Biosynthesis of the Cyanogenic Glucosides Linamarin and Lotaustralin in Cassava: Isolation, Biochemical Characterization, and Expression Pattern of CYP71E7, the Oxime-Metabolizing Cytochrome P450 Enzyme[^OA]

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Cassava (Manihot esculenta) is a eudicotyledonous plant that produces the valine- and isoleucine-derived cyanogenic glucosides linamarin and lotaustralin with the corresponding oximes and cyanohydrins as key intermediates. CYP79 enzymes catalyzing amino acid-to-oxime conversion in cyanogenic glucoside biosynthesis are known from several plants including cassava. The enzyme system converting oxime into cyanohydrin has previously only been identified in the monocotyledonous plant great millet (Sorghum bicolor). Using this great millet CYP71E1 sequence as a query in a Basic Local Alignment Search Tool-p search, a putative functional homolog that exhibited an approximately 50% amino acid sequence identity was found in cassava. The corresponding full-length cDNA clone was obtained from a plasmid library prepared from cassava shoot tips and was assigned CYP71E7. Heterologous expression of CYP71E7 in yeast afforded microsomes converting 2-methylpropanal oxime (valine-derived oxime) and 2-methylbutanal oxime (isoleucine-derived oxime) to the corresponding cyanohydrins, which dissociate into acetone and 2-butanone, respectively, and hydrogen cyanide. The volatile ketones were detected as 2,4-dinitrophenylhydrazine derivatives by liquid chromatography-mass spectrometry. A Ke of approximately 0.9 μM was determined for 2-methylbutanal oxime based on substrate-binding spectra. CYP71E7 exhibits low specificity for the side chain of the substrate and catalyzes the conversion of aliphatic and aromatic oximes with turnovers of approximately 21, 17, 8, and 2 min⁻¹ for the oximes derived from valine, isoleucine, tyrosine, and phenylalanine, respectively. A second paralog of CYP71E7 was identified by database searches and showed approximately 90% amino acid sequence identity. In tube in situ polymerase chain reaction showed that in nearly unfolded leaves, the CYP71E7 paralogs are preferentially expressed in specific cells in the endodermis and in most cells in the first cortex cell layer. In fully unfolded leaves, the expression is pronounced in the cortex cell layer just beside the epidermis and in specific cells in the vascular tissue cortex cells. Thus, the transcripts of the CYP71E7 paralogs colocalize with CYP79D1 and CYP79D2. We conclude that CYP71E7 is the oxime-metabolizing enzyme in cyanogenic glucoside biosynthesis in cassava.

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Cassava (Manihot esculenta) is one of the most important root crops in the world. The drought tolerance of cassava, combined with a high yield on poor soils and the possibility to leave the starch-rich tubers in the soil for extended time periods as a food and thus harvest on demand, renders cassava a key staple food especially in Africa (Nweke et al., 2002). All parts of the cassava plant contain the Val- and Ile-derived cyanogenic glucosides, linamarin and lotaustralin, in an approximately 97:3 ratio (Lykkesfeldt and Møller, 1994). The cyanogenic glucosides are primarily synthesized in the shoot apex (Andersen et al., 2000) and transported to the tubers, where they accumulate up to 1.5 g kg⁻¹ dry weight (Bokanga, 1994; Jørgensen et al., 2005a). In planta, cyanogenic glucosides and specific β-glucosidases able to cleave the β-glucosidic linkage are compartmentalized into different tissues or subcellular compartments, including the apoplast (Morant et al., 2008). This provides a two-component system that is activated upon cellular disruption (e.g. during food processing or by chewing insects). The cyanohydrin formed by the action of the β-glucosidase subsequently dissociates into a ketone and toxic HCN.
The presence of cyanogenic glucosides in cassava poses a major nutritive drawback in rural areas where cassava constitutes the staple crop available. Ingestion of incompletely processed cassava-derived products in combination with an unbalanced diet deficient in sulfur amino acids may give rise to chronic cyanide intoxication, as sulfur amino acids are required for cyanide detoxification (Rosling, 1994). In extreme cases, this may result in severe diseases such as tropical ataxic neuropathy and konzo (Banea-Mayambu et al., 1997; Oluwole et al., 2000). Acute cyanide intoxication inactivates the mitochondrial cytochrome aa₃ oxidase and thereby blocks cellular respiration (Nelson, 2006). Unfortunately, careful processing of cassava tubers to remove the cyanide-generating constituents results in concomitant loss of proteins, vitamins, and minerals and thus significantly reduces the nutritional value of this important crop (Maziya-Dixon et al., 2009).

The enzymes and corresponding genes for the entire cyanogenic glucoside biosynthetic pathway have only been identified in the monocotyledonous crop great millet (Sorghum bicolor; Jones et al., 1999). Great millet contains the aromatic cyanogenic glucoside dhurrin. The dhurrin pathway is genetically simple, as only three structural genes encode the entire pathway. Two membrane-bound, multifunctional cytochromes P450, CYP79A1 and CYP71E1, and a soluble UDPG-glucosyltransferase, UGT85B1, catalyze the conversion of Tyr into dhurrin (Halkier et al., 1995; Bak et al., 1998; Jones et al., 1999). CYP79A1 catalyzes the conversion of the amino acid Tyr into (Z)-p-hydroxyphenyl acetaldoxime in a reaction sequence that involves two consecutive N-hydroxylations, a dehydration and a decarboxylation reaction, and a final isomerization reaction (Halkier et al., 1995; Sibbesen et al., 1995). The next enzyme in the pathway, CYP71E1, converts (Z)-p-hydroxyphenyl acetaldoxime into p-hydroxymandelonitrile via a dehydration and a C-hydroxylation reaction (Halkier et al., 1998; Kahn et al., 1997; Bak et al., 1998). Finally, the p-hydroxymandelonitrile is glucosylated by UGT85B1 to yield dhurrin (Jones et al., 1999). The biosynthetic pathway is known to proceed as a highly channeled process (Moller and Conn, 1980), and the three biosynthetic enzymes are thought to be organized within a multienzyme complex, a metabolon (Nielsen and Moller, 2005; Nielsen et al., 2008), to secure highly efficient conversion of the parent amino acid into the cyanogenic glucoside and to facilitate channeling of toxic and reactive intermediates (for review, see Winkel, 2004; Jørgensen et al., 2005b).

Enzymes of the CYP79 family catalyze the first and rate-limiting steps in cyanogenic glucoside biosynthesis and have been identified from several cyanogenic plants (for review, see Bak et al., 2006), including cassava (Andersen et al., 2000). CYP79 enzymes are clearly evolutionarily conserved between monocotyledons and eudicotyledons and can be easily distinguished from other cytochromes P450 by unique amino acid substitutions in the otherwise generally conserved heme-binding domain and in the so-called “PERF” motif in the meander region (Bak et al., 2006; Paquette et al., 2009). CYP79 enzymes also catalyze the first committed step in biosynthesis of the crucifer-specific aliphatic, aromatic, and indole glucosinolates and camalexin (Rauhut and Glawischnig, 2009). The general assumption is that the glucosinolate pathway has evolved from the ancient cyanogenic glucoside pathway and that the amino acid-to-oxime step is catalyzed by orthologous enzymes and the two pathways branch at the oxime (Bak et al., 2006). In addition, the CYP79 biochemical activity is readily detected using radiolabeled amino acids as a substrate (Andersen et al., 2000). In contrast, identification of candidate CYP71E1 functional homologs or orthologs that catalyze the oxime-to-cyanohydrin step remains a major challenge because of multiple gene duplications and rearrangements resulting in numerous neofunctionalizations and subfunctionalizations within the CYP71 family. This is exemplified by the presence of 52 members of the CYP71 family in the diploid eudicotyledon Arabidopsis (Arabidopsis italiana) covering only two subfamilies, CYP71A and CYP71B, with amino acid sequence identities above 55% in each subfamily (Paquette et al., 2000, 2009; Nelson et al., 2004). In addition, CYP71E1 lacks obvious unique substitutions in otherwise conserved motifs in the amino acid sequence. Identification of a functional CYP71E1 homolog from plants producing aliphatic cyanogenic glucosides is further complicated by the lack of a sensitive method to detect the volatile reaction products. The lack of known CYP71E1 functional homologs raises the question of whether the oxime-metabolizing enzyme is evolutionarily conserved between monocotyledons and eudicotyledons as well or if the post-oxime step is a case of convergent rather than divergent evolution.

In cassava, the committed step from amino acid to oxime in loutastranol and linamarin biosynthesis is catalyzed by the isoenzymes CYP79D1 and CYP79D2 (Fig. 1; Andersen et al., 2000). These two enzymes are dually specific in that they catalyze the conversion of both Ile and Val into the corresponding oximes. The location of CYP79D1 and CYP79D2 transcripts has been examined in the first fully unfolded leaf and its petiole in the shoot tip of 2-month-old cassava plants. CYP79D1 and CYP79D2 are coexpressed in all tissues examined, with high expression in the outer cortex, endodermis, and pericycle cell layers and in tissues surrounding laticifers, xylem, and phloem cells in the petiole (Jørgensen et al., 2005a). The presence of two apparently functional redundant CYP79 homologs most likely reflects the fact that cassava is allopolyploid (Fregene et al., 1997).

In this study, we report the identification, biochemical characterization, and expression pattern in cassava petioles and young leaves of a bifunctional P450 enzyme designated CYP71E7, which catalyzes the conversion of Ile- and Val-derived oximes into their...
corresponding cyanohydrins in the biosynthesis of the cyanogenic glucosides lotaustralin and linamarin. This demonstrates that the oxime-metabolizing step is conserved in cyanogenic glycoside biosynthesis in monocotyledons and eudicotyledons.

### RESULTS

**Isolation and Heterologous Expression of CYP71E7 cDNA**

The biosynthetic pathway for the cyanogenic glucosides lotaustralin and linamarin in cassava is illustrated in Figure 1 (Koch et al., 1995; Andersen et al., 2000). The enzyme(s) catalyzing the conversion of the aliphatic oximes (Z)-2-methylbutanal oxime (ileox) and (Z)-2-methylpropanal oxime (valox) into the corresponding cyanohydrins has remained elusive. In *S. bicolor*, the CYP71E1 enzyme has been shown to catalyze conversion of the aromatic oxime p-hydroxyphenyl acetaldoxime (tyrox) into the corresponding cyanohydrin, p-hydroxymandelonitrile (Bak et al., 1998). Using the *S. bicolor* CYP71E1 amino acid sequence as a query in a BLASTp search, a cassava sequence with approximately 50% identity and approximately 68% similarity at the amino acid level was identified (Zhang et al., 2003; AY217351). Phylogenetic analysis grouped the cassava cytochrome P450 sequence with the CYP71E1 sequence from *S. bicolor* as well as with cytochrome P450 sequences from rice (*Oryza sativa*), wheat (*Triticum aestivum*), and sugarcane (*Saccharum officinarum*; data not shown), four species known to synthesize cyanogenic glucosides (Jones, 1998). Based on the cytochrome P450 nomenclature rules (http://drnelson.uthsc.edu/cytochromeP450.html), the cassava P450 sequence has been assigned as CYP71E7. Analysis of the genome sequence of the cassava line AM-560-2 (Cassava Genome Project 2010 [http://www.phytozome.net/cassava]) revealed the presence of a CYP71E7 paralog on scaffold 08265 located within 12,000 bp of CYP71E7. The two paralogs are 90% identical and 94% similar on the amino acid level, and the open reading frames are 92% identical.

To investigate whether CYP71E7 is the cassava oxime-metabolizing enzyme, a CYP71E7 cDNA was isolated by PCR from a cDNA library prepared from top shoots of cassava. This library had previously provided CYP79D1 and CYP79D2 (Andersen et al., 2000) encoding the multifunctional and dually specific CYP79D1 and CYP79D2. CYP71E7 catalyzes the conversion of the two (Z)-oximes to cyanohydrins by consecutive dehydration and C-hydroxylation reactions. Glucosylation of the cyanohydrins by a putative UDP-glucosyltransferase (UGT) provides lotaustralin and linamarin.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Biosynthesis of the Ile- and Val-derived cyanogenic glucosides lotaustralin and linamarin in cassava with emphasis on the CYP71E7-catalyzed reaction. The conversion of Ile and Val via N-hydroxyamino acids, N,N-dihydroxyamino acids, and (E)-oximes to (Z)-oximes is catalyzed by the multifunctional and dually specific CYP79D1 and CYP79D2. CYP71E7 catalyzes the conversion of the two (Z)-oximes to cyanohydrins by consecutive dehydration and C-hydroxylation reactions. Glucosylation of the cyanohydrins by a putative UDP-glucosyltransferase (UGT) provides lotaustralin and linamarin.
CYP71E7 is the Oxime-Metabolizing Cytochrome P450 in the Biosynthesis of Lotaustralin and Linamarin

Yeast microsomes harboring CYP71E7 were assayed for their ability to convert ileox and valox into the corresponding cyanohydrins, 2-hydroxy-2-methylbutyronitrile and acetone cyanohydrin. The design of the assay was based on dissociation of the labile cyanohydrins formed into hydrogen cyanide and ketones by alkanilization of the reaction mixture at the end of the incubation period (Fig. 3A) and subsequent trapping of the volatile ketones (2-butane and acetone) as 2,4-dinitrophenylhydrazones (Fig. 3B). After extraction, the 2,4-dinitrophenylhydrazones formed were identified and quantified by liquid chromatography-mass spectrometry (LC-MS). To reduce background levels due to contaminating aldehydes and ketones in the surrounding air and to retain the 2-butane and acetone produced, incubations were carried out in closed glass vials with an acidified solution of 2,4-dinitrophenylhydrazine (DNPH) placed in a center well.

In the presence of NADPH and oxygen, CYP71E7 converted ileox into 2-hydroxy-2-methylbutyronitrile with a turnover of 17 ± 1 \text{min}^{-1} and a \text{K}_m of 21 ± 11 \text{\mu M}. In agreement with this, product formation was detected already at 10 \text{\mu M} ileox and increased with substrate concentration up to 100 \text{\mu M} ileox (Fig. 3C, extracted ion chromatogram [EIC] 253). In the absence of NADPH or in assay mixtures using microsomes isolated from yeast transformed with an empty expression vector, hydrazone formation above background was not observed (Fig. 3C, EIC 253). The 2,4-dinitrophenylhydrazone assay enables detection of as little as 50 pmol of 2-butane with negligible background interference (Fig. 3C, EIC 253). The 2,4-dinitrophenylhydrazone of 2-butane was detected as two components, with the main component (83%) eluting at 10.6 min and representing the (E)-isomer and the minor component (17%) eluting at 9.8 min and representing the (Z)-isomer (Fig. 3C, EIC 253).

CYP71E7 catalyzed the conversion of valox into acetone cyanohydrin at a turnover of 21 ± 2 \text{min}^{-1} (Table I). The acetone obtained by dissociation of the acetone cyanohydrin produced a 2,4-dinitrophenylhydrazone that eluted as a single component at 6.4 min (Fig. 3C, EIC 239), as the carbonyl functional group of acetone carries two identical substituents. As for ileox, product formation was detected already at a substrate concentration of 10 \text{\mu M} (Fig. 3C, EIC 239). Acetone is omnipresent in air and absorbed to glassware in sufficient amounts to interfere with this highly sensitive assay. Accordingly, a high and slightly variable background level was observed even in blank samples including only buffer (Fig. 3C, EIC 239). The variable background level prevented calculation of an accurate \text{K}_m for valox. In addition to metabolizing the two aliphatic oximes, CYP71E7 was also able to convert tyrox and the Phe-derived phenylacetaldoxime (pheox) into the corresponding cyanohydrins, albeit with lower turnovers of 8.1 ± 0.3 and 1.3 ± 0.2 \text{min}^{-1}, respectively.

Spectral Analysis of Substrate Binding to CYP71E7

Binding of substrates within the active site of cytochromes P450 induces spin-state changes of the heme iron that can be recorded spectroscopically (Jeffcoate, 1978). Yeast microsomes harboring CYP71E7 produced a substrate-binding spectrum with a trough at 415 nm upon addition of ileox (Fig. 4A), which reflects the affinity of CYP71E7 for ileox, increasing concentrations of ileox (0.2–11.0 \text{\mu M}) were added to the yeast microsomes harboring CYP71E7. From this, a \text{K}_S of 0.9 ± 0.2 \text{\mu M} was calculated. The amplitude of the substrate-binding spectrum reached a maximum at 3 \text{\mu M} ileox (Fig. 4A). This demonstrated that ileox is a high-affinity ligand of CYP71E7. Cytochromes P450 are known to produce type II spectra upon binding of nitrogen-containing inhibitors such as \text{n-octylamine}. This spectral change is indicative of the proximity of the inhibitor amine to the active site heme iron (Jeffcoate, 1978). Saturation of CYP71E7 with \text{n-octylamine} (100 \text{\mu M}) resulted in the formation of a typical type IIb-binding spectrum with a trough at 410 nm and a peak at 435 nm (Fig. 4B, trace 1). A new baseline was recorded by the addition of 100 \text{\mu M} \text{n-octylamine} to the CYP71E7 microsomes in the reference cuvette (Fig. 4B, trace 2). To measure the ability of ileox to displace \text{n-octylamine} from the active site of CYP71E7, increasing concentrations of ileox were added to the \text{n-octylamine}-saturated CYP71E7 microsome solution. Addition of ileox produced a reverse type IIb spectrum with a peak at 410 nm and a trough at 430 nm (Fig. 4B, traces 3–5). The amplitude of the reverse type IIb spectrum reached maximum upon addition of...
Addition of valox and pheox to the yeast microsomes harboring CYP71E7 likewise resulted in substrate-binding spectra with a trough at 415 nm (data not shown), although higher substrate concentrations were required to produce the spectral shift compared with ileox. Only upon addition of more than 30 μM substrate did tyrox result in a weak trough at 415 nm (data not shown). Therefore, the broad substrate affinity of CYP71E7 is reflected in the ability to bind both aliphatic and aromatic oximes. The ability of ileox to efficiently replace n-octylamine shows that ileox is a high-affinity ligand to CYP71E7 and that n-octylamine is a competitive inhibitor that also is able to bind to the active site of CYP71E7.

**CYP71E7 and CYP79D1 Are Coexpressed in Cassava Leaf Petioles**

In tube in situ PCR was used to determine the cellular location of CYP71E7 transcripts in cassava. The analyses were performed on tissue sections from the petiole and leaf blade of the nearly unfolded leaf and of the leaf blade of the first fully unfolded leaf using 2-month-old cassava plants and with primers that enabled the detection of both CYP71E7 paralogs. The young leaf stages were selected because they contain the highest concentration of cyanogenic glucosides (Jørgensen et al., 2005a) and hence were expected to exhibit high expression levels of the mRNAs that encode the biosynthetic enzymes. Strong expression of CYP71E7 in the petiole was found in outer cortex cells and in the cell layer corresponding to the endodermis, around phloem cells and laticifers, as well as in cells surrounding the xylem as visualized by alkaline phosphatase staining (Fig. 5, CYP71E7). The same expression pattern is seen for CYP79D1 transcripts in sections from the same petiole (Fig. 5, CYP79D1). Alkaline phosphatase staining could not be detected in the absence of CYP71E7 or CYP79D1 primers in the reverse transcriptase reaction (negative control; Fig. 5, Control). In a separate set of experiments (Fig. 5), the in situ colocalization of the CYP71E7 and CYP79D1 transcripts in the petiole was visualized using fluorescein isothiocyanate (FITC)-labeled antibodies against digoxigenin (DIG) incorporated during the PCRs. As in the experiment with alkaline phosphatase, the outer cortex cells, endodermis cells, and...
cells surrounding the phloem and laticifers, and cells surrounding the xylem showed strong labeling. Negative control sections showed no background staining.

In the corresponding leaf blade of the first nearly unfolded leaf, expression of CYP71E7 and CYP79D1 was observed in most cells in the cortex and in the epidermis (Fig. 6, A and B, respectively). In fully unfolded leaves, strong expression of CYP79D1 was observed in the cortex cell layer just beside the epidermis and in specific cells in the vascular tissue (Fig. 6, C and D). Especially in the leaf section, red fluorescence was observed due to autofluorescence from the chloroplast. In a previous study, CYP79D1 and CYP79D2 were shown to have similar expression patterns (Jørgensen et al., 2005a).

The coexpression of CYP71E7 and CYP79D1 further substantiates that CYP71E7 is the oxime-metabolizing enzyme in cyanogenic glucoside biosynthesis in cassava.

**DISCUSSION**

A P450 enzyme, designated CYP71E7, catalyzing the conversion of Ile- and Val-derived oximes into the corresponding cyanohydrins in the biosynthesis of cyanogenic glucosides in cassava was identified based on amino acid sequence homology to CYP71E1, functional expression in yeast, substrate-binding spectra, and transcriptional colocalization with CYP79D1 and CYP79D2, the genes encoding the two enzymes that catalyze the initial steps in cyanogenic glucoside synthesis in cassava. The cyanohydrins (2-hydroxy-2-methylbutyronitrile and acetone cyanohydrin) produced by CYP71E7 are labile and decompose into volatile ketones (2-butanone and acetone) and HCN. To enable identification and quantification, the ketones were detected as 2,4-dinitrophenylhydrazone derivatives by LC-MS. This method enabled the detection of picomolar amounts of ketones formed in the CYP71E7-containing reaction and thus is more sensitive than the previously used colorimetric cyanide assay, which requires the presence of nanomolar amounts (Halkier and Möller, 1991).

**The Oxime-Metabolizing P450s in Cyanogenic Glucoside Biosynthesis Are Highly Efficient Enzymes**

Ileox is a high-affinity substrate for CYP71E7, with \( K_s \) of approximately 0.9 ± 0.2 \( \mu \)M, which is reflected in the conversion of ileox into the corresponding cyanohydrin, with a \( K_m \) of 21 ± 2 \( \mu \)M and a turnover rate of 17 ± 1 min\(^{-1}\). A similar conversion rate was observed for valox, with a turnover of 21 ± 2 min\(^{-1}\). The higher turnover of valox as compared with ileox is in agreement with a 9:3 ratio of linamarin to lotaustralin in cassava (Lykkesfeldt and Möller, 1994). The \( K_s \), \( K_m \), and turnover rates are in the same order of magnitude as those observed for the other known plant oxime-metabolizing cytochrome P450 involved in the biosynthesis of cyanogenic glucosides (CYP71E1) and as those for the structurally related glucosinolates (CYP83A1 and CYP83B1; Möller and Conn, 1979; Koch et al., 1992; Bak et al., 2001; Bak and Feyereisen 2001; Hansen et al., 2001; Naur et al., 2003). In contrast, the \( K_m \) values for CYP79D1 toward Ile and Val are several magnitudes higher, namely 1.3 and 2.2 mm, respectively (Andersen et al., 2000). The \( K_m \) values for bird’s foot trefoil (Lotus japonicus) CYP79D3 and CYP79D4 toward Ile and Val are also in the 1 to 3 \( \mu \)M range (Forslund et al., 2004). Earlier experiments using microsomes prepared from cassava, flax (Linum usitatissimum), and seaside arrow grass (Triglochin maritima) likewise demonstrated a \( K_m \) in the millimolar range toward the amino acid and a \( K_m \) in the micromolar range for the oxime (summarized by Koch et al., 1992). However, in *S. bicolor*, \( K_m \) values in the micromolar range were obtained for both Tyr and tyrox (Möller and Conn, 1979). It has been argued that an enzyme that catalyzes the first committed step in

![Figure 4. Substrate-binding properties of CYP71E7 as analyzed by optical difference spectroscopy. A, Trace 1, baseline recorded with CYP71E7-harboring microsomes in both sample and reference cuvettes; traces 2, 3, and 4, spectra after the addition of ileox (0.5, 1, and 3 \( \mu \)M, respectively) to the sample cuvette. B, Trace 1, spectrum of CYP71E7-harboring microsomes saturated with the cytochrome P450 inhibitor n-octylamine (100 \( \mu \)M); trace 2, baseline recorded upon the addition of equal amounts of n-octylamine (100 \( \mu \)M) to CYP71E7-harboring microsomes in both sample and reference cuvettes; traces 3, 4, and 5, displacement of n-octylamine by the addition of ileox (1, 10, and 30 \( \mu \)M, respectively) to the sample cuvette.](image-url)
the biosynthesis of a cyanogenic glucoside should have an elevated $K_m$ toward its substrate amino acid, to avoid depleting the free amino acid pool in the plant (Andersen et al., 2000). In contrast, the oxime-metabolizing enzyme is expected to have a low $K_m$ because the oximes must be metabolized efficiently in order to prevent the release of free oximes, which are known to be toxic to the plant (Grootwassink et al., 1990; Bak et al., 1999, 2001; Bak and Feyereisen, 2001; Hemm et al., 2003; Morant et al., 2007, 2010). The combination of the biosynthetic enzymes organized in a metabolon and a highly efficient oxime-metabolizing enzyme explains why no oxime intermediates, or derivatives thereof, are detected in cyanogenic plants (Bak et al., 1999; Tattersall et al., 2001; Kristensen et al., 2005). Alternatively, a functioning dependent dissociation of the metabolon might result in oxime production and serve to combat fungal attack (Møller, 2010).

In *S. bicolor*, the CYP71E1-catalyzed oxime-to-cyanohydrin conversion proceeds via an initial dehydration of the oxime to produce a nitrile that is then C-hydroxylated to yield the cyanohydrin (Fig. 1; Kahn et al., 1997; Bak et al., 1998). The CYP71E1-catalyzed dehydration is an unusual cytochrome P450 reaction and may represent a relic reaction type reflecting that P450s are ancient enzymes that originally were operating in an anaerobic environment (Nebert and Feyereisen, 1994). P450-catalyzed dehydrations of oximes are also known from human CYP3A4 (Boucher et al., 1994), where the reaction catalyzed by CYP3A4 has been shown to involve direct binding of the nitrogen atom of the oxime function to the heme iron of the P450 (Hart-Davis et al., 1998). The iron needs to be in its...
CYP71E7 Has Broad Substrate Specificity

Cyanogenic glucosides are produced from the protein amino acids Ile, Val, Leu, Tyr, and Phe and from the nonprotein amino acid cyclopentenyl Gly.

In addition to the aliphatic oxime intermediates in lotaustralin and linamarin biosynthesis, CYP71E7 catalyzes the conversion of tyrox and pheox, albeit with lower efficiency as compared with valox and ileox. This property is in agreement with results of previous studies using cassava microsomes that indicated that these had the ability to metabolize aliphatic as well as aromatic oximes into the corresponding cyanohydrins (Koch et al., 1992). Low substrate specificity at the oxime level was also observed using microsomes from bird’s foot trefoil (Forslund et al., 2004). Studies in transgenic plants likewise support low substrate specificity at the oxime level (Morant et al., 2007). These studies all provided evidence that the substrate specificity for cyanogenic glucosides and glucosinolate biosynthesis is determined at the parent amino acid level and that in both pathways the postoxime enzymes have low specificity for the side chain of the substrate. The lack of need for high substrate specificity of the postoxime enzymes can be explained by the assembly of the biosynthetic enzymes into a metabolon and by the absence of other oximes (Jørgensen et al., 2005b; Kristensen et al., 2005; Nielsen et al., 2008). The organization of the biosynthetic pathway within a metabolon imposes an evolutionary constraint for narrow substrate specificity only on the first enzyme in the pathway.

Evolutionary Origin of the Two CYP71E7 Paralogs

Cassava CYP79D1 and CYP79D2 catalyze the first committed steps in lotaustralin and linamarin biosynthesis and exhibit the same catalytic properties (Andersen et al., 2000). CYP79D1 and CYP79D2 possess approximately 85% amino acid sequence identity, and CYP79D1 and CYP79D2 show identical expression patterns based on in situ PCR (Jørgensen et al., 2005a). Cassava is an allopolyploid, and the relatively low sequence identity between CYP79D1 and CYP79D2 most likely reflects that they are homologs that originate from separate ancestral diploid parental genomes, rather than via a gene or genome duplication event. In support of this, CYP79D1 and CYP79D2 are located on separate scaffolds. In contrast, the two CYP71E7 paralogs are located on scaffold 08265 and are 90% identical and 94% similar on the amino acid level. In addition, both genes are located within a 12-kb region and in close proximity to CYP79D2, which is on the same scaffold. This indicates that the two paralogs have arisen by a recent gene duplication event and thus originate from the same ancestral diploid parental genotype. Hence, it might be expected that a homolog CYP71E7 could exist in the cassava genome originating from a parental genome different from the one harboring the two CYP71E7 paralogs. However, we have not been able to identify such a homolog in the current 4.1 version of the cassava genome.

The bird’s foot trefoil genome also contains two CYP79D paralogs. The sequence identity between these paralogs is approximately 95% at the amino acid level, and they catalyze the same enzyme reaction. However, their promoters have diverged and their expression patterns differ (Forslund et al., 2004). This indicates that the two CYP79D paralogs in bird’s foot trefoil have originated from a gene or whole genome duplication event and that subsequent subfunctionalization has led to differences in expression patterns that catalysis has maintained. The fact that bird’s foot trefoil is a paleopolyploid where the most recent autopolyploidy event happened more than 40 million years ago, preceding the speciation of bird’s foot trefoil and barrel clover (Medicago truncatula; Cannon et al., 2006), is in support of this assumption.

CYP71E7 and CYP79D1 Transcripts Colocalize in Cassava

CYP71E7 mRNA colocalizes with CYP79D1 mRNA in cassava petioles and leaves. This substantiates that CYP71E7 is the oxime-metabolizing enzyme in cyanogenic glucoside biosynthesis in cassava. The cyanogenic glucoside degrading β-glucosidase is located in the cell walls and in the laticifers in cassava (Elias et al., 1997); thus, expression of the biosynthetic genes in outer cortex, in endodermis, and in cells adjacent to the laticifers ensures that the cyanogenic glucosides and their bioactivators are in close proximity yet separated into different cell types or subcellular compartments. The observed expression of CYP71E7 and CYP79D1 in cells surrounding phloem could indicate that the cyanogenic glucosides synthesized in these cells are destined for transport.

Cyanogenic Glucosides Are Synthesized via an Evolutionarily Conserved Pathway in Monocotyledons and Eudicotyledons

S. bicolor is monocotyledonous and produces the aromatic cyanogenic glucoside dhurrin, while cassava is a eudicotyledon and synthesizes aliphatic cyanogenic glucosides. The identification of CYP71E7 and CYP71E1 as functional orthologs confirms that cyanogenic glucosides are synthesized via an evolutionarily conserved biosynthetic pathway (Jones et al., 2000; Bak et al., 2006). This will aid the identification of CYP71E1 orthologs from other eudicotyledonous cyanogenic plants such as the model legume bird’s foot trefoil...
and crop plants like white clover (Trifolium repens), almond (Prunus dulcis), sweet cherry (Prunus avium), lima bean (Phaseolus lunatus), and kidney bean (Phaseolus vulgaris). These plants all produce aliphatic cyanogenic glucosides, and CYP71E7 would be expected to serve as an ideal probe for the isolation of paralogs in those plants.

Identification and characterization of the enzymes involved in the biosynthesis, transport, and degradation of cyanogenic glucosides in cassava will provide the necessary molecular tools to enable the production of transgenic cassava lines in which the cyanogenic glucoside content of various tissues is optimized to achieve pest resistance while retaining acyanogenic tubers. Such acyanogenic cassava tubers will provide a healthier diet for millions of people, especially in developing countries.

**MATERIALS AND METHODS**

**Isolation and Expression of CYP71E7 in Saccharomyces cerevisiae**

The cassava (Manihot esculenta) CYP71E7 cDNA (accession no. AY217351) was PCR amplified using a plasmid cDNA library made from shoot tips of cassava plants of MCol22 (Andersen et al., 2000) as a template and primers 5'-gggggaggtgagcAGTCCGATCACCTAACAATCCTG-3' and 5'-caccacccggcccccACATATCCAAACC-3' harboring EcoRI and BamHI restriction sites (underlined). PCRs (total volume, 50 μL) were carried out in 10× Tris-HCl (pH 8.3), 50× KCl, and 2× MgSO₄ containing 2.5 units of Pwo DNA polymerase (Roche Diagnostics), 50 μM dATP, 50 μM dCTP, 50 μM dGTP, 50 μM dTTP, and 10 pmol of each of the primers listed above.

Thermal cycling parameters were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 65°C for 40 s, and a final 72°C for 5 min. The purified PCR product was ligated into the EcoRI and BamHI sites of pYeDP60 (Pompon et al., 1996) to yield pYeCYP71E7. The authenticity of the insert was verified by DNA sequencing. The unknown nucleotide at position 189 from the ATG start codon (Zhang et al., 2003) was hereby shown to be a T. pYeCYP71E7 was transformed into S. cerevisiae WAT11, expression of CYP71E7 was induced by Gal addition, and microsomes were obtained as described previously (Pompon et al., 1996). Recombinant CYP71E7 was quantified by carbon monoxide difference spectroscopy (Omura and Sato, 1964).

**Catalytic Activity of CYP71E7**

Yeast microsomes harboring recombinant CYP71E7 were assayed for the ability to convert ilexos and valox into the corresponding cyanohydrins using assay mixtures (total volume, 100 μL) containing approximately 0.20 μM CYP71E7, 10 mM Tricine (pH 7.9), 1 mM NADPH, and 0 to 100 μM 2-methylbutanolic oxime or 2-methylpropanolic oxime. The ilexox and valox administered to CYP71E7 are mixtures of approximately 70% (E)-isomers and 30% (Z)-isomers as determined by H NMR. Assay mixtures without NADPH or containing microsomes from S. cerevisiae WAT11 transformed with an empty pYeDP60 vector served as negative controls. Incubation (30 min, 28°C, 300 rpm) was performed in glass vials (1.5 mL) filled with a gas-tight silicone stopper and a center well composed of a plastic pipette tip melted at the end to form a closed cone inside the glass vial. Enzyme reaction was stopped by direct injection of NaOH (10 μL, 2.5 N). To trap volatile ketones, an acidified DNPH solution (50 μL; 6.66 mg of DNPH dissolved in 1.25 mL of HCl, 3.125 mL of water, and 0.625 mL of acetonitrile [Zwiener et al., 2002]) extracted three times with n-pentane to remove aldehyde and ketone contaminants) was injected into the center well, and incubation was continued (2 h, 50°C, 300 rpm) to trap the ketones as hydrazones in the center well. For LC-Ms analysis, hydrazones were extracted into n-pentane (600 μL), and the pellet obtained after removal of the n-pentane was dissolved in methanol (85%, 60 μL). The amount of hydrazone present was determined from the EIC based on the area under the appropriate signals compared with those generated by known standards (100 μL of 0, 1, 5, 10, 25, 50, and 100 μM solutions of acetone or 2-butanol) applied to the incubation vials and trapped by diffusion into the DNPH-containing center well. For calculation of turnover numbers, assays were performed in four replicates as described above, except that 0.026 μM CYP71E7 was applied with 100 μM substrate in the presence or absence of NADPH and incubation was reduced to 10 min. Turnover numbers were calculated based on the difference of oxime conversion in the presence and absence of NADPH. So values were calculated as the difference between the average turnover in the presence and absence of NADPH. The Kₘ value was calculated based on 10 different ileox concentrations ranging from 1 to 100 μM.

Assays to determine turnovers for tyrox and pheox were carried out as described above, except that 0.26 μM CYP71E7 was applied, incubation was reduced to 10 min, and termination of the reaction by NaOH (10 μL of 2.5 N NaOH, 10 min, 28°C) was followed by direct injection of DNPH solution (80 μL) into the assay mixture. After incubation (50°C, 1 h, 300 rpm), hydrazone products formed were extracted as above. Quantification of tyrox- and pheox-derived cyanohydrins was based on injection of standards (100 μL of 0, 1, 5, 10, 25, 50, and 100 μM solutions of p-hydroxymandelonitrile or mandelonitrile dissociated by NaOH addition and reacted with DNPH as described above). Authentic standards were produced by reacting p-hydroxybenzaldehyde and benzaldehyde (100 μL of 100 μM) directly with acidified DNPH.

**LC-Ms Analysis**

LC-MS was performed using a HP1100 HPLC apparatus (Agilent Technologies) coupled to a Bruker HCT-Ultra ion-trap mass spectrometer (Bruker Daltonics). An Xterra MS C18 column (Waters; 3.5 μm, 2.1 × 100 mm) was used at a flow rate of 0.2 mL min⁻¹. The mobile phases were as follows: A, 2% (v/v) formic acid in water; and B, methanol. The gradient program was as follows: 0 to 2 min, isocratic 65%; 2 to 10 min, linear gradient 65% to 100%; 10 to 12 min, isocratic 100%; 12 to 18 min, isocratic 65%. The spectrometer was run in positive atmospheric pressure chemical ionization mode using a vaporizer temperature of 400°C.

**Substrate Binding Monitored by Optical Difference Spectroscopy**

All spectra were obtained using a Perkin-Elmer spectrophotometer (Lambda 800) and microsomes harboring approximately 0.24 μM CYP71E7 resuspended in TEG buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 30% glycerol; total volume, 500 μL) in sample and reference cuvettes (1-cm light path). To monitor spectral changes upon substrate binding, a baseline was recorded using CYP71E7-expressing microsomes alone in both cuvettes. Binding spectra were recorded following the addition of ileox (0.5, 1, and 3 μM) or n-ocytolamine (100 μM) to the sample cuvette. A Kᵣ for ileox was calculated using SigmaPlot (Systat Software) from the increase in magnitude between trough and peak in the binding spectra recorded following the addition of increasing concentrations of ileox (nine concentrations between 0.2 and 11 μM). Displacement of the inhibitor n-ocytolamine from the CYP71E7 active site by ileox was measured as the spectral shifts observed upon the addition of increasing amounts of ileox (1, 10, and 30 μM) to CYP71E7-harboring microsomes in the presence of n-ocytolamine (100 μM) after recording a new baseline upon the addition of n-ocytolamine (100 μM) to the reference cuvette.

**In Tube in Situ PCR on Tissue Sections of Cassava Leaf Petioles**

In tube in situ PCR was carried out on sections of nearly unfolded leaves, fully unfolded leaves, and petioles from the first fully unfolded leaf of 2-month-old cassava plants of MC22 as described previously (Jørgensen et al., 2005a) using primers 5′-GATGTTGCTATCACAACC-3′ and 5′-GCTTGACATGACCTGTTG-3′ for CYP71E7 and 5′-CTCCTTCAAGAATCGTGT-3′ and 5′-ATTGGTCGTTGCGAATAAAC-3′ for CYP79D1. The specificity of the primers was verified by DNA sequencing of the PCR products. Prior to sectioning, all tissues were fixed in FAA (2% formaldehyde, 5% acetic acid, and 63% ethanol in phosphate-buffered saline [PBS]) for 5 h at 4°C and then washed three times with washing buffer (60% ethanol and 5% acetic acid in PBS). The tissue was embedded in 5% agarose in PBS to enable sectioning into 80-μm tissue sections on a Leica vibratome. The sections were treated overnight with 30 μL of RNase-free DNase (2 μL μL⁻¹) 290


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LITERATURE CITED


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