GENERATION OF ACYANOGENIC CASSAVA (MANIHOT ESCULENTA CRANTS): TRANSGENIC APPROACHES

DISSERTATION

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By

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ABSTRACT

Cassava (*Manihot esculenta*, Crantz) roots are the primary source of calories for more than 500 million people, the majority of whom live in developing countries of Africa. Cassava leaves and roots, however, contain potentially toxic levels of the cyanogenic glycoside, linamarin. Upon tissue damage linamarin is released from the vacuole and deglycosylated by the cell wall enzyme linamarase to produce acetone cyanohydrin. Acetone cyanohydrin is then broken down either spontaneously (pH > 5.0 or temperature > 35 °C) or by hydroxynitrile lyase (HNL) to produce acetone and hydrogen cyanide. During food processing essentially all free cyanide is removed by water extraction or volatilization. Consumption of residual cyanogens (linamarin or acetone cyanohydrin) in incompletely processed cassava roots, however, can result in cyanide poisoning due to conversion of the cyanogens to cyanide in the body. Our objective was to eliminate cyanogens from cassava so as to eliminate the need for food processing.

To achieve this goal we generated transgenic acyanogenic cassava plants in which the expression of the cytochrome P450 genes (CYP79D1 and CYP79D2), that catalyze the first-dedicated step in linamarin synthesis, was inhibited. Using a leaf-specific promoter to drive the antisense expression of the CYP79D1/CYP79D2 genes
we observed up to a 94% reduction in leaf linamarin content associated with an apparent complete inhibition of CYP79D1 and CYP79D2 expression. Significantly, the linamarin content of roots also was reduced by 99% in transgenic plants having between a 60% and 94% reduction in leaf linamarin content. Analysis of CYP79D1/CYP79D2 transcript levels in transgenic roots indicated they were unchanged relative to wild-type plants. These results suggest that linamarin is transported from leaves to roots and that a threshold level of leaf linamarin production is required for transport.

To investigate linamarin transport further we generated transgenic cassava plants in which the expression of CYP79D1 and CYP79D2 was inhibited using a tuber-specific patatin promoter to drive the antisense expression of the genes. Transformants with complete inhibition of CYP79D1 and CYP79D2 expression in the roots had no reduction in the root linamarin content compared to wild-type plants. Analysis of leaf CYP79D1/CYP79D2 transcript level and leaf linamarin content indicated they were unchanged relative to wild-type leaves. Thus it is apparent that, similar to Hevea brasiliensis, young cassava plants (3-4 month old) transport cyanogenic glucosides from the leaves to the roots.

It has been demonstrated that the major cyanogen present in poorly processed cassava roots is acetone cyanohydrin and that cassava roots have substantially lower levels of HNL (0-6%) than leaves (100%). The low level of root HNL activity is the apparent cause for high acetone cyanohydrin levels present in processed cassava foods. We postulated that elevated expression of HNL in roots would accelerate the
conversion of acetone cyanohydrin to cyanide and detoxification of cassava foods (from roots). To test this hypothesis we have over-expressed HNL in transgenic cassava plants under the control of a double 35S CaMV promoter. We show that HNL activity and protein levels increased more than 2-fold in leaves of transgenic plants relative to wild-type plants. These elevated HNL levels were correlated with substantially reduced levels of acetone cyanohydrin in homogenized root tissues. These results demonstrate that acetone cyanohydrin can be effectively eliminated in processed roots of plants over-expressing HNL. Importantly the over-expression of HNL in roots of transgenic cassava plants does not affect the steady-state linamarin levels in intact roots. Thus, transgenic plants with elevated HNL levels retain the herbivore deterrent attributes of cyanogens that may be of importance to subsistence farmers.
TO MY PARENTS, BROTHER AND SISTERS
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ABBREVIATIONS

ACN- acetone cyanohydrin; bp- base pairs; BSFTA- Bis (trimethylsilyl) trifluoroacetamide; Cab1- chlorophyll a/b; CN- cyanide; CYP- cytochrome P450; DNA- deoxyribonucleic acid; EDTA- ethylenediamine-tetraacetic acid; FEC- friable embryonic callus; g- gram; GC-MS- gas chromatography mass spectrometry; gdw- gram dry weight; gfw- gram fresh weight; HCN- hydrogen cyanide; HNL- hydroxynitrile lyase; hr- hour; Kan'- kanamycin resistance gene; kb- kilo bases; kDa- kilo Daltons; kgfw- kilo gram fresh weight; Mcol 2215- cassava cultivar *Manihot columbia* 2215; µCi- micro curie; µg- microgram; µL- micro liter; µM- micro molar; mg- milligram; mL- milliliter; mM- millimolar; mmol- millimoles; MS- Murashige and Skoog; mt- metric tons; nos- gene encoding nopaline synthetase; nptII- gene encoding neomycin phosphotransferase II; ng- nanogram; Pat-patatin; PCR- polymerase chain reaction; RNA- ribonucleic acid; rpm- revolutions per minute; RT-PCR- reverse transcription-PCR; *SBEII*- gene encoding starch branching enzyme II; SDS- sodium dodecyl sulfate; SIM- selective iron monitoring; T-DNA- transfer DNA; ter- terminator; TLC- thin layer chromatography; TIC- total iron chromatograph; WT- wild type; w/v- ratio of weight to volume
1.1 AGRONOMY AND VALUE OF CASSAVA

Cassava (*Manihot esculenta* Crantz), a member of the Euphorbiaceae family, is one of the most important food crops in the world, especially in the tropics. Its tuberous roots contain up to 85% of their dry weight as starch. This woody perennial shrub serves as a basic staple for over 500 million people worldwide, most of who live in developing regions of the world (Cock, 1982). Cassava is native to tropical South America and its evolutionary center of origin is thought to be along the southern border of the Amazon River basin in Brazil (Olsen and Schall, 1999). The Portuguese initially introduced cassava into Africa in the 16th century and then to Asia by Spanish traders during the 17th century (Jones, 1959; Leone, 1977). At present, cassava is grown in over 60 countries in Africa, Asia and Latin America.

Cassava grows to be 1 to 5 meters in height. The leaves are petiolated with 5-7 lobes in a palmate orientation while the fibrous root system can develop 5 –10 starchy roots with secondary thickening. Starch deposition in roots is first observed about 4 weeks after planting (Figure 1.1).
Figure 1.1: Schematic diagram of whole cassava plant (A) and cassava grown in pots in greenhouse conditions (B).
Cassava is the fourth most important crop in the developing countries surpassed only by maize, rice and sugarcane as a source of calories (Bradbury, 1988). It is a drought tolerant crop that has the ability to withstand extremely poor, exhausted soils. Under favorable conditions, cassava is one of the most efficient producers of edible carbohydrates. Between 1995 and 1997 the production of cassava was 165.3 million metric tons per year and it is projected to reach 215 million tons by 2005 and 290.8 million metric tons per year by 2020 (Table 1.1; Table 1.2). This projected growth rate of 1.95 percent per year in developing countries is third behind potatoes and maize but exceeds those projected for other major cereals such as rice and wheat (Table 1.3) (Scott et al, 2000). At 142 trillion kilocalories per year, cassava ranks first in edible energy among major root and tuber crops. The value of cassava grown in the developing countries is US $8.8 billion per year (1995 – 1997) and is estimated to increase to US $14 billion by year 2020 (Scott et al, 2000).
Table 1.1: Global cassava production from 1973 to 2020.

Global cassava production is expected to show continued growth over the next five years, with Africa leading the way. Five countries, Brazil, the Democratic Republic of the Congo, Indonesia, Nigeria and Thailand account for almost 70 percent of the world's cassava production. Source: FAO
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**Table 1.3**: Projected growth rates for major food crops in developing countries, 1993-2020.
Cassava is the crop of choice for subsistence and small-scale farmers in the tropics for many reasons. The primary reason is the ability of cassava to withstand drought and extremely poor, exhausted soil thus making cassava suitable for growth in marginal areas unable to sustain many other crops. Cassava is propagated vegetatively from stem cuttings thus the farmers do not have to sacrifice part of the harvest (roots) for the next planting season. A flexible harvesting time, 8 to 24 months, makes cassava an excellent famine foodstuff as well. Cassava is also known as a ‘food-bank’ as it is amenable to partial harvest where the subsistence farmer can harvest only the required amount of roots and restore to rest back in the soil for later harvest. Second only to sugarcane, cassava produces high amounts of calories per unit of land (Scott et al. 2000).

However, cassava is not a perfect crop and has its share of deficiencies. Though a good source of carbohydrates the roots lack appreciable amounts of protein. Due to the low protein content (1-2%) additional food sources are required to ensure a diet balanced with proteins (Cock, 1985). Cassava has the lowest amount of protein content among major crops at 0.9 grams per 100 grams of edible portion (Scott et al., 2000). Due to the low protein content additional food sources are required to ensure a diet balanced with proteins (Cock, 1985). During the lengthy period in the soil attack by insect pests and virus diseases can lead to 20-50% yield losses worldwide resulting in complete crop failure in some instances (Belotti et al., 1999; Thresh et al., 1994). Post harvest deterioration during transport, storage and marketing of cassava is another problem (Wenham, 1995). Even though roots can stay underground for
extended periods of time, once harvested the roots deteriorate very rapidly and are unmarketable within 48 hours. To extend the shelf life of fresh roots cassava can be peeled, precooked and vacuum packed prior to reaching the market or in the case of export to the USA and Europe coated with paraffin.

Another problem with cassava is the presence of cyanogenic glucosides, linamarin and lotaustralin, in all parts of the plants with exception of seeds (Conn, 1994). Linamarin accounts for 95% of the cyanogenic glucosides present in intact cassava tissue (Balagopalan et al., 1985). These cyanogenic glycosides can be broken down to produce cyanide. It is thought that the presence of cyanogenic glycosides protects the plant from herbivores, however, residual cyanogens may cause health problems for human consumers. The physiology, biochemistry and health effects of cyanogenic glycosides are discussed in more detail below. The primary objective of the work presented in this thesis is to produce an acyanogenic cassava plant using available molecular biology tools, tissue culture techniques and transformation procedures.

Attempts to address these shortcomings of cassava through conventional breeding have not been successful. This is likely due to the high heterozygosity of the allopolyploid plants, the low natural fertility, and a long growing season. Thus, traditional breeding is difficult, laborious and time consuming. Biotechnology holds the key to overcoming these limitations and producing cassava plants that are more nutritious, resistant to pests and viruses, more profitable and safer.
1.2 CASSAVA REGENERATION IN VITRO

Plant regeneration through tissue culture has been achieved in cassava using one of five methods. These methods are somatic embryogenesis, meristem culture, organogenesis, protoplast and friable embryonic callus systems.

**Somatic embryogenesis:** A somatic embryo is an independent bipolar structure that is not attached vascularity to the tissue of origin (Ammirato, 1987). These embryos can develop to zygotic embryos, and to plantlets. Primary embryos can be induced from different cassava tissues: shoot apical and axillary meristems (Stamp and Henshaw, 1987; Szabados et al., 1987; Puonti-Kaerlas et al., 1998), immature leaves (Stamp and Henshaw, 1987; Szabados et al., 1987; Raemakers et al., 1993; Li et al, 1995), and cotyledons of zygotic embryos (Konan et al., 1994a). Primary embryos will develop to become mature embryos with green cotyledons and eventually produce shoots. Cyclic systems of somatic embryo production can be maintained through continuous culture of somatic embryos in auxin-supplemented medium.

**Meristems culture:** Meristems on apical or axillary buds can be induced by cytokinin containing media to produce shoots (Kartha and Gamborg 1975; Ng et al., 1990).

**Organogenesis:** Plant regeneration through organogenesis refers to the emergence of adventitious organs (shoot or roots) directly from the explant without an intervening
callus phase. Li et al. (1996, 1998) were able to produce adventitious shoots from cotyledons of cassava somatic embryos using medium supplemented with cytokinins (BAP and IBA). These shoots were successfully transplanted to soil after being rooted in hormone-free medium.

**Friable embryogenic callus (FEC):** A type of less organized embryogenic tissue, FECs, can be produced from organized mature somatic embryos of cassava (Taylor et al. 1996). FECs can be maintained for over two years as suspension cultures under a three-week subculture regime. In the presence of maturation medium the suspension develops into mature embryos and germinates into plants after desiccation treatment (Raemakers et al. 1996; Taylor et al., 1996).

**Protoplasts:** Cassava regeneration from protoplasts has not been very successful. Despite the use of various media protoplasts only regenerated into green callus or adventitious roots at best (Sofiari et al., 1997; McDonnell and Gray, 1997). But more recently protoplasts isolated from FECs were found to divide and develop readily into callus and eventually lead to plantlets (Sofiari et al., 1998).
1.3 CASSAVA TRANSFORMATION

The most common ways to transform plant cells with foreign genes are Agrobacterium-mediated gene transfer and particle bombardment. Though low in transformation efficiency, other ways of gene transfer to plants have been established; electroporation (Fromm et al., 1986; Lurquin, 1997), silicon carbide whisker-mediated DNA transfer (Thompson et al., 1995), protoplast transformation (Shillito, 1999) and microinjecting DNA into cells or zygotes (Leduc et al., 1996).

1.3.1 Agrobacterium-mediated T-DNA transfer

*Agrobacterium tumefaciens* is a soil bacterium that causes crown gall disease mainly in dicotyledonous plants and some gymnosperms (Klee et al., 1987; Gelvin, 1991; Tinland, 1996) and has the ability to transfer a portion of a large plasmid (Ti-plasmid), the T-DNA, to the host plant (Winans, 1992; Tinland, 1996). In wild-type Agrobacterium, three genetic components of the bacterium are required for plant cell transformation. The tumor inducing or Ti-plasmid contains two components, the T-DNA and the virulence (*vir*) genes. The Ti-plasmid is usually 150 to 200 Kb in size. The third component, which resides in the Agrobacterium chromosome, consists of loci *chvA, chvB, chvE, att, cell* etc. (Zambryski, 1988; Chen et al., 1991; Tinland, 1996). In nature the oncogenic genes reside in the T-DNA between two 25-bp border
repeats (Wang et al., 1984). In the Agrobacterium ti-plasmid used for plant transformation these oncogenes have been either deleted or replaced.

The infection cycle of Agrobacterium is complex and involves a number of chemical signals emitted by both the pathogen and the host plant. The T-DNA transfer process is activated when Agrobacterium perceives certain phenolics and sugar compounds released by the wounded plant. The bacterium then attaches to the plant cells and transfers a portion of the Ti plasmid. The T-DNA does not encode any important genes for the transfer process although it has to be delimited by 25-bp direct repeats in order to be transferred (Zupan and Zambrsky, 1995; Gelvin, 2000). The processing and transfer of T-DNA is mediated by the products of the \textit{vir} genes located on the Ti plasmid. The \textit{vir} region is 30-35 Kb in size and organized into seven operons, \textit{VirA}, \textit{VirB}, \textit{VirC}, \textit{VirD}, \textit{VirE}, \textit{VirG} and \textit{VirH} (Zupan and Zambrsky, 1995; Tinland, 1996; Gelvin, 2000).

\textit{VirA} is a transmembrane dimeric sensor protein that detects signal molecules, released from wounded plants (Pan et al. 1993). The signals for \textit{VirA} activation include acidic pH, phenolic compounds, such as acetylsyringone (Winans et al., 1992), and monosaccharides that act synergistically with phenolic compounds (Ankenbauer et al., 1990; Cangelosi et al., 1990; Doty et al., 1996). Monosaccharide detection by \textit{VirA} is an important amplification system to respond to low levels of phenolic compounds. The induction of this system is only possible through the periplasmic sugar (glucose/galactose) binding protein ChvE (Ankenbauer et al., 1990; Cangelosi et al., 1990), which interacts with \textit{VirA} (Turk et al., 1993; Chang and Winans, 1992).
Activation of the **VirA** in turn phosphorylates **VirG**. Phosphorylated **VirG** activates the induction of **vir** gene transcription (Winnans, 1992; Tinland 1996) culminating with the generation of the T-strand, a single stranded copy of the T-DNA. The ss-T-DNA is transferred into the host plant cell nucleus and integrated into the genomic DNA. During the T-DNA transfer, the virulence protein D2 (VirD2) and E2 (VirE2) protect the ss-T-DNA against nucleases and act as a nuclear localization signal to target the incoming ss-T-DNA into the plant cell nucleus (Tinland et al., 1995; Zupan and Zambrysky, 1997).

Today Agrobacterium-mediated transformation is routinely used to transform cassava. Agrobactium has been successfully used to transfer genes to all the cassava tissue that has the ability to regenerate into plantlets. Sarria et al. (1995, 2000) showed that wild-type strains of *Agrobacterium tumefacience* were able to transfer to cassava somatic embryos a T-DNA carrying **nptII**, **bar** and **uidA** genes. A transient expression of GUS was shown in meristems, meristem-derived somatic embryos after co-cultivation with Agrobacterium (Puonti-Kaerlas et al., 1997; Konan et al., 1994b). Li et al. (1996) used Agrobacterium mediated transformation to make transgenic cassava plants via shoot organogenesis from somatic cotyledons. Lastly, Gonzalez et al. (1998) developed a method to transform cassava FECs using Agrobacterium. For this study **nptII** gene and **uidA** gene was used as the selectable marker and visible marker, respectively.
1.3.2 Biolistics method of transformation

The bioloistic transformation technique is mediated by microprojectile bombardment (Klein et al., 1987). DNA-coated microprojectiles are accelerated by explosive material or a high-pressure burst of gas to penetrate the plant cell wall and deliver the DNA for expression and/or integration into the plant genome. This technique is widely used to deliver foreign DNA to regenerable cells without the burdens of Agrobacterium related host specificity limitations. Schopke et al. (1996) and Raemakers et al. (1996) were the first to report the use of biolistics to transform cassava. In both instances somatic embryogenesis was utilized as the regeneration method. More recently Zhang et al (2000a, 2000b) reported the use of particle bombardment to produce stably transformed cassava.

1.4 CYANOGENESIS

1.4.1 Cyanogenesis in Sorghum

It has been estimated that over 2500 plant species have the capacity to release hydrogen cyanide from endogenous cyanogenic glucosides (Conn, 1981; Kakes, 1990; Jones, 1988; Seigler, 1998). Several agriculturally important crop plants are known to be cyanogenic, e.g. cassava, sorghum, barley, bamboo, lima beans, white clover, flax,
rubber tree, cherry, plum, peach, apricot, almond, macademia and mango (Conn, 1981; Lechtenberg and Nahrstedt, 1999; Moller & Seigler, 1999; Pourmohseni and Ibenthal, 1991; Pourmohseni, 1993). All known cyanogenic glucosides are O-β-glycosides of α-hydroxynitriles and consists of an amino acid backbone with a nitrile group and one or more glucosyl moieties. The majority of the cyanogenic glucosides identified are primarily synthesized from 5 parent amino acids: valine, isoleucine, tyrosine, phenylalanine and leucine.

Dhurrin synthesis in *Sorghum bicolor* has been used as the model system to study cyanogenic glucoside synthesis in plants. Following germination sorghum seedlings rapidly accumulate the cyanogenic glucoside, dhurrin, to levels up to 6% of the dry weight of the seedling (Halkier and Moller, 1989). Moller and Conn (1979) were successful in identifying the N-hydroxylation of tyrosine as the first dedicated step in dhurrin formation and an aldoxime as an intermediate formed later in the pathway (Figure 1.3). The microsomal fraction of the sorghum seedling is known to be the site of dhurrin synthesis (McFarlane et al. 1975). Oxygen consumption experiments demonstrated the incorporation of three oxygen atoms during dhurrin synthesis (Halkier and Moller, 1990; Halkier et al. 1991). Two oxygen molecules are consumed in hydroxylation steps leading to the synthesis of the aldoxime, and a third oxygen was incorporated in the conversion of the oxime to p-hydroxymandelonitrile (Figure 1.3). This third oxygen-requiring step was found to be a simple dehydration reaction.
The identification of two hydroxylation events in the synthesis of dhurrin implicated the involvement of two cytochrome P450 enzymes (Halkier and Moller, 1991). Using a microsomal fraction, it was observed that dhurrin synthesis could be inhibited by carbon monoxide, and this inhibition can be reversed by illumination with 450nm light. Furthermore, dhurrin synthesis was shown to be both NADPH and oxygen dependent. The first cytochrome P450-mediated hydroxylation reaction is the N-hydroxylation of tyrosine to form N-hydroxytyrosine. The second reaction is the C-hydroxylation converting p-hydroxyphenylacetonitrile into p-mandelonitrile.

Sibbesen et al. (1994) reported the purification of the cytochrome P450 that catalyzes the first dedicated step in dhurrin biosynthesis. This enzyme, cytochrome P450TYR, had an estimated molecular weight of 57 kDa and showed high specificity for its substrate L-tyrosine. Cytochrome P450TYR has been shown to be a multi-functional enzyme, capable of carrying out two sets of hydroxylations. Immediately following the purification, Koch et al. (1995) isolated a 1674 bp cDNA clone for cytochrome P450TYR that has an open reading frame encoding a protein of 61.9 kDa. Sequence comparison showed that this protein had a high identity (31%) to 3',5'-flavonoid hydroxylase (CYP75A1) from petunia and an avocado cytochrome P450 sequence of unknown function (CYP71A1). Based on the dissimilarity of the sequence to other cytochrome P450s gene sequences, the sorghum cytochrome P450TYR gene was assigned to a new family, CYP79.
1.4.2 Cyanogenesis in Cassava

1.4.2.1 Linamarin synthesis in Cassava

Cassava, due to the presence of cyanogenic glucosides, is potentially toxic. With the exception of seeds, the cyanogenic glucosides linamarin (95%) and lotaustralin (5%) are present in all parts of the mature cassava plant (Conn, 1994; Balagopalan et al., 1985). Linamarin and lotaustralin are derived from the amino acids valine and isoleucine, respectively. The biosynthesis of linamarin proceeds through a series of intermediates similar to those seen in dhurrin biosynthesis (Figure 1.3). Using cassava seedling microsomes it was observed that linamarin production was dependent upon the presence of NADPH, and that synthesis could be photoreversibly inhibited by carbon monoxide. Similar to the dhurrin synthesis pathway there seems to be two cytochrome P450s catalyzing the conversion of valine to acetone cyanohydrin (Koch et al., 1992). The first committed step in the synthesis of linamarin is the conversion of valine to 2-methylpropanol oxime and then to acetone cyanohydrin, the non-glycosylated form of linamarin (Figure 1.2). The initial N-hydroxylation of L-valine to N-hydroxyvaline, catalyzed by the first cytochrome P450 involved, is believed to be the rate-limiting step in linamarin synthesis. Glycosylation of acetone cyanohydrin by UDPG-glucosyltransferase, yields linamarin (Figure 1.2). White et al., (1994) showed that the conversion of valine to linamarin occurred in a NADPH-dependent reaction in partially purified vacuoles, suggesting that the cytochrome P450s involved in the
initial biosynthesis steps are localized in the tonoplast membrane and that the UDPG-glucosyltransferase is a vacuolar enzyme.

More recently, Andersen et al. (2000) isolated two full-length cDNA clones that encode cytochrome P450s catalyzing the conversion of valine to 2-methylpropanol oxime. These two cassava cytochrome P450s, CYP79D1 and CYP79D2, are 85% identical and share a 54% sequence identity with sorghum cytochrome P450 involved in the dhurrin cyanogenic glucoside pathway. The similarity of the reactions between the cassava linamarin synthesis pathway and the sorghum dhurrin synthesis pathway (Figure 1.3) led Andersen and colleagues to assume that the unknown cassava cyanogenic glucoside cytochrome P450 belonged to the CYP79 family. Based on this assumption they screened an immature leaf cDNA library with degenerate primers homologous to conserved region of the CYP79 gene family. Cassava partial cDNAs showing high homology to CYP79 family were then used to isolate two equally abundant, full-length cDNA clones. The clones have open reading frames encoding Cyt P450s of 61.2 kDa and 61.3 kDa. Functional analyses of these two Cyt P450s, performed in *Pichia pastoris* yeast, showed substrate specificity towards valine and isoleucine, the precursors for linamarin and lotaustralmin, respectively.
Figure 1.2: The linamarin biosynthetic pathway from cassava
Figure 1.2

L-Valine

\[ \text{O}_2 + \text{NADPH} \rightarrow \text{NADP}^+ \]

\[ \text{O}_2 + \text{NADPH} \rightarrow \text{NADP}^+ \]

Linamarin

CYP79D1 CYP79D2

Acetone cyanohydrin

\[ \text{O}_2 + \text{NADPH} \rightarrow \text{NADP}^+ \]

Putative CYP71E1

2-Methylpropanal oxime

UDPG-glucosyl transferase

(Figure 1.2)
Figure 1.3: Comparison of the cyanogenic glucoside pathways of sorghum and cassava.
1.4.2.2 Cyanide synthesis in Cassava

The generation of cyanide from linamarin involves the initial deglycosylation of linamarin by linamarase and then the cleavage of acetone cyanohydrin (Figure 1.4). Linamarase is localized in the cassava cell wall and is also abundant in laticifers (Mkpong et al., 1990) (Figure 1.5). Therefore, release of cyanide occurs only after tissue damage when linamarin comes in contact with linamarase, as is the case during herbivore attack or during food processing (Belloti and Arias, 1992; Cock, 1985; Balagopalan, 1985). The production of cyanide from acetone cyanohydrin is catalyzed by hydroxynitrile lyase (HNL), which also produces a ketone. This cleavage reaction also occurs spontaneously at temperatures greater than 35°C or pHs > 5 (White et al., 1994; Hughes et al., 1994) (Figure 1.4).

Cassava HNL, with a molecular weight of 29 kDa, has been shown to exist as a dimer when isolated from the apoplast of leaves (White et al., 1994). The HNL present in apoplastic extracts has a maximum activity of 24 mmol HCN/mg protein/hr in the presence of saturating concentrations of acetone cyanohydrin. In 1994, Hughes et al. (1994) identified a 1kb cDNA clone of HNL from cassava with an open reading frame encoding a protein of 258 amino acids. The cassava HNL and rubber tree HNL shared 78% amino acid similarity (Hasslacher et al., 1996). In 1996, White reported the identification of another HNL cDNA sequence from cassava. The cDNA was 1118 bp in length encoding a 258 amino acid protein. The sequence was identical to the Hughes et al. (1994) sequence with the exception of 13 amino acids. Closer analysis of
these dissimilar 13 amino acids shows 8 amino acids in White’s HNL sequence are similar to that of rubber tree HNL (Hasslacher et al. 1996) while Hughes’s HNL sequence does not. Southern blot analysis performed by both groups show the existence of one copy of the HNL gene in the cassava genome. Later Hughes recognized that they had made a sequencing error and in fact the correct sequence for the gene was identical to that described by White.

1.4.2.3 Compartmentalization of enzymes involved in cyanogenesis

The compartmentalization of linamarin in the vacuole and linamarase and HNL in the cell wall prevents the formation of toxic cyanide in undamaged cells (Figure 1.5). The breakdown of the physical barriers between substrates and the enzymes, following tissue damage, initiates cyanogenesis (Poulton, 1990).
Figure 1.4: Catabolism of linamarin to produce hydrogen cyanide
Figure 1.5: Compartmentalization of the cyanogenic glucoside pathway in cassava
1.4.3 Functions of cyanogens

Cyanogenesis has been shown to protect the plant against herbivore or fungal attack (Belloti and Arias, 1992; Hickel et al., 1996). Narstedt (1985) performed feeding deterrent studies using insect pests of cyanogenic plants to prove that cyanogenic glucosides function as herbivore deterrents. In this study the Mexican bean beetle (*Epilachna varivestis*) caused much greater crop damage to *Phaseolus vulgaris* when compared to *Phaseolus lunatus* that has 4-fold higher quantity of cyanogenic glucosides. In a study conducted by Arias and Bellotti (1984), they found that certain insect pests that prey upon cassava showed preference for low cyanogenic varieties over high cyanogenic varieties.

Cyanogenic glucosides may have an additional function in plants. Selmar et al. (1988) proposed that cyanogenic glucosides serve as nitrogen storage compounds as well as help in nitrogen mobilization in young plants. They observed a rapid increase of cyanogen transport following germination of rubber tree (*Hevea brasiliensis*), although an accompanying increase in HCN was not observed. Selmar suggests that linamarin is immediately converted into linustatin, a glucoside of linamarin which is unable to be hydrolyzed by linamarase, and is subsequently transported. This apoplastic cyanogen transport has been demonstrated in rubber tree and may also be operating in cassava (Koch et al. 1992; Selmar, 1994). In rubber tree, linamarin is converted to linustatin at the source site and transported apoplastically to the sink (Figure 1.6). At present, the sum contribution of the cyanogens produced in rubber
tree leaves and cotyledons to the root steady-state levels remains a mystery. At the sink, linustatin can be metabolized in two ways. Linustatin can be deglucosylated using a ‘simultaneous’ deglucosidase that produces gentibiose and acetone cyanohydrin, or it can be ‘sequentially’ deglucosylated to linamarin and then to acetone cyanohydrin (Figure 1.6). In the case of the simultaneous pathway acetone cyanohydrin can be glucosylated via UDP-glucosyltransferase to form linamarin.

Selmar showed that infiltration of $^{14}$C-labelled linustatin into rubber cotyledons led to no loss of $^{14}$C as volatile HCN. After 4 hours, 50% of the linustatin was converted to linamarin while another 12% was incorporated into residual compounds. After a preliminary phase during which the $^{14}$C-label accumulated in the cotyledon, up to 65% of the label was transported from the cotyledon. Although the initial $^{14}$C labeled compound in the roots was linustatin, 24 hours after label incorporation 83.4% of the $^{14}$C product was linamarin. Selmar and coworkers conclude that the presence of $^{14}$C-linamarin in roots is consistent with an apoplastic transport of linustatin.
Figure 1.6: Apoplastic transportation of glucosylated linamarin, linustatin, from the leaves to the roots.
Evidence for cyanogenic glucosides being mobile nitrogen storage compounds in cassava is far less compelling. Labelling studies of cassava by Bediako et al. (1981) with \( ^{14}\)C-valine showed that the primary site of linamarin synthesis is the leaf, while the roots and lower stem had minimal ability to incorporate labeled valine. A few years later, Ramanujam and Indira (1984) showed a 13-fold decrease in root cyanogens when girdling was performed on the cassava stem. To re-address this issue, Makame et al. (1987) performed a series of grafting experiments between roots and shoots of low- and high-cyanogenic cultivars of cassava. The results showed that, at least in part, cyanogen accumulation in the roots was due to the transportation of cyanogens from the leaves.

The total cyanogens present in the roots might not come completely from cyanogens synthesized in the leaves and subsequently moved to the roots. Jennifer McMahon-Smith (Ph.D. thesis, 1997) showed that the roots were capable of synthesizing linamarin. Fibrous roots from young plants of varieties CM996-6 and HMC-1 were able to synthesize linamarin at rates of 0.217 and 0.235 nmol/gfw/hr, respectively. The root linamarin content of these two varieties is 2.11 and 2.35 \( \mu \)mol/gfw. At this rate of root linamarin synthesis it would take >400 days to synthesize the linamarin present in roots. Thus it is apparent that the rate of linamarin synthesis in the root is not sufficient to account for all the linamarin present in the roots and that leaf-synthesized linamarin probably contributes to the linamarin present in roots (Du et al. 1995).
1.4.4 Cyanogen toxicity

1.4.4.1 Variation of cyanogen levels in Cassava

Unprocessed fresh cassava roots contain on average from 15 to 440 mg CN equivalents/kgfw, while high cyanide cultivars can contain up to 1500 mg CN equivalents/kgfw (O’Briens et al., 1991). Cultivars having root cyanide equivalents <100 mg/kgfw cyanogens are classified as low cyanogenic cultivars, whereas cultivars with cyanogen levels >500 mg/kgfw are referred to as high cyanogenic cultivars. A marked radial gradient in linamarin content exists from the outer peel to the inner parenchyma tissue. It has been shown that the outer peel can contain between 7- and 16-times the linamarin of parenchyma tissue of the same variety (Nartey, 1978; Bradbury and Egan, 1992).

The use of cassava leaves as a food source for humans and animals has prompted extensive analysis of the cyanogen content of leaves. The cyanogen levels in leaves range from 200 to 1,300 mg CN equivalents/kgfw. Gomez and Valdreso (1985) noted that the youngest leaves contain the highest linamarin levels. They found that the leaf cyanide potential was less than that of the root peel but greater than that of the inner parenchyma tissue. In a later study, Mkpong et al. (1990) found that leaf linamarin levels were about 20 times higher that in the parenchyma cells in the root cortex. On average leaf cyanogen content (446 mg/kgfw) and root parenchyma
cyanogen content (85 mg/kgfw) are about 77% and 14% of the cyanogen levels in the root peel (577 mg/kgfw), respectively (Table 1.4).
### Table 1.4: Average range of cyanogen content in different cassava tissue (tabulated from the reported cyanogen content of cassava tissue by Conn, 1979; Sinha and Nair, 1967; Cooke, 1978; Narrey, 1968; Makame et al. 1987; Bradbury and Egan, 1992, 1994; Rao and Hahn, 1984; Cooke and DeLakruz, 1982; Mkpong et al., 1990)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Average cyanogen content (mg/kgfw)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf</td>
<td>446</td>
<td>200 - 1040</td>
</tr>
<tr>
<td>Root Peel</td>
<td>577</td>
<td>186 - 1100</td>
</tr>
<tr>
<td>Root Parenchyma</td>
<td>85</td>
<td>12.3 - 330</td>
</tr>
<tr>
<td>Seed</td>
<td>4.6</td>
<td>0 - 8.5</td>
</tr>
</tbody>
</table>
1.4.4.2 Cassava cyanide related complications

The residual cyanogens, linamarin and acetone cyanohydrin, are the apparent source of cyanide toxicity to animals when converted to cyanide inside the body. For an adult human, consumption of 50 to 100 mg or 2 mmol of HCN within 24 hours can completely block cellular respiration leading to death (Rosling, 1993). Exposure to lower levels of cyanide can also cause a variety of symptoms, such as vomiting, nausea, palpitations, headaches and impaired vision (Rosling, 1988). The ingestion of large quantities of cassava or prolonged exposure to improperly processed cassava food has been associated with chronic cyanide toxicity in several areas of Africa (Tylleskar et al., 1992; Mlingi et al., 1992). During drought cassava associated cyanide poisoning is aggravated by the lack of firewood resulting in inadequate cooking and detoxification of cassava.

Cyanide intake from cassava-dominated diet is a contributing factor in two forms of nutritional neuropathies, tropical ataxic neuropathy described from Nigeria (Osuntokun, 1981) and epidemic spastic paraparesis described from Mozambique, Tanzania and Zaire (Ministry of Health, Mozambique 1984). Epidemic spastic paraparesis seems to be the same disease in all these countries where it is a current problem but it differs in several ways from tropical ataxic neuropathy described from Nigeria.
1.4.4.2.1 Tropical ataxic neuropathy

Clark (1935) was the first to suggest that cyanide exposure from cassava was the cause of tropical ataxic neuropathy (TAN). The clinical picture of this disease was dominated by damage to one of the sensory tracts in the spinal cord resulting in an uncoordinated gait called ataxia. The disease mainly occurred among adult males. In diseased persons high plasma thiocyanate levels were associated with high cyanide intake (Monekosso et al. 1966). A low dietary protein intake, resulting in a deficiency of sulphur, needed for cyanide detoxification, was proposed as a contributing factor in cyanide poisoning (Osuntokun et al. 1968, Osuntokun 1981). The occurrence of TAN has decreased during the last decade which may be explained by a change in the diet by populations at risk.

1.4.4.2.2 Epidemic spastic paraparesis (Konzo)

Konzo is mainly a disease of women and children. It is an acute disease, rapidly and permanently crippling the victim by damaging nerve tracts in the spinal cord that transmit signals for movement, causing a spastic paralysis of both legs (Howlett et al. 1990). Paraparesis refers to the paralysis of both legs and spastic implies that muscles of the legs are not flaccid. The legs can usually support affected persons sufficiently to allow them to stand, especially if supported by a stick, but the affected persons will have their knees crossed. Attempts to walk often result in
uncontrolled muscle jerks. In the most extreme case the arms are also affected similar to the legs and poisoned individuals may also have diminished vision and difficulties in speaking.

The occurrences of all reported incidence of epidemic spastic paraparesis are during seasons when populations experience food shortages (Howlett et al. 1990, Mlingi et al. 1991, Tylleskar et al. 1991). Cassava was the only food available in quantity in these areas experiencing food shortages and thus famine was avoided, but the roots were consumed without sufficient processing. The disease has, so far, not been reported from any population not consuming cassava or from cassava consuming populations having balanced diets.

1.4.4.2.3 Goiter

The thyroid gland, situated in the front of the neck, is normally not visible. Its main function is to produce the iodine-containing hormones that regulate the metabolism of the body. When the dietary intake of iodine is insufficient the thyroid gland starts to enlarge to enable a maximal extraction of iodine from the blood. The occurrence of such enlargement is known as goiter. The most severe public health effect of goiter is that the children born to iodine-deficient mothers will suffer from cretinism, a condition characterized by mental retardation and stunted growth (Delange et al., 1983).
Extensive studies in Zaire have established that goiter and cretinism due to iodine deficiency can be considerably aggravated by a continuous dietary cyanide exposure from insufficiently processed cassava. This effect is caused by thiocyanate, the detoxification product of cyanide. Thiocyanate has a similar size to the iodine molecule and interferes with the iodine intake in the thyroid gland (Bourdux et al. 1978). Populations in northern Zaire with very low iodine intake and having high thiocyanate levels resulting from consumption of inadequately processed cassava, suffered from very severe endemic goiter with a high prevalence of cretinism. When the population was given iodine supplementation by injection of iodized oil, the goiter problem decreased considerably in spite of continued consumption of insufficiently processed cassava (Ermans et al. 1981).

1.4.5 Detoxification of cyanide

1.4.5.1 Detoxification pathways

There are two known cyanide detoxification or assimilation pathways in cassava mediated by two different enzymes, rhodanese and β-cyanoalanine synthase. The conversion of cyanide to thiocyanate by the enzyme rhodanese (Figure 1.7A) is thought to be less significant in cassava than cyanide assimilation by the β-cyanoalanine synthase pathway. This assumption is based on the observation that there is low rhodanese activity and thiocyanate levels detected in tubers (Nambisan, 1994).
In addition, Kakes and Hakvoort (1992) compared rhodanese activity in cyanogenic and acyanogenic cultivars of white clover (*Trifolium repens*), as well as other plant species in which rhodanese activity had been reported. They found a lack of correlation between cyanogenic potential and rhodanese activity in all species tested.

The conversion of cyanide to β-cyanoalanine, through a condensation reaction with cysteine, catalyzed by β-cyanoalanine synthase (Figure 1.7B), is thought to be the major cyanide detoxification pathway in cassava. β-cyanoalanine is then hydrolyzed to asparagine by β-cyanoalanine hydrolase. The presence of both these enzymes in all cassava tissues indicates the importance of this pathway to detoxify cyanide (Nambisan & Sundaresan, 1994; Elias et al. 1997a; 1997b). Nartey (1969) reported that the primary product of HCN incorporation was asparagine, while β-cyanoalanine was not detected. Nartey attributes the lack of the intermediate (β-cyanoalanine) in cassava to the fact that some plant species preferentially store cyanide metabolites as β-cyanoalanine, while other species immediately convert β-cyanoalanine to asparagine. Comparative studies between 14 cyanogenic and acyanogenic higher plants for the presence of β-cyanoalanine synthase by Miller and Conn (1980) found that the cyanide potential of the plants was proportional to the activity of β-cyanoalanine synthase.

In cassava, the highest β-cyanoalanine synthase activity per unit protein was found in the root (36 µg H₂S/min/100mg protein) and the least in the leaf (13 µg H₂S/min/100mg protein) (Elias et al. 1997a,b). Similarly, root has a higher β-cyanoalanine hydrolase (25 µg ammonia/min/100mg protein) activity per unit protein than leaf (8.8
µg ammonia/min/100mg protein). Consistent with this observation is the presence of higher asparagine levels in the root than in the leaf (Nambisan B, unpublished). Elias et al. (1997b) also showed that the activity of asparaginase, which directs the conversion of asparagine to aspartic acid and ammonia, is 5.0 µg ammonia/min/100mg protein in cassava root compared to 7.1 µg ammonia/min/100mg protein in leaf. It therefore appears that in roots the degradation of cyanogenic glucosides leads to comparatively more accumulation of asparagine whereas in leaves further degradation of asparagine to ammonia apparently occurs. However, it is also noteworthy that the total protein content of leaves (90g protein/kgfw) is substantially higher than in roots (9g protein/kgfw). Thus, the total activities of the aforementioned cyanide assimilatory enzymes in leaves are greater than in roots.
Rhodanese

(A) $\text{HCN} + \text{S}_2\text{O}_3^- \rightarrow \text{SCN}^- + \text{SO}_4^{2-}$

Hydrogen Cyanide  Thiosulfate  Thiocyanate  Sulfate

$\beta$-cyanoalanine synthase

(B) $\text{HCN} + \text{Serine/Cysteine} \rightarrow \beta$-cyanoalanine + $\text{H}_2\text{S}$

+ $\text{H}_2\text{O}$  $\beta$-cyanoalanine Hydrase

Asparagine

**Figure 1.7**: Hydrogen cyanide metabolism in plants. (A) rhodanese pathway (B) $\beta$-cyanoalanine synthase pathway.
1.4.5.2 Removal of cyanogenic glucosides from cassava foods

To prevent cyanide poisoning, linamarin and lotaustralin have to be removed from cassava foods. The most efficient processing procedures include peeling, soaking (fermenting), chopping, grating, drying, and cooking.

**Peeling:** Many methods of processing cassava roots commence with the peeling of the roots, which reduces the cyanogenic glucoside content by at least 50%.

**Grating:** This process takes place after peeling and is sometimes applied to whole roots. Grating of the whole root ensures homogeneous distribution of the cyanide in the product, and will also make the nutrients contained in the peel available for use. In the grated product, the residual concentration of cyanogens will depend on the time during which linamarin and linamarase interact.

**Soaking:** Soaking of cassava roots is normally done prior to cooking. It extracts the soluble cyanide into the water. Although this process removes about 20% of the free cyanide within 4 hours, a significant reduction of total cyanide is achieved only after 4-5 days; helped by routine changing of the soaking water. It has been reported that peeled cassava roots soaked for 4-5 days followed by sun drying showed a reduction of cyanide of about 98.6% of the initial content in the roots.

Most marketed cassava products like “garri”, “fufu”, “pupuru”, “apu” etc., in Africa are obtained through fermentation. The importance of fermentation in cassava
processing is based on its ability to reduce the cyanogenic glucosides to relatively insignificant levels. Unlike alcoholic fermentation, the biochemistry and microbiology of cassava fermentation is only superficially understood, but it is believed that some cyanidrophilic/cyanide tolerant microorganisms are involved in the breakdown of the cyanogenic glucoside. Higher retention of starch in grated cassava leads to more efficient detoxification of cyanogens and cyanide by the bacteria. This could be due to the fermentative substrate provided by the starch.

**Boiling/Cooking:** Similar to soaking, there is a rapid reduction in the free cyanide from boiling cassava roots. About 90% of free cyanide is removed within 15 minutes in boiling water, whereas 55% of the cyanogenic glycoside is lost after 25 minutes (Cooke and Maduagwu, 1985).

**Drying:** Proper sun drying is achieved in 1-3 days in the dry season and up to 8 days in the rainy season. Gomez et al. (1984) indicated that more than 86% of HCN present in cassava roots was lost during sun drying.
1.5 OBJECTIVES

Cassava is the fourth most important crop in the developing countries and its roots are the primary source of calories for more than 500 million people, the majority of whom live in the developing countries of Africa. Cassava leaves and roots contain potentially toxic levels of the cyanogenic glycoside, linamarin, however. Upon tissue damage linamarin is released from the vacuole and deglycosylated by the cell wall enzyme linamarase to produce acetone cyanohydrin. Acetone cyanohydrin is then broken down either spontaneously (pH > 5.0 or temperature > 35 °C) or by hydroxynitrile lyase (HNL) to produce acetone and hydrogen cyanide. During food processing essentially all free cyanide is removed by water extraction or volatilization, however, short-cut processing techniques can yield toxic food products. Consumption of residual cyanogens (linamarin or acetone cyanohydrin) in incompletely processed cassava roots, however, can result in cyanide poisoning due to conversion of the cyanogens to cyanide in the body.

Our objective was to eliminate cyanogens from cassava so as to eliminate the need for extensive food processing. To achieve this goal we generated transgenic acyanogenic cassava plants in which the expression of the cytochrome P450 genes (CYP79D1 and CYP79D2), that catalyze the first-dedicated step in linamarin synthesis, was selectively inhibited in either leaves or roots. In this regard the CYP79D1 and CYP79D2 genes were expressed in antisense orientation under the control of leaf-specific Cab1 promoter or root-specific patatin promoter. Equally
importantly, utilizing the two types of cassava transformants we will also attempt to shed light on the existence of cyanogenic glucoside movement system within the cassava plants, similar to that of rubber tree.

Unlike leaves, HNL is not expressed in cassava roots (White et al. 1998). Since acetone cyanohydrin may be stabilized by the low pHs often encountered during root processing we hypothesized that the low levels of HNL in roots may effectively reduce the efficiency of cyanogenesis and cyanide removal. To test this hypothesis we have over-expressed HNL in transgenic cassava plants under the control of a double 35S CaMV promoter. Our objective was to elevate HNL levels that would result in substantially reduced levels of acetone cyanohydrin in homogenized root tissues. These transgenic plants should have the normal levels of linamarin thus retaining the herbivore deterrent attributed of cyanogens that is of importance to subsistence farmers.
CHAPTER 2

ANTISENSE EXPRESSION OF THE CYP79D1 AND CYP79D2 GENES UNDER THE CONTROL OF CAB1 PROMOTER

2.1 INTRODUCTION

In sub-Saharan Africa root crops and particularly cassava provide the majority of calories for human nutrition. Cassava ranks fourth in production among all tropical crops (162 million metric tons produced/year) and is valued for the food security that it provides. Cassava is drought tolerant, grows well in poor soils, and the roots (starch) can persist in soil for 1-2 years without decay, providing a reliable food source during periods of famine (Best et al., 1994). In addition, cassava leaves are consumed by many African cultures and are an excellent source of protein and vitamins (Latham, 1979; Ikoigbo, 1980).

The leaves, roots and stems of cassava, however, contain potentially toxic levels of cyanogenic glycosides (linamarin (95%) and lotaustralin (5%)). These cyanogens yield cyanide following enzymatic hydrolysis (Kakes, 1990; Koch et al., 1992; McMahon et al., 1995; White et al., 1994). Significantly, the cyanogen levels in
leaves (200-1,300 mg CN equivalents/kg dry weight) and roots (10-500 mg CN equivalents/kg dry weight) of many cassava cultivars are higher than the maximum recommended cyanide levels (10 mg CN equivalents/kg dry weight) for foods established by the FAO. In Africa, a number of cyanide-associated health disorders have been attributed to eating poorly processed cassava, particularly by nutritionally compromised individuals (Rosling, 1994). The severity of these disorders depends on the level and frequency of cyanogen exposure and the health of the consumer. Chronic, low-level cyanide exposure has been associated with the development of goiter and tropical ataxic neuropathy, whereas acute cyanogen poisoning, particularly during famines, has been associated with outbreaks of Konzo, a paralytic disorder, and in some cases death (Osuntokun, 1981; Tylleskar et al., 1992; Rosling et al., 1992).

To make cassava safe for human consumption cassava roots and leaves must be processed to remove the cyanogens. Typically, processing involves tissue maceration, soaking, rinsing and baking. These steps release linamarin from the cell vacuole allowing it to be de-glycosylated by linamarase which is localized in the cell walls and laticifers of cassava (Figures 1.4 and 1.5). The cyanogenic product of deglycosylation, acetone cyanohydrin, is then decomposed to yield cyanide and acetone. This process may occur spontaneously, at pHs > 5.0 or at elevated temperatures (> 35 °C), or enzymatically by hydroxynitrile lyase (HNL) (McMahon et al., 1995; White, 1996; White et al., 1998)). Significantly, HNL is abundant in leaves but is present at very low levels in roots (White et al., 1998). The low abundance of HNL in roots may account for food products that have potentially toxic levels of
acetone cyanohydrin. Finally, the conversion of acetone cyanohydrin to free cyanide during processing results in a safe food product since the cyanide is volatilized or removed by washing.

Surprisingly, many subsistence cultures prefer high cyanogenic cassava varieties. The reasons for cultivating more toxic varieties include, taste preference, reduction in herbivory, and protection against theft (Nweke et al., 2002). In many regions of Africa both high- and low-cyanogenic varieties are planted together to provide food security as well as a low cyanogen food source.

For cassava to become a reliably safe and acceptable cash crop as well as to reduce the cyanide intake of subsistence farmers there is a need for acyanogenic cassava cultivars. Recently, the genes encoding a small (CYP79D1 and CYP79D2) family of cytochrome P450s that catalyze the first-dedicated step in linamarin and lotaustralpin synthesis were isolated (Andersson et al., 2000). These two cassava cytochrome P450s, CYP79D1 and CYP79D2, share a nucleotide identity of 85% (Figure 2.1) and an amino acid homology of 80% (Figure 2.2). In addition, both cassava cytochrome P450s share a 54% amino acid sequence identity with sorghum cytochrome P450 involved in the sorghum cyanogenic glucoside pathway. The CYP79D1 and CYP79D2 clones have open reading frames encoding Cyt P450s of 61.2 kDa and 61.3 kDa, respectively. Andersson et al. (2000) reconstituted of CYP79D1 with sorghum NADPH-P-450 oxidoreductase in the presence of the lipid L-alpha-dioleyl phosphatidylcholin and $^{14}$C-labelled amino acids. The amino acids used in plants as precursors for cyanogenic glucoside synthesis were utilized. CYP79D1
showed substrate specificity towards valine and isoleucine, the precursors for linamarin and lotaustralin, respectively (Figure 2.3). This was further confirmed by the ability of transgenic *Pichia pastoris* yeast, expressing either CYP79D1 or CYP79D2, to convert $^{14}$C-valine to $^{14}$C labeled val-oxime (Figure 2.4).

This chapter will demonstrate the reduction in expression of CYP79D1 and CYP79D2 transcripts in plants expressing the CYP79D1 and CYP79D2 genes in an antisense orientation under the control of the leaf-specific Cab1 promoter. We demonstrate that both leaf and root levels of linamarin are reduced up to 94% and 99%, respectively, in CYP79D1/CYP79D2 antisense plants. Furthermore, there is no apparent reduction in root CYP79D1 or CYP79D2 transcript levels. These results suggest that linamarin may be transported from leaves to roots in young plants. These acyanogenic cassava plants may represent a safer and more marketable food product as well a tool to determine the role of cyanogens in protection against herbivory and in crop productivity.
**Figure 2.1:** Nucleotide sequence comparison between the CYP79D1 and CYP79D2 genes show an 85% identity. Cloning of the CYP79D1 and CYP79D2 was done using a common forward primer (D1/D2A) and two separate reverse primers specific for CYP79D1 (D1-2) and CYP79D2 (D2-3). Subsequent analysis of transcript abundance in the transgenic plants using RT-PCR was performed using gene specific primers for CYP79D1 (D1-F1 as forward primer and D1-R4 as reverse primer) and CYP79D2 (D2-F1 as forward primer and D2-R5 as reverse primer).
(Figure 2.1 cont.)

CYP79D2  CCCATCAGGATTT GCTAAGCAGGTTG GCTTCTGTCTCTAC CCCACTTCACCTGTTA ATTAAGCCAAAGA 1701

CYP79D1  CCCATCTGCTGCTT GCAAGCCTCGCCTG GCTTCTGTCTCTAC CCAAGTCACCTTGA AAGAGATCAAGATC TTATCGTTCTTAA 1676

CYP79D2  ATGTGAGTTGTTAGT AGTTTTAATAAAAAA ATTATGAGTTTATTA TGTGTAATTACGT-G GTAACCCTCAAATG GTCTGTT-ATTGAGA 1789

CYP79D1  ACGTCCCTTTAATT-A TGATTTGCTAAAAAC AA-ATAAAAATATTG GTAATCTTACAAGCT TCCTGTTTTATGAGA 1763

CYP79D2  GTT-TTTAATCCC TCAAAATTTTTTG TGGCTAAGATTTGTT CATCTTTGGAATCTT CGCAAT-TGTTTTC ATATACATCTTTA 1874

CYP79D1  GTTTAATTAAACTC TCAAAATTTTTG GGGTTAAAACTTT TTCTCTTGTCAATATAGTGTTCT TTAAATAGTTGCT TTA--------- 1829

CYP79D2  TTTCTTAAAAAAA AAAAAAATTTTTT AAAAAAATTTTTT AAAAAAATTTTTT AAAAAAATTTTTT A A 1920

CYP79D1  ----CTAGAAATTTT AAAAAAATTTTTT AAAAAAATTTTTT AAAAAAATTTTTT AAAAAAATTTTTT A 1845
Figure 2.2: Amino acid sequence comparison between CYP79D1 and CYP79D2 shows 80% homology. The heme-binding motif in CYP79D1 and CYP79D2 is underlined (TFSTGRRGCV).
### Figure 2.2

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|     | 60 | 70 | 80 | 90 | 100 |
|     | 110 | 120 | 130 | 140 | 150 |
|     | 160 | 170 |
| CYP79D1 | PPGPTPWPLIGNPEMRIYRPTFRLWQKLMKDNMTDCLIRFQGTNVPISCPVIAREIL |
|     | 60 | 70 | 80 | 90 | 100 |
|     | 110 | 120 | 130 | 140 | 150 |
|     | 160 | 170 |
| CYP79D2 | KKHDAVFSNRPKILCAKTMSGGYLTTIVVPYNDQVKKMRFKVTSEIIISPARHKWLHDKRA |
|     | 180 | 190 | 200 | 210 | 220 |
|     | 230 |
| CYP79D2 | EADQLFYINQYKSNKVNVRIAARHYGGNVIRKMMFSKRYFGKGMDDGGPGPEEIMH |
|     | 180 | 190 | 200 | 210 | 220 |
|     | 230 |
| CYP79D1 | KKHDAVFSNRPKILCAKTMSGGYLTTIVVPYNDQVKKMRFKVTSEIIISPARHKWLHDKRA |
|     | 180 | 190 | 200 | 210 | 220 |
|     | 230 |
| CYP79D1 | EADQLFYINQYKSNKVNVRIAARHYGGNVIRKMMFSKRYFGKGMDDGGPGPEEIMH |

|     | 240 | 250 | 260 | 270 | 280 |
|     | 290 |
| CYP79D2 | VDAIFTALKLYGFCISDLPLLQLDLGQKLIOMNKLNRQELDRQPRIQWRS |
|     | 240 | 250 | 260 | 270 | 280 |
|     | 290 |
| CYP79D1 | IDAVFTALKLYGFCISDLPLLQLDLGQKLIOMNKLNRQELDRQPRIQWRS |

|     | 300 | 310 | 320 | 330 | 340 |
|     | 350 |
| CYP79D2 | ERKEMDDLVITLQDSGKLLPDEIKNQIAEIATIDNPAVENAMGLINQPE |
|     | 300 | 310 | 320 | 330 | 340 |
|     | 350 |
| CYP79D1 | ERKEMDDLVITLQDSGKLLPDEIKNQIAEIATIDNPAVENAMGLINQPE |

|     | 360 | 370 | 380 | 390 | 400 |
|     | 410 |
| CYP79D2 | LLAKATEEILDREVKGDRLVQESDIPNLNYVKACAREAPRLHPVAYNVNFHVMEDAVIGD |
|     | 360 | 370 | 380 | 390 | 400 |
|     | 410 |
| CYP79D1 | LLAKATEEILDREVKGDRLVQESDIPNLNYVKACAREAPRLHPVAYNVNFHVMEDAVIGD |

|     | 420 | 430 | 440 | 450 | 460 |
|     | 470 |
| CYP79D2 | YFIPKGSWAILSRYGLGRNPKTWPDLKYDPERHLEVEVLTEHDLRFVFSTGRRGCV |
|     | 420 | 430 | 440 | 450 | 460 |
|     | 470 |
| CYP79D1 | YFIPKGSWAILSRYGLGRNPKTWPDLKYDPERHLEVEVLTEHDLRFVFSTGRRGCV |

|     | 480 | 490 | 500 | 510 | 520 |
|     | 530 | 540 |
| CYP79D2 | YFIPKGSWAILSRYGLGRNPKTWPDLKYDPERHLEVEVLTEHDLRFVFSTGRRGCV |
|     | 480 | 490 | 500 | 510 | 520 |
|     | 530 | 540 |
| CYP79D1 | YFIPKGSWAILSRYGLGRNPKTWPDLKYDPERHLEVEVLTEHDLRFVFSTGRRGCV |
Figure 2.3: Substrate selectivity of CYP79D1 (Andersson et al., 2000). Reconstituted CYP79D1 was incubated with $^{14}$C-labelled amino acids. After incubation, the reaction mixtures were extracted with ethyl acetate, and the extracts were analyzed by TLC (thin layer chromatography). CYP79D1 was able to convert valine and isoleucine to their respective oximes.
Figure 2.4: Functional expression of CYP79D1 and CYP79D2 in *Pichia pastoris* yeast (Andersson et al. 2000). Cells were incubated with $^{14}$C-L-valine and extracted with ethyl acetate. The ethyl acetate extracts were analyzed by TLC (thin layer chromatography). Lane 1, *P. pastoris* transformed with empty vector (control); lane 2, vector with CYP79D1; lane 3, vector with CYP79D2.
2.2 EXPERIMENTAL PROCEDURES

2.2.1 E. coli, and Agrobacterium strains

The *Escherichia coli* strain DH5alpha (Life Technologies, Grand Island, NY) was used for all recombinant DNA work. Selection for transformed E.coli cells was performed on Luria-Beritani (LB) media containing 100 mg/mL of ampicillin. Transformation of cassava was performed using modified *Agrobacterium tumefaciens* strain LBA4404 (Life Technologies, Grand Island, NY). Selection for transformed Agrobacterium cells was conducted on YM media supplemented with 50 mg/mL of kanamycin.

2.2.2 Cassava cultivar

Cassava cultivar *Manihot Columbia* 2215 was used for all transformations. Cassava tissue culture media, transformation protocol and regeneration media are explained subsequently in this section.

2.2.3 Cloning of CYP79D1 and CYP79D2

The 5’- 650 bp ends of both CYP79D1 and CYP79D2 were isolated from leaf genomic DNA of greenhouse grown cassava plants by PCR (polymerase chain reaction). Genomic DNA was extracted from the leaves according to Doyle et al.
(1990). Approximately 300 mg of leaf tissue was ground in 500 μL of 60°C extraction buffer (2% w/v CTAB (hexadecyltrimethylammonium bromide), 1.4 M NaCl, 0.2% w/v β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl pH 8.0). The mixture was then incubated for 30 minutes 60°C, centrifuged for 2 minutes at 13,000 rpm in a microfuge (Baxter Scientific, CT) and the supernatant was extracted with equal volume of chloroform:isoamyl alcohol (24:1). This was followed by a 2 minute centrifugation at 4,500 rpm. DNA was precipitated by adding 2:3 (v/v) of cold isoproponol to the upper phase, mixed gently and incubated at room temperature for 2 hours. After pelleting the DNA at 4,500 rpm for 2 minutes, the pellet was washed with 1 mL of 70% (v/v) ethanol/10 mM ammonium acetate. The DNA pellet obtained from the second 4,500 rpm spin for 2 minutes was air-dried and resuspended in 300 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 10 μg of RNase. After incubating the DNA for 30 minutes at 37°C the DNA was precipitated in 100 μL of 7.5 M ammonium acetate (pH 7.7) and 750 μL of cold ethanol at –20°C for 2 hours. DNA was precipitated with centrifugation of 13,000 rpm for 20 minutes at 4°C and suspended in 100 μL of 10 mM Tris buffer (pH 8.5).

The 85% nucleotide similarity between CYP79D1 and CYP79D2 prompted the use of gene specific reverse primers (D2-3 and D1-2) whereas a primer common for both genes was used as the forward primer (D1/D2A). At the 5’-end of the reverse primers a restriction site Smal was added while the 5’-end of the forward primer was tagged with a restriction site StsI to facilitate the subsequent cloning steps.
Common forward primer-

\[ \text{D1/D2A: } 5' \text{ GGAGCTCAATATGGCCATGAACG } 3' \] 
\[ SstI \]

Unique reverse primers –

\[ \text{D2-3: } 5' \text{ ATCCCGGGCACATTCTTGTTGCTC } 3' \]
\[ \text{D1-2: } 5' \text{ TTCCCGGGCACATTTTTATTTGCTT } 3' \] 
\[ Smal \]

PCR reactions were performed in a total volume of 50 µL containing: 1 X Vent buffer, 20-100 ng of leaf DNA, 0.1 mM each dNTP, 2 unit Vent polymerase (New England Biolabs, Beverly MA), 0.4 µM each primer (IDT, Coralville, IA). The DNA was amplified in a Perkin Elmer Cetus DNA thermal cycle using 35 PCR cycles of 30 seconds at 94°C, 1 minute at 65°C and 30 seconds at 72°C preceded by a 4 minute incubation at 96°C. PCR products were fractionated on 1% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and the DNA was isolated from the agarose gel by QIAEX II gel extraction kit (QIAGEN Inc, Valencia, CA) according to manufacturers protocol. Each specific DNA product was sequenced at the Ohio State University Neurobiotechnology Center DNA sequencing facility to prove its authenticity.
2.2.4 Vector construction and PCR screening

A modified Agrobacterium binary vector 4B:Cab1 constructed by Dr. Sue Lawrence was used as the starting vector for the expression of the CYP79 genes. 4B:Cab1 construct has the vector back-ground of pBI121 (Strategene) but the 2X 35S promoter and β-glucorinidase gene were substituted by a Cab1 leaf specific promoter (Brusslan et al., 1992) and glgC gene (Stark et al., 1992) (Figure 2.5). The T-DNA region also contains the nptII gene for kanamycin/paromomycin resistance.

The 5’ ends (650 bp each) of both CYP79D1 and CYP79D2 genes were cloned into the Agrobacterium binary vector, pBI121, in antisense orientation. The 5’- 650 bp pieces of CYP79D1 and CYP79D2 genes, as well as 4B:Cab1 vector were restricted with SstI and SmaI restriction enzymes, separated on a 1% agarose gel and excised as mentioned previously. 4B:Cab1 vector restricted with SstI and SmaI releases glgC gene from the vector. Approximately 300 ng of pBI121 and 1 µg of either 650 bp CYP79D1 or CYP79D2 gene fragments were ligated together using 10 units T4 DNA ligase for 12 hours at 14°C. The resulting modified vectors were called Cab1:D1AS and Cab1:D2AS respectively. Ten percent of the ligation mixture was used to transform DH5α E. coli competent (Life Technologies) cells. After incubating the recombinant DNA and the cells for 30 minutes on ice the suspensions were heat shocked for 25 seconds at 42°C followed by a 2 minute recovery time in ice and the addition of 900 µL of pre-warmed (to 42°C) SOC media (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl and 2.5 mM KCl). Cell suspensions were incubated
for 1 hour at 37°C and 200 rpm constant shaking. The cells were then spread on LB medium containing 100 mg/mL of ampicillin. Overnight cultures of the transformed E.coli colonies were used to purify the recombinant plasmid in quantity using QIAGEN plasmid midi kit (Qiagen Inc, Valencia, CA).
Figure 2.5: T-DNA region of 4B:Cab1 vector. This vector was constructed using pBI121 and was used in the work to form Cab1:D1AS and Cab1:D2AS modified vectors.
Primers specific for the 5’-end of Cab1 promoter (Cab1F2) and 3’-end of the nos terminator (NOSAR2) were used to PCR amplify Cab1:CYP79D1:ter cassette from Cab1:D1AS modified vector. An EcoRI restriction site was added to the 5’ end of the both primers to facilitate subsequent cloning. Cab1F2 and NOSAR2 primers had the sequence of CCGAATTCGGTTTACATTGATGCTCTC and CCGAATTCATAGATGACACCGCGC, respectively (underlined are the EcoRI sites).

PCR reactions were performed in a total volume of 50 µL containing: 20 mM Tris-HCl (pH 8.0), 1.5 mM MgCl2, 40-50 ng of Cab1:D1AS plasmid DNA, 0.2 mM each dNTP, 2.5 units of PLATINUM Taq DNA polymerase (Life Technologies, Grand Island, NY), 0.4 µM each primer (IDT, Coralville, IA). The DNA was amplified in a Perkin Elmer Cetus DNA thermal cycle by incubating the DNA mixture for 4 minutes at 94°C followed by 35 cycles of 30 seconds at 94°C, 1 minute at 65°C and 1.5 minutes at 72°C, and final 4 minute incubation at 94°C. PCR products were fractionated on 1% agarose gel in TAE buffer and the DNA was isolated from the agarose gel by QIAEX II gel extraction kit (QIAGEN Inc, Valencia, CA) according to manufacturers protocol. The DNA product was sequenced in both directions at the Ohio State University Neurobiotechnology Center DNA sequencing facility to prove its authenticity.

This Cab1:D1AS cassette with EcoRI ends and Cab1:D2AS vector were both restricted with EcoRI. Restricted Cab1:D2AS vector was de-phosphorylated using 2.5 units of calf intestinal alkaline phosphate (CIAP) (Life Technologies, Grand Island,
NY) to prevent self-annealing. Approximately 1 µg of Cab1:D2AS vector and 250 ng of the Cab1:D1AS cassette were ligated using 10 units T4 DNA ligase for 24 hours at 14°C. The resulting modified vectors were called Cab1:D1:D2AS. Ten percent of the ligation mixture was used to transform DH5α E. coli competent (Life Technologies, Grand Island, NY) cells. After incubating the recombinant DNA and the cells for 30 minutes on ice the suspensions were heat shocked for 25 seconds at 42°C followed by a 2 minute recovery time in ice and the addition of 900 µL of pre-warmed (to 42°C) SOC. Cell suspensions were incubated for 1 hour at 37°C and 200 rpm constant shaking. The cells were then spread on LB medium containing 100 mg/mL of ampicillin. The resulting colonies were grown over-night in LB medium under selection and Cab1:D1:D2AS vector was isolated from the cultures using QIAGEN plasmid midi kit (Qiagen Inc, Valencia, CA). The Cab1:D1:D2AS vector was PCR amplified using different primers and the resulting DNA products were sequenced at the Ohio State university Neurobiotechnology Center DNA sequencing facility to prove its validity. In addition Cab1:D1:D2AS vector was restricted with SstI restriction enzyme to separate a 1.3 kb fragment.

2.2.5 Agrobacterium transformation

Approximately 300 ng of Cab1:D1:D2AS vector was used to transform Agrobacterium tumefaciens strain LBA4404 (Life Technologies Inc, Rockville, MD). Transformation was carried out by electroporation of 20 µL of ElectroMAX LBA4404
cells (Life Technologies, Grand Island, NY) with Cab1:D1:D2AS vector. A Bio-Rad electroporator was used at the following settings: 1.8 kV, 25 uF, 200 ohms and 4.8 milliseconds. Electroporation was followed by incubation in 1 mL of YM medium (0.04% yeast extract, 1% mannitol, 1.7 mM NaCl, 0.8 mM MgSO₄·7H₂O, 2.2 mM K₂HPO₄·3H₂O, pH 7.0) at 225 rpm for 3 hours at 30°C. After the recovery period, 100 µL of cell suspension was spread on solid YM medium supplemented with 100 mg/L streptomycin and 50 mg/L kanamycin for 2-3 days at 30°C. The resulting colonies were PCR screened with primers specific for CYP79D1 and CYP79D2.

2.2.6 Cassava transformation

2.2.6.1 Plant Material

Shoot apical meristems of cassava cultivar MCol 2215, an important cultivar in northeast Colombia, were cultured on MS basal medium for production of in vitro plants (Murashige & Skoog, 1962; Arias-Garzon et al., 1993). Apical leaves were placed on supplemented MS8 medium (MS basal medium supplemented with 2% (w/v) sucrose, 8 mg/L 2,4-D, Gamborg’s B-5 vitamins (Gamborg et al., 1968), 50 mg/L casein and 0.5 mg/L CuSO₄ at pH 5.7) for induction of somatic embryos. Plant cultures were kept under 12 hr/day photoperiod (5 µmole photons m⁻² s⁻¹) at 28°C. Once embryos formed they were transferred to regeneration medium (MS basal medium supplemented with 1 mg/L thiamine, 100 mg/L myo-inositol, 2% (w/v) sucrose, 1.0 mg/L BAP and 1 mg/L GA at pH 5.7) to induce the development of cotyledonary leaves.
2.2.6.2 Transformation

Germinated somatic embryos were used for transformation (Arias-Garzon et al., 1994; Li, H-Q et al., 1996). Two-day old cultures of Agrobacterium containing the vectors described above were grown in YM liquid medium containing 100 mg/L streptomycin plus 12.5 mg/L tetracycline. The bacteria were then were co-cultivated with cassava germinated somatic embryos in MS medium supplemented with 100 μM acetylsyringone. Prior to co-cultivation the bacterial cultures were induced in MS basal medium containing 200 μM acetylsyringone for 2 to 4 hours at 28°C. After two days of co-cultivation at 27°C (in darkness) the tissue was transferred to MS8 media containing 500 mg/L carbenicillin and 75 mg/L paromomycin to kill Agrobacterium and to select for transformants. During this period plants were incubated at 28°C with a 12-hour photoperiod at 5-10 μmole photons m⁻²s⁻¹. Four weeks after co-cultivation clumps of somatic embryos were transferred to cassava regeneration medium containing the aforementioned antibiotics for approximately four more weeks. Once the individual somatic embryos germinated and formed shoots they were transferred to cassava micropropagation medium (MS salts, 2 % (w/v) sucrose, 0.04 mg/L benzylamino purine, 0.05 mg/L gibberellic acid, 0.02 mg/L NAA, 1 mg/L thiamine, 100 mg/L myo-inositol, pH 5.7) for induction of root growth.
2.2.7 Molecular analysis of transgenic plants

2.2.7.1 PCR analysis of the transgenic plants for the integration of the T-DNA

For PCR analysis, genomic DNA was isolated from 40 mg of 3-4 month old in-vitro grown leaves from either wild-type plants or paromomycin-resistant putative transformants according to Doyle et al. (1990), as previously mentioned. Wild-type plants used were not transformed but grown in tissue culture media (without selection) similar to the putative transformants. The presence of the nptII gene and the transgenes was detected by PCR amplification. PCR reactions were performed in a total volume of 50 µL containing: 1X Vent buffer, 20-100 ng of leaf DNA, 0.1 mM each dNTP, 1 unit Vent polymerase, 0.4 µM each primer (IDT, Coralville, IA).

Kanamycin resistant nptII gene, which is closer to the right border of the T-DNA, was amplified using NPT5’ (CCGCCGATGACGCGGGACAAGCC) and NPT3’ (GGTCCGCCACACCCAGCCGGCCA) primers. The CYP79D1 transgene, which is closer to the left border of the T-DNA, was amplified with primers specific for the Cab1 promoter/CYP79D1 junction (Cab1F1: CAATACCAAACCTTGGC) and CYP79D1/nos terminator junction (NOSAR1: ATCGCAAGACCGGCAACAGAG). The DNA was amplified using 10 touchdown PCR cycles of 30 seconds at 94°C, 1 minute at 69°C - 64°C and 30 seconds at 72°C followed by 25 cycles at 64°C annealing temperature. Each specific DNA product was sequenced to prove its authenticity. The presence or absence of the
VirG gene was confirmed by PCR using Agrobacterium VirG gene primers (GCCGACAGCAGGAGTTTAC and CCTGCCGTAAGTTTCACCTCACC).

2.2.7.2 Southern blot analysis of the transgenic plants

Total DNA was isolated from 3-4 month old in vitro grown plants according to Soni et al. (1994). Approximately 1 g of leaves was ground in 10 mL of extraction buffer (50 mM Tris-Cl pH8, 10 mM EDTA, 2% SDS, 100 mM LiCl, 10 µg/mL proteinase K) and incubated for 15 minutes at room temperature with occasional mixing. After centrifugation at 3500 rpm for 15 minutes, 200 µg of RNase was added to the supernatant and incubated for 30 minutes at 37°C. This was followed by equal volume extraction with phenol, phenol/chloroform and phenol/chloroform/isoamyl alcohol. DNA from the upper phase of the final extraction was precipitated with 0.25 X vol. 10 M ammonium acetate/2 X vol. –20°C ethanol at room temperature for 10 minutes. DNA was pelleted at 13,000 rpm for 20 minutes and the pellet was washed with 70% (v/v) ethanol. The air-dried pellet was resuspended in 0.5 mL of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

Southern blot analysis was performed following standard protocols (Sambrook et al., 1989). 10 µg of DNA was digested with KpnI and fractionated on a 0.8% agarose gel. KpnI does not restrict the T-DNA region of the modified vector used in this study. The gel was soaked in 250 mM HCl for 10 minutes, denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 45 minutes and neutralization buffer (1 M Tris-HCl,
1.5 M NaCl, pH 8.0) for 30 minutes. Each soaking step included constant gentle agitation followed by a brief rinse with distilled water. The DNA was transferred to nitrocellulose membrane using 20 X SSC buffer for 20 hours. Transferred DNA was cross-linked to the membrane by UV light exposure for 45 seconds. The pre-hybridization of the membrane was done for a minimum of 2 hours at 42°C in CHURCH buffer (0.5 M NAHPO₄ (pH 7.2), 1 mM EDTA, 1% (w/v) BSA, 7% (w/v) SDS). Hybridization of the membrane was conducted at 42°C with ³²P- labeled CYP79D1 probe (5’ end 650bp) for 16-18 hours. The probe was made using PCR primers specific to CYP79D1 and the PCR reaction was performed in a total volume of 50 µL containing: 1 X PCR buffer, 100 ng Cab1:D1AS vector DNA, 0.1 mM each dATP/dTTP/dGTP, 100 µCi of ³²P-dCTP (Amersham, Piscataway, NJ), 2.5 units of Taq polymerase (Life Technology, Grand Island, NY), 0.4 µM each primer (IDT, Coralville, IA), 1.5 mM MgCl₂. The DNA was amplified in a Perkin Elmer Cetus DNA thermal cycle using 35 PCR cycles of 30 seconds at 94°C, 45 seconds at 65°C and 30 seconds at 72°C preceded by 3 minutes incubation at 94°C. The labeled probe was cleaned using Qiagen PCR cleaning kit (Qiagen Inc, Valencia, CA) and boiled for 5 minutes before the addition to the membrane. After hybridization the membrane was washed with 1 X SSC containing 0.1% (w/v) SDS at 50°C for 20 minutes followed by two washes with 0.1 X SSC containing 0.1% (w/v) SDS each at 50°C for 20 minutes. The membrane was then exposed to a phosphorimager overnight.
2.2.7.3 RT-PCR analysis of the abundance of CYP79 transcripts in transgenic plants

Total RNA was isolated from 100 mg of 3-4 month old in vitro leaves or 40-50 mg of in vitro roots from putative (paromomycin-resistant) transformants using the Qiagen Plant RNA extraction kit (Qiagen Inc, Valencia, CA). Contaminant DNA was eliminated by incubation of the RNA with 1 unit of DNase (Invitrogen, Carlsbad, CA) for 15 minutes at room temperature. DNase was inactivated according to manufacturers recommendations. RT-PCR was performed using 6 µg of total RNA with RT-PCR kit from Invitrogen (Carlsbad, CA) according to the manufacturers recommendations. First strand synthesis on 6 µg of total RNA was performed using 1X reverse transcription buffer (50 mM Tris-HCl pH 8.0, 75 mM KCl, 3 mM MgCl₂), 0.3 mM dNTP, 0.5 µg oligo dT₁₂₋₁₈ primer, 200 units of Superscript II reverse transcriptase (Life Technology Inc, Rockville, MD) with two incubation of 65°C for 5 minutes and 42°C for 75 minutes.

The cDNA amplification was performed using 1X PCR buffer, 1.5 mM MgCl₂, 0.1 mM dNTP each, 2.5 units Taq polymerase, 0.4 µM each primer with 35 cycles of 30 seconds at 94°C, 1 minute at 56°C and 30 seconds at 72°C (Life Technology Inc, Rockville, MD). The primers used were specific for the 3’ end of CYP79D1 (D1-F1: GCTAAATCAACCAGAAATCCTGAAG and D1-R4: TGCAAGAGAAACAAGATAACCCC) and CYP79D2 (D2-F1: CTGATAAATCAACCAGAACTTCTGGCA and D2-R5: CTAACAACTCACATTCATCCCTCCCC ) genes (Figure 2.1). DNA products were
separated on a 1% agarose gel. Significantly, the 3’ primers are not complimentary to the antisense portions of transgenes introduced into cassava and thus should not amplify off the T-DNA. First strand cDNA was normalized between wild-type and transgenic plants on the basis of cassava starch branching enzyme II (SBE-II) RT-PCR product levels. In each case a negative control without DNA and a positive control amplifying the respective genes cloned in plasmids was performed.

2.2.8 Linamarin quantification using Gas Chromatography – Mass Spectrometry

The leaf and root linamarin content was measured from 3-5 independently isolated 3-4 month old in vitro wild-type or transgenic plants grown in tissue culture medium (same conditions as for PCR). Linamarin was extracted and derivatized from leaves and roots according to Mkpong et al. (1990), with phenyl β-D-glucoside added as an internal standard. In vitro leaves or roots were ground in liquid nitrogen and immediately lyophilized for 14-16 hours. Linamarin was extracted from 4 mg of tissue using acetonitrile. 20 µg phenyl- β-D-glucoside was added to the mixture prior to derivatization with Bis (trimethylsilyl) trifluoroacetamide (BSFTA) (Aldrich, Milwaukee, WI). Derivatization was performed at 90°C for 30 minutes, followed by 15 second centrifugation and the resulting supernatant was subjected to GC-MS analysis at the Central Chemical Instrumentation Center (CCIC) of the Ohio State University.
GC-MS, on a Thermo-Finnigan Trace 2000 instrument, was performed on a 30 meter long, 0.25 micron film thickness Restek XTI-5 (5% diphenyl – 95% dimethyl polysiloxane) capillary gas chromatography column using a split/splitless injector in the splitless mode at an injection temperature of 250°C. The GC-MS was operated under a pressure control mode using pressures that gave flow rates near 1 mL/minute. The GC oven temperature program was: 50°C for one minute after injection, ramp at 30°C/minute to 185°C, ramp at 6°C/minute to 230°C (linamarin elution), ramp at 12°C/minute to 300°C (internal standard elution), and ramp at 40°C/minute to 360°C for 3 minutes to clean the column. A dedicated EI (electron impact) ion source was used at an electron energy of –70eV. The source temperature was held at 250°C.

The mass analyzer used was a Trace MS single quadrupole mass spectrometer. For the confirmation runs using commercially available linamarin the mass spectrometer was operated in full scan mode collecting the full mass spectrum from mass 39 to mass 459 amu at a rate of 2.5 scans per second for the elution times between 9.0 and 20 minutes. For quantification runs the mass spectrometer was operated in Single Ion Monitoring (SIM) mode. For the linamarin region of the GCMS run the SIM masses monitored mass 132 for the time range of 11 to 15 minutes in the chromatogram. The internal standard signal was also monitored recording intensities at masses 189 and 393 for the time between 17 and 21 minutes in the chromatogram. During SIM runs, mass/intensity points are recorded to disk at 7.5 points per second to allow very high precision area measurements on the chromatographic peaks. The mass 132 signal was chosen for linamarin quantification.
because it is the single most characteristic ion unique to the tri-methyl-silylate-
linamarin derivative. Likewise, the two masses for the internal standard were chosen for the same reason. However, the characteristic mass signal at mass 189 was often so intense that it saturates the ion detector. Thus for most of the work only the lower intensity mass 393 signal was used for quantification.

Each of the runs was normalized for the internal standard (phenyl β-D-
glucoside) and linamarin is expressed as a % of the quantity present in wild-type untransformed plants.

2.2.9 Evaluation of Cab1 transformant in ammonium-free medium

Nodal cuttings of wild-type and transformants (Cab1-1 to Cab1-5) were grown in normal Murashige and Skoog media (Murashige and Skoog, 1962) in the presence or absence of ammonia. Normal MS media contains the following: ammonium nitrate (20.625 mM), boric acid (0.1 mM), calcium chloride (3 mM), cobalt chloride (0.0001 mM), cupric sulfate (0.0001 mM), ferric sulfate (0.1 mM), magnesium sulfate (1.5 mM), manganese sulfate (0.1 mM), potassium iodide (0.005 mM), potassium nitrate (18.81 mM), potassium phosphate (1.25 mM), sodium molybdate (0.001 mM), zinc sulfate (0.03 mM). MS media without ammonium nitrate (Caisson Laboratories, Rexburg, ID) was supplemented with 38 mM potassium nitrate.
2.3 RESULTS

2.3.1 Cloning of CYP79D1 and CYP79D2

The 5’ end of both CYP79D1 (accession number AF140613) and Cyp79D2 (accession number AF140614) were cloned using PCR primers specific for each sequence published in GenBank by Andersson et al. (2000) (Figure 2.6). Figure 2.1 shows the positions of the primers used. Sequencing results and restriction analysis was used to show that both these 85% identical genes were isolated from cassava leaves (data not shown).

2.3.2 Construction of the CYP79D1 and CYP79D2 anti-sense vector

Using pBI121 Agrobacterium binary vector backbone Cab1:D1:D2AS vector was made expressing the 5’-650bp ends of CYP79D1 and CYP79D2 in antisense orientation. Each of the antisense genes was flanked by leaf specific Cab1 promoter and a nos terminator (Figure 2.7). The final construction was made in a manner not to facilitate the formation of mRNA secondary structure by having the two cassettes in the same orientation. Restriction of Cab1:D1:D2AS vector with SstI releases a 1.3 kb DNA piece that is indicative of the construction of this vector in the intended orientation (Figure 2.8).
Figure 2.6: Cloning of CYP79D1 and CYP79D2. 5’-ends of both CYP79D1 and Cyp79D2 were cloned from cassava leaves using sequence specific PCR primers.
**Figure 2.7:** T-DNA region of the Cab1:D1:D2AS vector. Each of the antisense CYP79D1 (D1) and CYP79D2 (D2) genes is flanked by leaf specific Cab1 promoter and a nos terminator (Ter). The two CYP79 cassettes are in the same orientation. Sst1 restriction enzyme releases a 1.3 kb DNA piece (Figure 2.8). Cab1F1 and NOSAR1 are primers used to PCR analyze putative cassava transformants.
Figure 2.8: Cab1:D1:D2AS vector restricted with SstI releases a 1.3kb DNA piece. Digestion with SstI releases ter: Cab1 promoter: Cyp79D1 portion of Cab1:D1:D2AS vector.
2.3.3 Production of transgenic cassava

To generate acyanogenic plants *Agrobacterium*-mediated T-DNA transformation was used to introduce the 5’ ends (650 bp) of the CYP79D1 and CYP79D2 genes in the reverse orientation (antisense) under the control of leaf-specific, Cab1 promoters. In addition to the antisense CYP79D1 and CYP79D2 genes, the T-DNA included a bacterial *nptII* gene for antibiotic selection of transformants. Fifty-nine independent paromomycin-resistant putative transformants were obtained following transformation of 2445 cassava explants (Table 2.1 and Figure 2.9).
<table>
<thead>
<tr>
<th>Transformation #</th>
<th># of explants used</th>
<th>Paromomycin resistant plantlets</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>230</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>125</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>415</td>
<td>13</td>
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<tr>
<td>4</td>
<td>255</td>
<td>22</td>
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<tr>
<td>5</td>
<td>195</td>
<td>7</td>
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<tr>
<td>6</td>
<td>200</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>250</td>
<td>5</td>
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<tr>
<td>9</td>
<td>100</td>
<td>1</td>
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<tr>
<td>10</td>
<td>175</td>
<td>2</td>
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<tr>
<td>11</td>
<td>250</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>150</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2445</strong></td>
<td><strong>59 (2.4%)</strong></td>
</tr>
</tbody>
</table>

*Table 2.1:* Cassava transformation efficiency. Modified Agrobacterium carrying the Cab1:D1:D2AS vector was used to transform cassava germinated somatic embryos.
**Figure 2.9:** Cassava tissue culture. After co-cultivation of Agrobacterium with cassava explants, embryos are formed within 4 weeks in MS8 medium (A). Shoots generate from the embryos within 4 weeks in RM1 medium (B) and further 4-5 weeks in 4e normal micropropagation media induces root generation (C and D).
2.3.4. PCR analysis for the integration of the T-DNA

Ten of the forty nine paromomycin-resistant putants that were screened by PCR for the *nptII* gene were confirmed positive. Each putative transformant was obtained from a unique explant and not from a secondary somatic embryo. Similarly, untransformed wild-type plants used in all analyses were generated from independent explants via the same tissue culture procedures used to generate putative transformants (minus paromomycin selection). Thus, any observed differences in CYP79 transcript levels or linamarin abundance in transformed plants relative to wild-type plants could not be attributed to differences in plant culture.

To confirm the integration of the T-DNA the transformants were screened by PCR amplification for the *nptII* gene and the truncated CYP79D1 gene. The presence of *nptII* gene was analyzed using NPT5’ and NPT3’ primers. A 900 bp band was amplified from the putative cassava transformants and not from wild-type plants (Figure 2.10). DNA primers specific for the region between the Cab1 promoter/CYP79D1 junction and the CYP79D1/NOS terminator junction (Fig. 2.7) were used to amplify the CYP79D1 transgene. A diagnostic 700 bp region was amplified in the transformants (Figure 2.11A). The identity of all the bands was confirmed by DNA sequence analysis. The absence of *Agrobacterium* contamination was verified by the use of *Agrobacterium VirG* specific primers (Figure 2.11B). The presence of the *nptII* gene and CYP79D1 gene in the transformants coupled with the absence of the *VirG* gene demonstrated integration of the T-DNA in the transformants.
**Figure 2.10:** Analysis for the integration of the T-DNA cassette using primers specific to the *nptII* gene. Amplification of a 900 bp T-DNA fragment from only the transgenic plants confirms the integration of the T-DNA
Figure 2.11. Analysis of the integration of the T-DNA cassette using primers specific to the *Cab1* promoter and *nos* terminator. Amplification of a 700 bp T-DNA fragment from only the transgenic plants confirms the integration of the T-DNA (A). The absence of *Agrobacterium* contamination was verified by the use of *VirG* specific primers (B).
2.3.5 Southern blot analysis

Southern blot analysis was used to further confirm the PCR screening results for integration of the transgenes. Genomic DNA of wild-type MCol 2215 and five transformants described, Cab1-1 to Cab1-5, was restricted with \( Kpn1 \) and probed with \( ^{32}P \)-labelled CYP79D1. Southern blot analysis shows that between one and three independent T-DNA integration events had occurred into the genome of the various transformants (Fig. 2.12). The upper common CYP79D1 bands represent the wild-type genes. Two of the transformants (Cab1-3 and Cab1-4) had apparently similar banding patterns for the transgenes. The Cab1-3 and Cab1-4 transgenic plants were obtained from independent transformation events and were not derived from identical somatic embryos.
Figure 2.12: Southern blot analysis of wild-type and transgenic plants. 10 µg of leaf DNA was digested with *KpnI* and hybridized with a probe for the CYP79D1. Lane marked ‘positive’ is the modified binary vector restricted with *HindIII* and *SstI* to release the 650 bp CYP79D1 gene.
2.3.6 RT-PCR analysis for the CYP79D1 and CYP79D2 transcript abundance

The CYP79D1 and CYP79D2 transcript abundance of wild-type and transgenic plants was analyzed by RT-PCR amplification using primers specific for the 3’ ends of the CYP79D1 and CYP79D2 genes (Figure 2.1). These primers do not anneal to the 5’ portion of the genes used in the anti-sense construct. To normalize the CYP79D1 and CYP79D2 RT-PCR products from separate plants the PCR products were compared on the basis of the abundance of the cassava starch branching enzyme-II (SBE-II) RT-PCR product levels. Two transgenic plants (Cab1-1 and Cab1-2) completely lacked any detectable CYP79D1 and CYP79D2 transcripts and three transgenic plants (Cab1-3 through Cab1-5) had reduced levels of the CYP79D1 and CYP79D2 transcripts ranging from 20-80% of wild-type plants (Figure 2.13).
Figure 2.13: RT-PCR amplification of the CYP79D1 and CYP79D2 transcripts from leaves. Primers specific for the 3’ end of each gene were used (primer pairs D1-F1 / D1-R4 and D2-F1 / D2-R5 amplify CYP79D1 and CYP79D2, respectively). RT-PCR amplifications of CYP79D1, CYP79D2 and SBE-II transcripts were performed in parallel.
2.3.7 Analysis of the linamarin content in leaves

The untransformed and five transgenic cassava plants were analyzed for their leaf linamarin content by GC-MS analysis. Commercial linamarin was derivatized and used as a standard (elution time of 11-14 minutes) to identify its diagnostic selective ion monitoring (SIM) mass (132). An internal standard (phenyl β-D-glucoside) was included in all plants extractions prior to derivatization to determine the efficiency of the extraction and derivatization of linamarin. The SIM masses for phenyl β-D-glucoside were 189 and 393, respectively, and the elution time was 17-21 minutes. Transgenic plants used for the linamarin extractions and derivatizations were all approximately the same age (3-4 month old in vitro plantlets).

The leaf linamarin content was measured from 3-5 independently isolated in vitro wild-type or transgenic plants grown in tissue culture. Figure 2.14 shows the gas chromatograph for one of those trials (‘Leaf trail #1). The samples were monitored through Total Ion Chromatography (TIC) and analyzed using the selective ion for linamarin and phenyl β-D-glucoside for masses 132 and 393 respectively. Peak area of linamarin from each sample was normalized to the peak area of the internal standard (Table 2.2). Table 2.2 also shows leaf linamarin percentages of the transgenic plants compared to wild-type for ‘Leaf trial #1’. In this trial leaf linamarin content of the Cab1 transgenic plants were reduced to 5-19 % of the leaf linamarin content of wild-type cassava plants.
**Figure 2.14:** GC-MS analysis of leaf linamarin content of wild-type and Cab1 transgenic cassava plants (Cab1-1 through Cab1-5) (Leaf trial #1). Retention time (RT) and Mass Area (MA) are denoted in each chromatograph. RT for linamarin and phenyl β-D-glucoside was 14.09 and 19.03 minutes, respectively. Linamarin content as a percentage of that present in wild-type, after normalizing for the internal control, is noted in parenthesis for each plant.
(Figure 2.14)

Untransformed (100%)

Cab1-1 (15.4%)  Cab1-2 (5.5%)  Cab1-3 (18.2%)  Cab1-4 (19%)  Cab1-5 (6%)

RT: 14.09  MA: 1047939
RT: 19.03  MA: 10527
RT: 14.08  MA: 104644
RT: 19.02  MA: 6820
RT: 14.09  MA: 145020
RT: 19.03  MA: 8019
RT: 14.08  MA: 160889
RT: 19.02  MA: 8519
RT: 14.09  MA: 83815
RT: 19.03  MA: 14102

Relative Abundance

Time (min)
<table>
<thead>
<tr>
<th>Sample</th>
<th>Linamarin peak area ($10^3$)</th>
<th>Internal std peak area ($10^3$)</th>
<th>Normalized</th>
<th>Percent linamarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2215 (WT)</td>
<td>1047</td>
<td>10.5</td>
<td>1047</td>
<td>100.0 %</td>
</tr>
<tr>
<td>Cab1-1</td>
<td>104</td>
<td>6.8</td>
<td>161</td>
<td>15.4 %</td>
</tr>
<tr>
<td>Cab1-2</td>
<td>55</td>
<td>10.0</td>
<td>58</td>
<td>5.5 %</td>
</tr>
<tr>
<td>Cab1-3</td>
<td>145</td>
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<td>190</td>
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</tr>
<tr>
<td>Cab1-4</td>
<td>161</td>
<td>8.5</td>
<td>199</td>
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</tr>
<tr>
<td>Cab1-5</td>
<td>84</td>
<td>14.1</td>
<td>62.5</td>
<td>6.0 %</td>
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</table>

**Table 2.2:** Leaf linamarin peaks of wild-type and cab1 transformants normalized for the internal control. The last column expresses linamarin as a % compared to wild-type linamarin levels. This is the percent linamarin data for ‘Leaf trial #1’ in table 2.3.
To obtain absolute linamarin values, known amounts of commercial linamarin was subjected to GC-MS analysis similar to the leaf samples. Figure 2.15 shows the peak area as a function of varying amounts (2 – 120 µg) of linamarin. Using figure 2.15 the linamarin content of wild-type plants was found to be approximately 80 µmoles/g dry weight.

Similar analysis was performed 3-5 times independently on 3-4 month old *in vitro* leaves of untransformed and transformed plants. Tabulation of these leaf linamarin trials is shown in table 2.3. As shown in table 2.3 and figure 2.16 leaf-linamarin content was reduced by 60-94% (relative to wild type) in the 5 transgenic plants analyzed. The highest reduction in leaf linamarin was observed in Cab1-5 (94%) and lowest in Cab1-4 (60%) compared to the wild-type plants.
Figure 2.15: GC-MS linamarin peak area as a function of linamarin amounts (2–120 µg). Commercial linamarin dissolved in acetonitrile and derivatized as mentioned in the methods section was used. Phenyl β-D-glucoside was used as internal standard. Broken line (----) shows the leaf linamarin amount in wild-type plants.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Leaf trial #1</th>
<th>Leaf trial #2</th>
<th>Leaf trial #3</th>
<th>Leaf trial #4</th>
<th>Leaf trial #5</th>
<th>Average*</th>
<th>Standard Deviation</th>
</tr>
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<tr>
<td>Untransformed</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
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<tr>
<td>Cab1-1</td>
<td>15.4</td>
<td>8.0</td>
<td>8.4</td>
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<td>-</td>
<td>11.3</td>
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</tr>
<tr>
<td>Cab1-2</td>
<td>5.5</td>
<td>8.1</td>
<td>46.5</td>
<td>32.0</td>
<td>55.0</td>
<td>29.4</td>
<td>10.06</td>
</tr>
<tr>
<td>Cab1-3</td>
<td>18.2</td>
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<td>-</td>
<td>23.5</td>
<td>5.50</td>
</tr>
<tr>
<td>Cab1-4</td>
<td>19.0</td>
<td>18.0</td>
<td>56.0</td>
<td>48.0</td>
<td>61.4</td>
<td>40.5</td>
<td>9.34</td>
</tr>
<tr>
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<td>6.0</td>
<td>7.0</td>
<td>5.7</td>
<td>-</td>
<td>-</td>
<td>6.2</td>
<td>0.39</td>
</tr>
</tbody>
</table>

* percent (%) linamarin

**Table 2.3:** Average leaf linamarin quantity of untransformed and transformed (Cab1-1 to Cab1-5) 3-4 month old *in vitro* plants. Each trial was normalized for the internal control and untransformed plant’s linamarin quantity was set at 100%. Calculation of leaf linamarin content for ‘Leaf trial #1’ is shown in table 2.2. Similar calculation was performed for ‘Leaf trial #2’ and ‘Leaf trial #3’.
Figure 2.16: Average leaf linamarin content in wild-type and Cab1 CYP79D1/CYP79D2 antisense transformed plants. Each sample was normalized on the basis of the internal standard (phenyl β-D-glucoside). Linamarin is expressed as a percentage of the quantity present in wild-type untransformed plant (column 1). These are the averages of 3-5 individual trials (table 2.3).
2.3.8 Analysis of the linamarin content in roots

The untransformed and five transgenic cassava plants were analyzed for their root linamarin content by GC-MS analysis. Due to undetectable amounts of linamarin present in the roots of the transgenics (Cab1-1 through Cab1-5) the TIC mode of data acquisition (used for leaf) was not feasible. As a result the mass spectrometer was operated in single ion monitoring mode (SIM) for root linamarin analysis. The SIM mode of data acquisition increases sensitivity dramatically, increases quantitative precision, and eliminates centroid errors caused by co-eluting nearby mass signals. For the linamarin region of the GCMS run the SIM masses monitored just mass 132 for the time range of 11 to 15 minutes in the chromatogram. Then the internal standard signal is monitored recording intensities at masses 189 and 393 for the time between 17 and 21 minutes in the chromatogram. During SIM runs, mass/intensity points are recorded to disk at 7.5 points per second to allow very high precision area measurements on the chromatographic peaks.

The root linamarin content was measured from 3 independently isolated in vitro wild-type or transgenic plants grown in tissue culture. Figure 2.17 shows the gas chromatograph for one of those trials (Root trial #1). Peak area of linamarin from each sample was normalized to the peak area of the internal standard (Table 2.4). Table 2.4 also shows root linamarin percentages of the transgenic plants compared to wild-type for ‘Root trial #1. This trial shows the root linamarin content of Cab1 transgenic plants
to be reduced down to <1.5% for the root linamarin content of wild-type cassava plants.
Figure 2.17: GC-MS analysis of root linamarin content of wild-type and Cab1 transgenic cassava plants (Cab1-1 through Cab1-5) (Root trial #1). Retention time (RT) and Mass Area (MA) are denoted in each chromatograph. RT for linamarin and phenyl β-D-glucoside was 13.91 and 18.91 minutes, respectively. Linamarin content as a percentage of that present in wild-type plants, after normalizing for the internal control, is noted in parenthesis for each plant.
(Figure 2.17)
Table 2.4: Root linamarin peaks of wild-type and cab1 transformants normalized for the internal control. The last column expresses linamarin as a % of wild-type linamarin levels. This is the percent linamarin data for ‘Root trial 1’ in table 2.5.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Linamarin peak area (10^6)</th>
<th>Internal std peak area (10^6)</th>
<th>Normalized</th>
<th>Percent linamarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2215 (WT)</td>
<td>38.9</td>
<td>3.9</td>
<td>38.9</td>
<td>100.0 %</td>
</tr>
<tr>
<td>Cab1-1</td>
<td>0.73</td>
<td>4.8</td>
<td>0.59</td>
<td>1.5 %</td>
</tr>
<tr>
<td>Cab1-2</td>
<td>0.2</td>
<td>5.0</td>
<td>0.16</td>
<td>0.4 %</td>
</tr>
<tr>
<td>Cab1-3</td>
<td>0.2</td>
<td>6.7</td>
<td>0.12</td>
<td>0.3 %</td>
</tr>
<tr>
<td>Cab1-4</td>
<td>0.29</td>
<td>5.3</td>
<td>0.21</td>
<td>0.55 %</td>
</tr>
<tr>
<td>Cab1-5</td>
<td>0.07</td>
<td>3.9</td>
<td>0.07</td>
<td>0.18 %</td>
</tr>
</tbody>
</table>
To obtain absolute linamarin values known amounts of commercial linamarin was subjected to GC-MS analysis similar to the root samples. Figure 2.18 shows the peak area as a function of varying amounts (0.5 – 4 µg) of linamarin. Using figure 2.18 the linamarin content of wild-type plants was found to be approximately 3.6 µmoles/g dry weight. This value is in agreement with root linamarin levels reported by Mkpong et al. (1990) (2.96 µmoles/g dry weight), Wheatley et al. (1993) (0.4 – 2 µmoles/g fresh weight) and Nambisan and Sundaresan (1994) (3 – 5.5 µmoles/g dry weight).

Similar analyses were performed 3 times independently on 3-4 month old in vitro roots of untransformed and transformed plants. Surprisingly, the linamarin content of roots of transgenic plants having reduced leaf linamarin levels was reduced to even a greater extent than in leaves. Tabulation of these root linamarin trials is shown in table 2.5. As shown in table 2.5 and figure 2.19, root linamarin content was reduced by 99% in all transgenic plants analyzed (relative to wild-type plants).
Figure 2.18: GC-MS linamarin peak area as a function of linamarin amounts (0.5 – 4 µg). Commercial linamarin dissolved in acetonitrile and derivatized as mentioned in the methods section was used. phenyl β-D-glucoside was used as internal standard. Broken line (----) shows the root linamarin amount in wild-type plants.
### Table 2.5: Average root linamarin quantity of untransformed and transformed 3-4 month old in vitro plants. Each trial was normalized for the internal control and untransformed plant’s linamarin quantity was set at 100%. Calculation of root linamarin content from ‘Root trial #1’ is shown in table 2.4. Similar calculation was performed for ‘Root trial #2’ and ‘Root trial #3’

<table>
<thead>
<tr>
<th>Sample</th>
<th>Root trial #1</th>
<th>Root trial #2</th>
<th>Root trial #3</th>
<th>Average</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransformed</td>
<td>100.0</td>
<td>100.00</td>
<td>100.00</td>
<td><strong>100.00</strong></td>
<td>-</td>
</tr>
<tr>
<td>Cab1-1</td>
<td>1.5</td>
<td>0.30</td>
<td>1.15</td>
<td><strong>0.98</strong></td>
<td><strong>0.62</strong></td>
</tr>
<tr>
<td>Cab1-2</td>
<td>0.4</td>
<td>0.24</td>
<td>0.20</td>
<td><strong>0.29</strong></td>
<td><strong>0.12</strong></td>
</tr>
<tr>
<td>Cab1-3</td>
<td>0.3</td>
<td>0.24</td>
<td>0.23</td>
<td><strong>0.26</strong></td>
<td><strong>0.04</strong></td>
</tr>
<tr>
<td>Cab1-4</td>
<td>0.55</td>
<td>0.25</td>
<td>0.21</td>
<td><strong>0.33</strong></td>
<td><strong>0.17</strong></td>
</tr>
<tr>
<td>Cab1-5</td>
<td>0.18</td>
<td>0.26</td>
<td>1.75</td>
<td><strong>0.73</strong></td>
<td><strong>0.10</strong></td>
</tr>
</tbody>
</table>
Figure 2.19: Average root linamarin content in wild-type and Cab1 CYP79D1/CYP79D2 antisense transformed plants. Each sample was normalized on the basis of the internal standard (phenyl β-D-glucoside). Linamarin is expressed as a percentage of the quantity present in wild-type untransformed plant (column 1). These are the averages of 3 individual trials (Table 2.5).
2.3.9 RT-PCR analysis of CYP79D1 and CYP79D2 transcript levels in roots

One possible explanation for the reduced linamarin content in roots of transgenic plants having reduced CYP79D1 and CYP79D2 expression in leaves was a reduction in root expression of the CYP79 D1 and CYP79D2 genes as well. To determine whether this was the case we quantified root CYP79D1 and CYP79D2 transcript abundance by RT-PCR. As shown in figure 2.20, there was no significant reduction of CYP79D1 and CYP79D2 transcript levels in 3-4 month old in vitro roots of Cab1-1 through Cab1-5 plants. Thus, the reduction in root linamarin does not reflect an apparent reduction in CYP79D1 and CYP79D2 expression.
**Figure 2.20:** RT-PCR analysis of CYP79D1 and CYP79D2 transcript levels in roots. CYP79D1 and CYP79D2 transcripts were amplified using primers specific for the 3’ end of the genes (D1-F1 / D1-R4 and D2-F1 / D2-R5 primer pairs amplify CYP79D1 and CYP79D2, respectively).
2.3.10 Growth analysis in ammonia free MS media

Cab1 transgenic plants were not able to survive in soil. Many attempts at transplanting Cab1 transgenic plants from normal micropropogation media to soil failed. The transformants would survive in soil for 2 weeks and then proceed to wilt and eventually die within 3 weeks. To analyze the relationship between the inability of the transformants to survive in the soil and the role cyanogens might play as a mobile nitrogen source, wild-type and transformants were grown in ammonia free medium.

Four weeks after placing the nodal cutting in ammonium free MS media Cab1 transformants showed greater shoot and root growth retardation than wild-type plants (Figure 2.21). Shoot growth of wild-type plants were hindered in the ammonia free medium but failed in comparison to the interference of the shoot growth of the transformants. This effect was further exaggerated in the growth analysis after 6 weeks (Figure 2.22). In addition to the hindrance of the shoot growth in ammonium free media, Cab1 transformants showed retardation of root growth. Furthermore Cab1 transformants in normal MS media produced higher number of roots compared to the transformants in ammonium free medium (Figure 2.22).
Figure 2.21: Growth analysis of wild-type and transformants (Cab1-1 / Cab1-5) after 4 weeks in MS media with ammonia (A) and without ammonia (B).
Figure 2.22: Growth analysis of wild-type and transformants (Cab1-1 / Cab1-5) after 6 weeks in MS media with ammonia (A) and without ammonia (B).
2.4 DISCUSSION

To generate acyanogenic cassava plants we targeted the genes (CYP79D1 and CYP79D2) encoding the cytochrome P450s that catalyze the first dedicated-step in linamarin and lotaustralin synthesis for reduced levels of expression. Using an antisense strategy, we introduced the 5’ ends (650 bp) of the CYP79D1 and CYP79D2 genes into cassava in reverse orientation, via Agrobacterium-mediated, Ti-plasmid transformation. To reduce the cyanogen content of leaves, as well as to determine the potential effects of reduced leaf linamarin synthesis on root cyanogen levels we introduced the CYP79D1 and CYP79D2 antisense constructs into cassava plants under the control of the leaf-specific Cab1 promoter.

The five transformants with altered CYP79D1 and CYP79D2 transcripts had up to a 94% reduction in leaf linamarin content. Interestingly, there was not a direct correlation between CYP79D1/CYP79D2 transcript levels and linamarin content. This dichotomy may reflect additional metabolic flux controls which determine rates of linamarin synthesis, storage and export.

Significantly, the same transformants which had between 94% and 60% reductions in their leaf linamarin content had root linamarin contents which were less than 1% of wild type. The root linamarin content of in-vitro grown wild-type cassava was 3.5 (± 0.4) μmoles/g dry weight. This value is in agreement with root linamarin levels reported by Mkpong et al. (1990) (2.96 μmoles/g dry weight), Nambisan and Sundaresan (1994) (3 – 5.5 μmoles/g dry weight) and Wheatley et al. (1993) (0.4 – 2
µmoles/g fresh weight) for mature field grown cassava roots. Analysis of CYP79D1/CYP79D2 transcript levels in transgenic roots indicated that they were unchanged relative to wild-type plants and hence reductions in root CYP79D1/CYP79D2 transcripts did not apparently account for the reduction in root linamarin content. An alternative explanation for the reduced linamarin content of roots in Cab1 CYP79D1/CYP79D2 antisense plants is a reduction in linamarin transport from leaves to roots. The transport of cyanogenic glucosides has been well characterized in the related species, Hevea brasiliensis (Selmar, 1993). In young Hevea plants, linamarin is transported apoplastically from the leaves to the roots following its glycosylation to linustatin (Koch et al., 1992; Selmar et al., 1988). At the sink site linustatin is apparently metabolized by one of two mechanisms involving either a simultaneous or sequential deglycosylation. In the simultaneous deglycosylation pathway, linustatin is presumably deglycosylated to produce gentiobiose and acetone cyanohydrin. In the sequential pathway one glucose is removed at a time generating linamarin as an intermediate which is then deglycosylated to produce acetone cyanohydrin (Selmar, 1993). Significantly, linustatin has not been detected in cassava plants in quantities sufficient to facilitate cyanogen transport by the mechanism observed in Hevea. However, Bediako et al. (1981) observed transport of 14C-labeled linamarin from leaves to roots in cassava. Furthermore, grafts between roots of high-cyanogenic cultivars and shoots of low- and high-cyanogenic cultivars, indicated that root linamarin levels were in part determined by cyanogen transported from leaves (Makame et al., 1987). In addition, girdling of
stems near the base of cassava plants resulted in a 13-fold increase in the accumulation of linamarin above the girdling site (Ramanujam et al., 1984).

It also is apparent, however, that cassava root protoplasts and microsomal fractions are capable of synthesizing linamarin (McMahon et al., 1994). The contribution of root linamarin synthetic activity to the overall steady-state linamarin levels remains to be determined, however, particularly in young plants. These results suggest that the reduced root linamarin content of Cab1 CYP79D1/CYP79D2 antisense plants may be attributed to a reduction in transport of cyanogens to roots. If this explanation is correct, however, then there also must be a threshold leaf linamarin content to allow for linamarin transport to roots since plants having 60% of the wild-type leaf linamarin content have less than 1% of wild-type root linamarin content. In support of this is the observed shoot and root growth retardation of all five Cab1 transformants compared to wild-type plants in ammonium free growth medium. Currently further studies are underway to characterize cyanogen transport in the CYP79D1/CYP79D2 antisense transgenic plants as a function of root age and CYP79D1 and CYP79D2 expression.

In summary, we have generated acyanogenic cassava as an alternative food source to the low- and high-cyanogenic varieties currently cultivated. In addition, acyanogenic plants provide a more marketable and consistently cyanogen-free food product, potentially providing additional sources of income generation for subsistence farmers. The absence of cyanogenic glycosides may, however, necessitate the use of additional control practices and strategies to reduce crop losses due to herbivory or
theft. For these circumstances plants expressing high levels of the enzyme hydroxynitrile lyase in roots may provide an alternative to cyanogen-free cassava (White et al., 1998; Arias-Garzon et al., 2000).
CHAPTER 3

ANTISENSE EXPRESSION OF THE CYP79D1 AND CYP79D2 GENES UNDER
THE CONTROL OF THE PATATIN PROMOTER

3.1 INTRODUCTION

Cyanogenesis has been shown to protect the plant against herbivore or fungal
attack (Belloti and Arias, 1993; Hickel et al., 1996). In addition, Selmar et al. (1988)
have proposed that cyanogenic glucosides serve as nitrogen storage compounds and
help mobilize nitrogen in young plants of rubber, cassava and sorghum. In *Hevea
brasiliensis* linamarin is converted to linustatin at the source or site of synthesis and
transported apoplastically to the sink tissue. Linustatin is a cyanogenic diglucoside that
is not metabolized by the apoplastic linamarase which can only hydrolyze mono-
glucosides (see Chapter 1 for further review). Expression of the CYP79D1 and
CYP79D2 genes in an antisense orientation in transgenic cassava under the control of
the leaf-specific Cab1 promoter resulted in both leaf and root levels of linamarin being
reduced up to 94% and 99%, respectively in 3-4 month old CYP79D1/CYP79D2
antisense plants (Chapter 2). These results suggested that linamarin may be transported
from leaves to roots in young cassava plants.
To further investigate the influence of leaf linamarin synthesis and transport on root linamarin levels we expressed the CYP79D1 and CYP79D2 genes in an antisense orientation under the control of the *Solanum tuberosum* tuber-specific, class I patatin promoter. Patatin is a family of glycoproteins that account for up to 40% of the total soluble protein in potato tubers. Patatin glycoproteins are divided into two classes based on the presence (class II) or absence (class I) of a 5’-untranslated region, 22-bp in length. The promoter sequences of both class-I and class-II patatin genes are highly homologous up to base pair position –87 and then diverge (Mignery et al. 1988). Mignery and colleagues (1988) have shown that class I transcripts are 50-100 fold more abundant in the potato tuber than class II transcripts. Preliminary work done in our laboratory by Dr. Sue Lawrence demonstrated the expression of β-glucorinidase gene driven by the patatin promoter in cassava roots but not in cassava leaves. These results suggest that it would be possible to drive the antisense expression of the CYP79 genes specifically in the roots.

In this chapter, we demonstrate that root-specific inhibition of CYP79D1 and CYP79D2 transcripts does not lead to a reduction of linamarin content in roots of 3-4 month old *in vitro* plants. The CYP79D1 and CYP79D2 transcript levels and linamarin levels in the leaves of these transformants were unaffected. These results in conjunction with results of chapter 2, demonstrate that the linamarin present in roots is synthesized and transported from leaves to the roots in young (3-4 month old) cassava plants.
3.2 EXPERIMENTAL PROCEDURES

3.2.1 E. coli, Agrobacterium and Cassava strains and cultivar

The *Escherichia coli* strain DH5α (Life Technologies, Grand Island, NY) was used for all recombinant DNA work. Selection for transformed E.coli cells was performed on Luria-Beritani (LB) media containing 100 mg/mL of ampicillin. Transformation of cassava was performed using modified *Agrobacterium tumefacience* strain LBA4404 (Life Technologies, Grand Island, NY). Selection for transformed Agrobacterium cells was conducted on YM media supplemented with 50mg/mL of kanamycin. Cassava cultivar *Manihot Columbia* 2215 was used for all transformations. Cassava tissue culture media, transformation protocol and regeneration media are explained subsequently in this section.

3.2.2 Vector construction and PCR screening

A modified Agrobacterium binary vector 4B:Pat constructed by Dr. Sue Lawrence was used as the starting vector for the expression of the CYP79 genes. The 4B:Pat construct has the vector back ground of pBI121 (Strategene) but the 2X35S promoter and β-glucorinidase gene have been substituted by the class I patatin tuber-specific promoter (Rocha-Sosa et al., 1989) and the *glgC* gene (Stark et al., 1992) (Figure 3.1). The T-DNA region also contains nptII gene for kanamycin/paromomycin resistance.
**Figure 3.1:** T-DNA region of 4B:Pat vector. This vector was constructed using pBI121 and was used in the work to form Pat:D1AS and Pat:D2AS modified vectors.
The *glgC* gene was subsequently removed and the 5’ ends (650 bp each) of both CYP79D1 and CYP79D2 genes were cloned into the Agrobacterium binary vector, pBI121, in an antisense orientation under the control of the patatin promoter. The 5’-650 bp pieces of CYP79D1 and CYP79D2 genes (amplified and tagged with *SstI* and *SmaI* restriction site as described in Chapter 2), as well as 4B:Pat vector were digested with *SstI* and *SmaI* restriction enzymes, separated on a 1% agarose gel and excised using QIAEXII gel extraction kit (Qiagen Inc, Valencia, CA). The 4B:Pat vector was digested with *SstI* and *SmaI* releasing the *glgC* gene from the vector. Approximately 300 ng of vector and 1 µg of either the 650 bp CYP79D1 or CYP79D2 genes were ligated together using 10 units T4 DNA ligase for 12 hours at 14°C. The resulting modified vectors were called Pat:D1AS and Pat:D2AS, respectively. Ten percent of the ligation mixture was used to transform DH5α E. coli competent cells (Life Technologies, Grand Island, NY). After incubating the recombinant DNA and the cells for 30 minutes on ice the cells were heat shocked for 25 seconds at 42°C followed by a 2 minute recovery time in ice and the addition of 900 µL of pre-warmed (to 42°C) SOC media (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl and 2.5 mM KCl). Cell suspensions were incubated for 1 hour at 37°C and 200 rpm constant shaking. The cells were then spread on LB medium containing 100 mg/mL of ampicillin. Overnight cultures of the transformed E.coli colonies were used to purify the recombinant plasmid in quantity using QIAGEN plasmid midi kit (Qiagen Inc, Valencia, CA).

Primers specific for the 5’-end of patatin promoter (PatF2) and 3’-end of the nos terminator (NOSAR2) were used to PCR amplify Pat:CYP79D1:ter cassette from Pat:D1AS modified vector. An *EcoRI* restriction site was added to the 5’ end of the
both primers to facilitate subsequent cloning. PatF2 and NOSAR2 primers had the sequence of GCCGAATTCTAGTTAATGCGTATTAG and CCGAATTCCATAGATGACACCGCGC respectively (underlined are the EcoR1 sites).

PCR reactions were performed in a total volume of 50 µL containing: 20 mM Tris-HCl (pH 8.0), 1.5 mM MgCl₂, 40-50 ng of Pat:D1AS plasmid DNA, 0.2 mM each dNTP, 2.5 units of PLATINUM Taq DNA polymerase (Life Technologies, Grand Island, NY), and 0.4 µM each primer (IDT, Coralville, IA). The DNA was amplified in a Perkin Elmer Cetus DNA thermal cycle by incubating the DNA mixture for 4 minutes at 94°C followed by 35 cycles of 30 seconds at 94°C, 1 minute at 65°C and 1.5 minutes at 72°C, and final 4 minutes incubation at 94°C. PCR products were fractionated on 1% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and the DNA was isolated from the agarose gel by QIAEX II gel extraction kit (QIAGEN Inc, Valencia, CA) according to manufacturers protocol. The DNA product was sequenced in both directions at the Ohio State University Neurobiotechnology Center DNA sequencing facility to confirm its authenticity.

This Pat:CYP79D1:ter cassette with EcoR1 ends and Pat:D2AS vector were both digested with EcoR1. Digested Pat1:D2AS vector DNA was then de-phosphorylated using 2.5 units of calf intestinal alkaline phosphatase (CIAP) (Life Technologies, Grand Island, NY) to prevent self-annealing. Approximately 1 µg of Pat:D2AS vector and 250 ng of the Pat:D1AS cassette were ligated using 10 units T4 DNA ligase for 30 hours at 14°C. The resulting modified vectors were called Pat:D1:D2AS. Ten percent of the ligation mixture was used to transform DH5α E. coli
competent cells (Life Technologies, Grand Island, NY). After incubating the recombinant DNA and the cells for 30 minutes on ice the suspensions were heat shocked for 25 seconds at 42\(^\circ\)C followed by a 2 minute recovery time on ice followed by the addition of 900 \(\mu\)L of pre-warmed (to 42\(^\circ\)C) SOC media (2\% (w/v) tryptone, 0.5\% (w/v) yeast extract, 10 mM NaCl and 2.5 mM KCl). Cell suspensions were incubated for 1 hour at 37\(^\circ\)C and 200 rpm constant shaking. The cells were then spread on LB medium containing 100 mg/mL of ampicillin. The resulting colonies were grown overnight in LB medium under selection and Pat:D1:D2AS vector was isolated from the cultures using QIAGEN plasmid midi kit (Qiagen Inc, Valencia, CA). The Pat:D1:D2AS vector was PCR amplified using different primers and the resulting DNA products were sequenced at the Ohio State University Neurobiotechnology Center DNA sequencing facility to prove its validity. In addition Pat:D1:D2AS vector was digested with \textit{SstI} restriction enzyme to separate a 1.9 kb fragment encoding ter:patatin promoter:CYP79D1 portion.

3.2.3 Agrobacterium transformation

Approximately 300 ng of Pat:D1:D2AS vector was used to transform \textit{Agrobacterium tumefaciens} strain LBA4404 (Life Technologies Inc, Rockville, MD). Transformation was carried out by electroporation of 20 \(\mu\)L of ElectroMAX LBA4404 cells (Life Technologies, Grand Island, NY) with Pat:D1:D2AS vector. A Bio-Rad electroporator was used at the following settings: 1.8 kV, 25 \(\mu\)F, 200 \(\Omega\) and 4.8 milliseconds. Electroporation was followed by incubation in 1 mL of YM medium (0.04\%
yeast extract, 1% (w/v) mannitol, 1.7 mM NaCl, 0.8 mM MgSO$_4$.7H$_2$O, 2.2 mM K$_2$HPO$_4$.3H$_2$O, pH 7.0) at 225 rpm for 3 hours at 30°C. After the recovery period 100 μL of cell suspension was spread on solid YM medium supplemented with 100 mg/L streptomycin and 50 mg/L kanamycin for 2-3 days at 30°C. The resulting colonies were screened for the presence of the CYP79 genes by PCR using primers specific for CYP79D1 and CYP79D2.

3.2.4 Cassava transformation

3.2.4.1 Plant Material

Shoot apical meristems of cassava cultivar MCol 2215 were cultured on MS basal medium for production of in vitro plants (Murashige et al., 1962; Arias-Garcon et al., 1993). Apical leaves were placed on supplemented MS8 medium (MS basal medium supplemented with 2% (w/v) sucrose, 8 mg/L 2,4-D, Gamborg’s B-5 vitamins (Gamborg et al., 1968), 50 mg/L casein and 0.5 mg/L CuSO$_4$ at pH 5.7) for induction of somatic embryos. Plant cultures were kept under 12 hr/day photoperiod (5 μmole photons m$^{-2}$s$^{-1}$) at 28°C. Once embryos formed they were transferred to regeneration medium (MS basal medium supplemented with 1 mg/L thiamine, 100 mg/L myo-inositol, 2% (w/v) sucrose, 1.0 mg/L BAP and 1 mg/L GA at pH 5.7) to induce the development of cotyledonary leaves.
3.2.4.2 Transformation

Cassava transformation using germinated somatic embryos was performed as described in chapter 2.

3.2.5 Molecular analysis of transgenic plants.

3.2.5.1 PCR analysis of the transgenic plants for the integration of the T-DNA

For PCR analysis, genomic DNA was isolated from 40 mg of 3-4 month old, \textit{in vitro}-grown leaves from either wild-type plants or paromomycin-resistant putative transformants according to Doyle et al. (1990). Wild-type plants used were not transformed but grown in tissue culture media (without selection) similar to the putative transformants. The presence of the transgenes was detected by PCR amplification. PCR reactions were performed in a total volume of 50 μL containing: 1X Vent buffer, 20-100 ng of leaf DNA, 0.1 mM each dNTP, 1 unit Vent polymerase, 0.4 μM each primer (IDT, Coralville, IA). The CYP79D1 transgene was amplified with primers specific for the patatin promoter/CYP79D1 junction (PatF1: TTTCTCAACTTGTGTACGGTCC) and CYP79D1/nos terminator junction (NOSAR1: ATCGCAAGACCGCAACAGGATTC). The DNA was amplified using 10 touchdown PCR cycles of 30 seconds at 94\textdegree C, 1 minute at 69\textdegree C - 64\textdegree C and 30 seconds at 72\textdegree C followed by 25 cycles at 64\textdegree C annealing temperature. Each specific DNA product was sequenced to prove its authenticity. The presence or absence of the
Agrobacterium-specific *virG* gene was confirmed by PCR using Agrobacterium *virG* gene primers (VirGF1: GCCGACAGCACCCAGTTCAC and VirGR1: CCTGCCGTAAGTTTCACCTCACC).

### 3.2.5.2 Southern blot analysis of the transgenic plants

Total DNA was isolated from 3-4 month old *in-vitro* grown plants according to Soni et al. (1994). Approximately 1 g of leaves was ground in 10 mL of extraction buffer (50 mM Tris-Cl pH 8.0, 10 mM EDTA, 2% (w/v) SDS, 100 mM LiCl, 10 µg/mL proteinase K) and incubated for 15 minutes at room temperature with occasional mixing. After centrifugation at 3500 rpm for 15 minutes, 200 µg of RNase was added to the supernatant and incubated for 30 minutes at 37°C. This was followed by equal volume extraction with phenol, phenol/chloroform and phenol/chloroform/isoamyl alcohol. DNA from the upper phase of the final extraction was precipitated with 0.25 X volume 10M ammonium acetate/2 X volume −20°C ethanol at room temperature for 10 minutes. DNA was pelleted at 13,000 rpm for 20 minutes and the pellet was washed with 70% ethanol. The air-dried pellet was resuspended in 0.5 mL of TE buffer (10mM Tris-Cl, 1mM EDTA, pH 8.0).

Southern blot analysis was performed following standard protocol (Sambrook et al., 1989). 10 µg DNA was digested with *SalI*, fractionated on a 0.7% agarose gel. *SalI* does not restrict the T-DNA region of the modified vector used in this study. The gel was soaked in 250 mM HCl for 10 minutes, denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 45 minutes and neutralization buffer (1 M Tris-HCl, 1.5 M NaCl) for 30
minutes. Each soaking step included constant gentle agitation followed by a brief rinse with distilled water. The DNA was transferred to nylon membrane using 20 X SSC buffer for 20 hours. Transferred DNA was cross-linked to the membrane by UV light exposure for 45 seconds. The pre-hybridization of the membrane was done for a minimum of 2 hours at 42°C in CHURCH buffer (0.5 M NAHPO₄ (pH 7.2), 1 mM EDTA, 1% (w/v) BSA, 7% (w/v) SDS). Hybridization of the membrane was conducted at 42°C with ^32P- labeled CYP79D1 probe (5' end 650bp) for 16-18 hours. The probe was made using PCR primers specific to CYP79D1 and the PCR reaction was performed in a total volume of 50 µL containing: 1 X PCR buffer, 100 ng Pat1:D1AS vector DNA, 0.1 mM each dATP/dTTP/dGTP, 100 µCi of ^32P-dCTP (Amersham, Piscataway, NJ), 2.5 units of Taq polymerase (Life Technology, Grand Island, NY), 0.4 µM each primer (IDT, Coralville, IA), 1.5 mM MgCl₂. The DNA was amplified in a Perkin Elmer Cetus DNA thermal cycle using 35 PCR cycles of 30 seconds at 94°C, 45 seconds at 65°C and 30 seconds at 72°C preceded by 3 minutes incubation at 94°C. The labeled probe was cleaned using Qiagen PCR cleaning kit (Qiagen Inc, Valencia, CA) and boiled for 5 minutes before being added to the membrane. After hybridization the membrane was washed with 1 X SSC containing 0.1% SDS at 50°C for 20 minutes followed by two washes with 0.1 X SSC containing 0.1% SDS each at 50°C for 20 minutes. The membrane was then exposed to a phosphorimager overnight.
3.2.5.3 RT-PCR analysis of the abundance of CYP79 transcripts in transgenic plants

Total RNA was isolated from 100 mg of 3-4 month old *in vitro* leaves or 40-50 mg of *in vitro* roots from putative (paromomycin-resistant) transformants using the Qiagen Plant RNA extraction kit (Qiagen Inc, Valencia, CA). Contaminant DNA was eliminated by incubation of the RNA with 1 unit of DNase (Invitrogen, Carlsbad, CA) for 15 minutes at room temperature. DNase was inactivated according to manufacturers recommendations. RT-PCR was performed using 6 µg of total RNA with RT-PCR kit from Invitrogen (Carlsbad, CA) according to the manufacturers recommendations. First strand synthesis on 6 µg of total RNA was performed using 1X reverse transcription buffer, 0.3 mM dNTP, 0.5 µg oligo dT(12-18) primer, 200 units of Superscript II reverse transcriptase (Life Technology, Grand Island, NY) with incubations of 65°C for 5 minutes followed by 42°C for 75 minutes.

The cDNA amplification was performed using 1 X PCR buffer, 1.5 mM MgCl₂, 0.1 mM dNTP each, 2.5 units Taq polymerase, 0.4 µM each primer with 35 cycles of 30 seconds at 94°C, 1 minute at 56°C and 30 seconds at 72°C (Life Technology, Grand Island, NY). The primers used were specific for the 3’ end of CYP79D1 (D1-F1: GCTAAATCAACCAGAAATCCTGAAG and D1-R4: TGCAAGAGAAACAGATAACCCC) and CYP79D2 (D2-F1: CTGATAAATCAACCAGAAACTTCTGGCA and D2-R5: CTAACAACACTCACATCCCTCCCTCCC ) genes (Figure 2.1). DNA products were
separated on a 1% agarose gel. As assurance for the formation of first strand cDNA, starch-branching enzyme II (SBEII) transcript was amplified from the roots and leaves of the patatin transgenics. In each case a negative control without DNA and a positive control amplifying the respective genes cloned in plasmids was performed.

3.2.6 Linamarin quantification using Gas Chromatography – Mass Spectrometry

The leaf and root linamarin content was measured from 3 independently isolated 3-4 month old *in vitro* wild-type or transgenic plants grown in tissue culture. Linamarin was extracted and derivatized from leaves and roots according to Mkpong et al. (1990), with phenyl β-D-glucoside added as an internal standard. *In vitro* leaves or roots were ground in liquid nitrogen and immediately lyophilized for 14-16 hours. Linamarin was extracted from 4 mg of tissue using acetonitrile. 20 µg phenyl- β-D-glucoside was added to the mixture prior to derivatization with Bis (trimethylsilyl) trifluoroacetamide (BSFTA) (Aldrich, Milwaukee, WI). Derivatization was performed at 90°C for 30 minutes, followed by a 15 second centrifugation. The resulting supernatant was subjected to GC-MS analysis at the Central Chemical Instrumentation Center (CCIC) of the Ohio State University.

GC-MS, on a Thermo-Finnigan Trace 2000 instrument, was performed on a 15-meter long, 0.25 micron film thickness Restek XTI-5 (5% diphenyl - 95% dimethyl polysiloxane) capillary gas chromatography column using a split/splitless injector in the splitless mode at an injection temperature of 250°C. The GC-MS was operated under a pressure control mode using pressures that gave flow rates near 1mL/minute. The GC
oven temperature program was: 50°C for one minute after injection, ramp at
30°C/minute to 185°C, ramp at 6°C/minute to 230°C (linamarin elution), ramp at
12°C/minute to 300°C (internal standard elution), and ramp at 40°C/minute to 360°C for
3 minutes to clean the column. A dedicated EI (electron impact) ion source was used at
an electron energy of -70eV. The source temperature was held at 250°C.

The mass analyzer used was a Trace MS single quadrupole mass spectrometer.
The TIC mode (used for leaf linamarin quantification in chapter 2) was used for data
acquisition. For the linamarin region of the GCMS run mass 132 was monitored for the
time range of 11 to 15 minutes in the chromatogram. The internal standard signal was
monitored recording intensities at masses 189 and 393 for the time between 16 and 20
minutes in the chromatogram. Each of the runs was normalized for the internal standard
(phenyl β-D-glucoside) and linamarin is expressed as a % of the quantity present in
wild-type untransformed plants.
3.3 RESULTS

3.3.1 Making of the CYP79D1 and CYP79D2 anti-sense construct

Using pBI121 Agrobacterium binary vector backbone, the Pat:D1:D2AS vector was constructed expressing the 5’-650 bp ends of CYP79D1 and CYP79D2 in antisense orientation. Each of the antisense genes was flanked by tuber-specific patatin promoter and a nos terminator (Figure 3.2). The final construction was made in a manner to prevent the formation of mRNA secondary structure by having the two cassettes in the same orientation. Restriction of Pat:D1:D2AS vector with SstI releases a 1.9 kb DNA piece that is indicative of the construction of this vector in the intended orientation (Figure 3.3).

3.3.2 Production of transgenic cassava

To generate acyanogenic plants Agrobacterium-mediated T-DNA transformation was used to introduce the 5’ ends (650 bp) of the CYP79D1 and CYP79D2 genes in the reverse orientation (antisense) under the control of tuber-specific, patatin promoters. In addition to the antisense CYP79D1 and CYP79D2 genes, the T-DNA included a bacterial nptII gene for antibiotic selection of transformants. Ninety-nine independent paromomycin-resistant putative transformants were obtained following transformation of 3560 cassava explants (Table 3.1).
Figure 3.2: T-DNA region of the Pat:D1:D2AS vector. Each of the antisense CYP79D1 and CYP79D2 genes is flanked by a tuber-specific patatin promoter and a nos terminator (ter). The two cassettes are in the same orientation. \textit{SstI} restriction enzyme releases a 1.9kb DNA piece (Figure 3.3). PatF1 and NOSAR1 are the primers used for PCR amplification to analyze putative cassava transformants.
Figure 3.3: Pat:D1:D2AS vector restricted with SstI releases a 1.9kb DNA piece encoding terminator:patatin promoter:CYP79D1 portion.
<table>
<thead>
<tr>
<th>Transformation #</th>
<th># of explants used</th>
<th>Paromomycin resistant plantlets</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>250</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>250</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>130</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>360</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>180</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>200</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>400</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>470</td>
<td>26</td>
</tr>
<tr>
<td>9</td>
<td>400</td>
<td>11</td>
</tr>
<tr>
<td>10</td>
<td>220</td>
<td>19</td>
</tr>
<tr>
<td>11</td>
<td>200</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>500</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>3560</td>
<td><strong>99 (2.8%)</strong></td>
</tr>
</tbody>
</table>

**Table 3.1:** Cassava transformation efficiency. Modified Agrobacterium carrying the Pat:D1:D2AS vector was used to transform cassava germinated somatic embryos.
3.3.3 PCR analysis for the integration of the T-DNA

Each putative transformant was obtained from a unique explant and not from a secondary somatic embryo. Similarly, untransformed wild-type plants used in all analyses were generated from independent explants via the same tissue culture procedures used to generate putative transformants (minus paromomycin selection). Thus, any observed differences in CYP79 transcript levels or linamarin abundance in transformed plants relative to wild-type plants could not be attributed to differences in plant culture.

To confirm the integration of the T-DNA the transformants were screened by PCR amplification for the truncated CYP79D1 gene. DNA primers specific for the region between the patatin promoter/CYP79D1 junction and the CYP79D1/NOS terminator junction (Fig. 3.2) were used to amplify the CYP79D1 transgene. A diagnostic 700 bp region was amplified in the transformants (Figure 3.4A). The identities of all the bands were confirmed by DNA sequence analysis. The absence of Agrobacterium contamination was verified by the use of Agrobacterium virG specific primers (Figure 3.4B). The presence of the CYP79D1 gene in the transformants coupled with the absence of the virG gene demonstrated integration of the T-DNA in the transformants.
Figure 3.4: Analysis for the integration of the T-DNA cassette using primers specific to the patatin promoter and nos terminator. Amplification of a 700bp T-DNA fragment from only the transgenic plants confirms the integration of the T-DNA (A). The absence of Agrobacterium contamination was verified by the use of *virG* specific primers (B).
3.3.4 Southern blot analysis

Southern blot analysis was used to further confirm the transformation results obtained by PCR. Genomic DNA of wild-type Mcol 2215 and three transformants described, Pat1 to Pat3, was restricted with \textit{SalI} and probed with $^{32}$P-labelled CYP79D1. Southern blot analysis shows that between one and two independent T-DNA integration events had occurred into the genome of the various transformants (Fig. 3.5). The upper common CYP79D1 bands represent the wild-type genes. Two of the transformants, Pat2 and Pat3, had one integration event and Pat1 showed two integration events.
Figure 3.5: Southern blot analysis of wild-type and patatin transgenic plants. 10µg of leaf DNA was digested with SalI and hybridized with a probe for the CYP79D1. Lane 1 is wild-type DNA and lanes 2-4 are transformants Pat1 – Pat3, respectively. Lane marked ‘+’ is the modified binary vector restricted with EcoRI to release the 1.9kb cassette of patatin promoter:650bp CYP79D1:terminator.
3.3.5 RT-PCR analysis for the CYP79D1 and CYP79D2 transcript abundance

The CYP79D1 and CYP79D2 transcript abundance of wild-type and transgenic plants was analyzed by RT-PCR amplification using primers specific for the 3’ ends of the CYP79D1 and CYP79D2 genes (Figure 2.1). These primers do not anneal to the 5’ portion of the genes used in the anti-sense construct. In order to verify proper first strand synthesis from reverse transcriptase reaction cassava starch branching enzyme (SBEII) transcript was amplified from in vitro roots. For figure 3.6 shows the complete lack of any detectable CYP79D1 and CYP79D2 transcript in the roots of the transgenic cassava plants (Pat1 –3).

Analysis of the CYP79 transcript levels in the leaves of patatin CYP79 antisense transgenic plants showed partial reduction in the CYP79D1 and CYP79D2 transcript levels in the Pat1 transformed plants but no apparent reduction in the Pat2 or Pat3 transformed plants (Figure 3.7). Thus any alterations of linamarin content in the roots of the Pat2 and Pat3 transgenic plants are not due to change in the abundance of CYP79 transcripts in the leaves.
**Figure 3.6:** RT-PCR amplification of the CYP79D1 and CYP79D2 transcripts from roots. Primers specific for the 3’ end of each gene were used (primer pairs D1-F1 / D1-R4 and D2-F1 / D2-R5 amplify CYP79D1 and CYP79D2, respectively). Control RT-PCR amplification of SBEII transcript was performed as explained in chapter 2.
Figure 3.7: RT-PCR amplification of the CYP79D1 and CYP79D2 transcripts from leaves. Primers specific for the 3’ end of each gene were used (primer pairs D1-F1 / D1-R4 and D2-F1 / D2-R5 amplify CYP79D1 and CYP79D2, respectively). RT-PCR amplifications of CYP79D1, CYP79D2 and SBE-II transcripts were performed in parallel.
3.3.6 Analysis of the linamarin content in leaves

The wild-type and Pat1-3 transgenic cassava plants were analyzed for their leaf linamarin content by GC-MS analysis. An internal standard (phenyl β-D-glucoside) was included in all plants extractions prior to derivatization to determine the efficiency of the extraction and derivatization of linamarin. Transgenic plants used for the linamarin extractions and derivatizations were all approximately the same age (3-4 month old in vitro plantlets). The leaf linamarin content was measured from 3 independently isolated in vitro wild-type or transgenic plants grown in tissue culture.

Figure 3.8 shows the gas chromatograph profiles for one of those trials (‘Leaf trial #1’). The samples were monitored through Total Ion Chromatography (TIC) and analyzed using selective ion for linamarin and phenyl β-D-glucoside for masses 132 and 393 respectively. Peak area of linamarin from each sample was normalized to the peak area of the internal standard of the wild-type plant (Table 3.2). All linamarin peak areas in this chapter (leaves, roots and 0.5-160 µg linamarin) were normalized to internal peak area to facilitate direct comparisons. This was done to better ascertain and compare the absolute linamarin amounts. Table 3.2 also shows the relative leaf linamarin content of the transgenic plants compared to wild-type for ‘Leaf trial #1’. In this trial leaf linamarin content of the patatin transgenic plants was not significantly reduced compared to wild-type cassava plants.
Figure 3.8: GC-MS analysis of leaf linamarin content of wild-type and patatin-CYP79 transgenic cassava plants (Pat-1 through Pat-3) (Leaf trial #1). Retention time (RT) and Mass Area (MA) are denoted in each chromatograph. RT for linamarin and phenyl β-D-glucoside was 14.15 and 17.73 minutes, respectively. Linamarin content is expressed as a percentage of wild-type after normalizing for the internal control.
(Figure 3.8)

Untransformed (100%)

Pat1 (98%)

Pat2 (102%)

Pat3 (89%)
<table>
<thead>
<tr>
<th>Sample</th>
<th>Linamarin peak area (10^3)</th>
<th>Internal std peak area (10^3)</th>
<th>Normalized*</th>
<th>Percent wild type linamarin content</th>
</tr>
</thead>
<tbody>
<tr>
<td>2215(WT)</td>
<td>26.6</td>
<td>16.8</td>
<td>26.6</td>
<td>100 %</td>
</tr>
<tr>
<td>Pat1</td>
<td>35.3</td>
<td>22.8</td>
<td>26.0</td>
<td>98 %</td>
</tr>
<tr>
<td>Pat2</td>
<td>29.4</td>
<td>18.3</td>
<td>27.0</td>
<td>102 %</td>
</tr>
<tr>
<td>Pat3</td>
<td>24.3</td>
<td>17.3</td>
<td>23.6</td>
<td>89 %</td>
</tr>
</tbody>
</table>

* Linamarin peak area of all samples was normalized to the peak area of the internal standard.

**Table 3.2:** Relative leaf linamarin content normalized for the internal standard (for leaf trial #1). The last column expresses linamarin as a % compared to wild-type linamarin levels. This is the percent linamarin data for ‘Leaf trial #1’ in table 3.3.
To determine absolute leaf linamarin values, known amounts of commercial linamarin was subjected to GC-MS analysis. Figure 3.9 shows the peak area as a function of varying amounts (2 – 160 µg) of linamarin. Using values obtained from figure 3.9 we determined that the linamarin content of wild-type leaves was approximately 73 µmoles/g dry weight. A wild-type leaf linamarin level of 73 µmoles/g dry weight is in agreement with levels reported by Nambisan and Sundaresan (1994) (52 – 74 µmoles/g dry weight) and Mkpong et al. (1990) (74 µmoles/g dry weight).

Similar linamarin analyses were performed independently 3 times on 3-4 month old in vitro leaves of wild-type and pat1-3 transformed plants. As shown in table 3.3 and figure 3.10 average leaf-linamarin content was not reduced (relative to wild type) in the 3 transgenic plants analyzed.
Figure 3.9: GC-MS linamarin peak area as a function of linamarin amounts (2 –160 µg). Commercial linamarin dissolved in acetonitrile and derivatized as mentioned in the methods section was used. Phenyl β-D-glucoside was used as internal standard. Broken line (-----) shows the amount of leaf linamarin present in wild-type cassava plant.
Table 3.3: Average leaf linamarin content of untransformed and transformed (Pat1-3) 3-4 month old *in vitro* plants. Each trial was normalized for the internal standard and untransformed plant’s linamarin quantity was set at 100%. Calculation of leaf linamarin content for ‘Leaf trial #1’ is shown in table 3.2. Similar calculations were performed for ‘Leaf trial #2’ and ‘Leaf trial #3’.

<table>
<thead>
<tr>
<th>Leaf Sample</th>
<th>Leaf trial #1</th>
<th>Leaf trial #2</th>
<th>Leaf trial #3</th>
<th>Average*</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransformed</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100 %</td>
<td>0</td>
</tr>
<tr>
<td>Pat1</td>
<td>98</td>
<td>91</td>
<td>107</td>
<td>99 %</td>
<td>6.5</td>
</tr>
<tr>
<td>Pat2</td>
<td>102</td>
<td>84</td>
<td>102</td>
<td>96 %</td>
<td>8.4</td>
</tr>
<tr>
<td>Pat3</td>
<td>89</td>
<td>116</td>
<td>98</td>
<td>101 %</td>
<td>11.1</td>
</tr>
</tbody>
</table>

* percent (%) linamarin
Figure 3.10: Average leaf linamarin content in wild-type and patatin CYP79D1/CYP79D2 antisense transformed plants. Each sample was normalized on the basis of the internal standard (phenyl β-D-glucoside). Linamarin is expressed as a percentage of the quantity present in wild-type untransformed plants (column 1). These are the averages of three individual trials (table 3.3).
3.3.7 Analysis of the linamarin content in roots

The untransformed and three transgenic cassava plants were analyzed for their root linamarin content by GC-MS analysis. The root linamarin content was measured from 3 independently isolated *in vitro* wild-type or transgenic plants grown in tissue culture. Figure 3.11 shows the gas chromatograph for one of those trials (Root trial #1). Peak area of linamarin from each sample was normalized to the peak area for the internal standard of wild-type plant (Table 3.4). Table 3.4 also shows root linamarin percentages of the transgenic plants compared to wild-type for ‘Root trial #1’. This trial shows the root linamarin content of patatin transgenic plants were not altered compared to wild-type cassava plants.
**Figure 3.11:** GC-MS analysis of root linamarin content of wild-type and patatin CYP79 antisense transgenic cassava plants (Pat-1 through Pat-3) (Root trial #1). Retention time (RT) and Mass Area (MA) are denoted in each chromatograph. RT for linamarin and phenyl β-D-glucoside was 14.15 and 17.72 minutes, respectively. Linamarin content as a percentage of that present in wild-type, after normalizing for the internal control, is noted in parenthesis for each plant.
(Figure 3.11)

Untransformed (100%)

RT: 14.15  MA: 4142

RT: 17.72  MA: 31684

Pat1 (84%)

RT: 14.14  MA: 6999

RT: 17.72  MA: 64504

Pat2 (104%)

RT: 14.15  MA: 8437

RT: 17.72  MA: 61775

Pat3 (106%)

RT: 14.14  MA: 3594

RT: 17.71  MA: 26105

Time (min)
<table>
<thead>
<tr>
<th>Sample</th>
<th>Linamarin peak area (10^5)</th>
<th>Internal std peak area (10^5)</th>
<th>Normalized*</th>
<th>Percent linamarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2215(WT)</td>
<td>4.1</td>
<td>31.7</td>
<td>4.1</td>
<td>100 %</td>
</tr>
<tr>
<td>Pat1</td>
<td>7.0</td>
<td>64.5</td>
<td>3.4</td>
<td>84 %</td>
</tr>
<tr>
<td>Pat2</td>
<td>8.4</td>
<td>61.8</td>
<td>4.3</td>
<td>104 %</td>
</tr>
<tr>
<td>Pat3</td>
<td>3.6</td>
<td>26.1</td>
<td>4.4</td>
<td>106 %</td>
</tr>
</tbody>
</table>

* Linamarin peak area of all samples was normalized to the peak area of internal control.

**Table 3.4:** Root linamarin peaks are normalized for the internal standard (for ‘Root trial #1’). The last column expresses linamarin as a % of wild-type linamarin levels. This is the percent linamarin data for ‘Root trial #1’ in table 3.5.
To obtain absolute linamarin values known amounts of commercial linamarin was subjected to similar GC-MS analysis. Figure 3.12 shows the linamarin peak area as a function of varying amounts (0.5 – 15 µg) of linamarin and demonstrates a linear response to the amount of linamarin injected. Using the values obtained from figure 3.12 the linamarin content of in-vitro grown wild-type roots was found to be approximately 3.0 µmoles/g dry weight. This value is in agreement with root linamarin levels reported by Mkpong et al. (1990) (2.96 µmoles/g dry weight), Wheatley et al. (1993) (0.4 – 2 µmoles/g fresh weight) and Nambisan and Sundaresan (1994) (3 – 5.5 µmoles/g dry weight).

Similar analyses were performed 3 times independently on 3-4 month old in vitro roots of untransformed and transformed plants. Tabulation of these root linamarin trails is shown in table 3.5. As shown in table 3.5 and figure 3.13, root linamarin content was not altered in any of the patatin transgenic plants relative to wild type.
Figure 3.12: GC-MS linamarin peak area as a function of linamarin amounts (0.5 – 15 µg). Commercial linamarin dissolved in acetonitrile and derivatized as mentioned in the methods section was used. Phenyl β-D-glucoside was used as internal standard. Broken line (-----) shows the amount of root linamarin present in wild-type plant.
Table 3.5: Average root linamarin quantity of untransformed and transformed 3-4 month old *in vitro* plants. Each trial was normalized for the internal standard and untransformed plant’s linamarin quantity was set at 100%. Calculation of root linamarin content from ‘Root trial #1’ is shown in table 3.4. Similar calculations were performed for ‘Root trial #2’ and ‘Root trial #3’.
Figure 3.13: Average root linamarin content in wild-type and patatin CYP79D1/CYP79D2 antisense transformed plants. Each sample was normalized on the basis of the internal standard (phenyl β-D-glucoside). Linamarin also is expressed as a percentage of the quantity present in wild-type untransformed plant. These are the averages of three individual trials (table 3.5).
3.4 DISCUSSION

To determine the effects of inhibition of the expression of the CYP79D1 and CYP79D2 genes in roots on root linamarin content, we introduced the CYP79D1 and CYP79D2 into cassava in an antisense orientation under the control of the tuber-specific patatin promoter. Three transformants (3-4 month old in vitro plants) with complete inhibition of root CYP79 transcripts did not exhibit any reduction in root linamarin levels. All three transformants had wild type levels of linamarin in the roots. The CYP79D1 and CYP79D2 leaf transcript levels though not altered in Pat2 and Pat3 were partially reduced in Pat1. But the linamarin levels in leaves of the three transformants were unaffected.

In chapter 2 we demonstrated that the selective inhibition of CYP79D1 and CYP79D2 expression in leaves lead to the near complete elimination of linamarin in roots. These results in conjunction with the results presented in this chapter demonstrate that the linamarin present in the roots was synthesized in the leaves and subsequently transported to the roots. Even though the research presented in this thesis strongly suggests that the linamarin present in roots is synthesized in and transported from leaves Jennifer McMahon (PhD thesis, 1997) and Bokanga et al. (1994) demonstrated that the roots are capable of synthesizing linamarin. Fibrous roots of young plant of varieties CM996-6 and HMC-1 were able to synthesize linamarin at rates of 0.217 and 0.235 nmol/gfw/hr, respectively. The root linamarin content of these two varieties is 2.11 and 2.35 µmol/gfw, respectively. At this rate of root linamarin synthesis it would take >400
days to synthesize the linamarin measured in the roots, however. Thus, it was apparent that the rate of linamarin synthesis in the root is not sufficient to account for all the linamarin present in the roots and that leaf-synthesized linamarin undoubtedly contributes to the accumulation of linamarin in the roots.

Many of the cyanogenic plants produce cyanogenic glucosides in sufficient quantities to be toxic and, as a result, are often avoided by herbivores (Nahrstedt, 1985; Jones, 1998). Nevertheless, there has been some controversy over the effectiveness of cyanogenesis as a herbivore defense system (Hruska, 1988; Gleadow and Woodrow, 2002). There a number of studies in which cyanogenic glucosides are shown to have little or no effect on herbivores (Scriber, 1978; Ferreira et al., 1997; Glander et al., 1989). In fact, *Phenacoccue manihot*, which feeds on cassava, actually uses linamarin as a phagostimulant (Calatayud and Le Ru, 1996; Mowat and Clawson, 1996).

Cyanogenic compounds also function in the metabolism and transport of nitrogen (Lieberei et al. 1985; Selmar et al., 1988, Selmar, 1993; Gleadow et al., 1998). This function of cyanogenic glucosides, however, should not be seen as an alternative to their role in herbivore defense.

Transport of cyanogens in cassava may be apoplastic as demonstrated in rubber tree (Selmar, 1988; Selmar, 1993) (For review of apoplastic cyanogen transport see section 1.4.3). In addition dhurrin-6-glucoside, the diglucoside of cyanogenic dhurrin, has been isolated from the apoplastic exudates of young seedlings of sorghum (Selmar et al., 1996), further strengthening the hypothesis that cyanogenic diglucosides are the
transportable form of cyanogens within some cyanogenic plants (see figure 1.6 for model of cyanogen transport).

Upon translocation of cyanogenic glucosides to the roots, linamarin is hydrolyzed to acetone cyanohydrin which may then spontaneously decompose to produce cyanide. The cyanide is then presumably converted to \( \beta \)-cyanoalanine, through a condensation reaction with cysteine, which is catalyzed by \( \beta \)-cyanoalanine synthase. \( \beta \)-cyanoalanine is then converted to asparagine by \( \beta \)-cyanoalanine hydrolase (Figure 1.7). Interestingly the highest \( \beta \)-cyanoalanine synthase activity on a protein basis was found in the roots (36 \( \mu \)g H\(_2\)S/min/100mg protein) and the least in the leaves of cassava (13 \( \mu \)g H\(_2\)S/min/100mg protein). Similarly, cassava root has a higher \( \beta \)-cyanoalanine hydrolase (25 \( \mu \)g ammonia/min/100mg protein) activity relative to leaf (8.8 \( \mu \)g ammonia/min/100mg protein) (Elias et al. 1997a,b). The conversion of cyanide to asparagine has been demonstrated using radiolabeled precursors in sorghum and cassava (Nartey, 1969, 1973). Asparagine then can be readily metabolized to aspartate and ammonia by the asparaginase catalyzed deamination reaction. Ammonia in turn is assimilated into amino acids via the glutamine synthetase/glutamate synthase cycle (Lea et al. 1990; 1992). Alternatively, it maybe hypothesized that acetone cyanohydrin which is produced by root linamarase activity, may be directly assimilated into amino acid(s). This possibility is intriguing since roots lack hydroxynitrile lyase and presumably would have slow rates of conversion of acetone cyanohydrin to cyanide. Currently, however, there is no known direct assimilatory pathway for acetone cyanohydrin to amino acids.
Complementing the importance of cyanogens as herbivore deterrents is the activity of cyanogens as a mobile nitrogen source. Mobility of cyanogens bestows upon cyanogenic plants the ability to respond swiftly to biotic and abiotic stress conditions by increasing the pool of available amino acids through the rapid conversion of cyanide to amino acids.
CHAPTER 4

OVER-EXPRESSION OF HYDROXYNITRILE LYASE IN TRANSGENIC CASSAVA (MANIHOT ESCULENTA, CRANTZ) ROOTS ACCELERATES CYANOGENESIS

4.1 INTRODUCTION

In humans, the ingestion of large quantities of improperly processed cassava food products has been associated with chronic cyanide disorders such as hyperthyroidism and tropical ataxic neuropathy, particularly in Africa (Tylleskar et al., 1992; Mlingi et al., 1992). Konzo, a permanent paralysis of the legs is the result of acute cyanide poisoning and has typically been associated with drought and famine when short-cut methods are used to process cassava. The effects of cyanide toxicity may also be acerbated during periods of drought when some cassava cultivars may have elevated cyanogen levels (Cardoso et al., 1999; Ernesto et al., 2002).

The biochemistry and physiology of cyanogenesis in cassava has been well characterized (reviewed in McMahon et al., 1995). Rupture of the vacuole initiates cyanogenesis by releasing linamarin which is then hydrolyzed by linamarase, a cell-wall localized β-glycosidase (McMahon et al., 1995). The deglycosylated product,
acetone cyanohydrin, can spontaneously decompose at pHs >5.0 or temperatures >35°C or is enzymatically broken down by hydroxynitrile lyase (HNL) to produce acetone and HCN (Cutler and Conn, 1981; Wajant et al., 1994, 1996; White et al., 1994, 1995, 1998; Yemm and Poulton, 1986; Zheng and Poulton, 1995). The HCN generated during hydrolysis is volatilized or it is extracted with water during processing and does not contribute to food toxicity. Since acetone cyanohydrin is chemically unstable it was generally thought that residual linamarin was the primary source of cyanide in processed cassava roots. In 1992, Tylleskar et al. demonstrated that the major cyanogen present in poorly processed cassava roots was acetone cyanohydrin and not linamarin.

Cassava HNL, with molecular weight of 29 kDa, has been shown to exist as a dimer from apoplast extracts of leaves (White et al., 1994). In 1994, Hughes et al. (1994) identified 1kb cDNA clone of HNL from cassava with an open reading frame encoding a protein of 258 amino acids (Figure 4.1). The cassava HNL and rubber tree HNL shared 78% amino acid similarity (Hasslacher et al., 1996). In 1996, White reported the identification of another HNL cDNA sequence from cassava. The cDNA was 1118 bp in length encoding a 258 amino acid protein. The amino acid sequence was identical to the Hughes et al. (1994) sequence with the exception of 13 amino acids. Closer analysis of these dissimilar 13 amino acids showed that 8 amino acids in White’s HNL sequence were similar to those of rubber tree HNL (Hasslacher et al. 1996) while Hughes’s HNL sequence was not. Southern blot analysis performed by both groups show the existence of one copy of the HNL gene in the cassava genome.
Later Hughes recognized that they had made a sequencing error and in fact the correct sequence was deposited in NCBI as identical to that described by White (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=1359930).

In 1998, using northern and western blots White et al. demonstrated that cassava roots have substantially lower levels of HNL (0-6%) than leaves (100%). The low level of root HNL activity was attributed to reduced transcription of the HNL gene in roots (White, 1996; White et al., 1998). The organ specific localization of HNL in cassava is identical to that found in sorghum and linen (Linum usitatissimum) (Wajant et al., 1994), where HNL is only present in the leaves and not in stems or roots. Due to the instability of the cyanohydrins in some plant species or even in specific organs cyanogenesis may occur only spontaneously.

It was hypothesized that the high acetone cyanohydrin levels present in processed cassava foods could be attributed to the absence of HNL activity in roots. We postulated that elevated expression of HNL in roots would accelerate the conversion of acetone cyanohydrin to cyanide and the detoxification of cassava foods by cyanide volatilization (from roots). We demonstrate here that the over-expression of HNL in roots of transgenic cassava plants does not affect the steady-state linamarin levels in intact roots but substantially reduces the accumulation of acetone cyanohydrin and accelerates its conversion to cyanide following tissue disruption. This will lead to a potentially safer food product.
**Figure 4.1:** Nucleotide and amino acid sequences of hydroxynitrile lyase (HNL) of cassava (from http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=1359930). Primers used for subsequent molecular analysis of the HNL transformant are marked in boxes (HNL5’ and HNL3’).
4.2 EXPERIMENTAL PROCEDURES

The following procedures were performed by Dr. Diana Arias-Garzon for the fulfillment of her Ph. D. thesis (Arias-Gazon, 1997):

- Vector construction (pKYLX-HNL) and transformation
- Cassava transformation using pKYLX-HNL
- PCR analysis of the putative transformed plants
- HNL enzymatic analyses of leaves
- Western blot analysis of HNL abundance in leaves
- Measurement of root linamarase activity

To complete this project, I performed the following experimental procedures:

- Southern blot verification of transgenic plants
- Measurement of root linamarin quantity
- Quantification of free cyanide and acetone cyanohydrin in roots following maceration

4.2.1 Vector construction and transformation

The modified Agrobacterium binary vector pKYLX-HNL was constructed for the expression of HNL gene in transgenic cassava (Manihot esculenta, Crantz). The original pKYLX binary vector contains an nptII gene for kanamycin/paromomycin resistance. A cassava hydroxynitrile lyase cDNA was cloned between the 2X35S promoter and a pea rubisco small subunit 3’ terminator to create pKYLX-HNL (Figure 161)
4.2). The modified binary vector was transformed into *Agrobacterium tumefaciens* strain LBA4404 (Life Technology, Grand Island, NY) by electroporation and used to transform germinated cassava somatic embryos (Arias-Garzon, 1997).

### 4.2.2 Cassava Transformation

Cassava ex-plant cultivation, somatic embryo generation, Agrobacterium mediated cassava transformation, and selection of transgenic plants were all performed according to methods described in chapter 2.

### 4.2.3 Molecular analysis

Genomic DNA was extracted from 30 mg of *in vitro* leaves of independent putative transformed and control plants (Sweeney et al., 1994). The presence of the *nptII* and the HNL cDNA genes was detected by polymerase chain reaction (PCR) amplification. PCR reactions were performed in a total volume of 50 µL containing: 5 µL of 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.4, 15 mM MgCl₂, 1 mg mL⁻¹ gelatin), 100 ng of leaf DNA of transformed and untransformed *in vitro* plants, 0.5 mM MgCl₂, 0.05 mM dNTP’s, 2.5 units Taq polymerase (Life Technology, Grand Island, NY), and 0.1 µM each of NOSNPT5’ (5’CCGCCGATGACGCGGGACAAGCC3’) and NOSNPT3’ (5’GGTCCGCCACACCCAGCCGGCCA3’) for amplification of the *nptII* gene or HNL5’ (5’AAAGTCGACATGGTAACTGCACATTTTGTT3’) and HNL3’
(5’AAAGAATTCTCAAGCATATGCATCAGCCAC3’) primers for amplification of the HNL gene (Figure 4.1). The reaction to amplify the nptII gene also contained 2.5 µL of DMSO. The DNA was amplified using 30 PCR cycles of 1 minute denaturation at 94°C, followed by 1 minute annealing at 64°C (nptII) or 55°C (HNL) and 1 minute extension at 72°C.

The absence of residual Agrobacterium contamination was tested in all plants by using two primers that amplify a 1093 bp fragment of the virA gene located outside of the T-DNA (Pavingerova et al., 1997) following a protocol described by Moore et al. (1992).

Southern blot analysis was performed on total DNA isolated from 8-month-old greenhouse grown plants according to Soni et al. (1994). The DNA (10 µg) was digested with XbaI, transferred to a nitrocellulose membrane (Sambrook et al., 1989). Hybridization was conducted at 42°C with 32P-labelled HNL cDNA probe. The probe was made using PCR primers HNL5’ and HNL3’ (Figure 4.2). PCR reaction was performed in a total volume of 50 µL containing: 1X PCR buffer, 100 ng of pKYLX-HNL vector DNA, 0.1 mM each dATP/dTTP/dGTP, 100 µCi of 32P-dCTP (Amersham, Piscataway, NJ), 2.5 units of Taq polymerase (Life Technology, Grand Island, NY), 0.4 µM each primer (IDT, Coralville, IA), 1.5 mM MgCl2. The DNA was amplified using 35 PCR cycles of 30 seconds at 94°C, 45 seconds at 65°C and 30 seconds at 72°C preceded by 3 minutes incubation at 94°C. The labeled probe was cleaned using Qiagen PCR cleaning kit (Qiagen Inc, Valencia, CA) and boiled for 5 minutes before the addition to the membrane. The membrane was washed at 50°C in
1X SSC buffer/0.1% (w/v) SDS once followed by 0.1X SSC buffer/0.1% (w/v) SDS twice (20 minutes per wash).

4.2.4 Crude protein extractions and HNL enzymatic analysis

In vitro cassava leaves from 5-month old putative transformed and untransformed plants (100 mg) were frozen in liquid nitrogen and ground with a mortar and pestle in 0.5 mL of 0.05 M sodium phosphate buffer pH 5.0, 3 mM DTT, 1% (w/v) polyvinyl pyrrolidine at 4°C and filtered through miracloth. The cell wall material was pelleted by centrifugation at 13,000 x g for 15 minutes at 4°C. The supernatant was collected and centrifuged again at 13,000 x g for 5 minutes at 4°C to remove residual debris. Supernatant protein concentrations were determined by the bicinchoninic acid (BCA) method (Akins and Tuan, 1992) using bovine serum albumin as a standard.

Hydroxynitrile lyase assays were performed in a final volume of 1 mL containing 50 mM sodium phosphate buffer pH 5.0, 20 μg total leaf protein, and 28 mM acetone cyanohydrin (Sigma). After 30 minutes incubation at 28°C in capped tubes, 10 μL of the reaction mixture was added to 20 mL of 50 mM sodium phosphate pH 4.0 and HCN was determined using the Spectroquant 14800 cyanide detection kit (EM Science, Gibbstown, NJ). A reaction with no cassava protein added was also carried out to determine the rate of spontaneous acetone cyanohydrin breakdown. This value was subtracted from the enzyme-catalyzed reactions.
4.2.5 Western Blot analysis

Western blot analysis was performed using 15 μg of soluble leaf protein separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to Inmobilon-P (Millipore) using a Sartorius semi-dry horizontal electroblotter at 20V for 1.5 hours. Immunoblots were performed according to the method of Harlow and Lane (1988). Polyclonal antibodies were raised against purified HNL by the Ohio State University Antibody Center (Columbus, OH). Cross-reacted bands were visualized using goat anti-mouse IgG alkaline phosphatase conjugate (Promega, Madison, WI) and a colorimetric assay according to the procedure of Harlow and Lane (1988).

4.2.6 Quantification of free cyanide and acetone cyanohydrin in roots following maceration

Eight month old greenhouse grown transgenic and wild-type plants were used for cyanide and acetone cyanohydrin assays. Root parenchyma (1 gram) was homogenized in 5 mL of 0.1 M sodium phosphate buffer pH 5.0 (the low pH was used to stabilize acetone cyanohydrin and reduce its spontaneous breakdown) for 30 seconds and incubated at 30°C for 0 to 120 minutes in capped tubes. Starch was pelleted and removed by centrifugation at 7,500 x g for 2 minutes. The supernatant was immediately subjected to two assays using the spectroquant cyanide assay.
described above. Liberated cyanide, a measure of acetone cyanohydrin decomposition, was measured by adding 0.5 mL of supernatant to 3.5 mL of 0.1 M sodium phosphate pH 5.0, followed by cyanide quantification using the spectroquant assay. Total acetone cyanohydrin plus cyanide was determined by adding 0.1 mL of supernatant to 0.6 mL of 0.2M NaOH and 3.3 mL of 0.1 mM sodium phosphate buffer pH 5.0, followed by cyanide quantification using the spectroquant assay (O’Briens, 1991). The addition of NaOH converts all acetone cyanohydrin into free cyanide. The amount of acetone cyanohydrin present in the HNL transformants was calculated as the difference between the two assays \{(acetone cyanohydrin + cyanide assay) – (cyanide assay)\}.

**4.2.7 Measurement of root linamarase activity and linamarin content**

The crude linamarase activity of roots was assayed using \(\rho\)-nitrophenol-\(\beta\)-glucopyranoside as a substrate according to Mkpong et al. (1990). Root linamarin was quantified by homogenizing 1g of roots in 5 mL of 0.1 M sodium phosphate pH 4.0 followed by pelleting starch at 7,500 x g for 2 minutes. The supernatant was then boiled for 5 minutes to normalize all samples for the linamarase subsequently added. A 20-fold excess of isolated cassava linamarase (total linamarase activity added equals, 1.0 mmole glucose hr\(^{-1}\)) was added to the root extract followed by incubation at 30°C in closed tubes for 5 minutes. The resulting acetone cyanohydrin plus cyanide was quantified as described above. Exogenously added cassava linamarase was purified by homogenizing 1g of leaves in 0.1 M sodium phosphate (pH 3.5) followed
by centrifugation at 24,000 X g for 30 minutes (Mkpong et al., 1990). The proteins in
the supernatant were precipitated by addition of ammonium sulfate to 40% saturation
followed by dialysis against 0.05 M sodium phosphate (pH 7.0). Linamarase was
isolated by separating the proteins on a Sepharose S-200 column in a buffer containing
0.1M sodium phosphate (pH 7.0) and 200 mM NaCl using a Bio-Rad Econo System
chromatography system (Hercules, CA). Linamarase was the first protein peak that
eluted from the column (data not shown).
4.3 RESULTS AND DISCUSSION

4.3.1 Plant transformation efficiency

Transgenic cassava plants were generated by *Agrobacterium*-mediated transformation of somatic embryos with pKYLX-HNL (Figure 4.2) and using paromomycin as a selective agent. Seventy-seven independent paromomycin-resistant putative transformants were obtained from transformation of 2447 explants for an apparent transformation efficiency of 3.6 % (based on paromomycin resistance) (Arias-Garzon, 1997).

4.3.2 Molecular analysis of the transformants

Three of the paromomycin-resistant HNL transformed plants are described in this chapter. To verify integration of the T-DNA into the paromomycin-resistant plants the presence of the *nptII* gene and HNL cDNA was confirmed by PCR analysis (Figure 4.3). Amplification of genomic DNA yielded a 850 bp DNA fragment from the *nos* promoter-*nptII* cassette using NOSNPT5’ and NOSNPT3’ primers. In addition, an 800 bp fragment corresponding to the HNL cDNA was amplified by PCR using the HNL5’ and HNL3’ primers (Figures 4.1 and 4.3). The identity of each band was confirmed by DNA sequence analysis. Genomic DNA obtained from untransformed cassava did not amplify the endogenous HNL gene presumably due to the presence of a 7.0 kb intron (White, 1996).
In order to determine whether Agrobacterium cells were present in the transformed plants, primers specific for the \textit{virA} gene of \textit{Agrobacterium} were used to amplify this gene by PCR. The \textit{virA} gene is present in the Ti plasmid but is not transferred to the plant genome by the T-DNA. The \textit{virA} primers successfully amplified the \textit{virA} from \textit{Agrobacterium} but no \textit{virA} PCR products were obtained from any of the cassava transformants (data not shown). The presence of the \textit{nptII} gene and the HNL cDNA gene in the transformants coupled with the absence of the \textit{virA} gene demonstrates integration of the T-DNA in the transformants.

These results were further confirmed by Southern blot analysis of cassava DNA probed with the HNL cDNA. Restriction enzyme \textit{XbaI} used in the Southern blot analysis cleaves the T-DNA between the HNL gene and rubisco terminator. The results of the Southern blot demonstrate the independent integration of the T-DNA (HNL cDNA) into each transformant (Figure 4.4). Transformant HNL-2 had the largest number of T-DNA integration events with three apparent copies of the HNL gene integrated into the genome. The HNL-1 and HNL-3 transformants each had one apparent HNL T-DNA copy integrated into the genome.
Figure 4.2: T-DNA region of the modified binary vector pKYLX-HNL (13 Kb) used for stable transformation of cassava. HNL gene is flanked by a 2X35S CaMV promoter and a rubisco terminator. Probe refers to the HNL gene sequence used for the Southern blot analysis. The location of the relevant PCR primers is noted.
**Figure 4.3:** Amplification of 850bp *nos* promoter-*nptII* gene fragment (A) and 800bp HNL cDNA (B) from genomic DNA isolated from transformed (HNL-1 to 3) and untransformed plants (WT). For PCR conditions see methods. M equals DNA size markers.
**Figure 4.4:** Southern blot analysis of HNL transformed and untransformed plants. The analysis was performed on 10 µg of greenhouse grown cassava leaf DNA restricted with *XbaI* and probed with $^{32}$P-labelled HNL cDNA.
4.3.3 Root HNL protein levels and leaf HNL enzyme activity

Previously, we have demonstrated that HNL was not detectable in cassava roots either by western blot analysis or by enzyme assay (White et al., 1998). As a result of the absence of detectable HNL in wild-type roots and the low overall protein levels present in roots we compared HNL protein levels and activities using leaves of wild-type and transgenic plants. Our justification for using leaves was the expectation that the 35S CaMV promoter, used to drive the expression of the HNL gene, would be as strongly expressed in leaves as in roots. In addition, we have previously determined by transient GUS assays using particle gun bombarded cassava leaves and roots that the 2X 35S CaMV promoter drives gene expression equally well in leaves as in roots (data not shown). As shown in Figure 4.5, western blot analysis indicated that the HNL level of each transformant was elevated relative to untransformed plants. Significantly, the protein loadings (15 µg protein) used for the western blot analyses had previously been shown to give a linear response to HNL by immuno-detection (White et al., 1998) (Figure 4.5). To compare the HNL protein levels present in leaves the western blot was scanned and analyzed by densitometry. HNL levels present in leaves of transgenic lines HNL-1, HNL-2 and HNL-3 were 1.2, 2.3 and 1.4-fold greater than those observed in untransformed plants, respectively (Figure 5). Interestingly, the highest level of HNL protein was observed in transformant HNL-2 which also had the greatest number of HNL gene copies (3 versus 1) integrated into its genome.
Similarly, we compared the HNL activity of leaves from wild-type and transformed plants. The hydroxynitrile lyase activity of untransformed plant leaves was 1.7 mmol HCN mg protein$^{-1}$ hr$^{-1}$. This value is about three-fold higher than for leaf crude extracts than previously reported and reflects an improvement in the cyanide detection assay. Significantly, the HNL activity of crude leaf extracts of transformants HNL-1, HNL-2 and HNL-3 was 2.4, 4.0 and 3.8 mmol HCN mg protein$^{-1}$ hr$^{-1}$, respectively, representing a 40-135% increase in HNL activity (Figures 4.6). Interestingly these increases in HNL activity in the respective transgenic strains reflected the increases observed in HNL protein abundance (Figures 4.5).
**Figure 4.5:** Two immunoblots of hydroxynitrile lyase using 15 µg total protein from transformed and untransformed leaf tissue.
Figure 4.6: Hydroxynitrile lyase activity in wild-type and transformed leaves.
4.3.4 Reduction of acetone cyanohydrin levels in homogenized roots of transgenic plants

As previously indicated, the major cyanogen present in poorly processed cassava roots is acetone cyanohydrin (Tylleskar et al., 1992). The substantially lower levels of HNL in roots compared to leaves (White et al., 1998) is thought to account for the high acetone cyanohydrin levels present in processed cassava foods. To determine whether the over-expression of HNL in transgenic plants enhanced root cyanogenesis (conversion of acetone cyanohydrin to cyanide) we measured residual acetone cyanohydrin and cyanide in homogenized roots as a function of incubation time post homogenization. Under these conditions the cell wall localized linamarase will convert linamarin into acetone cyanohydrin which will either accumulate over time at the low pH (5.0) used to homogenize wild-type roots or decompose to cyanide in transgenic plants due to the expression of HNL.

The data for the analysis of acetone cyanohydrin levels of 8 month-old green house grown wild-type and transgenic cassava roots is shown in table 4.1. The average acetone cyanohydrin present 0-120 minutes after homogenization, as a percentage of wild-type levels, is shown in graphical form in figure 4.7. After 60 minutes of 30°C incubation time the three transgenic roots had acetone cyanohydrin levels that were 41 to 65% lower than those of wild-type cassava roots. This reduction in residual acetone cyanohydrin present was even more apparent at 120 minute incubation following homogenization. After 120 minutes the levels of acetone cyanohydrin present in the
transgenic roots were 35 to 58% of wild-type levels (Table 4.1 and Figure 4.7). The smallest reduction (58%) in acetone cyanohydrin levels after 120 minutes of 30°C incubation time is transformant HNL-3.

After incubation at 30°C post homogenization, the greatest reduction in residual acetone cyanohydrin was observed in the roots of transformant HNL-2. When compared to wild-type levels, there was a 65% reduction in acetone cyanohydrin levels (35% of what is present in wild-type; Figure 4.7). Consistent with this observation, HNL-2 had the highest number of T-DNA integrations (Figure 4.4) and highest leaf HNL enzyme activity (4.0 mmol HCN mg protein^{-1} hr^{-1}; Figure 4.6) of the three transformants.
<table>
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<th>Trial #3</th>
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B. HNL-1

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C. HNL-2

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D. HNL-3

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Table 4.1: Root acetone cyanohydrin quantity evaluation of 8-month-old green house grown wild-type (A) and transgenic, HNL-1 (B), HNL-2 (C), HNL-3 (D), cassava plants. Minutes refer to the 30°C incubation time after maceration.
Figure 4.7: Average acetone cyanohydrin present in the 8 month-old HNL transgenic cassava roots compared to wild-type roots homogenized under similar conditions. Acetone cyanohydrin levels of transformants are shown as a percentage level present in wild-type cassava.
As shown in figure 4.8, transformed roots from plants having elevated HNL levels in their leaves had nearly three-fold lower levels of acetone cyanohydrin (0.98 µmoles acetone cyanohydrin gfw⁻¹, HNL-2) than roots from untransformed plants (2.81 µmoles acetone cyanohydrin gfw⁻¹ after 120 minutes incubation at 30°C). Significantly, the linamarin content (3.52 – 3.78 µmoles gfw⁻¹) and linamarase activity (1.3 mmoles p-nitrophenol mg protein⁻¹ hour⁻¹) of the transformed and untransformed plants were identical. Therefore, the observed differences in acetone cyanohydrin levels between wild-type and transgenic roots (following homogenization) could not be attributed to strain-dependent differences in acetone cyanohydrin production. Furthermore, the relative reduction in acetone cyanohydrin levels in homogenized roots was correlated with the relative leaf HNL activity measured in transgenic and wild-type plants (Figures 4.5 and 4.6). In fact after the first assay (30 minutes post homogenization) there was virtually no net increase in acetone cyanohydrin levels in transformant HNL-2 (Figures 4.8). These results demonstrate that expression of HNL driven by the 35S CaMV promoter accelerates root cyanogenesis reducing cyanogen toxicity.
**Figure 4.8:** Acetone cyanohydrin levels in homogenized roots from wild-type and transformed eight month-old greenhouse grown plants. ACN equals acetone cyanohydrin.
With the importance of cyanogenic glucoside as a mobile form of nitrogen not thoroughly studied, it might be crucial for the well being of a cassava plant to have a normal movement of cyanogenic glucosides within the plant. These HNL transformants do not have altered cyanogenic glucoside contents and therefore nitrogen transport via linamarin is likely to be unaltered. In fact these high HNL expressing plants can be easily transferred to and grow in soil unlike the linamarin-free plants which apparently cannot supply reduced nitrogen to the roots.

Prior to rupturing of the edible root cells, these HNL transformants have similar linamarin and acetone cyanohydrin levels as untransformed cassava, thus, giving them the same herbivore deterrent capabilities. In the past it was thought that all of the residual cyanogen present in cassava foods was in the form of linamarin. More recently it has been demonstrated that the major cyanogen present in poorly processed cassava roots was indeed acetone cyanohydrin, not linamarin (Tylleskar et al., 1992). The presence of higher levels of HNL in the root provides a faster conversion of acetone cyanohydrin to cyanide. Processing of HNL transgenic cassava as a food source will lead to quicker and more thorough detoxification of cyanide, producing a safer food product for human consumption.
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