CYP83B1 Is the Oxime-metabolizing Enzyme in the Glucosinolate Pathway in *Arabidopsis*

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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-hydroxyphenylacetaldothiohydroximate, S-(p-hydroxyphenylacetylthiohydroximoyl)-L-cysteine, the next proposed intermediate in the glucosinolate pathway. The production was shown to be dependent on a cytochrome P450 monoxygenase. 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 “P450nox” 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-[1,2,14C]phenylethane into benzyglucosinolate (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 β-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 monoxygenase. 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 1-cys-
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to 3-aminophenol was added, and after an additional 1 h of growth, the cultures were harvested and frozen in liquid nitrogen.

Phylogenetic Trees

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 pSP91 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′-GAATTCATATGGCTATCTTGCTCTGTTTC-3′) and primer 2 (antisense direction; 5′-GGTACGATGATCCGTTGAAGTCTGTTGAT-3′). The PCR product 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 56 °C, and 30 s at 72 °C. The PCR fragment was digested with EcoRI and PstI, ligated into pBluescript II SK (Stratagene), and transferred from pBluescript II SK to an NdeI/SphI-digested pSP91 g10L vector. The CYP71B7 gene was PCR-amplified from EST 5G6 using primer 3 (sense direction; 5′-GAATTCATATGGCTATCTTGCTCTGTTTC-3′) and primer 4 (antisense direction; 5′-GGTACGATGATCCGTTGAAGTCTGTTGAT-3′). The PCR product was set up as described above. The PCR was incubated for 2 min at 94 °C, 23 cycles of 15 s at 94 °C, 20 s at 56 °C, and 30 s at 72 °C. The PCR fragment was digested with EcoRI and BamHI, ligated into pBluescript II SK, and transferred from pBluescript II SK to an NdeI/BamHI-digested pSP91 g10L vector. The CYP83B1 coding region was amplified from the EST 148G2T7 by PCR with primer 5 (sense direction; 5′-GAATTCATATGGCTATCTTGCTCTGTTTC-3′) and primer 6 (antisense direction; 5′-GGTACGATGATCCGTTGAAGTCTGTTGAT-3′). The PCR product 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 30 s at 72 °C. The PCR fragment was digested with EcoRI and BamHI, ligated into pBluescript II SK, and transferred from pBluescript II SK to an NdeI/BamHI-digested pSP91 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 ampicillin. 2 ml of culture was used to inoculate 200 ml of modified TB medium containing 50 mg/ml ampicillin (14). The cultures were grown at 37 °C at 250 rpm until A600 was 0.5-0.7. Then 1 ml thiamine and 75 μg/ml β-aminolevulinate were added, and after an additional 1 h of growth, the cultures were induced with 1 mM isopropanol.β-thiogalactoside and subsequently grown at 28 °C for 40 h at 125 rpm. Spheroplasts were prepared as previously described (14) except that glycero was omitted in the final buffer. The amount of expressed functional cytochrome P450 was monitored by Fe(II)CO versus Fe(II) difference spectroscopy and quantified using an extinction coefficient of 91 mM−1 cm−1.
C43(ΔE3) 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-hydroxyphenylacetoxime 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-hydroxyphenylacetoxime 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, pH 7.5, and 0.2 mM of either p-hydroxyphenylacetoxime, 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-14C]-p-hydroxyphenylacetoxime 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 and the water phases were analyzed by TLC. As a control boiled microsomes from S. alba—biosynthetically active microsomes from S. alba—were used (Figure 1, column 6–10). The product formation was significantly reduced when NADPH was not added to the reaction mixture (columns 8 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 1. Production of the cysteine conjugate (cy-cj), the N-acetylcysteine conjugate (N-Ac-cys-cj.), and an unidentified compound (X) by S. alba microsomes. [U-14C]-p-Hydroxyphenylacetoxime 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.

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-hydroxyphenylacetothiohydroximoyl)-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-hydroxyphenylacetothiohydroximoyl)-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-14C]-p-hydroxyphenylacetoxime 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-hydroxyphenylacetothiohydroximoyl)-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 2D, can only take place if
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 reaction mixtures as evidenced by TLC (Figure 1, columns 1 and 6). The activity of the oxime-metabolizing enzyme in 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.

*Phylogenetic Analysis—* The activity of the oxime-metabolizing enzyme in microsomes from 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 bioinformatic 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. 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.

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 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 (S)-(p-hydroxyphenylacetohydroximoyl)-L-cysteine showing [M + Na]⁺ at m/z 335, [M + H]⁺ at m/z 313, and [M + Na]⁺ at m/z 107.

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 Protist 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.

2 S. Paquette and S. Bak, personal communication.

3 S. Bak, personal communication.
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-¹⁴C]-p-hydroxyphenylacetaldoloxime and cysteine or N-acetyl cysteine were added to spheroplasts of E. coli expressing CYP83B1 reconstituted with NADPH:cytochrome P450 reductase in the presence of cysteine (Figures 4, 5, and 7), the N-acetyl cysteine conjugate (N-Ac-cys.cj.) was produced in E. coli—CYP71B7, CYP83B1, or empty vector. The spectra were recorded at 12 °C.

In the present study, we have characterized biochemically the conversion of p-hydroxyphenylacetaldoloxime to S-(p-hydroxyphenylacetoxydihydroximoyl)-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-hydroxyphenylacetaldoloxime into p-hydroxybenzylglucosinolate (22).
S-(Hydroximoyl)-L-cysteine is the most likely S-alkyl thiophosphoramide in the glucosinolate pathway for several reasons. First, in vitro feeding studies have shown that among other sulfur donors, cysteine is most efficiently incorporated into glucosinolates (23). Second, the enzyme that converts S-alkyl thiophosphoramide to thiophosphoramide 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 thiophosphoramide, 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-hydroxyphenylacetoxime 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 nitrite oxides (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-3-oxide) formed by dimerization of nitrite 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).

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.

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, P450mox, catalyzes the oxime-metabolizing step in the biosynthetic pathway of glucosinolates (Figure 9). According to the hypothesis, P450mox would oxidize the oxime to a reactive or 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 sulfate transferase. 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.

Figure 8. The conversion of oxime to thiophosphoramic 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/P450ox) 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 “P450mox” that converted the oxime into a toxic compound that the plant detoxified into a glucosinolate.
The CYP71 family is the largest cytochrome P450 family in *Arabidopsis* 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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