

Transgenic Tobacco and Arabidopsis Plants Expressing the Two Multifunctional Sorghum Cytochrome P450 Enzymes, CYP79A1 and CYP71E1, Are Cyanogenic and Accumulate Metabolites Derived from Intermediates in Dhurrin Biosynthesis¹

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Novel cyanogenic plants have been generated by the simultaneous expression of the two multifunctional sorghum (*Sorghum bicolor* [L.] Moench) cytochrome P450 enzymes CYP79A1 and CYP71E1 in tobacco (*Nicotiana tabacum* cv Xanthi) and Arabidopsis under the regulation of the constitutive 35S promoter. CYP79A1 and CYP71E1 catalyze the conversion of the parent amino acid tyrosine to *p*-hydroxymandelonitrile, the aglycone of the cyanogenic glucoside dhurrin. CYP79A1 catalyzes the conversion of tyrosine to *p*-hydroxyphenylacetaldoxime and CYP71E1, the subsequent conversion to *p*-hydroxymandelonitrile. *p*-Hydroxymandelonitrile is labile and dissociates into *p*-hydroxybenzaldehyde and hydrogen cyanide, the same products released from dhurrin upon cell disruption as a result of pest or herbivore attack. In transgenic plants expressing CYP79A1 as well as CYP71E1, the activity of CYP79A1 is higher than that of CYP71E1, resulting in the accumulation of several *p*-hydroxyphenylacetaldoxime-derived products in the addition to those derived from *p*-hydroxymandelonitrile. Transgenic tobacco and Arabidopsis plants expressing only CYP79A1 accumulate the same *p*-hydroxyphenylacetaldoxime-derived products as transgenic plants expressing both sorghum cytochrome P450 enzymes. In addition, the transgenic CYP79A1 Arabidopsis plants accumulate large amounts of *p*-hydroxybenzyl glucosinolate. In transgenic Arabidopsis expressing CYP71E1, this enzyme and the enzymes of the pre-existing glucosinolate pathway compete for the *p*-hydroxyphenylacetaldoxime as substrate, resulting in the formation of small amounts of *p*-hydroxybenzylglucosinolate. Cyanogenic glucosides are phytoanticipins, and the present study demonstrates the feasibility of expressing cyanogenic compounds in new plant species by gene transfer technology to improve pest and disease resistance.

Plants are sessile organisms. Accordingly, they respond to environmental challenges and attacks from herbivores and microbial pathogens by defending themselves rather than escaping. Natural products play an important role in these responses. Phytoalexins are defense compounds elicited by pathogen attack. Their formation from remote precursors is dependent on *de novo* enzyme synthesis and follows a lag phase of several hours after infection. In contrast, phytoanticipins are inactive defense compounds that are produced in specific plant tissues and at specific developmental stages independent of pathogen attack. These natural products are instantly activated upon tissue damage or pathogen attack. The activation is mediated by compartmentalized plant enzymes released as a result of loss of cell integrity. Phytoanticipins are therefore among the first chemical barriers to potential herbi-

vores and pathogens (VanEtten et al., 1994; Osbourn, 1996).

Cyanogenic glucosides constitute an important group of phytoanticipins. In sorghum (*Sorghum bicolor* [L.] Moench) the biosynthetic pathway of the cyanogenic glucoside dhurrin has been elucidated using a microsomal system that catalyzes the conversion of the parent amino acid L-Tyr to the aglycone *p*-hydroxymandelonitrile (Møller and Seigler, 1999). The pathway involves a number of unusual and labile intermediates. At the genetic level, the pathway is surprisingly simple because the conversion from Tyr to the aglycone is catalyzed by two multifunctional cytochromes P450 (P450s) each encoded by a single structural gene (Fig. 1). CYP79A1 catalyzes the conversion of Tyr to (*Z*)-*p*-hydroxyphenylacetaldoxime (Koch et al., 1995; Sibbesen et al., 1995), which is subsequently converted by CYP71E1 to the cyanohydrin *p*-hydroxymandelonitrile (Kahn et al., 1997; Bak et al., 1998). Finally, the labile *p*-hydroxymandelonitrile is stabilized by glucosylation via a soluble UDP-Glc (UDPG)-glucosyl transferase to produce dhurrin (Jones et al., 1999) (Fig. 1). To carry out hydroxylation reactions, P450s are de-

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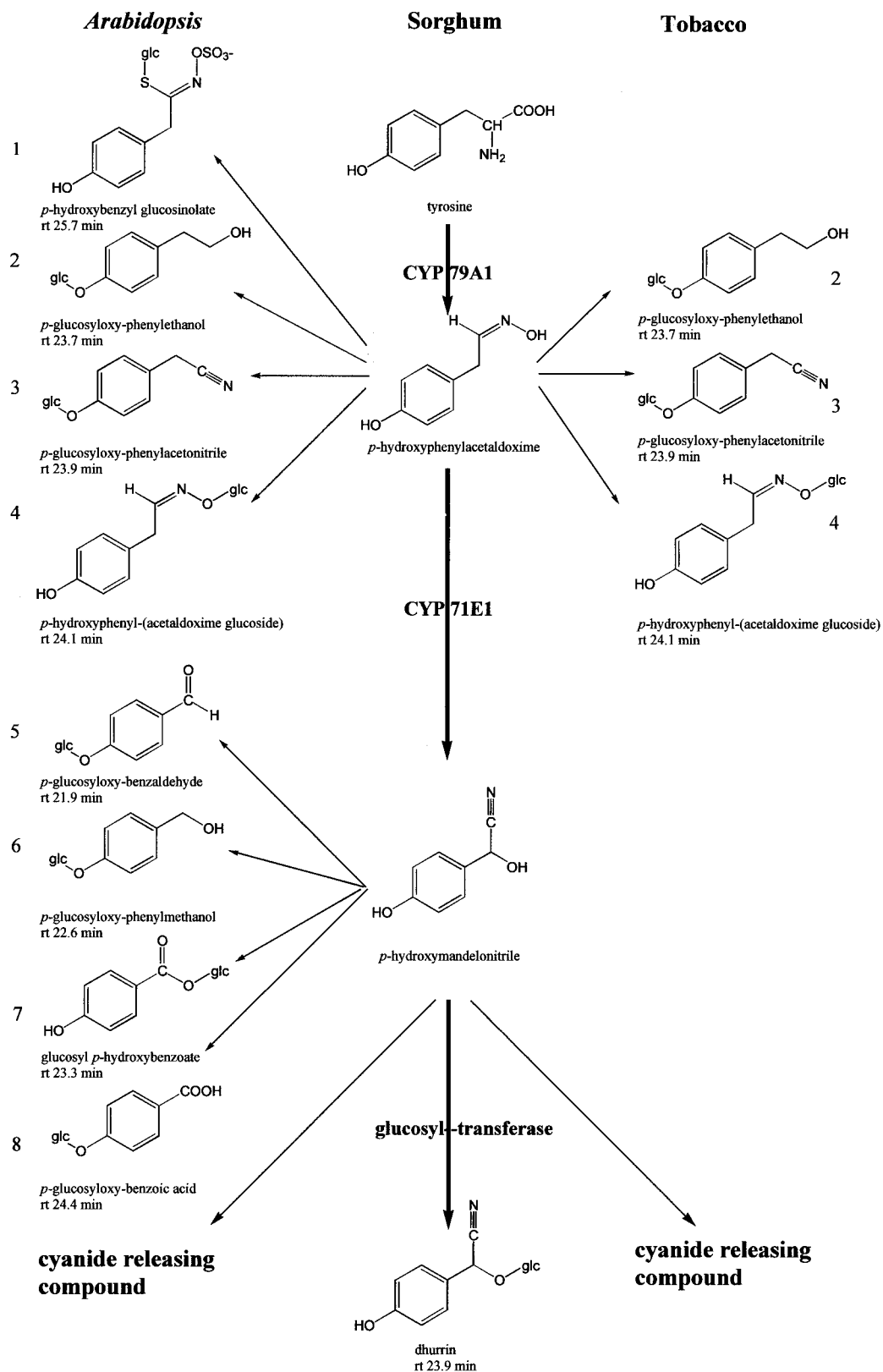


Figure 1. The biosynthetic pathway of the cyanogenic glucoside dhurrin. In sorghum, the pathway is catalyzed by CYP79A1 and CYP71E1, two multifunctional membrane-bound P450s, and by a soluble UDPG-glucosyltransferase. The glucosylated intermediates and metabolites that are detected in transgenic Arabidopsis and tobacco expressing one or both P450s are indicated. The retention time (rt) of the TMS derivatives observed upon GC analysis is shown below each structure.

pendent on reducing equivalents from NADPH. The ubiquitous plant enzyme P450 reductase mediates electron transfer. Generally, plants contain a single P450 reductase that donates electrons to each of the individual P450s in the endoplasmic reticulum membrane, but two P450 reductase genes have been identified in *Arabidopsis* (Urban et al., 1997).

Upon tissue damage, cyanogenic glucosides are hydrolyzed by β -glucosidases and α -hydroxynitrile lyases to produce the active defense compounds (Conn, 1981; Poulton, 1990; Hughes et al., 1994; Hu and Poulton, 1999). In the case of dhurrin, these are hydrogen cyanide and *p*-hydroxybenzaldehyde. These components may serve to deter a broad spectrum of potential pathogens. Nevertheless, numerous attempts to correlate the variation in levels of cyanogenic glucosides with resistance to specific pathogens have failed. In fact, the opposite effect has been observed in the rubber tree (Lieberei et al., 1989), barley (Pourmohseni and Ibenthal, 1991), and flax (Lüdtke and Hahn, 1953) in which increased levels of cyanogenic glucosides render these plants less resistant to fungal attack. These observations reflect a complex interplay between co-occurrence and possible covariation of numerous other defense compounds in plants as well as co-evolution. Successful pathogens are able to circumvent the protective effects of phytoanticipins either by limiting the extent of tissue damage and thus minimizing the release of toxic components or by developing tolerance or ability to sequester or metabolize the toxic constituents. Genetic engineering offers the possibility to transfer the ability to produce a specific defense compound from one plant species to an unrelated species. Because natural pests have not co-evolved with the new trait, it is possible that the compound introduced will render the transformed plant less vulnerable to attack by its natural pests.

The genetic simplicity of the biosynthetic pathway of the cyanogenic glucoside dhurrin in sorghum combined with the ubiquitous occurrence of Tyr provide an obvious model system to study the effects of introduction of natural compounds belonging to the phytoanticipin group into new plant species by gene transfer technology. We have previously shown that expression of CYP79A1 cDNA from sorghum in *Arabidopsis* results in the production of large amounts of *p*-hydroxybenzylglucosinolate documenting the feasibility to efficiently channel *p*-hydroxyphenylacetaldoxime into the pre-existing glucosinolate pathway. In the present study, we have expressed CYP79A1 and CYP71E1 in tobacco (*Nicotiana tabacum* cv Xanthi) and *Arabidopsis* plants and have demonstrated that the transgenic plants are cyanogenic due to the formation of *p*-hydroxymandelonitrile, the aglycone of dhurrin. In addition, several glucosides that relate to dhurrin biosynthesis and catabolism were identified.

RESULTS

Expression of Sorghum CYP79A1 and CYP71E1 in Tobacco and *Arabidopsis*

For expression of CYP79A1 and CYP71E1 in tobacco and *Arabidopsis*, two sets of transformation vectors were constructed and introduced by Ti plasmid-mediated transformation. In construct pPZP111.79, the coding sequence of sorghum CYP79A1 was fused to the constitutive cauliflower mosaic virus 35S promoter and polyadenylation site (Bak et al., 1999). When pPZP111.79 was introduced into tobacco and *Arabidopsis*, microsomes isolated from selected transgenic lines catalyzed the conversion of Tyr to *p*-hydroxyphenylacetaldoxime as evidenced by comigration with an authentic standard on thin-layer chromatography (TLC) (Fig. 2) and gas chromatography mass spectrometry (GC-MS) (data not shown). In addition to *p*-hydroxyphenylacetaldoxime, tyrosol (*p*-hydroxyphenylethanol) (Kindl and Schiefer, 1971) was identified in both microsomes isolated from wild-type and tobacco plants expressing CYP79A1.

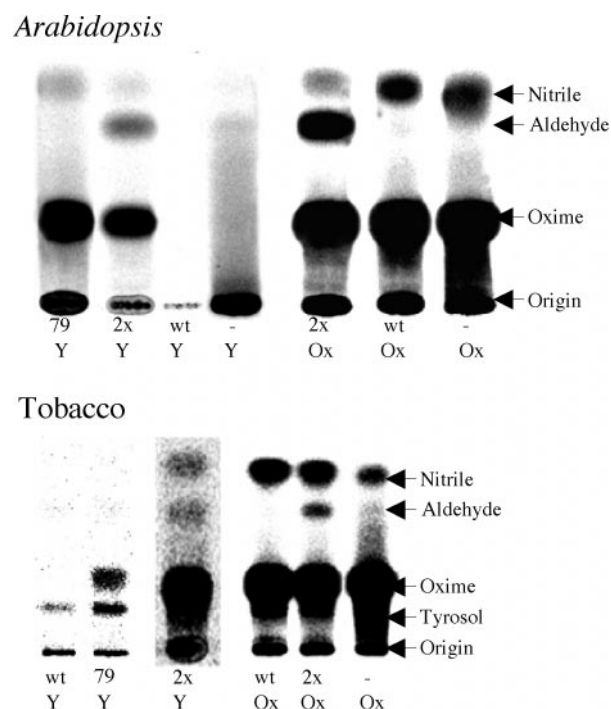


Figure 2. Expression of CYP79A1 and CYP71E1 in transgenic *Arabidopsis* and tobacco. Microsomes isolated from selected transgenic lines were incubated with either radiolabeled Tyr (Y) or radiolabeled *p*-hydroxyphenylacetaldoxime (Ox). After incubation, the reaction mixtures were extracted with ethyl acetate and the ethyl acetate extracts analyzed by TLC. —, Purity of the radiolabeled *p*-hydroxyphenylacetaldoxime precursor used. Plants expressing CYP79A1 (79) catalyze the conversion of Tyr to *p*-hydroxyphenylacetaldoxime (Oxime). Plants expressing both CYP79A1 and CYP71E1 (2X) catalyze the conversion of Tyr to *p*-hydroxyphenylacetaldoxime and further conversion of *p*-hydroxyphenylacetaldoxime to *p*-hydroxymandelonitrile. *p*-Hydroxymandelonitrile is detected as its decomposition product *p*-hydroxybenzaldehyde (Aldehyde).

The second construct, pPZP111.79.71E1, simultaneously introduces CYP79A1 and CYP71E1 each under regulation of a 35S promoter and polyadenylation site. CYP71E1 catalyzes the additional conversion of *p*-hydroxyphenylacetaldoxime to *p*-hydroxymandelonitrile. This cyanohydrin is labile and spontaneously decomposes to *p*-hydroxybenzaldehyde and hydrocyanic acid (HCN), if not stabilized by glucosylation. Selected tobacco and Arabidopsis lines transformed with pPZP111.79.71E1 express both the CYP79A1 and CYP71E1 enzymatic functions, as evidenced by administration of either radiolabeled Tyr or *p*-hydroxyphenylacetaldoxime to isolated microsomes (Fig. 2). The metabolism of radiolabeled Tyr in the transgenic tobacco and Arabidopsis lines resulted in the accumulation of relatively large amounts of *p*-hydroxyphenylacetaldoxime compared with *p*-hydroxybenzaldehyde. This is in contrast to the results obtained with microsomes isolated from etiolated sorghum seedlings in which *p*-hydroxybenzaldehyde was obtained as the major component with only trace amounts of *p*-hydroxyphenylacetaldoxime and *p*-hydroxyphenylacetoneitrile detected (Halkier and Møller, 1991).

The enzymatic activities of microsomes isolated from selected transgenic tobacco and Arabidopsis lines demonstrate correct targeting of the two sorghum P450s to the endoplasmic reticulum membranes. Furthermore, the microsomal data show that endogenous Arabidopsis and tobacco P450 reductases support and donate reducing equivalents to the sorghum P450s.

The Formation of Tyr-Derived Aglucones

To facilitate transport to and storage in the vacuoles, nucleophilic xenobiotics are generally subjected to glucosylation in higher plants (Sandermann, 1992). Accordingly, the content of Tyr-derived glucosides in transgenic plants compared with wild-type plants was investigated using methanol extracts subjected to hydrolysis with either β -glucosidase or Viscozym L. The aglucones released were separated and identified by GC-MS and TLC. To facilitate the detection of metabolites specifically derived from Tyr, TLC analysis was carried out using extracts prepared from detached Arabidopsis or tobacco leaves to which radiolabeled Tyr had been administered 18 h before extraction. In methanol extracts from Arabidopsis plants expressing CYP79A1 and subsequently treated with β -glucosidase, *p*-hydroxyphenylacetoneitrile, and tyrosol were identified by GC-MS (data not shown). When both sorghum CYP79A1 and CYP71E1 were expressed in Arabidopsis, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, and *p*-hydroxyphenylmethanol were identified in addition to the two aglucones identified in plants expressing CYP79A1 alone (data not shown). When methanol extracts derived from plant material radiolabeled

with Tyr were subjected to analysis by TLC followed by autoradiography, the same β -glucosidase generated aglucones were detected. When the radiolabeled methanol extracts were digested with Viscozym L, *p*-hydroxyphenylacetaldoxime in addition to the aglucones identified after β -glucosidase treatment could be detected by TLC. Viscozym L is a mixture of fungal carbohydrases and has a broader substrate specificity than β -glucosidase. Accordingly, the accumulated oxime was considered to have been glycosylated at the oxime function. In addition to glycosylation, oximes are also known to be subjected to sulfurylation (Grootwassink et al., 1990). Viscozym L contains esterase activities that putatively may also have converted a sulfurylated oxime into the free oxime.

Expression of sorghum CYP79A1 in tobacco identified the same aglucones as in Arabidopsis after treatment with β -glucosidase and Viscozym L. In contrast to Arabidopsis, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, and *p*-hydroxyphenylmethanol could not be detected in glucosidase-treated extracts from tobacco plants expressing both sorghum P450s (Fig. 3). As observed in Arabidopsis, *p*-hydroxyphenylacetaldoxime could only be released after digestion with Viscozym L. In wild-type and control Arabidopsis and tobacco plants, none of the above Tyr-derived aglucones could be detected with the exception of traces of tyrosol. The TLC data from the Tyr tracer studies confirm that the aglucones identified by GC-MS are derived from Tyr and relate to the expression of the sorghum P450s.

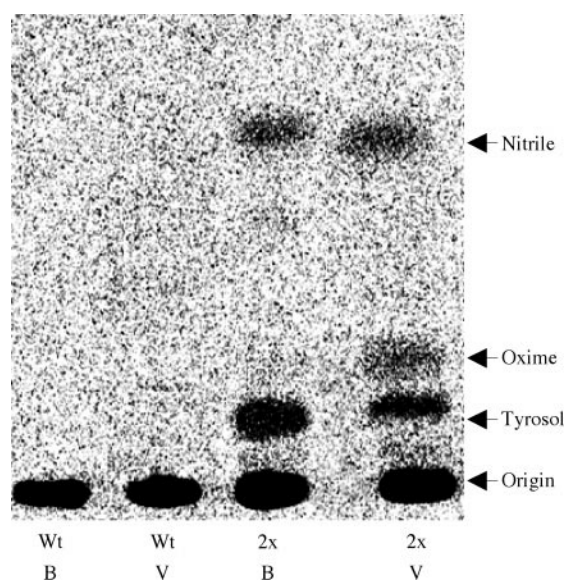


Figure 3. Tyr-derived glycosylated metabolites in transgenic tobacco plants expressing CYP79A1 and CYP71E1 (2 \times) as analyzed after deglycosylation, using either β -glucosidase (B) or Viscozym L (V). Aglucones were extracted into ethyl acetate and separated by TLC.

Identification of New Glucosides

The glucoside profile of the transgenic plants was analyzed by GC-MS performed in both chemical ionization (CI) and electron impact (EI) mode of their trimethylsilyl (TMS) derivatives. TMS-derivatized glucosides exhibit a characteristic fragmentation ion at m/z 451 that originates from the Glc moiety. Further fragmentation due to the loss of a trimethylsilyloxy radical results in a m/z 361 ion of relative high abundance (Ehmann, 1974). This diagnostic m/z 361 ion was used to monitor the occurrence of new glucosides in the transgenic plants. The GC-CIMS total ion current profile of a TMS-derivatized methanol extract from a transgenic Arabidopsis plant that expresses CYP79A1 and CYP71E1 and the corresponding m/z 361 ion trace are shown in Figure 4, A and B, respectively. Monitoring of the diagnostic m/z 361 ion trace compared with the total ion current selectively reduces much of the background noise in the spectra from the methanol extracts. The structures shown in Figure 1 have been numbered 1 through 8. The numbering is used to indicate the elution of the corresponding TMS derivatives during GC analysis (Fig. 4).

We have previously demonstrated by HPLC and GC-MS analyses that expression of sorghum CYP79A1 in Arabidopsis resulted in the production of large amounts of *p*-hydroxybenzylglucosinolate (1) (25.7-min retention time, Figs. 1 and 4D) (Bak et al., 1999). *p*-Hydroxybenzylglucosinolate is not a naturally occurring glucosinolate in Arabidopsis and is formed from *p*-hydroxyphenylacetaldoxime that has been channeled into the pre-existing glucosinolate biosynthetic pathway. Downstream to the oxime, the enzymes catalyzing glucosinolate biosynthesis exert low substrate specificity with respect to the structure of the oxime side chain (Bak et al., 1999). In the present study, the two phenolic glucosides *p*-glucosyloxy-phenylethanol (2) (23.7-min retention time) and *p*-glucosyloxy-phenylacetoneitrile (3) (23.9-min retention time), and *p*-hydroxyphenylacetaldoxime glucosylated at the oxime function (4) (24.1-min retention time) were also demonstrated to accumulate. Analysis by GC-MS of silylated methanol extracts from Arabidopsis plants expressing sorghum CYP79A1 as well as CYP71E1 showed the additional presence of *p*-glucosyloxy-benzaldehyde (5) (21.9-min retention time), *p*-glucosyloxy-phenylmethanol (6) (22.6-min retention time), glucosyl *p*-hydroxybenzoate (7) (23.3-min retention time), and *p*-glucosyloxy-benzoic acid (8) (24.4-min retention time) (Figs. 1 and 4E).

The occurrence of these glucosides compares well with the aglucones identified in extracts following carbohydrase treatments. Only novel glucosides that relate to the engineered Tyr metabolism were identified in the transgenic plants, reflecting the high substrate specificity of CYP79A1 and CYP71E1 (Kahn et al., 1999).

Soluble extracts from sorghum seedlings have been shown previously to glucosylate *p*-hydroxybenzoic

acid (Reay et al., 1974). Expression of the *ubiC* gene of *Escherichia coli* encoding chorismate pyruvate lyase in transgenic tobacco chloroplasts led to an up to 860-fold increase in *p*-hydroxybenzoic acid content as determined after acid hydrolysis of the plant material (Siebert et al., 1996). Using NMR spectroscopy, 95% of the *p*-hydroxybenzoic acid was demonstrated to accumulate as either the phenolic glucoside or the ester glucoside. Of the remaining 5%, one-half occurred as free *p*-hydroxybenzoic acid, whereas the rest was bound to the cell wall.

When CYP79A1 was expressed in tobacco, the same three *p*-hydroxyphenylacetaldoxime-derived glucosides identified in Arabidopsis were identified: *p*-glucosyloxy-phenylethanol, *p*-glucosyloxy-phenylacetoneitrile, and *p*-hydroxyphenyl-(acetaldoxime glucoside) (Figs. 1 and 4G). *p*-Hydroxybenzylglucosinolate was not identified. This was expected, because tobacco plants do not possess the ability to produce glucosinolates. In tobacco plants expressing both sorghum P450s (Fig. 4H) as documented by the microsomal data (Fig. 2) only the same three *p*-hydroxyphenylacetaldoxime-derived glucosides were identified as in the CYP79A1-expressing plants, indicating that the expression level of CYP71E1 was lower in tobacco compared with Arabidopsis, preventing detection of additional products by GC-MS. To verify that CYP71E1 was actively expressed, extracts prepared from detached tobacco leaves to which radiolabeled Tyr had been administered were analyzed. These analyses revealed that free *p*-hydroxyphenylacetaldoxime, *p*-hydroxyphenylacetoneitrile, tyrosol, and low amounts of *p*-hydroxybenzaldehyde accumulated in tobacco plants expressing both CYP79A1 and CYP71E1 (Fig. 5).

Tobacco Plants Expressing Sorghum CYP79A1 and CYP71E1 Are Cyanogenic

The cyanide potential of transgenic tobacco plants expressing both sorghum P450s reached as much as 40 pmol HCN/mg fresh weight. No cyanide could be detected in either wild-type or tobacco plants expressing only CYP79A1. To distinguish between the presence of free cyanide in the transgenic plants and the release of cyanide from an engineered metabolite, the colorimetric cyanide assay was carried out using fresh leaf samples as well as methanol extracts (Fig. 6). Free cyanide would be lost as HCN during extract preparation, e.g. during the lyophilization step. The observed retention of the cyanide potential in the methanol extracts thus demonstrates that the tobacco plants expressing both sorghum P450s are able to produce and accumulate a metabolite that releases free cyanide upon hydrolysis. The cyanide potential of the transgenic tobacco plants is very low, as also indicated by the GC-MS (Fig. 4H) and TLC (Fig. 5) analyses. Direct identification of the cyanogenic com-

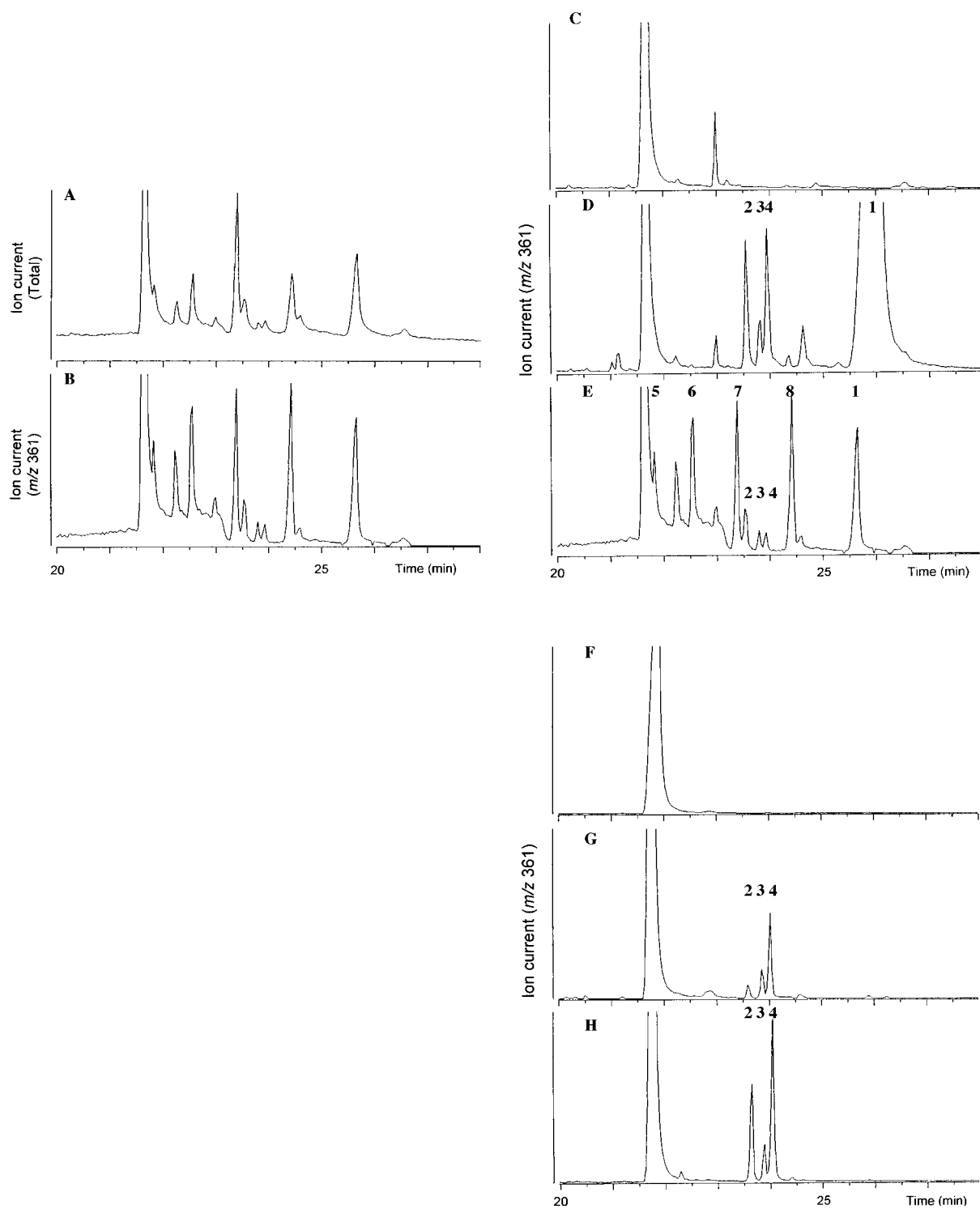


Figure 4. GC-CIMS analysis of Tyr-derived glucosides in transgenic Arabidopsis and tobacco lines. A and B, Comparison of the total ion trace versus that of m/z 361 using methanol extract prepared from an Arabidopsis plant expressing CYP79A1 and CYP71E1 (2 \times). C to E, m/z 361 ion trace of wild-type Arabidopsis (C) and transgenic Arabidopsis expressing CYP79A1 (79) (D) or CYP79A1 + CYP71E1 (2 \times) (E). F to H, m/z 361 ion trace of wild-type tobacco (F) and transgenic tobacco expressing CYP79A1 (79) (G) or CYP79A1 + CYP71E1 (2 \times) (H). Suc 21.7 min. *p*-Hydroxybenzylglucosinolate, 25.7 min (1); *p*-glucosyloxy-phenylethanol, 23.7 min (2); *p*-glucosyloxy-phenylacetoneitrile, 23.9 min (3); *p*-hydroxyphenyl-(acetaldoxime glucoside), 24.1 min (4); *p*-glucosyloxy-benzaldehyde, 21.9 min (5); *p*-glucosyloxy-phenylmethanol, 22.6 min (6); glucosyl *p*-hydroxybenzoate, 23.3 min (7); and *p*-glucosyloxy-benzoic acid, 24.4 min (8).

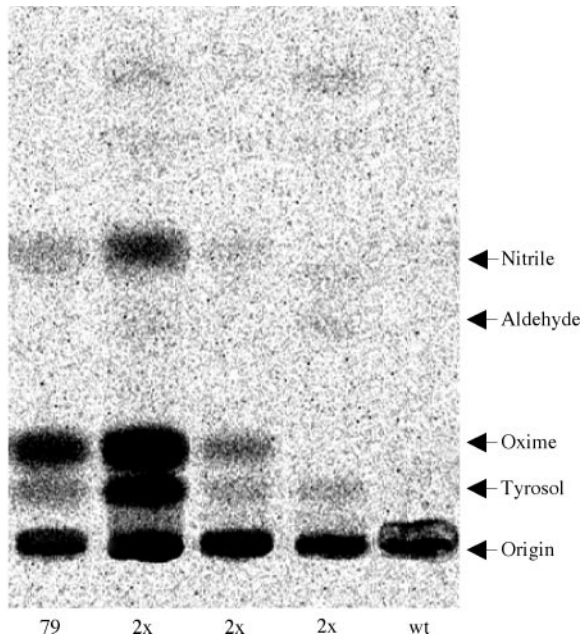


Figure 5. Intermediates in dhurrin biosynthesis accumulate in transgenic tobacco. Radiolabeled Tyr was administered to detached tobacco leaves from the highest expresser of CYP79A1 (79), from the highest and two additional expressers of both CYP79A1 and CYP71E1 (2×), and from a control line (wt). After 18 h of incubation, intermediates were extracted and analyzed by TLC.

pond therefore has not been achieved. Thus, neither dhurrin nor *p*-glucoxymandelonitrile nor its degradation products *p*-hydroxybenzaldehyde- β -D-glucoside were detectable by GC-MS.

Transgenic Arabidopsis plants that express CYP79A1 were found to accumulate up to 4.7 nmol *p*-hydroxybenzylglucosinolate/mg fresh weight (Bak et al., 1999). The level of *p*-hydroxybenzylglucosinolate production in Arabidopsis is therefore 2 orders of magnitude higher than the cyanide constituent found in tobacco. When indole glucosinolates and *p*-hydroxybenzylglucosinolate are degraded by endogenous myrosinases present in the Arabidopsis tissue, a labile isothiocyanate ion is released. Under the experimental conditions, this ion spontaneously disintegrates into stoichiometric amounts of the corresponding alcohol and SCN^- (Bak et al., 1999). Because SCN^- interferes with the colorimetric cyanide assay (Epstein, 1947), this assay cannot be used to quantify the amounts of cyanide released from the Arabidopsis plants expressing both sorghum P450s. Because the Arabidopsis plants contain *p*-glucosyloxy-*p*-henylacetonitrile that co-elutes with dhurrin and large amounts of *p*-hydroxybenzylglucosinolate that co-elute with *p*-glucosyloxy-mandelonitrile, GC analysis could not be used to establish whether these plants accumulate either of these two cyanogenic glucosides.

DISCUSSION

Expression Levels of Sorghum CYP79A1 and CYP71E1 Are Unbalanced in the Transgenic Tobacco and Arabidopsis Plants

In sorghum, the biosynthetic pathway of the Tyr-derived cyanogenic glucoside dhurrin is highly channeled (Møller and Conn, 1980). When sorghum microsomes are incubated with Tyr, *p*-hydroxybenzaldehyde accumulates as the predominant product (McFarlane et al., 1975). *p*-Hydroxybenzaldehyde is the dissociation product of *p*-hydroxyman-delonitrile, the aglycone of dhurrin. Biosynthetic experiments using sorghum microsomes have demonstrated that CYP79A1 constitutes the rate-limiting step (McFarlane et al., 1975; Møller and Conn, 1980). When microsomes isolated from Arabidopsis or tobacco lines that express both sorghum P450s were incubated with Tyr, large amounts of *p*-hydroxy-phenylacetaldoxime accumulate in addition to relative small amounts of *p*-hydroxybenzaldehyde (Fig. 2). Although CYP79A1 and CYP71E1 each are controlled by identical 35S promoters and terminators in the transgenic plants, the resulting catalytic activity of CYP71E1 appears to be limiting. During sample preparation and *in vitro* reconstitution, CYP71E1 is more labile compared with CYP79A1 (Kahn et al., 1999). CYP71E1 is a

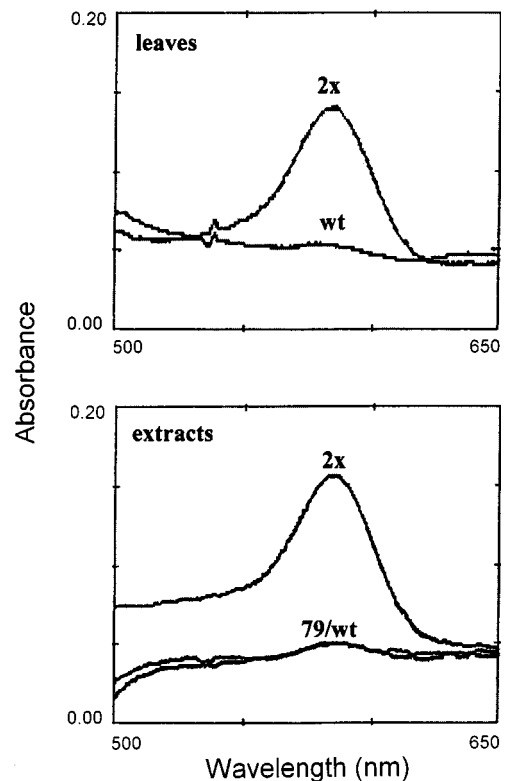


Figure 6. Cyanide potential of leaves and methanol extracts of transgenic and wild-type tobacco. Cyanide released was measured colorimetrically. 2×, Tobacco expressing both CYP79A1 and CYP71E1; 79, tobacco expressing CYP79A1; wt, tobacco control plant transformed with pZP111.

non-classical P450 (Kahn et al., 1999) and, compared with CYP79A1, may pose more strict requirements, e.g. to the lipid matrix and redox state of the environment to exert its maximal activity. These requirements may not be fully met in the transgenic plants. The lability of CYP71E1 has prevented determination of its turnover number, which even at optimal conditions may be lower than that of CYP79A1 (Kahn et al., 1999). The ability to detect free *p*-hydroxyphenylacetaldoxime in tobacco plants expressing both CYP79A1 and CYP71E1 (Fig. 5) and the identification of the three *p*-hydroxyphenylacetaldoxime-derived glucosides in tobacco and Arabidopsis plants that express both sorghum P450s (Fig. 4, E and H) augment the conclusion from the microsomal data that the final CYP79A1 and CYP71E1 activities are unbalanced. Specific antibodies are available for both CYP79A1 (Halkier et al., 1995) and CYP71E1 (Bak et al., 1998), but it has not been possible to estimate the relative levels of the two enzymes in the transgenic plants by immunoblotting due to the overall low expression levels (data not shown). The accumulation of *p*-hydroxyphenylacetaldoxime in the microsomes and the presence of its derived glucosides strongly indicate that in the transgenic plants the level of CYP79A1 activity is higher than that of CYP71E1.

Alternatively, the limiting CYP71E1 activity could be due to inefficient coupling of tobacco and Arabidopsis P450 reductase to CYP71E1. However, addition of isolated sorghum P450 reductase to the tobacco and Arabidopsis microsomes did not affect the level of *p*-hydroxybenzaldehyde compared with *p*-hydroxyphenylacetaldoxime. This indicates that the tobacco and Arabidopsis P450 reductases are adequate (data not shown).

Glucosides-Derived from *p*-Hydroxymandelonitrile

The microsomal data show expression of both sorghum P450s in Arabidopsis as well as in tobacco plants transformed with pPZP111.79.71E1 (Fig. 2). This demonstrates that the aglycone of dhurrin, *p*-hydroxymandelonitrile, is produced in both plants. Glucosylation at the α -hydroxy position results in the formation of dhurrin or the epimer taxiphyllin (Rosen et al., 1975). Glucosylation of the phenolic hydroxy group results in the formation of *p*-glucosyloxy-mandelonitrile (Reay and Conn, 1970). If the labile *p*-hydroxymandelonitrile is not stabilized by glucosylation, it will decompose and establish equilibrium with *p*-hydroxybenzaldehyde and HCN (Reay and Conn, 1974). The identification of glucosides other than dhurrin in the Arabidopsis plants (Fig. 4, D and E) argues that the UDPG-glucosyltransferase that glucosylate and stabilize *p*-hydroxymandelonitrile is limiting. *p*-Glucosyloxy-benzaldehyde was identified in the Arabidopsis plants (Fig. 4E). This demonstrates that *p*-hydroxymandelonitrile was indeed produced in

planta. The inability to detect *p*-glucosyloxybenzaldehyde in the tobacco extracts most likely reflects the low levels present of this compound and its elution close to the highly abundant Suc (Fig. 4H). *p*-Glucosyloxy-mandelonitrile (25.6-min retention time) could not be identified in the transgenic tobacco or in the Arabidopsis plants. In Arabidopsis, *p*-glucosyloxy-mandelonitrile and *p*-hydroxybenzylglucosinolate co-elute and a reliable diagnostic fragmentation ion that can distinguish between these two metabolites is not available. Accordingly, the presence of *p*-glucosyloxy-mandelonitrile can neither be confirmed nor rejected based on the GC-MS analysis. At the experimental conditions used for GC-MS analysis, dhurrin and taxiphyllin cannot be distinguished from *p*-glucosyloxy-phenylacetoneitrile as they have the same retention time and the same major fragmentation ions using the EI mode as well as the CI mode. This may be due to thermal decomposition during sample preparation and analysis of these labile compounds (Stenhagen and Alborn, 1989).

The use of radiolabeled Tyr as a precursor and cleavage of the radiolabeled glucosides formed using carbohydases demonstrate that the identified aglycones *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, and *p*-hydroxybenzylalcohol are derived from Tyr and their formation depends on the expression of both sorghum P450s. Therefore, *p*-hydroxybenzoic acid and *p*-hydroxybenzylalcohol are derived from *p*-hydroxymandelonitrile via *p*-hydroxybenzaldehyde. *p*-Hydroxybenzaldehyde can be oxidized by an NAD- or NADP-dependent aldehyde dehydrogenase to *p*-hydroxybenzoic acid or reduced to *p*-hydroxybenzylalcohol by an alcohol dehydrogenase. Esters of *p*-hydroxybenzoic acid are known to possess antimicrobial activity and their formation can be induced by fungal elicitors (Schnitzler et al., 1992). Therefore, the observed accumulation of different *p*-hydroxybenzaldehyde-derived glucosides in the transgenic plants expressing CYP79A1 and CYP71E1 most likely represents general metabolic defense reactions that take place upon tissue damage of all cyanogenic plants accumulating Tyr-derived cyanogenic glucosides. The classification of cyanogenic glucosides as phytoanticipins that give rise to an immediate defense response is therefore a simplification of their role in plant defense response reactions.

In Arabidopsis Plants, the Glucosinolate Pathway Competes with CYP71E1 for the *p*-Hydroxyphenylacetaldoxime

Expression of the two sorghum P450s in Arabidopsis resulted in the detection of several new glucosylated products that reflected the enzymatic activities of the two P450s.

High levels of *p*-hydroxybenzylglucosinolate accumulate in the transgenic Arabidopsis plants (Fig. 4, D

and E). This reflects that the enzymes in the glucosinolate pathway downstream of the oxime compete with CYP71E1 for the *p*-hydroxyphenylaldoxime produced by CYP79A1. As *p*-hydroxyphenylacetaldoxime has not been detected in the cytosol in sorghum we speculate that CYP79A1 and CYP71E1 form a binary complex to prevent release of *p*-hydroxy-phenylacetaldoxime to the cytosol. If in the transgenic Arabidopsis plants, the level of CYP79A1 enzyme is higher than that of CYP71E1, then the CYP79A1 enzyme in excess can couple with enzymes in the glucosinolate pathway. The production of *p*-hydroxy-benzylglucosinolate in the transgenic Arabidopsis plants expressing only CYP79A1 is therefore much more pronounced compared with that observed in Arabidopsis plants expressing CYP79A1 as well as CYP71E1 (Fig. 4) In the latter plants, a major portion of the *p*-hydroxyphenylacetaldoxime formed is converted into *p*-hydroxy-mandelonitrile as manifested by the accumulation of *p*-hydroxymandelonitrile-derived glucosides. In agreement with this interpretation, the relative levels of *p*-hydroxy-phenyl-(acetaldoxime glucoside) to the levels of *p*-glucosyloxy-phenylethanol and *p*-glucosyloxyphenylacetoneitrile are lower in the Arabidopsis plants expressing both CYP79A1 and CYP71E1 than in the plants expressing only CYP79A1 (compare Fig. 4, D and E).

Transgenic tobacco lines with sorghum P450 activities as high as those obtained in Arabidopsis could not be generated. Of the 35 independent transgenic tobacco lines generated from transformation using the pPZP111.79.71E1 construct, only 10 lines had a detectable cyanide potential. In all of the 11 transgenic lines analyzed from Arabidopsis, *p*-glucoxybenzaldehyde could readily be detected. When introduced into plants, oximes are subjected to a number of detoxification reactions (Grootwassink et al., 1990; Bak et al., 1999). In Arabidopsis, the glucosinolate pathway may be considered an additional route of detoxification that efficiently lowers the levels of free *p*-hydroxyphenylacetaldoxime produced by CYP79A1, thereby permitting a higher CYP79A1 expression level in Arabidopsis compared with tobacco. This is further supported by the observation that free *p*-hydroxyphenylacetaldoxime, tyrosol, and *p*-hydroxyphenylacetoneitrile accumulate in tobacco (Fig. 5) but not in Arabidopsis (data not shown). The use of kanamycin sulfate as selection agent for generation of tobacco plants expressing CYP79A1 as well as CYP71E1 did not lead to isolation of transformants. However, the use of the stronger selection agent G-418 did enable selection of transformants but with a much lower frequency compared with using the empty pPZP111 transformation vector. This suggests that transformants with high expression levels are deleterious, and only lines with low levels can be generated. In contrast to transformation of Arabidopsis by the infiltration method, transformation of tobacco by the leaf disc method relies on the ability to

regenerate a plant from a single transformed cell. In tobacco, the lack of the glucosinolate biosynthetic pathway as a sink for *p*-hydroxyphenylacetaldoxime may be more deleterious at the initial single-cell stage.

The expression of sorghum CYP79A1 and CYP71E1 in tobacco as well as in Arabidopsis resulted in novel cyanogenic plants. In sorghum, expression of CYP79A1 and CYP71E1 results in the accumulation of high levels of the cyanogenic glucoside dhurrin (Halkier and Møller, 1989). In the transgenic plants, the expression of sorghum CYP79A1 and CYP71E1 resulted in much lower levels of cyanogenic compounds. This may be explained by the use of the constitutive 35S promoter and a construct that would insert the two P450s at the same position in the genome. Using this strategy, a balanced activity level of the two P450s was not achieved, resulting in the accumulation of free *p*-hydroxyphenylacetaldoxime in the transgenic plants. Further studies should incorporate the use of other promoters to obtain more appropriately balanced activity levels. An alternative approach would be to cross a transgenic plant exhibiting a high CYP71E1 expression level with a plant with a more moderate CYP79A1 expression level to achieve a balanced metabolite flow through the two P450s. Recently, the cDNA encoding the UDPG-glucosyltransferase that in sorghum specifically glucosylates *p*-hydroxymandelonitrile to dhurrin has been isolated (Jones et al., 1999). Because *p*-hydroxymandelonitrile is unstable we speculate that CYP79A1 and CYP71E1 form a multi-enzyme complex with the UDPG:*p*-hydroxymandelonitrile-*O*-glucosyltransferase in planta. Introduction of this cDNA into transgenic plants expressing CYP79A1 and CYP71E1 may lead to a better channeling of *p*-hydroxyphenylacetaldoxime and *p*-hydroxymandelonitrile to dhurrin and thus enable accumulation of higher dhurrin levels. Transgenic Arabidopsis and tobacco plants expressing the glucosyltransferase as well as CYP79A1 and CYP71E1 are under characterization (D.B. Tattersall, P.R. Jones, S. Bak, P.B. Høj, and B.L. Møller, unpublished data). Nevertheless, the ability of the transgenic tobacco and Arabidopsis plants obtained in the present study to produce and accumulate a range of novel metabolites that relate to cyanogenic glucoside synthesis and degradation demonstrates that phytoanticipins as well as their active degradation products can be introduced into new plant species. Future studies will determine the effect of the altered profile of natural products in these transgenic plants on herbivory and pests.

MATERIALS AND METHODS

Vector Constructions

Arabidopsis (ecotype Columbia) and tobacco (*Nicotiana tabacum* cv Xanthi) were transformed using three different vectors. The vector pPZP111.79 contains the CYP79A1 cDNA under the control of the 35S promoter and polyade-

nylation site (Bak et al., 1999). The vector pPZP111.79.71E1 contains *CYP79A1* and *CYP71E1* each under control of the 35S promoter. To obtain this construct, the *CYP71E1* cDNA (Bak et al., 1998) was excised with *KpnI* and *XbaI* and ligated into the *KpnI* and *XbaI* sites of pRT101 (Töpfer et al., 1987) to generate pRT101.71E1. *CYP71E1* including the introduced 35S promoter and polyadenylation signal was excised from pRT101.71E1 with *HindIII* and ligated into the *HindIII* site of pPZP221 (Hajdukiewicz et al., 1994) to generate pPZP221.71E1. *CYP71E1* including the 35S promoter and polyadenylation site was then excised from pPZP221.71E1 using *HindIII*, blunt-ended, and ligated into the dephosphorylated *SmaI* site of pPZP111.79 to generate pPZP111.79.71E1. The pPZP111.79.71E1 vector harbors the two cytochromes in opposite orientation. The empty vector pPZP111 was used as a control.

Plant Transformation

The three constructs pPZP111, pPZP111.79, and pPZP111.79.71E1 were introduced into *Agrobacterium tumefaciens* C58C1/pGV3850 by electroporation. Arabidopsis was transformed using the vacuum infiltration method. Seeds were germinated on Murashige and Skoog medium containing 2% (v/v) Suc, 50 mg L⁻¹ kanamycin sulfate, and 0.8% (v/v) agar. Transgenic plants were selected, transplanted to peat, and grown in a controlled environment (20°C, 70% relative humidity) in an Arabidopsis growth chamber (AR-60L, Percival, Boone, IA) at a photosynthetic flux of 100 to 120 μmol photons m⁻² s⁻¹ with a 12-h photoperiod. Primary transformants were selfed, and selected homozygotes were used for further analysis.

Tobacco plants were transformed with the same constructs according essentially to the leaf disc method of Svab et al. (1995). Transformants were selected using kanamycin sulfate (100 mg L⁻¹) and tested for expression of the neomycin phosphotransferase (NPT) II protein using the NPT II ELISA kit (5 Prime → 3 Prime, Boulder, CO) prior to transfer to peat and growth in a greenhouse. Only false positive transformants were obtained when kanamycin sulfate was used as a selection agent in combination with *A. tumefaciens* transformed with pPZP111.79.71E1. Use of the gentamycin analog G-418 (50 mg L⁻¹) enabled the selection of 35 independent transgenic lines as evidenced by the expression of the NPT II product and confirmed by segregation analysis on kanamycin sulfate (100 mg L⁻¹) of the progeny of selfed primary transformants.

Plants transformed with the empty vector pPZP111 were designated control plants, those with the vector pPZP111.79 were designated 79, and those transformed with pPZP111.79.71E1 were designated 2×.

Biosynthetic Activity in Transgenic Plants as Determined Using Isolated Microsomes

Microsomes were prepared from leaf tissue from selected Arabidopsis plants homozygous for the transgene(s). The leaf material (0.3 g) was homogenized in 0.3 g of polyvinylpyrrolidone and 10 mL of buffer A (250

mM Tricine [N-(2-hydroxy-1,1-Bis[hydroxymethyl]ethyl)-glycine], 250 mM Suc, 100 mM ascorbic acid, 50 mM NaHSO₃, 2 mM dithiothreitol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5 mg mL⁻¹ bovine serum albumin, pH 8.2) using a prechilled mortar and pestle. The isolation buffer was degassed and flushed with argon three times before use. The homogenate was centrifuged (10 min, 15,000g), and a microsomal pellet was obtained by centrifugation (30 min, 100,000g) of the resulting supernatant. The pellet was resuspended in isolation buffer B (50 mM NaCl, 100 mM Tricine, 250 mM Suc, 2 mM dithiothreitol, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, pH 8.2) and pelleted by centrifugation (30 min, 100,000g). The final pellet was resuspended in 100 μL of buffer B.

Microsomes from tobacco were prepared essentially as those from Arabidopsis except that 1.25 g of leaf tissue from approximately 1-month-old plants from the highest expressers were used and homogenized in 1.25 g of polyvinylpyrrolidone and 40 mL of buffer B. Tobacco microsomes were finally resuspended in 100 μL of buffer B. Microsomes isolated from tobacco plants older than 1 month did not exhibit the *CYP71E1* activity, most likely because microsomal activity was strongly inhibited by the high amounts of alkaloids and phenolics present at the later growth stages.

Arabidopsis or tobacco microsomes (37 μL) were incubated (total volume 50 μL) with 25 ng of NADPH and 500 nCi of L-[U-¹⁴C]Tyr or 500 nCi of L-[U-¹⁴C]p-hydroxyphenylacetaldoxime (Bak et al., 1998). After incubation (30°C, 30 min), the reaction mixtures were extracted twice with ethyl acetate. The combined organic phases were concentrated in vacuo and analyzed by TLC (Silica gel 60 F254, Merck, Rahway, NJ) using ethyl acetate:toluene (1:5) as eluant. Radioactive bands were visualized using a STORM 840 phosphor imager (Molecular Dynamics, Sunnyvale, CA).

Identification of Tyr-Derived Metabolites

Detached leaves of transgenic Arabidopsis plants or 1-month-old tobacco plants were mounted in vials containing 500 nCi of L-[U-¹⁴C]Tyr. Water was administered as required after the leaves had absorbed the tracer. After 18 h of incubation, the leaves were boiled in 85% (v/v) methanol for 2 min to extract Tyr-derived metabolites. The extract was filtered, lyophilized, and the dry matter resuspended in 300 μL of water. The water phase was clarified by repeated extractions with ethyl acetate, and the combined ethyl acetate phases were concentrated in vacuo to provide an extract containing free metabolites present in the plants. To facilitate elucidation structures of the glucosides present in the aqueous phase, these were converted into their respective aglucones and sugars by treatment (1 h, 30°C) of aliquots (50 μL) of the aqueous phase with 0.1 mg of β-D-glucosidase type II (Sigma, St. Louis) or 1 μL of Viscozym L (Novo Nordisk A/S) in 50 mM MES (2-[N-morpholino]-ethanesulfonic acid), pH 6.5 (total volume 200 μL). After incubation, the released aglucones were extracted into ethyl acetate as described above. Extracts con-

taining free metabolites or released aglycones were analyzed by TLC with ethyl acetate:toluene (1:5) as eluant. In addition, the β -glucosidase treated samples were analyzed by GC-MS.

GC-MS Analysis

Derivatized glucosides were identified by GC-EIMS and GC-CIMS as described previously (Bak et al., 1999), using authentic standards in the case of Suc, *p*-hydroxybenzylglucosinolate, *p*-glucosyloxy-phenylacetone nitrile, *p*-glucosyloxy-benzaldehyde, *p*-glucosyloxy-benzoic acid, glucosyl *p*-hydroxybenzoate, *p*-glucosyloxy-mandelonitrile, and dhurrin. *p*-Glucosyloxy-phenylethanol, *p*-glucosyloxy-phenylmethanol, and *p*-hydroxyphenyl-(acetaldoxime glucoside) were identified based on their ionization fragmentation pattern using GC-EIMS and GC-CIMS before and after treatment with carbohydrases.

Determination of the Cyanide Potential of Leaves and Extracts

To determine the cyanide releasing potential of fresh leaves, the tissue (40 mg) was frozen in liquid N₂ in an Eppendorf tube, homogenized while frozen, and incubated (2 h, 30°C) with 200 μ L of 50 mM MES, pH 6.5, and 0.1 mg of β -glucosidase. Although endogenous β -glucosidases are present in the apoplast, which will come in contact with stored glucosides upon tissue damage, additional β -glucosidase was added to ensure complete hydrolysis. The reaction was stopped by addition of 40 μ L of 6 N NaOH, and the cyanide released quantified colorimetrically (Halkier and Møller, 1991). The cyanide potential of the water phases obtained from methanol extracts prepared as above was determined by incubation (30 min, 30°C) of aliquots in 50 mM MES, pH 6.5, and 0.1 mg of β -glucosidase (total volume of 200 μ L). The reaction was stopped by addition of 40 μ L of 6 N NaOH, and the cyanide released quantified as above.

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