CYP83A1 and CYP83B1, Two Nonredundant Cytochrome P450 Enzymes Metabolizing Oximes in the Biosynthesis of Glucosinolates in Arabidopsis[1]

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In the glucosinolate pathway, the postoxime enzymes have been proposed to have low specificity for the side chain and high specificity for the functional group. Here, we provide biochemical evidence for the functional role of the two cytochromes P450, CYP83A1 and CYP83B1, from Arabidopsis in oxime metabolism in the biosynthesis of glucosinolates. In a detailed analysis of the substrate specificities of the recombinant enzymes heterologously expressed in yeast (*Saccharomyces cerevisiae*), we show that aliphatic oximes derived from chain-elongated homologs of methionine are efficiently metabolized by CYP83A1, whereas CYP83B1 metabolizes these substrates with very low efficiency. Aromatic oximes derived from phenylalanine, tryptophan, and tyrosine are metabolized by both enzymes, although CYP83B1 has higher affinity for these substrates than CYP83A1, particularly in the case of indole-3-acetaldoxime, where there is a 50-fold difference in K_m value. The data show that CYP83A1 and CYP83B1 are nonredundant enzymes under physiologically normal conditions in the plant. The ability of CYP83A1 to metabolize aromatic oximes, albeit at small levels, explains the presence of indole glucosinolates at various levels in different developmental stages of the *CYP83B1* knockout mutant, *rnt1-1*. Plants overexpressing CYP83B1 contain elevated levels of aliphatic glucosinolates derived from methionine homologs, whereas the level of indole glucosinolates is almost constant in the overexpressing lines. Together with the previous characterization of the members of the CYP79 family involved in oxime production, this work provides a framework for metabolic engineering of glucosinolates and for further dissection of the glucosinolate pathway.

Glucosinolates are amino acid-derived natural plant products, containing a thio-Glc moiety and a sulfonate moiety bound to an oxime function. They are implicated in plant-insect and plant-pathogen interactions, and for humans, they have attracted attention as cancer-preventive agents and flavor compounds (for review, see Halkier, 1999; Rask et al., 2000). In recent years, significant advances have been made in our understanding of the biosynthetic pathway of glucosinolates, which can be divided into the chain elongation pathway, the formation of the core structure, i.e. the conversion of precursor amino acid to parent glucosinolate, and secondary modifications of side chain and Glc moiety of the parent glucosinolate (Wittstock and Halkier, 2002). In the biosynthesis of the core structure, cytochromes P450 belonging to the CYP79 family catalyze the first committed step, i.e. the conversion of amino acids to oximes (for review, see Halkier et al., 2002). Cytochromes P450 belonging to the CYP83 family have been shown to catalyze the conversion of aromatic oximes (Bak and Feyereisen, 2001; Bak et al., 2001; Hansen et al., 2001b). The remaining pathway leading to the parent glucosinolate proceeds through a thiohydroximic acid that is first glucosylated to give a desulphoglucosinolate that is finally sulfonated.

Glucosinolates are related to another group of natural plant products, cyanogenic glucosides, because both are derived from amino acids that are converted to oximes by cytochromes P450 belonging to the CYP79 family. The oximes form the branching point between the two pathways. In the biosynthetic pathway of the Tyr-derived cyanogenic glucoside dhurrin in sorghum (*Sorghum bicolor*), the substrate specificity of the involved enzymes widens as the pathway proceeds (Jones et al., 1999). The first enzyme, CYP79A1, accepts only one amino acid (Tyr) as substrate. The postoxime enzymes, CYP71E1 and sbHMNGT, retain high specificity for the functional group, oxime and α-hydroxynitrile, respectively, but are more promiscuous as to the nature of the side chain of the parent amino acid (Jones et al., 1999; Kahn et al., 1999; Jones and Vogt, 2001). Because oximes are not common constituents of plants, it seems reasonable that selection pressure for the side chain specificity for oximes

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would not be maintained (Kahn et al., 1999). As in the cyanogenic pathway, the CYP79s in the glucosinolate pathway represent the substrate-specific step, whereas the postoxime enzymes have been reported to have high substrate specificity for the functional group and low specificity for the side chain. This is based on biochemical evidence obtained with purified plant extracts (for review, see Halkier, 1999) and by metabolic engineering of novel glucosinolates in planta by production of exogenous oximes (Bak et al., 1999; Mikkelsen and Halkier, 2003).

Recombinant CYP83B1 has been characterized in vitro and shown to metabolize aromatic oximes derived from Trp, Phe, and Tyr to the corresponding *S*-alkyl thiohydroximates (Bak and Feyereisen, 2001; Bak et al., 2001; Hansen et al., 2001b). The CYP83A1 homolog with 65% identity at the amino acid level to CYP83B1 (Paquette et al., 2000) has been shown to metabolize the same aromatic oximes (Bak and Feyereisen, 2001). The substrate specificity of CYP83A1 and CYP83B1 with respect to aliphatic oximes is not known.

Two tDNA mutants of the *CYP83B1* gene have been identified, *rnt1-1* (Winkler et al., 1998) and *sur2* (Delarue et al., 1998). The mutants have a phenotype caused by increased levels of the phytohormone indole-3-acetic acid (IAA; Delarue et al., 1998; Barlier et al., 2000). The increased level of IAA implies that blockage of the CYP83B1-catalyzed metabolism of indole-3-acetaldoxime in the biosynthetic pathway leading to indole glucosinolates results in accumulation of indole-3-acetaldoxime, which is a precursor of IAA. Because indole-3-acetaldoxime is the branch point between at least two biosynthetic pathways, alterations in the level of either CYP83A1 or CYP83B1 might have dramatic consequences on the metabolite profile of the plants.

In the present paper, we have made a kinetic analysis of the substrate specificity of CYP83A1 and CYP83B1 to determine the functional role of these enzymes in the biosynthetic pathway of glucosinolates. We demonstrate that aliphatic oximes derived from chain-elongated homologs of Met are metabolized very efficiently by CYP83A1 and that CYP83B1 does so with much lower efficiency. Both enzymes metabolize aromatic oximes, although CYP83B1 exhibits the highest affinity for these substrates. In addition, *rnt1-1* and transgenic plants with ectopic overexpression of the *CYP83A1* and *CYP83B1* genes have been characterized with respect to their glucosinolate content.

RESULTS

Metabolism of Aliphatic Oximes by CYP83A1 and CYP83B1

The substrate specificity of CYP83A1 and CYP83B1 in biosynthesis of aliphatic glucosinolates was investigated by testing aliphatic oximes derived from chain-elongated Met derivatives as substrates of the recombinant enzymes. CYP83A1 was incubated with 4-methylthiobutanaldoxime in the presence of Cys and NADPH, and the aqueous phase after extraction with organic solvents was analyzed by liquid chromatography (LC)-mass spectrometry (MS). A unique product accumulated in the reaction mixture as evidenced by a new peak in the LC profile (Fig. 1A). The peak was dependent on the presence of enzyme and NADPH. The product was proposed to be the *S*-alkylthiohydroximate designated *S*-(4-methylthiobutylhydroximoyl)-L-Cys, the conjugate between the oxidized aliphatic oxime and Cys, analogous to the conjugates produced when using aromatic oximes as substrates and other thiol-containing compounds as nucleophile (Bak and Feyereisen, 2001; Bak et al., 2001; Hansen et al., 2001b). Conjugates of oxidized aromatic oximes and Cys have been shown previously to undergo an internal cyclization reaction yielding a thiazoline compound (Hansen et al., 2001b). The retention time and fragmentation pattern of the molecular ion at the product peak was in accordance with the product being 2-(3-methylthiopropyl)-thiazoline-4-carboxylic acid, the cyclization product of *S*-(4-methylthiobutanhydroximoyl)-L-Cys. CYP83B1 produced only minute amounts of this compound close to the detection limit (approximately 25 pmol) of the system (Fig. 1B). 5-Methylthiopentanaldoxime, 6-methylthiohexanaldoxime, and 7-methylthioheptanaldoxime were also tested as substrates for CYP83A1 and CYP83B1 and gave similar results, demonstrating that aliphatic oximes are substrates for CYP83A1 and to a much lesser extent for CYP83B1 (data not shown).

Determination of K_m values for CYP83A1 and CYP83B1

The functional role of CYP83A1 and CYP83B1 in biosynthesis of aromatic glucosinolates was investigated in a detailed kinetic analysis. ^{14}C-labeled oximes were used as substrates, Cys was included as nucleophile, and the apparent K_m values were determined. Generally, CYP83B1 has a higher affinity for aromatic oximes than CYP83A1, as evidenced by lower apparent K_m values (Table I). This is particularly true for indole-3-acetaldoxime, where there is a 50-fold difference in the K_m value for the two enzymes (Bak and Feyereisen, 2001). For the other aromatic oximes, there is a 3-fold difference in K_m value between the two enzymes, with the K_m value being lowest for CYP83B1. This difference becomes more marked when looking at the catalytic efficiency (K_{cat}/K_m; Table I), where for phenylacetaldoxime there is a 6-fold difference between CYP83A1 and CYP83B1.

For determination of K_m values for aliphatic oximes, ^{35}S-labeled glutathione was used instead of Cys. When CYP83A1 was incubated with 4-methylthiobutanaldoxime in the presence of [^{35}S]-glutathione as the nucleophile, an NADPH-dependent product was formed as evidenced by a band on a TLC plate

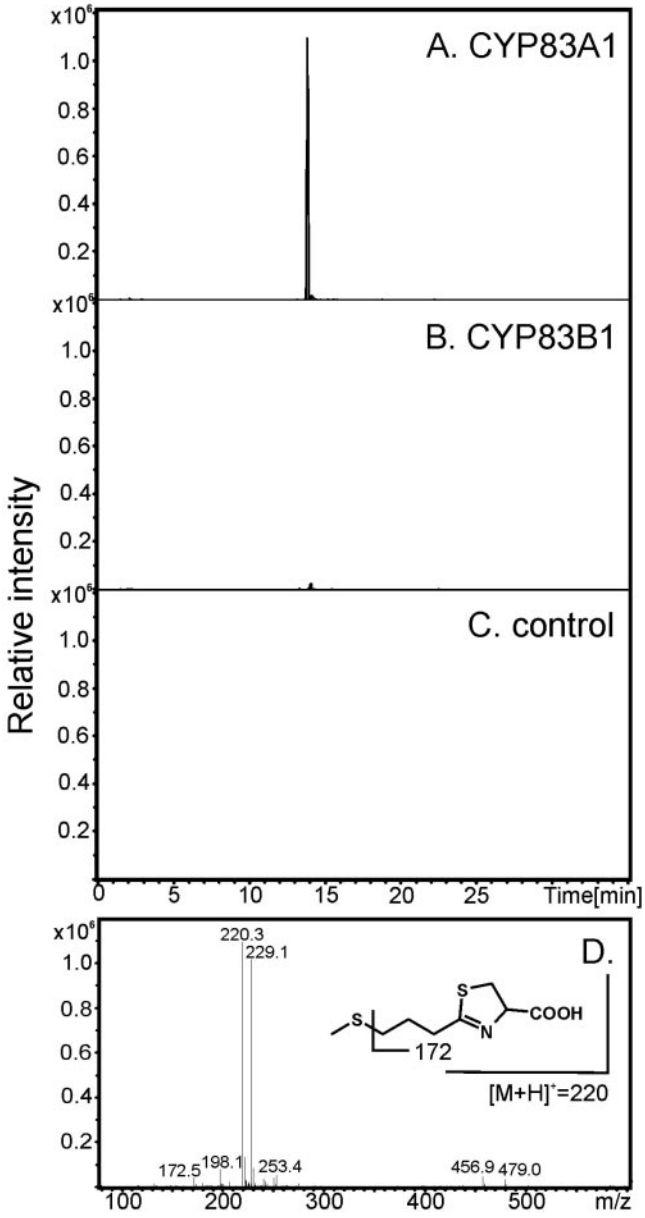

Figure 1. Production of conjugate between Cys and oxidized aliphatic oximes by CYP83A1 and CYP83B1. Reaction mixtures containing 4-methylthiobutanaldoxime, Cys, and recombinant CYP83A1 or CYP83B1 were incubated in the presence of NADPH for 30 min at 29°C and extracted twice with CH_2Cl_2. The aqueous phase was applied to LC-MS analysis. A to C, Reconstructed ion chromatogram at mass-to-charge ratio (*m/z*) 220 of reaction mixtures containing, respectively, CYP83A1 and CYP83B1, and a control reaction without enzyme. D, Mass spectrum of the peak at 13.8 min in A showing $[M + H]^+$ at *m/z* 220 corresponding to the expected cyclization product of the cysteine conjugate, 2-(3-methylthiopropyl)-thiazoline-4-carboxylic acid, and a fragment ion at *m/z* 172. The ion at *m/z* 229 is not specific for the enzymatic reaction and is found in all samples.

(Fig. 2). No product was detected in reconstitution experiments using CYP83B1. The product is most likely the *S*-alkylthiohydroximate designated *S*-(4-methylthiobutylhydroximoyl)-glutathione, analogous to the conjugates produced when using Cys as sulfur donor. The product comigrated with glutathione conjugates produced from radiolabeled aromatic oximes in the applied TLC system (data not shown). Another strong band appeared in the CYP83A1 lane. This band is dependent on NADPH, the oxime, and glutathione and may represent a breakdown product of *S*-(4-methylthiobutylhydroximoyl)-glutathione. The K_m values of recombinant CYP83A1 for aliphatic oximes were estimated to be in the range of 20 to 150 μM. Because the product band could not be separated from a smear of radioactivity supposed to be oxidation products of glutathione, accurate estimation of K_m values was not performed. K_m could not be estimated for CYP83B1 due to the low metabolism of aliphatic oximes by CYP83B1.

Characterization of 35S::CYP83 Plants in Wild-Type and *rnt1-1* Background

The effect of ectopic overexpression of CYP83A1 and CYP83B1 under the control of the 35S promoter was analyzed with respect to glucosinolate profiles and morphological phenotypes. The 35S::CYP83A1 plants did not show any apparent visual phenotype in the wild-type background. The glucosinolate profile of the 35S::CYP83A1 plants in the wild-type background had similar levels of indole and aliphatic glucosinolates as wild-type plants, both at the seedling stage and with fully expanded rosette leaves (Fig. 3). Approximately one-half of the 35S::CYP83B1 lines displayed a characteristic visual phenotype, such as early flowering and faciated stems (Fig. 4). Typically, the 35S::CYP83B1 plants developed the first inflorescence 4 to 6 d before wild type. Individual 35S::CYP83B1 lines contained up to 2 times more aliphatic glucosinolates, whereas indole glucosinolate levels were comparable with wild type (Fig. 5). The lines with the highest levels of aliphatic glucosinolates also had the most severe phenotype.

We analyzed the glucosinolate content in the *rnt1-1* mutant, which is a knockout of *CYP83B1*. Ten-day-old *rnt1-1* seedlings contained reduced levels of both indole and aliphatic glucosinolates. In *rnt1-1* seedlings, indole glucosinolate levels were reduced by 50%, whereas aliphatic glucosinolates levels were reduced by only 30% compared with wild-type levels. *rnt1-1* seedlings are characterized by excessive proliferation of roots. In accordance, the reduced levels of indole glucosinolates observed may be underestimated because roots generally contain more indole and less aliphatic glucosinolates than the aerial parts of the wild-type plant (Petersen et al., 2002). In the *rnt1-1* mutant, rosette leaves at the stage just before bolting contained indole glucosinolate levels comparable with wild-type rosette leaves at the same developmental stage. This was unexpected because a mutant with a null allele in *CYP83B1* is expected to cause a reduction in the level of indole glucosino-

Table I. *K_m values for CYP83A1 and CYP83B1 using aromatic oximes*

The K_m values (micromolar) were determined in reaction mixtures containing recombinant CYP83A1 and CYP83B1, radiolabeled oximes derived from Trp, Tyr, and Phe, respectively, and Cys. After 1 min of incubation, the reaction was stopped by addition of ethanol to 50% (v/v) and subjected to thin-layer chromatography (TLC) analysis. Radiolabelled bands were visualized and quantified using a Storm PhosphoImager.

Substrate	CYP83A1			CYP83B1		
	K_m	K_{cat}	K_{cat}/K_m	K_m	K_{cat}	K_{cat}/K_m
Indole-3-acetaldoxime[a]	150 ± 15	140 ± 10	0.93	3.1 ± 0.4	52 ± 2	16
p-Hydroxyphenylacetaldoxime	156 ± 16	26 ± 1.1	0.17	65 ± 8	14 ± 1.4	0.22
Phenylacetaldoxime	556 ± 92	25 ± 3.8	0.045	188 ± 23	47 ± 2.1	0.25

[a] Bak and Feyereisen, 2001.

lates. On the contrary, the level of aliphatic glucosinolates in rosette leaves of *rnt1-1* was increased approximately 2-fold. This increase was primarily caused by a 4- to 5-fold accumulation of 3-methylsulphinylglucosinolate (Fig. 5). This indicates that the content of aliphatic glucosinolates is affected by the metabolic status of the plant. Furthermore, we analyzed transgenic plants, where CYP83A1 was introduced into the *rnt1-1* background under control of the 35S promoter. Comparison between the effect of overexpressing CYP83A1 in *rnt1-1* and wild-type genetic backgrounds was made possible because the 35S::CYP83A1 line in the wild-type background was generated from a segregating *rnt1-1/RNT1-1* population. Overexpression of CYP83A1 in the *rnt1-1* background restored the content of both the aliphatic and indole glucosinolates to near wild-type levels at both the seedling stage and at the stage just before bolting. This confirms the in vitro data that CYP83A1 is able to metabolize indole-3-acetaldoxime. Similarly, overexpression of CYP83A1 in the wild-type background gave a wild-type glucosinolate profile (see above).

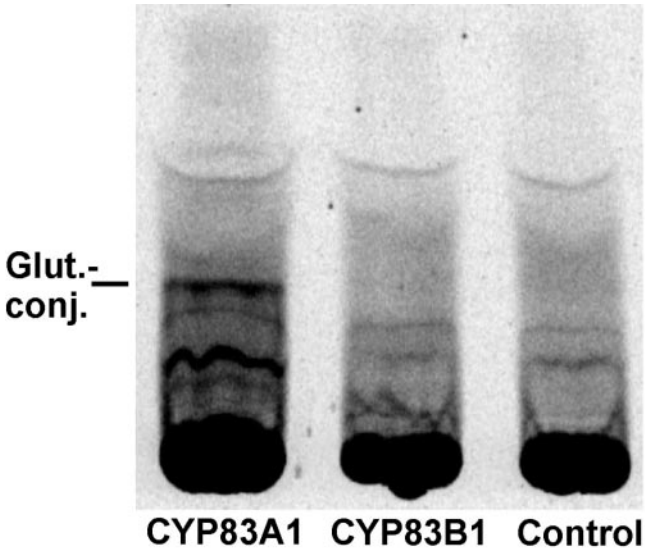

Figure 2. Production of conjugate between [^{35}S]glutathione and aliphatic oximes by CYP83A1 and CYP83B1. Recombinant CYP83A1 and CYP83B1 were incubated with 4-methylthiobutanaldoxime and [^{35}S]glutathione in the presence of NADPH at 29°C for 5 min and stopped by the addition of ethanol to 50% (v/v). Enzyme was omitted in control samples. After incubation, reaction mixtures were analyzed by TLC. The marked band represents a proposed *S*-(4-methylthiobutylhydroximoyl)-glutathione identified based on the R_F value of conjugates between aromatic oximes and glutathione.

Reverse Transcriptase (RT)-PCR Analysis

Expression levels of *CYP79* genes and *CYP83A1* were monitored by semiquantitative RT-PCR performed on RNA isolated from, respectively, rosette leaves of 5-week-old plants and 10-day-old seedlings of wild type and *rnt1-1*. For each primer set, the optimal number of cycles was determined based upon 20 to 30 cycles for *Actin1* and 32 to 38 cycles for the individual CYP79s (for details, see "Materials and Methods"). PCR with *Actin1* primers was done to ensure that equal amounts of the RT reactions were used as well as equal loading. For *CYP83A1*, the expression levels in both wild-type and *rnt1-1* seedlings and in rosette leaves from 5-week-old wild type were similar, whereas it was increased 1.5- ± 0.13-fold in rosette leaves from 5-week-old *rnt1-1* (Fig. 6). For *CYP79B2* and *CYP79B3*, expression levels were 1.3- ± 0.12-fold and 2.8- ± 0.32-fold higher in *rnt1-1* seedlings than in wild type, respectively, and 2.1- ± 0.07-fold and 2.1- ± 0.20-fold higher for rosette leaves of 5-week-old *rnt1-1* than for wild type, respectively. For *CYP79F1*, the only significant difference was a 1.5- ± 0.21-fold higher expression level in *rnt1-1* seedlings than in wild-type seedlings, whereas the expression level of *CYP79F2* was 2.3- ± 0.51-fold and 1.7- ± 0.28-fold higher in *rnt1-1* seedlings and 5-week-old plants than similar wild type, respectively.

DISCUSSION

We have demonstrated that CYP83A1 efficiently metabolizes aliphatic oximes derived from chain-elongated homologs of Met, whereas CYP83B1 is very inefficient in metabolism of aliphatic oximes. Both CYP83A1 and CYP83B1 metabolize aromatic oximes, but CYP83B1 does it more efficiently. The ability of CYP83A1 and CYP83B1 to primarily me-

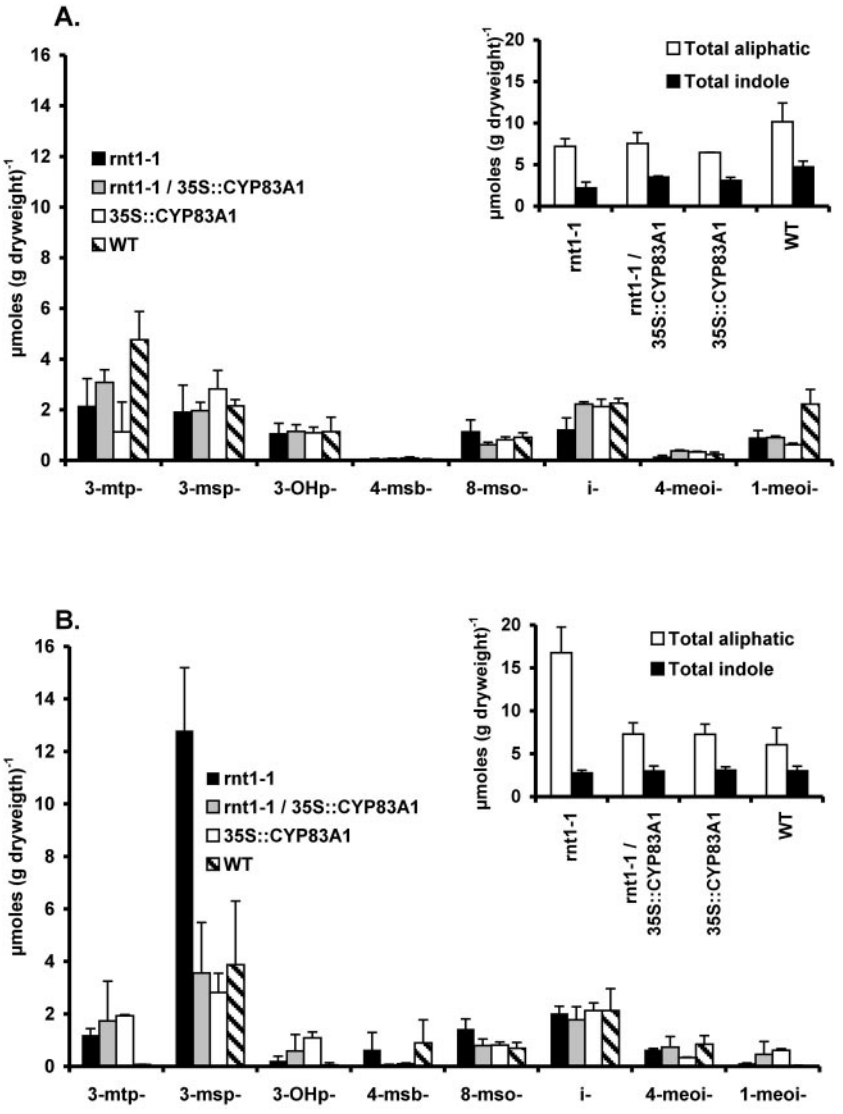

Figure 3. Major glucosinolates of 35S::CYP83A1 plants in *rnt1-1* and wild-type background. Glucosinolate content of wild type, *rnt1-1*, and 35S::CYP83A1 in *rnt1-1* and wild-type backgrounds was analyzed by HPLC. The experiments were done in triplicate. A, Ten-day-old seedlings. B, Rosette leaves harvested at the stage just before bolting. 3-mtp-, 3-Methylthiopropylglucosinolate; 3-msp-, 3-methylsulphinylpropylglucosinolate; 3-OHp-, 3-hydroxypropylglucosinolate; 4-msb-, 4-methylsulphinylbutylglucosinolate; 8-mso-, 8-methylsulphinyloctylglucosinolate; i-, indolylglucosinolate; 4-meoi-, 4-methoxyindolylglucosinolate; and 1-meoi-, 1-methoxyindolylglucosinolate. The inserts show total aliphatic and indole glucosinolate content for the respective lines.

tabolize aliphatic and aromatic oximes, respectively, is in agreement with the amplitude of the reverse type II difference spectra obtained with aliphatic and aromatic primary amines (Bak and Feyereisen, 2001). The ability of CYP83B1 to metabolize aliphatic oximes at low efficiency in vitro is unlikely to be of significance in vivo under normal physiological conditions because unphysiologically high concentrations of substrates were used in the assays. The situation is reversed for aromatic oximes because the low K_m and high K_{cat}/K_m values of recombinant CYP83B1 for aromatic oximes indicate that these oximes are preferentially metabolized by CYP83B1 in vivo. Although CYP83B1 is 17 times more efficient in metabolism of indole-3-acetaldoxime than CYP83A1 in terms of catalytic efficiency and 6-fold more efficient toward phenylacetaldoxime, the difference in catalytic efficiency toward *p*-hydroxyphenylacetaldoxime is negligible. This shows that CYP83B1 has the highest efficiency for indole-3-acetaldoxime, whereas the differences are less pronounced for the two other aromatic oximes. However, under abnormal condition such as in *CYP83B1* knockout plants, CYP83A1 metabolizes aromatic oximes as evidenced by the presence of indole glucosinolates in these plants. The relatively higher K_m values for Tyr- and Phe-derived oximes suggest that these compounds are not naturally occurring substrates for the CYP83 enzymes. This is supported by the observation that Arabidopsis does not contain Tyr- or Phe-derived glucosinolates. However, the ability of CYP83A1 and CYP83B1 to metabolize Tyr- and Phe-derived oximes suggests that Arabidopsis has the potential of making Tyr- and Phe-derived glucosinolates. In support of this is the accumulation of *p*-hydroxy-benzylglucosinolate in Arabidopsis by overexpression of sorghum CYP79A1 (Bak et al., 1999) and of benzylglucosinolates by overexpression of Arabidopsis CYP79A2 (Wittstock and Halkier, 2000). Sorghum CYP79A1 catalyzes the conversion of Tyr to

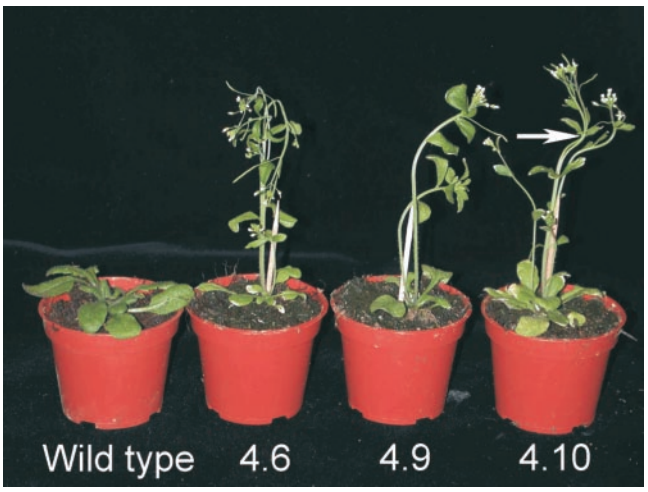

Figure 4. Phenotypes of 4-week-old 35S::CYP83B1 Arabidopsis plants. Approximately one-half of the 35S::CYP83B1 lines had characteristic visual phenotypes, such as early bolting and faciated stems (indicated with an arrow). The plants shown are representatives of the lines with the most severe phenotypes.

p-hydroxyphenylacetaldoxime in the biosynthesis of the cyanogenic glucoside dhurrin (Koch et al., 1995). The in vivo function of CYP79A2 is not currently understood, but CYP79A2 has been shown in vitro to catalyze the conversion of Phe to phenylacetaldoxime and not the conversion of homo-Phe to its corresponding oxime, for which no CYP79 has yet been identified (Wittstock and Halkier, 2000).

The glucosinolate profiles of transgenic plants overexpressing either CYP83A1 or CYP83B1 exhibited wild-type levels of indole glucosinolates. This suggests that CYP83B1 is not rate limiting and that the level of indole glucosinolates is controlled at the level of indole-3-acetaldoxime. Alternatively, the pool of indole glucosinolates may be controlled by breakdown, e.g. by myrosinase. The levels of aliphatic glucosinolates fluctuated to a high extent between the various lines and at different developmental stages. This was unexpected because the aliphatic glucosinolates are primarily developmentally regulated, whereas the indole glucosinolates are stress inducible (Mikkelsen et al., 2003). The increased level of aliphatic glucosinolates in fully expanded rosette leaves of *rnt1-1* suggests that the production of aliphatic oximes is up-regulated in the mutant. At this stage, however, *CYP79F1* transcription is not higher in *rnt1-1* than in wild type, which suggests that some regulation takes place in the chain elongation step. Furthermore, it has been shown previously that accumulation of aliphatic glucosinolates and the expression levels of the corresponding *CYP79F*s are not closely linked (Mikkelsen et al., 2003). Alternatively, the higher content of aliphatic glucosinolates could be due to the smaller leaf size in the *rnt1-1* mutant, which would give a higher concentration per gram dry weight. In the *cyp83B1* point mutant *atr4-1*, it was found that both the indole-3-acetaldoxime-forming *CYP79B2* and *CYP79B3* and Trp biosynthetic genes were up-regulated (Smolen and Bender, 2002). This is supported by the present data, which show that transcription of *CYP79s* is generally up-regulated in the *rnt1-1* mutant. The up-regulation of *CYP79B2/CYP79B3* is consistent with the finding that these genes are stress inducible (Mikkelsen et al., 2003) because the *atr4-1* and the *rnt1-1* plants are certainly stressed. Alternatively, it might indicate that the plants try to compensate for the decreased level of indole glucosinolates by producing more indole-3-acetaldoxime, which is less efficiently metabolized by CYP83A1 and, as a consequence, gives rise to higher IAA levels. Increasing indole-3-acetaldoxime production will most likely enhance the superroot phenotype of *rnt1-1*. However, the plants appear to be able to overcome this problem by increasing *CYP83A1* transcription. Interestingly, this phenomenon does

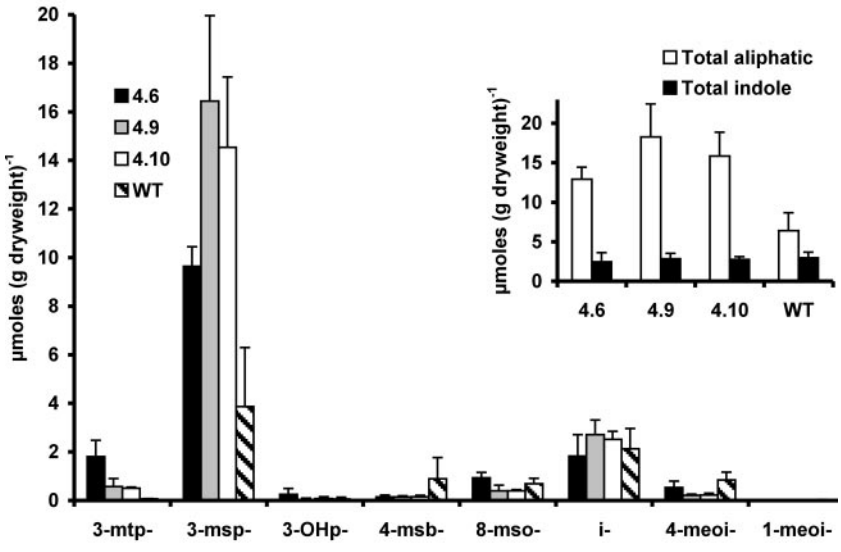

Figure 5. Major glucosinolates in individual 35S::CYP83B1 lines of Arabidopsis plants. The glucosinolate content of rosette leaves at the bolting stage was analyzed by HPLC. The experiments were done in triplicate. 3-mtp-, 3-methylthiopropylglucosinolate; 3-msp-, 3-methylsulphinylpropylglucosinolate; 3-OHp-, 3-hydroxypropylglucosinolate; 4-msb-, 4-methylsulphinylbutylglucosinolate; 8-mso-, 8-methylsulphinyloctylglucosinolate; i-, indolylglucosinolate, 4-meoi-, 4-methoxyindolylglucosinolate; and 1-meoi-, 1-methoxyindolylglucosinolate. The insert shows total aliphatic and indole glucosinolate content of the respective lines.

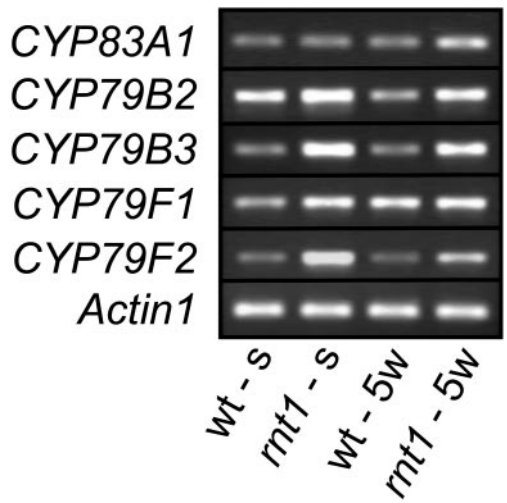

Figure 6. Expression of *CYP79s* and *CYP83A1* in seedlings and mature rosette leaves of wild-type and *rnt1-1* Arabidopsis. Semiquantitative RT-PCR analysis was performed on RNA extracted from, respectively, 10-d-old seedlings and 5-week-old rosette leaves of wild-type and *rnt1-1* plants. *Actin1* was used as control of equal RNA loading and RT reaction efficiency. The experiments were performed in triplicate. s, Seedling; 5w, 5 weeks old.

not occur in *rnt1-1* seedlings, which suggests that a certain threshold value needs to be reached before *CYP83A1* transcription is increased. In contrast to the present data, cDNA microarray analyses of *rnt1-1* seedlings have shown that *CYP83A1* transcripts are down-regulated 3.5-fold compared with wild type (Xu and Galbraith, personal communication, cited in Bak and Feyereisen, 2001). The down-regulation may be attributed to cross-hybridization between the *CYP83A1* target and the *CYP83B1* probe due to lack of *CYP83B1* target in *rnt1-1*, which would result in decreased *CYP83A1* signal.

Similarly, it is surprising that the level of aliphatic glucosinolates was increased in fully expanded rosette leaves of 35S::CYP83B1 lines because the kinetic analysis showed that CYP83B1 is unlikely to be involved in biosynthesis of aliphatic glucosinolates in vivo. Generally, it would not be expected that upregulation of an enzyme in the middle of a channeled biosynthetic pathway would influence the levels of the end product. The increased level of aliphatic glucosinolates in the 35S::CYP83B1 lines suggests that the plants are stressed, possibly due to channeling of indole-3-acetaldoxime away from the indole-3-acetaldoxime→IAA pathway. Because IAA is produced in the micromolar range, whereas glucosinolates have concentrations in the millimolar range, a small increased flux of indole-3-acetaldoxime into indole glucosinolates is not expected to be detectable in the levels of indole glucosinolates.

During the review of this paper, Hemm and coworkers reported four *CYP83A1* ethyl methanesulfonate mutants (called *ref2* mutants) with decreased levels of aliphatic glucosinolates and increased levels of indole glucosinolates (Hemm et al., 2003). It is surprising that these mutants were not totally devoid of aliphatic glucosinolates because three of the four *ref2* alleles introduce stop codons. This suggests that CYP83B1 in planta is able to metabolize aliphatic oximes. The *ref2* mutants were isolated in a screen for plants having altered fluorescence under UV light (Ruegger and Chapple, 2001). The mutants contain reduced levels of phenylpropanoid pathway-derived products (Hemm et al., 2003). This is likely to due to pleiotropic effects derived from the accumulation of aliphatic oximes. Interference with the biosynthetic pathway of aliphatic glucosinolates at the CYP79 level has been shown previously to result in unexpected pleiotropic effects. In *CYP79F1* knockout mutants, increased levels of IAA and altered IAA to cytokinin ratios (Reintanz et al., 2001; Tantikanjana et al., 2001) were measured, and the mutants had a dramatic increase in auxiliary buds. This is likely to be due to the increased levels of the CYP79F1 substrates, e.g. the metabolites dihomo-Met and trihomo-Met that accumulate to high levels (Hansen et al., 2001a). This suggests that Arabidopsis is af-

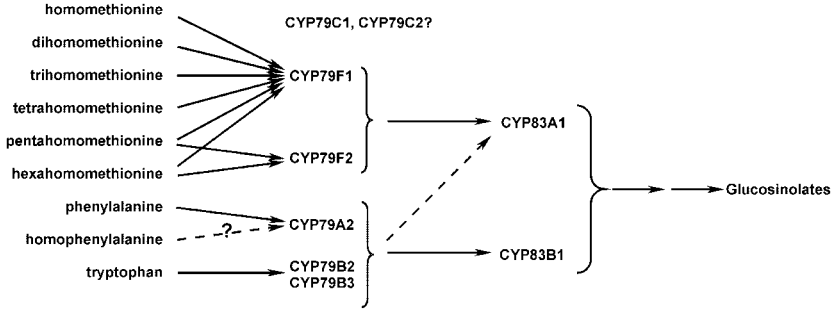

Figure 7. Cytochromes P450 involved in the biosynthesis of the core structure of glucosinolates in Arabidopsis. Characterization of the substrate specificity of the cytochromes P450 enzymes belonging to the CYP79 and CYP83 families has elucidated the first two steps in the biosynthesis of the core structure of glucosinolates in Arabidopsis. The CYP79Cs have not yet been assigned a function, and the suggested CYP79 with substrate specificity for homo-Phe is hypothetical. The dashed arrow from aromatic oximes to CYP83A1 is with little if any importance in the normal plant but may function in mutant backgrounds.

fected by changes in levels of intermediates in the biosynthesis of aliphatic glucosinolates, which might explain the phenotype seen in some of the 35S::CYP83B1 lines.

The observation that IAA accumulates in *sur2* (Delarue et al., 1998; Barlier et al., 2000) is a strong indication that there is metabolic cross-talk between the biosynthetic pathways leading to IAA and indole glucosinolates with indole-3-acetaldoxime as the branch point (Bak et al., 2001). Furthermore, indole-3-acetonitrile, a breakdown product of indole glucosinolates, can be hydrolyzed to IAA (Bak et al., 2001; Vorwerk et al., 2001). This suggests a role for indole glucosinolates as a source for IAA mobilization. Recently, in vivo feeding data have indicated that indole-3-acetaldoxime may be a precursor for other indole-derived natural products such as the phytoalexin brassinin (Pedras et al., 2001). This further emphasizes the role of indole-3-acetaldoxime as a metabolic branch point for various biosynthetic pathways. The low K_m value of CYP83B1 for indole-3-acetaldoxime is in accordance with a strict regulation of this metabolite.

Several biosynthetic pathways for indole-3-acetaldoxime have been described. The cytochromes P450 CYP79B2 and CYP79B3 both have been shown to produce indole-3-acetaldoxime from Trp (Hull et al., 2000; Mikkelsen et al., 2000). In addition, a plasma membrane-bound peroxidase-like enzyme has been shown to catalyze this reaction in Arabidopsis (Ludwig-Müller and Hilgenberg, 1992), and a flavine-dependent monooxygenase has been suggested to participate in the conversion of Trp to indole-3-acetaldoxime via a tryptamine pathway (Zhao et al., 2001; Tobena-Santamaria et al., 2002). Based on the evolutionary relationship to other CYP79 enzymes, CYP79B2- and CYP79B3-dependent synthesis of indole-3-acetaldoxime may be specific for production of indole glucosinolates, whereas other enzyme systems may produce indole-3-acetaldoxime for other biosynthetic pathways, e.g. IAA. This hypothesis is consistent with the finding that a *cyp79B2/cyp79B3* double mutant is devoid of indole glucosinolates but has only slightly decreased IAA levels (Zhao et al., 2002). This would require that the biosynthetic pathways are strictly regulated, perhaps through channeling of intermediates in metabolons or by compartmentalization of intermediates and enzymes. In agreement with this, oximes do not accumulate in wild-type plants; hence, the phenotype of *CYP83B1* knockout mutants could be explained by unregulated release of indole-3-acetaldoxime into the cytoplasm leading to IAA accumulation. Alternatively, there could be significant cross-talk between the different pathways with regulation at the postoxime enzymes.

In summary, we have demonstrated that the oxime-metabolizing enzymes CYP83A1 and CYP83B1 are nonredundant under normal physiological conditions and that the two enzymes are responsible for the metabolism of oximes giving rise to glucosinolates in Arabidopsis. Characterization of the substrate specificities of the two oxime-metabolizing enzymes provides important information to understand the fluxes of intermediates in the biosynthesis of the core structure of glucosinolates in Arabidopsis. Together with the previous characterization of the substrate specificity of the members of the CYP79 family involved in oxime production, this work provides a framework for metabolic engineering of glucosinolates and for further dissection of the glucosinolate pathway (Fig. 7).

MATERIALS AND METHODS

Oxime Substrates

^{14}C-labeled oxime substrates were produced from ^{14}C-labeled amino acids using the appropriate recombinant CYP79s, i.e. *p*-hydroxyphenylacetaldoxime from [^{14}C]Tyr by CYP79A1 (Halkier et al., 1995), phenylacetaldoxime from [^{14}C]Phe by CYP79A2 (Wittstock and Halkier, 2000), and indole-3-acetaldoxime from [^{14}C]Trp by CYP79B1 (Naur et al., 2003). The aliphatic oximes were synthesized as described previously (Dawson et al., 1993; Chen et al., 2003).

Measurements of Enzymatic Activity of Recombinant CYP83A1 and CYP83B1

Microsomes of yeast (*Saccharomyces cerevisiae*) cells expressing recombinant CYP83A1 and CYP83B1 in the yeast strain WAT11 co-expressing the Arabidopsis NADPH:cytochrome P450 reductase ATR1 were made as previously described (Bak and Feyereisen, 2001, and refs. therein). The cytochromes P450 were quantified by CO difference spectroscopy (Omura and Sato, 1964).

For kinetic analysis, reaction mixtures were set up in a total volume of 50 μL with 5.2 nM CYP83A1 or 9.6 nM CYP83B1, and 5 mM Cys, 0.5 to 5 times the K_m value of the oxime substrate (except for phenylacetaldoxime where the highest concentration used was 800 μM due to low solubility of this compound), plus 10 to 50 nCi ^{14}C-labeled oxime and 50 mM Tricine (pH 8.1). Reactions were started by the addition of NADPH to 3 mM, incubated at 29°C for 1 min, and stopped by the addition of 50 μL of 96% (w/v) ethanol. Aliquots of reaction mixtures were applied to TLC plates and eluted in isopropanol:EtOAc:water (7:1:2 [v/v]). Radiolabeled bands were visualized on a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and quantified using the ImageQuant software. Kinetic parameters were calculated using the SigmaPlot software (SPSS, Inc., Chicago).

Assays using [^{35}S]glutathione as nucleophile were done essentially as described above, except that radiolabeled oximes were excluded, and Cys was exchanged for 50 nCi [^{35}S]glutathione.

LC-MS

For LC-MS analysis, reaction mixtures were set up in a total volume of 500 μL with 5 nM CYP83A1 or CYP83B1, 5 mM Cys, 800 μM aliphatic oxime, and 50 mM Tricine (pH 8.1). Reactions were started by addition of NADPH to 3 mM and incubated for 30 min at 29°C. Reactions were stopped by the addition of 500 μL of CH_2Cl_2 and extracted twice. The aqueous phase was dried in vacuo, resuspended in 25 μL of water, and used for LC-MS analysis.

Electrospray ionization LC-MS analysis of CYP83 reaction products was done on an HP1100 LC coupled to a Bruker Esquire-LC ion trap mass spectrometer (Bruker Instruments, Billerica, MA) as described previously (Hansen et al., 2001b).

Construction of Transgenic Plants

The *rnt1-1* mutant functionally complemented by ectopic expression of the CYP83A1 cDNA under control of the cauliflower mosaic virus 35S promoter was line 2.9.5 (Bak and Feyereisen, 2001). The 2.9.5 line is *rnt1-1/*

rnt1-1 and homozygous for 35S::CYP83A1. A line that ectopically expresses the CYP83A1 cDNA in a wild-type background and where the transgene is inserted in the same position as in line 2.9.5 was generated by segregating *rnt1-1* out of the line that gave rise to 2.9.5. Thus, the resulting line, 2.9.11.5, is *RNT1/RNT1* and homozygous for 35S::CYP83A1. Lines 4.1 to 4.12 ectopically expressed the CYP83B1 cDNA under control of the cauliflower mosaic virus 35S promoter (Bak et al., 2001).

Plant Material and Growth Conditions

Seeds of Arabidopsis (ecotype Wassilewskija) were sown in humid peat (Enhets K-jord, Weibulls, Sweden), supplemented with 1 g L^{-1} soil of Bactimos (Wettable Products, Abbott Laboratories, Chicago) in 12- × 15-cm polystyrene trays, allowing water uptake from the bottom. Seeds were sown at densities of 400 or 4 seeds per 100 cm^2 when destined for 10-d-old and mature plants, respectively, and kept in a controlled-environment Arabidopsis Chamber (Percival AR-60L, Boone, IA), with a photosynthetic flux of 100 to 120 μmol photons m^{-2} s^{-1}, 70% relative humidity, and a photoperiod of 12/12 h, 20°C. The plants were watered at intervals of 4 d.

Analysis of Glucosinolates by HPLC

The content and composition of glucosinolates were determined by HPLC analysis of the desulphoglucosinolates as previously described (Petersen et al., 2001), with the exceptions that the sample amount was 20 mg (dry weight) instead of 100 mg (dry weight). Benzylglucosinolate (Merck, Rahway, NJ) or *p*-hydroxybenzylglucosinolate (Bioraf, Åkirkeby, Denmark) were added as internal standards at the start of the extraction procedure, and samples without internal standard were included on a regular basis. The HPLC was done on an LC-10ATvp (Shimadzu, Columbia, MD) equipped with a Supelcosil LC-ABZ 59142 RP-amid C_{16} column (25 cm × 4.6 mm, 5 mm) from Supelco (Holm & Halby, Brøndby, Denmark) and an SPD-M10AVP (Shimadzu) diode array detector with a flow rate of 1 mL min^{-1}. Desulphoglucosinolates were eluted by the following gradient: water (2 min), a linear gradient of 0% to 60% (w/v) methanol (48 min), a linear gradient of 60% to 100% (w/v) methanol (3 min), and 100% (w/v) methanol (14 min). Analyses of the different plant tissues were done in triplicate, and identification and quantification of individual glucosinolates were done as previously described (Petersen et al., 2001).

RT-PCR

RNA was extracted from Arabidopsis rosette leaves and seedlings using the TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from 2 μg of total RNA by using the Thermoscript RT system (Invitrogen) according to the manufacturer's instructions. PCR was performed in a total volume of 50 μL in PCR buffer (Invitrogen) containing 200 μM dNTPs, 1.5 mM $MgCl_2$, 50 pmol of the forward and reverse primers, and 2.5 units of *Taq*DNA polymerase (Roche, Basel). The PCR programs were as follows: 2 min at 94°C, 26 cycles (*Actin1*; U39449), 27 cycles (*CYP83A1*; At4g13770), 35 cycles (*CYP79B2*; At4g39950), (*CYP79B3*; At2g22330), *CYP79F1*; At1g16410), or 38 cycles (*CYP79F2*; At1g16400) of: 94°C for 10 s, 57°C (*Actin1*), or 54°C (all other primers) for 10 s and 72°C for 45 s. The following primers were used (all listed 5′ to 3′): *Actin1* forward, TGGAACTGGAATGGTTAAGGCTGG; *Actin1* reverse, TCTCCAGAGTCGAGCACAATACCG; *CYP83A1* forward, CGAGAGATAAGGAAGATGG; *CYP83A1* reverse, CCACTACAATATCCAAGATG; *CYP79B2* forward, AACCCACCATTAAGGAGC; *CYP79B2* reverse, TCATAAAATATATACGGCGTCG; *CYP79B3* forward, AAACCAACCATTAAGGAACT; *CYP79B3* reverse, TCCTCGCCGTACGTCACCG; *CYP79F1* forward, TTTTTAGACACCATCTTGTTTTCTTCTTC; *CYP79F1* reverse, AAAGCTCAATGGGTAGAAT; *CYP79F2* forward, AAAGCTCAATGCGTCGAAT; and *CYP79F2* reverse, GCGTCGAAACACATCACAGAG. Most primer sets were designed to be intron spanning and with these primers, no PCR products from genomic DNA were detected. All primers successfully amplified a band of the correct size when cDNA clones were used as template. Loading buffer (6×) was added to the PCR reactions, and 10 μL was analyzed by gel electrophoresis on a 1% (w/v) agarose gel. Bands were visualized by ethidium bromide staining and quantified on a Gel Doc 2000 Transilluminator (Bio-Rad, Hercules, CA). PCR with *Actin1* specific primers was used to ensure that an equal amount of RNA was used for all samples and to ensure that RT reactions were equally effective. RT-PCR analysis was performed in triplicate.

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LITERATURE CITED

Bak S, Feyereisen R (2001) The involvement of two P450 enzymes, CYP83B1 and CYP83A1, in auxin homeostasis and glucosinolate biosynthesis. Plant Physiol **127:** 108–118

Bak S, Olsen CE, Petersen BL, Møller BL, Halkier BA (1999) Metabolic engineering of *p*-hydroxybenzylglucosinolate in *Arabidopsis* by expression of the cyanogenic CYP79A1 from *Sorghum bicolor*. Plant J **20:** 663–671

Bak S, Tax FE, Feldmann KA, Galbraith DW, Feyereisen R (2001) CYP83B1, a cytochrome P450 at the metabolic branch point in auxin and indole glucosinolate biosynthesis in *Arabidopsis*. Plant Cell **13:** 101–111

Barlier I, Kowalczyk M, Marchant A, Ljung K, Bhalerao R, Bennett M, Sandberg G, Bellini C (2000) The SUR2 gene of *Arabidopsis thaliana* encodes the cytochrome P450 CYP83B1, a modulator of auxin homeostasis. Proc Natl Acad Sci USA **19:** 14819–14824

Chen S-X, Glawischnig E, Jørgensen K, Naur P, Jørgensen B, Olsen CE, Hansen CH, Rasmussen H, Pickett JA, Halkier BA (2003) Cytochrome P450 CYP79F1 and CYP79F2 genes catalyze the first step in the biosynthesis of short-chain and long-chain aliphatic glucosinolates in *Arabidopsis*. Plant J **33:** 923–937

Dawson GW, Hick AJ, Bennett RN, Donald A, Pickett JA, Wallsgrove RM (1993) Synthesis of glucosinolate precursors and investigations into the biosynthesis of phenylalkyl- and methylthioalkylglucosinolates. J Biol Chem **268:** 27154–27159

Delarue M, Prinsen E, Van Onckelen H, Caboche M, Bellini C (1998) *Sur2* Mutations of *Arabidopsis thaliana* define a new locus involved in the control of auxin homeostasis. Plant J **14:** 603–611

Halkier BA (1999) Glucosinolates. *In* R Ikan, ed, Naturally Occurring Glucosides. John Wiley & Sons Ltd., New York, pp 193–223

Halkier BA, Hansen CH, Mikkelsen MD, Naur P, Wittstock U (2002) The role of cytochromes P450 in biosynthesis and evolution of glucosinolates. *In* JT Romeo, ed, Recent Advances in Phytochemistry, Vol 36. Pergamon, Amsterdam, pp 223–248

Halkier BA, Nielsen HL, Koch B, Møller BL (1995) Purification and characterization of recombinant cytochrome P450TYR expressed at high levels in *Escherichia coli*. Arch Biochem Biophys **322:** 369–377

Hansen CH, Du L, Naur P, Olsen CE, Axelsen KB, Hick AJ, Pickett JA, Halkier BA (2001b) CYP83B1 is the oxime-metabolizing enzyme in the glucosinolate pathway in *Arabidopsis*. J Biol Chem **276:** 24790–24796

Hansen CH, Wittstock U, Olsen CE, Hick AJ, Pickett JA, Halkier BA (2001a) Cytochrome P450 CYP79F1 from *Arabidopsis* catalyzes the conversion of dihomomethionine and trihomomethionine to the corresponding aldoximes in the biosynthesis of glucosinolates. J Biol Chem **276:** 11078–11085

Hemm MR, Ruegger MO, Chapple C (2003) The Arabidopsis ref2 mutant is defective in the gene encoding CYP83A1 and shows both phenylpropanoid and glucosinolate phenotypes. Plant Cell **15:** 179–194

Hull AK, Vij R, Celenza JL (2000) Arabidopsis cytochrome P450s that catalyze the first step of tryptophan-dependent indole-3-acetic acid biosynthesis. Proc Natl Acad Sci USA **97:** 2379–2384

Jones P, Vogt T (2001) Glycosyltransferases in secondary plant metabolism: tranquilizers and stimulant controllers. Planta **213:** 164–174

Jones PR, Møller BL, Høj PB (1999) The UDP-glucose:*p*-hydroxymandelonitrile-*O*-glucosyltransferase that catalyzes the last step in synthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor*: isolation, cloning, heterologous expression and substrate specificity. J Biol Chem **274:** 35483–35491

Kahn RA, Fahrendorf T, Halkier BA, Møller BL (1999) Substrate specificity of the cytochrome P450 enzymes CYP79A1 and CYP71E1 involved in the biosynthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor* (L.) Moench. Arch Biochem Biophys **363:** 9–18

Koch BM, Sibbesen O, Halkier BA, Svendsen I, Moller BL (1995) The primary sequence of cytochrome P450tyr, the multifunctional *N*-hydroxylase catalyzing the conversion of L-tyrosine to *p*-hydroxyphenylacetaldehyde oxime in the biosynthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor* (L.). Moench Arch Biochem Biophys **323:** 177–186

Ludwig-Müller J, Hilgenberg W (1992) Tryptophan oxidizing enzyme and basic peroxidase isoenzymes in *Arabidopsis thaliana* (L.) Heynh: are they identical? Plant Cell Physiol **33:** 1115–11125

Mikkelsen MD, Halkier BA (2003) Metabolic engineering of valine- and isoleucine-derived glucosinolates in *Arabidopsis* expressing CYP79D2 from cassava. Plant Physiol **131:** 773–779

Mikkelsen MD, Hansen CH, Wittstock U, Halkier BA (2000) Cytochrome P450 CYP79B2 from *Arabidopsis* catalyzes the conversion of tryptophan to indole-3-acetaldoxime, a precursor of indole glucosinolates and indole-3-acetic acid. J Biol Chem **275:** 33712–33717

Mikkelsen MD, Petersen BL, Glawischnig E, Jensen AB, Andreasson E, Halkier BA (2003) Modulation of CYP79 genes and glucosinolate profiles in *Arabidopsis* by defense signaling pathways. Plant Physiol **131:** 298–308

Naur P, Hansen CH, Bak S, Hansen BG, Jensen NB, Nielsen HL, Halkier BA (2003) CYP79B1 from *Sinapis alba* converts tryptophan to indole-3-acetaldoxime. Arch Biochem Biophys **409:** 235–241

Omura T, Sato R (1964) The carbon monoxide-binding of liver proteins: II. Solubilization, purification and properties J Biol Chem **239:** 2379–2385

Paquette SM, Bak S, Feyereisen R (2000) Intron-exon organization and phylogeny in a large superfamily, the paralogous cytochrome P450 genes of *Arabidopsis thaliana*. DNA Cell Biol **19:** 307–317

Pedras MSC, Montaut S, Xu YM, Khan AQ, Loukaci A (2001) Assembling the biosynthetic puzzle of crucifer metabolites: indole-3-Acetaldoxime is incorporated efficiently into phytoalexins but glucobrassicin is not. Chem Commun **17:** 1572–1573

Petersen BL, Andreasson E, Bak S, Agerbirk N, Halkier BA (2001) Characterization of transgenic *Arabidopsis thaliana* with metabolically engineered high levels of *p*-hydroxybenzylglucosinolate. Planta **212:** 612–618

Petersen BL, Chen S, Hansen CH, Olsen CE, Halkier BA (2002) Composition and content of glucosinolates in developing *Arabidopsis thaliana*. Planta **214:** 562–571

Rask L, Andréasson E, Ekbom B, Eriksson S, Pontoppidan B, Meijer J (2000) Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Mol Biol **42:** 93–113

Reintanz B, Lehnen M, Reichelt M, Gershenzon J, Kowalczyk M, Sandberg G, Godde M, Uhl R, Palme K (2001) bus, a bushy *Arabidopsis* cyp79f1 knockout mutant with abolished synthesis of short-chain aliphatic glucosinolates. Plant Cell **13:** 351–367

Ruegger M, Chapple C (2001) Mutations that reduce sinapoylmalate accumulation in Arabidopsis thaliana define loci with diverse roles in phenylpropanoid metabolism. Genetics **159:** 1741–1749

Smolen G, Bender J (2002) *Arabidopsis* cytochrome P450 cyp83B1 mutations activate the tryptophan biosynthetic pathway. Genetics **160:** 323–332

Tantikanjana T, Yong JW, Letham DS, Griffith M, Hussain M, Ljung K, Sandberg G, Sundaresan V (2001) Control of axillary bud initiation and shoot architecture in *Arabidopsis* through the SUPERSHOOT gene. Genes Dev **15:** 1577–1588

Tobena-Santamaria R, Bliek M, Ljung K, Sandberg G, Mol JN, Souer E, Koes R (2002) FLOOZY of petunia is a flavin mono-oxygenase-like protein required for the specification of leaf and flower architecture. Genes Dev **16:** 753–763

Vorwerk S, Biernacki S, Hillebrand H, Janzik I, Muller A, Weiler EW, Piotrowski M (2001) Enzymatic characterization of the recombinant *Arabidopsis thaliana* nitrilase subfamily encoded by the NIT2/NIT1/NIT3-gene cluster. Planta **212:** 508–516

Winkler RG, Frank MR, Galbraith DW, Feyereisen R, Feldmann KA (1998) Systematic reverse genetics of transfer-DNA-Tagged lines of *Arabidopsis*: isolation of mutations in the cytochrome P450 gene superfamily. Plant Physiol **118:** 743–750

Wittstock U, Halkier BA (2000) Cytochrome P450 CYP79A2 from *Arabidopsis thaliana* L. catalyzes the conversion of L-phenylalanine to phenylacetaldoxime in the biosynthesis of benzylglucosinolate. J Biol Chem **275:** 14659–14666

Wittstock U, Halkier BA (2002) Glucosinolate research in the Arabidopsis era. Trends Plant Sci **7:** 263–270

Zhao Y, Christensen SK, Fankhauser C, Cashman JR, Cohen JD, Weigel D, Chory J (2001) A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science **291:** 306–309

Zhao Y, Hull AK, Gupta NR, Goss KA, Alonso J, Ecker JR, Normanly J, Chory J, Celenza JL (2002) Trp-dependent auxin biosynthesis in *Arabidopsis*: involvement of cytochrome P450s CYP79B2 and CYP79B3. Genes Dev **16:** 3100–3112