1 (P450ox) from *S. bicolor.* The tree was constructed by alignment of the amino acid sequences using ClustalW. Phylogenetic analysis was performed with the Protdist and Fitch (Fitch-Margoliash and least squares method) programs of the Phylip package. Despite their original name, the CYP83s belong correctly to the CYP71 family. CYP76C4, CYP98A3, and CYP84A1 are included as outgroups. CYP71B7, CYP71B6, and CYP83B1 were tested earlier as candidates for the oxime-metabolizing enzyme in the glucosinolate pathway.

Phylogenetic Analysis— The activity of the oxime-metabolizing enzyme in microsomes in *S. alba* was too low to pursue a biochemical approach to identify the cytochrome P450 involved, which we for evolutionary reasons expected to be related to the oxime-metabolizing enzyme in the cyanogenic pathway. Considering the rapid advance of the genome-sequencing project of *A. thaliana*, we took a bioinformatics approach to search for homologues of CYP71E1 (P450ox), which presently is the only oxime-metabolizing enzyme identified in the biosynthetic pathway of cyanogenic glucosides (12). In addition to the cytochromes designated CYP71s, members of the CYP83 family belong correctly to the CYP71 family based on sequence similarity.[2] With the sequences available at the given time (summer/autumn 1999), we combined BLAST searches and sequence alignments, expression data (19), and mutant phenotypes (20)[3] to identify candidates for the oxime-metabolizing enzyme in *A. thaliana*. Among the candidate genes found in the closest related subfamilies, *CYP71B7*,

[2] S. Paquette and S. Bak, personal communication.
[3] S. Bak, personal communication.

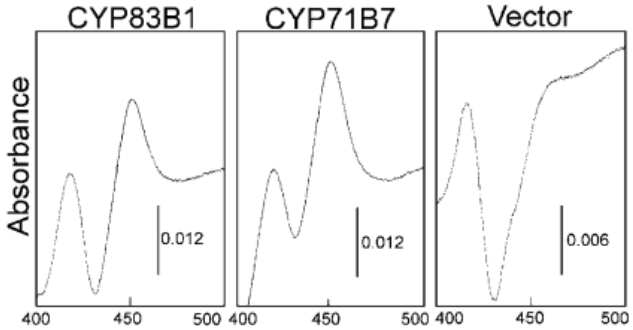

Figure 5. Carbon monoxide difference spectra of CYP71B7 and CYP83B1. The Fe^{2+}·CO *versus* Fe^{2+} difference spectra were measured on spheroplasts of *E. coli* expressing CYP71B7, CYP83B1, or empty vector. The spectra were recorded at 12 °C.

CYP71B6, and *CYP83B1* existed as full-length ESTs and were expressed in *E. coli*. After completion of the genome-sequencing project of *A. thaliana*, a phylogenetic tree of the CYP71 family was made (Figure 4).

Heterologous Expression of CYP71B6, CYP71B7, and CYP83B1 in E. coli— CYP71B6, CYP71B7, and CYP83B1 were expressed in *E. coli* strain C43(DE3) by use of the expression vector pSP19 g10L. CO difference spectrum with the characteristic peak at 450 nm was obtained for CYP71B7 and CYP83B1 indicating the presence of functional cytochrome P450 (Figure 5). Based on the peak at 450 nm, the expression levels of CYP71B7 and CYP83B1 were estimated to be 50 and 430 nmol of cytochrome P450 (liter of culture)$^{-1}$, respectively. The expression of CYP71B6 in *E. coli* did not result in production of a protein giving a peak at 450 nm in a CO difference spectrum; this, however, does not exclude the presence of a functional protein (4). When [U-^{14}C]*p*-hydroxyphenylacetaldoxime and cysteine or *N*-acetylcysteine were added to spheroplasts of *E. coli* expressing CYP83B1 reconstituted with NADPH:cytochrome P450 reductase in the presence of NADPH, a compound accumulated in the reaction mixtures that co-migrated with the cysteine conjugate (Figure 6, *column 4*) and the *N*-acetylcysteine conjugate (Figure 6, *column 9*) produced by the *S. alba* microsomes as evidenced by TLC. Formation of these products was furthermore shown to be dependent on NADPH as the electron source (Figure 6, *columns 5* and *10*). The products were not detected in reaction mixtures containing *E. coli* spheroplasts harboring either the CYP71B6 or CYP71B7 (data not shown) or empty vector (Figure 6, *columns 1* and *6*). The compounds produced from *p*-hydroxyphenylacetaldoxime by recombinant CYP83B1 using cysteine or *N*-acetylcysteine as a sulfur donor were identified by LC-MS analysis as the cyclization product of *S*-(*p*-hydroxyphenylacetohydroximoyl)-L-cysteine or the (uncyclizing) *S*-(*p*-hydroxyphenylacetohydroximoyl)-*N*-acetylcysteine (data not shown), respectively. Recombinant CYP83B1 has a pH optimum of 8.1, and kinetic studies showed that it has a turnover number of 16 min^{-1} and a K_m of 68 μM for *p*-hydroxyphenylacetaldoxime.

When radiolabeled oxime and NADPH were incubated with recombinant CYP83B1 in the absence of a sulfur donor, a radiolabeled compound accumulated in the reaction mixture that co-migrated with the unidentified compound that accumulated in the *S. alba* microsomal reaction mixtures under the same conditions as evidenced by TLC (Figure 6, *columns 3* and *8*). Several attempts to identify the compound (X) were unsuccessful. Accumulation of the unidentified product (X) and of radiolabeling retained at the application site was greatly reduced when a sulfur donor was added (Figure 6, *columns 4* and *9*). No detectable amounts of 1-nitro-2-(*p*-hydroxyphenyl)ethane accumulated in the reaction mixtures

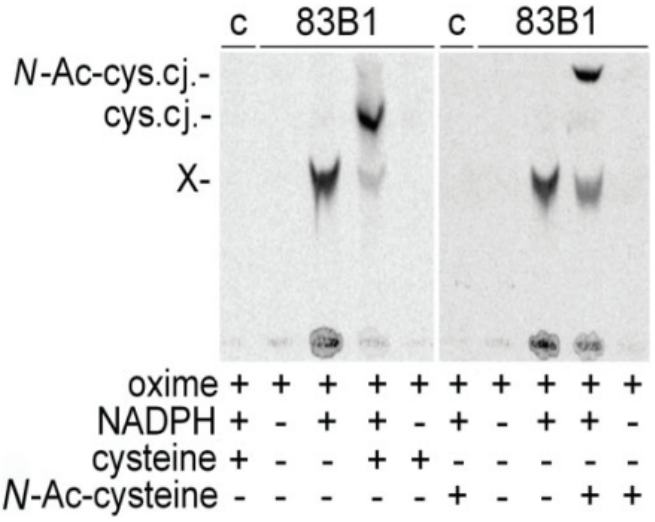

Figure 6. Production of the cysteine conjugate (*cys.cj.*), the *N*-acetylcysteine conjugate (*N-Ac-cys.cj.*), and an unidentified compound (*X*) by CYP83B1 (*83B1*). [U-^{14}C]*p*-Hydroxyphenylacetaldoxime was incubated with recombinant CYP83B1 reconstituted with NADPH:cytochrome P450 reductase in the presence of cysteine (*columns 2-5*) or *N*-acetylcysteine (*columns 7-10*) as a sulfur donor. The reaction mixtures were extracted with ethyl acetate, and the water phases were analyzed by TLC. In the absence of a sulfur donor a compound (*X*) accumulated (*columns 3* and *8*). Empty vector was used as a control (*c*) (*columns 1* and *6*).

containing recombinant CYP83B1. Furthermore, neither 1-nitro-2-(*p*-hydroxyphenyl)ethane nor *p*-hydroxyphenylacetonitrile was metabolized by CYP83B1 (data not shown). This indicates that neither the nitro compound nor nitrile is involved in the reaction catalyzed by CYP83B1.

Substrate Binding to CYP83B1— We analyzed spectrally the binding of substrate and potential intermediates to CYP83B1. Recombinant CYP83B1 was partially purified from *E. coli* spheroplasts by temperature-induced phase partitioning, which produced a detergent rich-phase containing the majority of the cytochrome P450. Recombinant CYP83B1 produced a type I spectrum with *p*-hydroxyphenylacetaldoxime (λ_{max} 390 nm and λ_{min} 427 nm), *p*-hydroxyphenylacetonitrile (λ_{max} 391 nm and λ_{min} 427 nm), and 1-nitro-2-(*p*-hydroxyphenyl)ethane (λ_{max} 391 nm and λ_{min} 427 nm) indicating a shift from a low to a high spin state upon substrate binding (21) (Figure 7, *a*, *b*, and *c*). The amplitude increased in size upon incubation and reached a stable maximum after ~30 min. No substrate binding spectrum was obtained upon the addition of *p*-hydroxyphenylacetamide (data not shown). Although neither the nitrile nor nitro compound was metabolized by CYP83B1, the formation of type I binding spectra demonstrated that CYP83B1 was able to bind these analogues.

DISCUSSION

In the present study, we have characterized biochemically the conversion of *p*-hydroxyphenylacetaldoxime to *S*-(*p*-hydroxyphenylacetohydroximoyl)-L-cysteine in microsomes from *S. alba* and shown that the enzyme activity was dependent on a cytochrome P450 monooxygenase. By combined use of bioinformatics, published expression data, and knock-out phenotypes, we identified CYP83B1 as the oxime-metabolizing enzyme in the glucosinolate pathway. We have used the tyrosine-derived oxime as substrate, although *A. thaliana* does not produce the tyrosine-derived *p*-hydroxybenzylglucosinolate. We have, however, previously shown that *A. thaliana* is capable of converting *p*-hydroxyphenylacetaldoxime into *p*-hydroxybenzylglucosinolate (22).

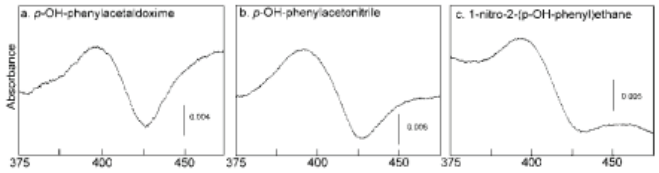

Figure 7. Substrate binding spectra obtained with recombinant CYP83B1. Each cuvette contained 0.25 nmol of recombinant CYP83B1 partly purified into a Triton X-114 detergent-rich phase. 0.2 mM of substrate was added to the sample cuvette, and spectra were recorded at 12 °C after 45 min.

Figure 8. The conversion of oxime to thiohydroximic acid in the glucosinolate pathway. It is presently not known whether the oxime is oxidized to an *aci*-nitro compound or a nitrile oxide by CYP83B1.

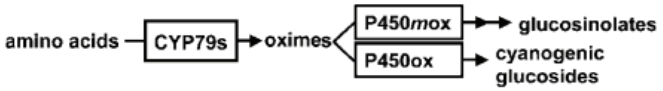

Figure 9. The evolutionary relationship between cyanogenic glucosides and glucosinolates. Identification of the oxime-metabolizing enzyme in the glucosinolate pathway (CYP83B1/P450*m*ox) as related to the oxime-metabolizing enzyme in the cyanogenic pathway (CYP71E1/P450ox) is consistent with the hypothesis that the P450ox in the cyanogenic pathway was mutated into a "P450*m*ox" that converted the oxime into a toxic compound that the plant detoxified into a glucosinolate.

S-(Hydroximoyl)-L-cysteine is the most likely *S*-alkyl thiohydroximate in the glucosinolate pathway for several reasons. First, *in vivo* feeding studies have shown that among other sulfur donors, cysteine is most efficiently incorporated into glucosinolates (23). Second, the enzyme that converts *S*-alkyl thiohydroximate to thiohydroximate is likely to be a C-S lyase, and the characterized C-S lyases from plants hydrolyze *S*-substituted cysteine derivatives and have an absolute requirement for the presence of the α-hydrogen atom and an unsubstituted amino group in the cysteine moiety (10, 24). We have succeeded for the first time in obtaining a mass spectrum providing evidence for enzymatic synthesis of the proposed intermediate *S*-(*p*-hydroxyphenylacetohydroximoyl)-L-cysteine. *In vitro S*-(*p*-hydroxyphenylacetohydroximoyl)-L-cysteine undergoes cyclization, which indicates that the proposed C-S lyase is tightly coupled to the sulfur-conjugating enzyme *in vivo*.

For the oxime to be converted into the *S*-alkyl thiohydroximate, it needs to be oxidized prior to conjugation with the sulfur donor. We have shown the production of 1-nitro-2-(*p*-hydroxyphenyl)ethane from *p*-hydroxyphenylacetaldoxime in microsomes from *S. alba*, *T. majus*, and *Z. mays*. Although recombinant CYP83B1 is able to bind the nitro to the active site, no nitro production or metabolism by the enzyme was detected, indicating that nitro compounds are not intermediates in the glucosinolate pathway. The form of the oxidized oxime might be the corresponding *aci*-nitro compounds (8) or nitrile oxides[4] (Figure 8), which are interconvertible by a simple dehydration/hydration reaction and which are very reactive compounds and subject to nucleophilic attack by *e.g.* cysteine. Based on the expected strong reactivity of the oxidized oxime, conjugation of the sulfur donor is likely to be under strict control. The proposed cysteine conjugation might be carried out by a glutathione *S*-transferase. Alternatively, CYP83B1 may have a binding site not only for the oxime but also for cysteine. In the absence of added sulfur donor, an unidentified compound (X) accumulated in the reaction mixture. This compound might be a furoxan (1,2,5-oxadiazole 2-oxide) formed by dimerization of nitrile oxide, or it might be a conjugate derived from a nucleophile present in the reaction mixture. Further studies are in progress to elucidate the mechanism for the formation of the cysteine conjugate.

Cyanogenic glucosides and glucosinolates are related groups of natural plant products derived from amino acids and with oximes as intermediates. Cyanogenic glucosides occur throughout the plant kingdom. This indicates that cyanogenesis arose as a very early evolutionary event. In contrast, glucosinolates are restricted to the order Capparales and the genus *Drypetes* in the distant order Euphorbiales (25). Cytochromes P450 belonging to the CYP79 family have been shown to catalyze the conversion of amino acids to oximes in both the cyanogenic and the glucosinolate pathway (3-7, 26), supporting the speculation that the biosynthesis of glucosinolates has evolved from the cyanogenic pathway (27).

[4] A. Kjær, personal communication.

If evolution of glucosinolates is based on a "cyanogenic predisposition," this raises the question of how glucosinolates evolved. In the biosynthetic pathway of the cyanogenic glucoside dhurrin in *S. bicolor*, the oxime-metabolizing enzyme P450ox (CYP71E1) converts the oxime to a α-hydroxynitrile (26). Our working hypothesis has been that a *mutated* homologue of P450ox, P450*m*ox, catalyzes the oxime-metabolizing step in the biosynthetic pathway of glucosinolates (Figure 9). According to the hypothesis, P450*m*ox would oxidize the oxime to a toxic or reactive compound such as an *aci*-nitro or a nitrile oxide, which the plant subsequently would have to detoxify. The post-oxime enzymes include the proposed glutathione *S*-transferase and C-S lyase in addition to the known glucosyltransferase and sulfotransferase. These enzyme groups are known to be involved in general detoxification reactions. This makes it likely that post-oxime enzymes have been recruited from the detoxification processes but now are specialized for glucosinolate production. Several mutants of CYP83B1 have been reported (20, 28, 29). Recently, CYP83B1 was described as a regulator of auxin production by controlling the flux of indole-3-acetaldoxime into indole-3-acetic acid and indole glucosinolates (29). Characterization of recombinant CYP83B1 expressed in yeast showed that indole-3-acetaldoxime is a high affinity substrate for CYP83B1. The product obtained was shown to form adducts with a number of thiol compounds (29). It has been suggested that the glucosinolate pathway evolved from an indole-3-acetic acid biosynthetic pathway and not necessarily from the cyanogenic pathway (29). Cyanogenic glucosides are derived from only five protein amino acids (Tyr, Phe, Leu, Ile, and Val). The same few protein amino acids are also precursors for glucosinolates together with tryptophan, alanine, methionine, and chain-elongated derivatives of methionine and phenylalanine, which are not precursors of cyanogenic glucosides. This suggests that CYP79s of the glucosinolate pathway have acquired new substrate specificities after having diverged from the "cyanogenic" CYP79s. The biosynthesis of tryptophan-derived and chain-elongated amino acid-derived glucosinolates seems to be recent evolutionary events because indole glucosinolates are present in only four families in the order Capparales, namely in the Brassicaceae, Resedaceae, Tovaraceae, and Capparaceae (30), and because glucosinolates from chain-elongated amino acids are only found in Brassicaceae, Resedaceae, and Capparaceae (1). Considering the taxonomical distribution of cyanogenic glucosides and glucosinolates in the plant kingdom and of indole glucosinolates in only four families in the Capparales order (30), it appears likely that glucosinolates have evolved from a cyanogenic predisposition.

The CYP71 family is the largest cytochrome P450 family in *A. thaliana* with 47 members that cluster into different subgroups (Figure 4). In the *Arabidopsis* genome, another member of the CYP83 family, *CYP83A1*, with 65% identity to *CYP83B1* at the amino acid level has been identified. The high sequence similarity suggests that CYP83A1 may be an oxime-metabolizing enzyme in the glucosinolate pathway. The two CYP83s form a little subgroup that is located close to the CYP71E1, which is consistent with an evolutionary relationship between the oxime-metabolizing enzymes in the cyanogenic and the glucosinolate pathway.

Within the last few years considerable advances in our understanding of glucosinolate biosynthesis have been achieved. Identification of the oxime-metabolizing enzymes in both the cyanogenic and the glucosinolate pathways combined with identification of substrate-specific oxime-producing CYP79s provides important molecular tools for metabolic engineering of glucosinolate profiles and for introduction of cyanogenic glucosides and other oxime-derived compounds in a glucosinolate or a non-glucosinolate background.

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