

Engineering cyanogen synthesis and turnover in cassava (*Manihot esculenta*)

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Abstract

Cassava is the major root crop for a quarter billion subsistence farmers in sub-Saharan Africa. It is valued for its ability to grow in adverse environments and the food security it provides. Cassava contains potentially toxic levels of cyanogenic glycosides (linamarin) which protect the plant from herbivory and theft. The cyanogens, including linamarin and its deglycosylated product, acetone cyanohydrin, can be efficiently removed from the root by various processing procedures. Short-cuts in processing, which may occur during famines, can result in only partial removal of cyanogens. Residual cyanogens in cassava foods may cause neurological disorders or paralysis, particularly in nutritionally compromised individuals. To address this problem and to further understand the function of cyanogenic glycosides in cassava, we have generated transgenic cassava in which cyanogenic glycoside synthesis has been selectively inhibited in leaves and roots by antisense expression of *CYP79D1/D2* gene fragments. The *CYP79D1/D2* genes encode two highly similar cytochrome P450s that catalyze the first-dedicated step in cyanogenic glycoside synthesis. Transgenic plants in which the expression of these genes was selectively inhibited in leaves had substantially reduced (60–94% reduction) linamarin leaf levels. Surprisingly, these plants also had a greater than a 99% reduction in root linamarin content. In contrast, transgenic plants in which the *CYP79D1/D2* transcripts were reduced to non-detectable levels in roots had normal root linamarin levels. These results demonstrate that linamarin synthesized in leaves is transported to the roots and accounts for nearly all of the root linamarin content. Importantly, transgenic plants having reduced leaf and root linamarin content were unable to grow in the absence of reduced nitrogen (NH_3). Cassava roots have previously been demonstrated to have an active cyanide assimilation pathway leading to the synthesis of amino acids. We propose that cyanide derived from linamarin is a major source of reduced nitrogen for cassava root protein synthesis. Disruption of linamarin transport from leaves in *CYP79D1/D2* anti-sense plants prevents the growth of cassava roots in the absence of an alternate source of reduced nitrogen. An alternative strategy for reducing cyanogen toxicity in cassava foods is to accelerate cyanogenesis and cyanide volatilization during food processing. To achieve this objective, we have expressed the leaf-specific enzyme hydroxynitrile lyase (HNL) in roots. HNL catalyzes the breakdown of acetone cyanohydrin to cyanide. Expression of HNL in roots accelerated cyanogenesis by more than three-fold substantially reducing the accumulation of acetone cyanohydrin during processing relative to wild-type roots.

Introduction

Cassava is one of the major root starch crops grown in the tropics. While native to South America, 51% of world's cassava is currently

grown in sub-Saharan Africa (Nweke *et al.*, 2002; Scott *et al.*, 2002). Due to its drought tolerance, ability to grow in poor soils, and resistance to herbivory cassava is well suited for cultivation by subsistence farmers (Nweke *et al.*, 2002). In

addition, cassava roots can persist in the soil for 1–2 years without decaying, providing food security especially during periods of drought (Nweke *et al.*, 2002). Cassava leaves also are consumed by many African cultures and are an excellent source of protein and vitamins (Bokanga 1994). In Africa, however, cassava rarely reaches its yield potential. This is due primarily to disease, and limited fertilizer and irrigation inputs. In addition, investments in breeding programs to enhance yield and food quality have been substantially less than those dedicated to other crops (Byrne, 1984; Dixon *et al.*, 1994; Kawano *et al.*, 1998).

Cyanogenesis in cassava

The leaves and roots of cassava contain potentially toxic levels of cyanogenic glycosides [linamarin (95%) and lotaustralin (5%)] (Conn, 1979, 1994; Balagopalan *et al.*, 1988). These cyanogens (linamarin, lotaustralin and acetone cyanohydrin, the deglycosylated form of linamarin) have been demonstrated to protect the plant from herbivory by animals and generalized insect feeders as well as protect cassava from theft (Nahrstedt, 1985; Bellotti and Arias, 1993; Bellotti and Riss, 1994; Nweke *et al.*, 2002). Significantly, cyanogen levels in leaves (200–1,300 mg CN equivalents/kg dry weight) and roots (10–500 mg CN equivalents/kg dry weight) are higher than the maximum levels (10 mg CN equivalents/kg dry weight) recommended for foods by the FAO. Therefore, cassava foods must be processed to remove cyanogens prior to consumption. In Africa, a number of cyanide-associated health disorders have been attributed to eating poorly processed cassava, particularly by nutritionally compromised (low protein intake) individuals (Cliff *et al.*, 1985; Delange *et al.*, 1994). The severity of these disorders depends on the level and frequency of cyanogen exposure and the state of nutrition of the consumer. Chronic, low-level cyanide exposure resulting from eating poorly processed cassava has been associated with the development of goiter and tropical ataxic neuropathy (Osuntokun, 1981; Tylleskar *et al.*, 1992; Oluwole *et al.*, 2000). Acute cyanogen poisoning can result from eating poorly processed high-cyanogen cassava varieties and is most commonly associated with famines. Acute cyanide poisoning can cause Konzo, a paralytic

disorder, and in some cases death (Howlett *et al.*, 1990; Mlingi *et al.*, 1991; Akintonwa and Tunwashe, 1992; Ernesto *et al.*, 2002; Sreeja *et al.*, 2003). When properly processed, however, cassava is a safe, valuable and secure crop, accounting for up to 40% of the caloric intake of sub-Saharan Africans (Nweke *et al.*, 2002).

To reduce the cyanogen content of cassava foods the roots and leaves must be thoroughly processed (Figure 1). Processing typically involves pounding or tissue maceration (Nweke *et al.*, 2002). Tissue disruption releases linamarin from the vacuole facilitating its de-glycosylation by linamarase, a cell wall and/or laticifer-localized β -glucosidase (Mkpong *et al.*, 1990; Hughes *et al.*, 1992; McMahon *et al.*, 1995). The cyanogenic product, acetone cyanohydrin, is then decomposed to yield cyanide and acetone. Cyanide generation from acetone cyanohydrin may occur spontaneously at pHs > 5.0 or temperatures > 35 °C, or is catalyzed by the apoplastic and leaf-specific enzyme, hydroxynitrile lyase (HNL) (Hughes *et al.*, 1994; White *et al.*, 1994). The free cyanide is then



Figure 1. Cooking and cyanide volatilization is the last step in gari (fermented cassava root starch) preparation in Nigeria.

extracted with water or volatilized into the atmosphere to complete the detoxification process. In leaves, linamarase and HNL have similar catalytic efficiencies and protein levels providing an efficient mechanism for cyanogenesis and food detoxification (McMahon *et al.*, 1995, White *et al.*, 1998). Cassava roots, however, have much lower linamarase activities than leaves and no HNL activity, presumably accounting for the accumulation of potentially toxic levels of acetone cyanohydrin in poorly processed cassava roots (White *et al.*, 1994, 1998).

At the elevated pHs and temperatures present in the human body acetone cyanohydrin will rapidly decompose to release cyanide. The released cyanide is bound primarily by met-hemoglobin until it becomes saturated (Lundquist *et al.*, 1985; Rosling *et al.*, 1993). Unbound cyanide is converted to the less toxic product isothiocyanate *via* the enzyme rhodanese and is excreted in the urine. Importantly, the synthesis of isothiocyanate requires cysteine (reduced sulphur) which may be limiting for nutritionally compromised individuals thus exacerbating the effects of cyanide poisoning (Cliff *et al.*, 1985; Rosling, 1994).

Many subsistence cultures prefer high-cyanogen cassava varieties. The reasons for cultivating more toxic varieties include, taste preference, reduction in herbivory, and protection against theft (Cock, 1985; Best and Hargrove, 1994, Nweke *et al.*, 2002). In many regions of Africa both high- and low-cyanogen cassava varieties are inter-planted to provide food security (high-cyanogen varieties) as well as to provide a more readily consumable or marketable cassava food products (low-cyanogen varieties). For cassava to become a routinely safe and acceptable food and cash crop it is necessary to insure the reduction of cyanogens to safe levels. While this objective can be achieved by proper processing, even with the most cyanogenic cultivars, it also may be possible to achieve this objective by developing cyanogen-free cultivars or cultivars that eliminate cyanogens and volatilize cyanide more efficiently.

Transgenic strategies for reducing cyanogens in cassava

Recently, the genes encoding a small family of cytochrome P450s (*CYP79D1* and *CYP79D2*) that

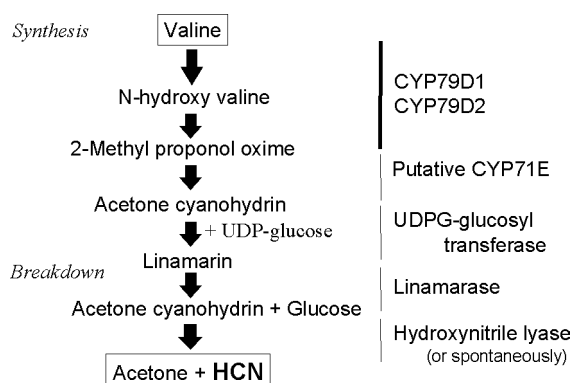


Figure 2. Cyanogenic glycoside (linamarin) synthesis and turnover pathways in cassava.

catalyze the first-dedicated step in linamarin and lotaustralin synthesis were isolated using a yeast complementation system (Andersen *et al.*, 2000). The *CYP79D1/D2* proteins are highly similar (85%), multifunctional enzymes that catalyze the conversion of either valine (95%, high affinity substrate) or isoleucine (5%, low affinity substrate) to their respective oximes. Subsequently, a second cytochrome P450 (*CYP71E*) converts the oxime to the respective nitrile (Bak *et al.*, 2002). The final step in the synthesis of linamarin is the addition of glucose to acetone cyanohydrin catalyzed by an UDP-glucosyl transferase. The linamarin biosynthetic and cyanogenesis pathways are summarized in Figure 2.

We have explored two strategies to reduce the cyanogen toxicity of cassava food products; (a) inhibition of the expression of the *CYP79D1/D2* genes and (b) over-expression of HNL in roots. The objective of the first strategy was to block linamarin synthesis and the objective of the second strategy was to accelerate cyanogenesis and cyanide volatilization during processing.

Inhibition of linamarin synthesis

Previous work on the synthesis of linamarin in cassava indicated that both leaves and roots were capable of linamarin synthesis (McMahon and Sayre, 1994; Du *et al.*, 1995; McMahon, 1997). It had also been shown by several investigators that linamarin can be transported from the leaves to the roots (Bediako *et al.*, 1981; Makame *et al.*, 1987; Selmar *et al.*, 1988; Koch *et al.*, 1992; Selmar,

1994). To determine the most effective strategy for reducing root linamarin content and to characterize the role of linamarin transport in determining root linamarin levels we generated transgenic cassava in which the expression of the *CYP79D1/D2* genes was selectively inhibited in leaves or roots only (Siritunga, 2002; Siritunga and Sayre, 2003). The tissue-specific inhibition of *CYP79D1/D2* expression was accomplished by the antisense expression of the *CYP79D1/D2* genes driven either by the leaf- or root-specific, *cab1* and patatin promoters, respectively (Brusslan and Tobin, 1992, Kim *et al.*, 1994; Zoureliduo *et al.*, 2002). Siritunga and Sayre (2003) detailed the generation of transgenic *cab1-CYP79D1/D2* antisense cassava plants by *Agrobacterium*-mediated transformation of somatic embryos. Integration of the T-DNA was confirmed by PCR and Southern blot analysis while RT-PCR analyses of *CYP79D1/D2* transcript abundance in *cab1-CYP79D1/D2* antisense plants indicated that *CYP79D1/D2* transcript levels could be reduced to intermediate or non-detectable levels in leaves. As expected *CYP79D1/D2* transcript levels were unaltered in roots.

CYP79D1/D2 transcript levels have also been reduced to non-detectable levels in transgenic roots in which the 5' ends (650 bp) of the *CYP79D1* and *CYP79D2* genes were expressed in the reverse orientation (antisense) under the control of the tuber-specific, patatin promoter. Molecular analysis for three paromomycin-resistant patatin-*CYP79* plants (Pat-1, Pat-2 and Pat-3) is described in this paper. Each of the putative *CYP79D1/D2* antisense transformants and wild-type plants was obtained from a unique explant regenerated from germinating somatic embryos (minus paromomycin selection for wild-type plants) using standard procedures developed in our lab (Siritunga and Sayre, 2003). Transgenic plants containing the patatin-*CYP79D1/D2* antisense constructs were identified and confirmed by PCR amplification and DNA sequence analysis of the *nptII* and the truncated *CYP79D1* gene (data not shown). The absence of *Agrobacterium* contamination was verified by PCR using *Agrobacterium VirG* specific PCR primers. The *VirG* gene is present in the Ti-plasmid but is not transferred to the plant genome by the T-DNA. The *VirG* primers successfully amplified the *VirG* gene from *Agrobacterium* but no *VirG* PCR products were obtained from any of the cassava transformants

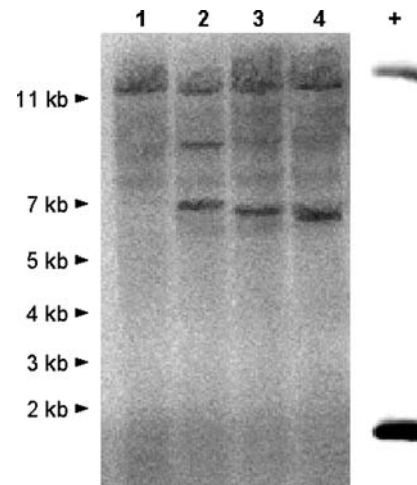


Figure 3. Southern blot analysis of wild-type and patatin-*CYP79D1/D2* antisense transgenic plants. 10 μ g of leaf DNA was digested with *SalI* and hybridized with the *CYP79D1* gene. Lane 1, wild-type DNA; Lanes 2-4, transformants Pat-1, Pat-2 and Pat-3, respectively. Lane marked '+' is the modified binary vector restricted with *EcoRI* to release the 1.9 kb cassette of patatin promoter:*CYP79D1*:terminator.

(data not shown). The transformation of cassava was further confirmed by Southern blot analysis of cassava DNA probed with radio-labeled *CYP79D1* cDNA (Figure 3). Two of the transformants described here had one T-DNA integration event, while the Pat-1 transformant had two copies of the *CYP79* gene integrated into the genome.

RT-PCR analysis of *CYP79D1* and *CYP79D2* transcript levels using DNA primers complementary to the 3' end of *CYP79D1* and *CYP79D2* transcripts shows the apparent absence of *CYP79D1/D2* transcripts in the roots of Pat-1, -2 and -3 transgenic cassava plants (Figure 4A). These primers do not anneal to the 5' portion of the genes used in the anti-sense construct and therefore would not amplify the T-DNA. In addition, control PCR experiments lacking reverse transcriptase gave no PCR products indicating the products generated by RT-PCR were not generated from DNA templates (data not shown). The cassava starch branching enzyme-II (SBE-II) RT-PCR product levels were used to normalize the mRNA template used for RT-PCR amplification. Similar RT-PCR analyses performed using total RNA isolated from leaves shows near wild-type levels of the *CYP79D1* and *CYP79D2* transcripts indicating that the patatin-*CYP79D1/D2* antisense

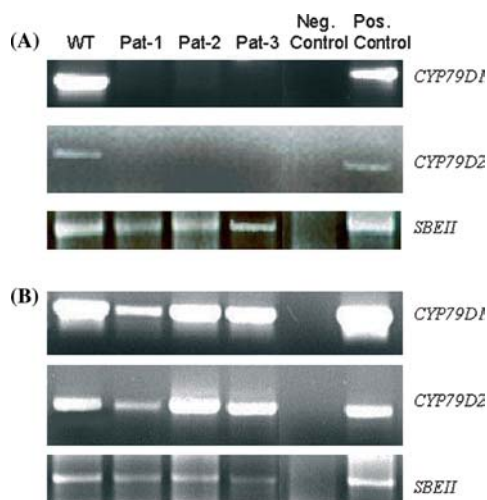


Figure 4. RT-PCR amplification of the *CYP79D1* and *CYP79D2* transcripts from roots (A) and leaves (B) of wild-type and patatin-*CYP79D1/D2* antisense transgenic plants. The primers used were specific for the 3' end of *CYP79D1* (GCTAAATCAACCAGAAATCCTGAAG and TGCAAGA GAAACAAGATAACCCC) and *CYP79D2* (CTGATAAAT CAACCAGAACTTCTGGCA and CTAACAACATCATCCCTCCC) genes. Control RT-PCR amplification of the starch branching enzyme II (*SBEII*) transcript was performed in parallel. Negative control: no DNA, Positive control: PCR amplification of the *CYP79D1* or *CYP79D2* gene.

constructs did not affect *CYP79D1/D2* steady-state transcript levels in leaves (Figure 4B).

Three to four-month old *in vitro* plants were used for linamarin quantification by GC-MS (Siritunga and Sayre, 2003). The leaf and root steady-state linamarin levels were unaltered in patatin-*CYP79D1/D2* transformants in which *CYP79D1/D2* transcript steady-state levels were selectively reduced to non-detectable levels in roots (Figure 5B). In comparison, the leaf linamarin content of Cab1-*CYP79D1/D2* transformants,

having substantially reduced *CYP79D1/D2* transcripts levels in leaves, was reduced between 60% and 94% (Siritunga and Sayre, 2003). The cab-1 *CYP79D1/D2* anti-sense transformants had a greater reduction in root linamarin content, to less than 1% of wild-type levels (Figure 5A) (Siritunga and Sayre, 2003). This reduction in root linamarin content was not associated with a reduction in root *CYP79D1/D2* transcript levels. These results demonstrated that in 3–4 month old cassava plants a reduction in leaf cyanogens levels causes a reduction in root cyanogen levels.

The cab1-*CYP79D1/D2* antisense plants exhibited normal growth patterns when grown (*in vitro*) in MS salts containing both nitrate (40 mM) and reduced nitrogen (20 mM NH_3) but failed to produce roots when grown on MS media in which the ammonia was replaced with nitrate (60 mM N) (Siritunga, 2002). In addition, cab1-*CYP79D1/D2* antisense plants all died when transferred to potting soil lacking reduced nitrogen. In contrast, patatin-*CYP79D1/D2* antisense plants grew normally when grown in modified MS media lacking ammonia or in potting soil. These results suggested that linamarin synthesized in leaves was transported to roots as a source of reduced nitrogen. Consistent with this hypothesis it has been reported that the total nitrogen present in linamarin is 2.4-fold higher than that present in other nitrogen-containing compounds in phloem exudates (Calatayud and Le Ru, 1996).

A possible biochemical pathway for the conversion of linamarin into asparagine is shown in Figure 6. According to this model, cyanide generated from linamarin is re-assimilated by β -cyanoalanine synthase and hydrated to produce asparagine. Consistent with this model, the activities of β -cyanoalanine synthase and β -cyanoalanine

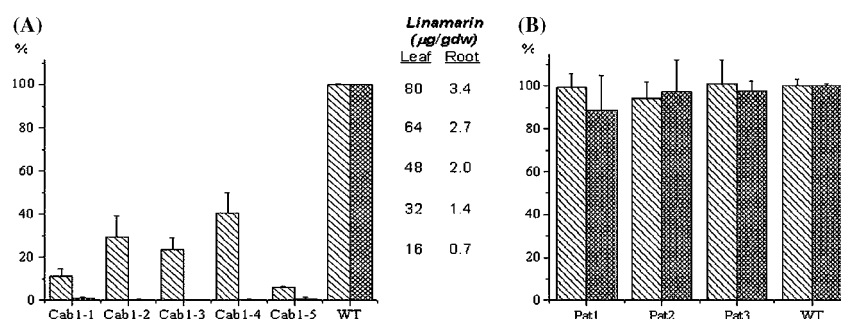


Figure 5. Linamarin content of roots (▨) and leaves (▧) of transgenic cassava plants in which the expression of the *CYP79D1/D2* genes was selectively inhibited in leaves (A) or roots (B).

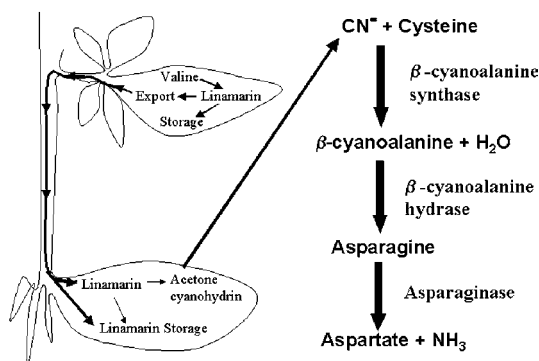


Figure 6. Proposed pathway for the transport of linamarin to roots and its metabolism to produce asparagine.

hydrase are 1.5 to 3-fold greater in roots than in leaves (Elias *et al.*, 1997a,b). These elevated cyanide assimilatory enzyme activities in roots are even more remarkable given the fact that roots have only 20% of the protein content of leaves.

The assimilation of cyanide into amino acids (asparagine and aspartate) in cassava has been previously demonstrated using ^{14}CN (Nartey, 1969). Nartey observed that germinating cassava seedlings exposed to ^{14}CN incorporated 49% of the radioactive label into the amide C of asparagine and 6% in aspartate. In addition, some label was found incorporated in glutamine and glutamate. The deamination of asparagine to produce aspartate and ammonia is significant since the ammonia can be assimilated into other amino acids *via* the glutamine synthetase/glutamate synthase cycle (Lea *et al.*, 1990, 1992). Alternatively, it may be 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 HNL and presumably would have slow rates of conversion of acetone cyanohydrin to cyanide. Currently, however, there is no known pathway for direct assimilation of acetone cyanohydrin into amino acids.

To generate a cassava plant having cyanogen-free roots and which has sufficient linamarin to support amino acid synthesis may require producing plants that either have sufficient linamarin transport levels to support amino acid synthesis but not linamarin accumulation or that partition all or most of the linamarin transported to the roots towards amino acid synthesis.

Over-expression of HNL in roots

An alternative strategy to reduce residual cyanogens in roots is to accelerate cyanogenesis and cyanide volatilization during root processing (Siritunga *et al.*, 2004). Analyses of cassava food products in Africa demonstrated that the cyanide toxicity increased substantially when short-cut processing technologies were used instead of traditional, long-term processing approaches (Tylleskar *et al.*, 1992). The cyanide toxicity associated with poorly processed cassava was largely attributed to residual acetone cyanohydrin. No free cyanide was detected in any cassava food products regardless of the efficiency of processing. These results were unexpected since it was assumed that cassava roots contained HNL. In 1998, we reported that cassava roots have virtually no HNL activity accounting for the high acetone cyanohydrin levels in poorly processed cassava roots (White *et al.*, 1998). To accelerate the conversion of acetone cyanohydrin to cyanide we generated transgenic cassava in which we over-expressed HNL in leaves (2-fold increase) and roots (13-fold increase) (Siritunga *et al.*, 2004). The transgenic plants were generated by *Agrobacterium*, Ti-plasmid mediated transformation. The expression of the cassava HNL transgene was driven by the 35S promoter. Under conditions favorable for acetone cyanohydrin accumulation, i.e. low pH (5.0) and moderate temperatures 25 °C, we observed that 80% of the root linamarin was converted to acetone cyanohydrin within 2 h following tissue maceration. In wild-type plants none of the acetone cyanohydrin was converted to cyanide under these conditions. In contrast, acetone cyanohydrin levels were substantially reduced in transgenic plants expressing HNL in their roots. The reduced acetone cyanohydrin levels in HNL-expressing plants was the result of HNL catalyzed cyanide generation (Figure 7). Importantly, plants over-expressing HNL in their roots had wild-type levels of linamarin and linamarase. Therefore, the HNL over-expressing plants retain the herbivore deterrence properties associated with the presence of linamarin but eliminate cyanogens more efficiently. Transgenic plants expressing HNL in their roots are particularly useful for subsistence farmers since unlike cyanogen-free cassava plants HNL over-expressing plants are not as likely to

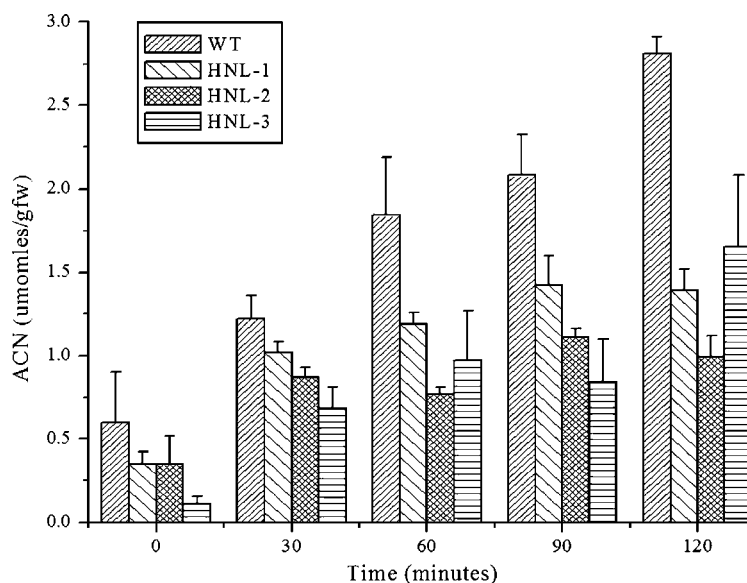


Figure 7. Acetone cyanohydrin levels in wild-type and transgenic cassava expressing HNL in roots. The acetone cyanohydrin produced in 2 h accounts for 80% of the total potential acetone cyanohydrin that could be produced based on measurements of root linamarin content.

require pesticide use and retain the anti-theft traits (linamarin and its associated bitterness) that thieves avoid.

In summary, we have successfully engineered transgenic cassava having linamarin-free roots by selective inhibition of linamarin synthesis in leaves. We have described a novel cyanide assimilation pathway for amino acid synthesis from linamarin and have proposed that linamarin functions not only as an herbivore deterrent but as a mobile form of reduced nitrogen that is essential for root nitrogen metabolism. Finally, our objective of generating a safer cassava food product was realized by engineering cassava to over-express HNL in roots accelerating cyanogenesis and cyanide volatilization.

References

- Akintonwa, A. and Tunwashe, O. 1992. Fatal cyanide poisoning from cassava-based meal. *Human Exp. Toxic.* 11: 47–49.
- Andersen, M., Bush, P., Svendsen, I. and Moller, B. 2000. Cytochromes P450 from cassava catalyzing the first steps in the biosynthesis of the cyanogenic glycosides linamarin and lotaustralin. *J. Biol. Chem.* 275: 1966–1975.
- Bak, S., Olsen, C.E., Halkier, B.A. and Moller, B.L. 2002. Transgenic tobacco and *Arabidopsis* plants expressing the two multifunctional sorghum cytochrome P450 enzymes, CYP79A1 and CYP71E1, are cyanogenic and accumulate metabolites derived from intermediates in Dhurrin biosynthesis. *Plant Physiol.* 123: 1437–1448.
- Balagopalan, C., Padmaja, G., Nanda, S. and Morthy, S. 1988. Cassava nutrition and toxicity. In: Cassava in Food, Feed and Industry. CRC Press, Boca Raton, Florida.
- Bediako, M., Tapper, B. and Pritchard, G. 1981. Metabolism synthetic site and translocation of cyanogenic glucoside in cassava. In: E. Terry (Ed.) Proceedings of the first triennial root crops symposium of the International society for tropical root crops, IDRC Canada, pp. 143–148.
- Bellotti, A. and Arias, B. 1993. The possible role of HCN on the biology and feeding behavior of the cassava burrowing bug (*Cyrtomenus bergi* Froeschner). In: W.M. Roca and A.M. Thro (Eds.) Proceedings of the first international scientific meeting of the Cassava Biotechnology Network, 25–28 August 1992. Cali, Colombia: pp 406–409, Centro Internacional de Agricultura Tropical.
- Bellotti, A. and Riss, L. 1994. Cassava cyanogenic potential and resistance to pests and diseases. *Acta Hort.* 375: 141–151.
- Best, R. and Hargrove, T. 1994. Cassava: the latest facts about an ancient crop. CIAT Publication, Cali, Colombia.
- Bokanga, M. 1994. Processing of cassava leaves for human consumption. *Acta Hort.* 375: 203–207.
- Brusslan, J. and Tobin, E. 1992. Light-independent developmental regulation of *cab* gene expression in *Arabidopsis thaliana* seedlings. *Proc. Natl. Acad. Sci. USA* 89: 7791–7795.
- Byrne, D. 1984. Breeding cassava. *Plant Breeding Rev.* 2: 73–134.
- Calatayud, P.A. and Le Ru, B. 1996. Study of the nutritional relationships between cassava and mealybug and its host plant. *Bull. Soc. Zool. Fr. Evol. Zool.* 121: 391–398.
- Cliff, J., Lundquist, P., Mårtenssen, J., Rosling H. and Sörbo, B. 1985. Association of high cyanide and low sulphur intake in cassava-induced spastic paraparesis. *Lancet* ii: 1211–1213.

- Cock, J. 1985. Cassava: New potential for a neglected crop. Westfield Press, London.
- Conn, E. 1979. Cyanogenic glycosides. *Int. Rev. Biochem.* 27: 21–43.
- Conn, E. 1994. Cyanogenesis—a personal perspective. *Acta Hort.* 375: 31–43.
- Delange, F., Ekpechi, L. and Rosling, H. 1994. Cassava cyanogenesis and iodine deficiency disorder. *Acta Hort.* 375: 289–293.
- Dixon, A., Asiedu, R. and Bokanga, M. 1994. Breeding of cassava for low cyanogenic potential: problems, progress and prospects. *Acta Hort.* 375: 153–161.
- Du, L., Bokanga, M., Moller, B. and Halkier, B. 1995. The biosynthesis of cyanogenic glucosides in roots of cassava. *Phytochem.* 39: 323–326.
- Elias, M., Sudhakaran, P. and Nambisan, B. 1997a. Purification and characterization of α -cyanoalanine synthase from cassava tissues. *Phytochem.* 46: 469–472.
- Elias, M., Nambisan, B. and Sudhakaran, P. 1997b. Catabolism of linamarin in cassava. *Plant Sci.* 126: 155–162.
- Ernesto, M., Cardoso, A., Nicala, D., Mirione, E., Massaza, F., Cliff, J., Haque, M. and Bradbury, J. 2002. Persistent konzo and cyanogen toxicity from cassava in northern Mozambique. *Acta Trop.* 82: 357–362.
- Howlett, W., Brubaker, G., Mlingi, N. and Rosling, H. 1990. Konzo, an epidemic upper motor neuron disease studied in Tanzania. *Brain* 113: 223–235.
- Hughes, M.A., Brown, K., Pancoro, A., Murray, B.S., Oxtoby, E. and Hughes, J. 1992. A molecular and biochemical analysis of the structure of the cyanogenic beta-glucosidase (linamarase) from cassava (*Manihot esculenta* Crantz). *Arch. Biochem. Biophys.* 295: 273–279.
- Hughes, J., Carvahlo, F. and Hughes, M. 1994. Purification, characterization and cloning of α -hydroxynitrile lyase from cassava (*Manihot esculenta* Crantz). *Arch. Biochem. Biophys.* 311: 496–502.
- Kawano, K., Narintaraporn, K., Narintaraporn, S., Sarakarn, S., Limsila, A. and Watan-Anonta, W. 1998. Yield improvement in a multistage breeding program for cassava. *Crop Sci.* 38: 325–332.
- Kim, S., Gregory, D. and Park, W. 1994. Nuclear protein factors binding to a class-I patatin promoter region are tuber-specific and sucrose-inducible. *Plant Mol. Biol.* 26: 603–615.
- Koch, B., Nielsen, V., Halkier, B., Olsen, C. and Møller, B. 1992. The biosynthesis of cyanogenic glycosides in seedlings of cassava (*Manihot esculenta* Crantz). *Arch. Biochem. Biophys.* 292: 141–150.
- Lea, P.J., Blackwell, R.D. and Joy, K.W. 1992. In: K. Mengel and D.H. Pillbeam (Eds.) *Nitrogen Metabolism in Plants* Clarendon press, Oxford, pp. 153–186.
- Lea, P.J., Robinson, S.A. and Stewart, G.R. 1990. In: (B.J. Mifflin and P.J. Lea, (Eds.)) *The Biochemistry of Plants* vol. 16, Academic Press, San Diego. pp 121–159.
- Lundquist, P., Rosling, H. and Sörbo, B. 1985. Determination of cyanide in whole blood, erythrocytes and plasma. *Clin. Chem.* 31: 591–595.
- Makame, M., Akoroda, M. and Hahn, S. 1987. Effects of reciprocal stem grafts on cyanide translocation in cassava. *J. Agr. Sci.* 109: 605–608.
- McMahon, J. 1997. Physiological and biochemical analysis of factors regulating the synthesis of linamarin in the tropical plant cassava (*Manihot esculenta* Crantz), Ph.D. thesis, The Ohio State University, Columbus, Ohio.
- McMahon, J. and Sayre, R. 1994. Regulation of cyanogenic potential in cassava (*Manihot esculenta* Crantz). In: W.M. Roca and A.M. Thro (Eds.) *Proceedings of the Second International Scientific Meeting of the Cassava Biotechnology Network*, pp. 423–438, Bogor, Indonesia.
- McMahon, J., White, W. and Sayre, R. 1995. Cyanogenesis in cassava (*Manihot esculenta*). *J. Exp. Bot.* 46: 731–741.
- Mkpong, O., Yan, H., Chism, G. and Sayre, R. 1990. Purification, characterization, and localization of linamarase in cassava. *Plant Physiol.* 93: 176–181.
- Mlingi, N., Kimatta, S. and Rosling, H. 1991. Konzo, a paralytic disease observed in southern Tanzania. *Tropical Doctor* 21: 24–25.
- Nahrstedt, A. 1985. Cyanogenic compounds as protecting agents for organisms. *Plant Syst. Evol.* 150: 35–47.
- Nartey, F. 1969. Studies on cassava *Manihot utilisima*, biosynthesis of asparagines- ^{14}C from ^{14}C -labelled hydrogen cyanide and its relations with cyanogenesis. *Physiol. Plantarum.* 22: 1085–1096.
- Nweke, F., Spencer, D. and Lynam, J. 2002. *The Cassava transformation: Africa's Best-Kept Secret*. Mich. St. Univ. Press, East Lansing, USA.
- Oluwole, O., Onabolu, A., Link, H. and Roslin, H. 2000. Persistence of tropical ataxic neuropathy in a Nigerian community. *J. Neurol. Neurosurg. Psych.* 69: 96–101.
- Osuntokun, B. 1981. Cassava diet, chronic cyanide intoxicification and neuropathy in Nigerian Africans. *World Rev. Nutr. Diet.* 36: 141–173.
- Rosling, H. 1994. Measuring effect in humans of dietary cyanide exposure from cassava. *Acta Hort.* 375: 271–283.
- Rosling, H., Mlingi, N., Tylleskar, T. and Banea, M. 1993. Causal mechanisms behind human diseases induced by cyanide exposure from cassava. In: W.M. Roca and A.M. Thro (Eds.) *Proceedings of the first international scientific meeting of the Cassava Biotechnology Network*, 25–28 August 1992. Cali, Colombia, pp. 366–375. Centro Internacional de Agricultura Tropical.
- Scott, G., Best, R., Rosegrant, M. and Bokanga, M. 2002. Roots and tubers in the global food system: a vision statement to the year 2020. A co-publication of the International Potato Center, Centro Internacional de Agricultura Tropical, International Food Policy Research Institute, International Institute of Tropical Agriculture and International Plant Genetic Resources Institute. Lima, Peru.
- Selmar, D. 1994. Translocation of cyanogenic glycosides in cassava. *Acta Hort.* 375: 61–68.
- Selmar, D., Lieberei, R. and Biehl, R. 1988. Mobilization and utilization of cyanogenic glycosides: the linustatin pathway. *Plant Physiol.* 86: 711–716.
- Siritunga, D. 2002. Generation of acyanogenic cassava (*Manihot esculenta*, Crantz): Transgenic approaches. Ph. D. thesis, The Ohio State University, Columbus, OH.
- Siritunga, D., Arias-Garcon, D., White, W. and Sayre, R. 2004. Over-expression of hydroxynitrile lyase in cassava roots accelerates cyanogenesis and detoxification. *Plant Biotech. J.* 2: 37–43.
- Siritunga, D. and Sayre, R. 2003. Generation of cyanogen-free transgenic cassava. *Planta* 217: 367–373.
- Sreeja, V., Nagahara, N., Li, Q. and Minami, M. 2003. New aspects in pathogenesis of konzo: neural cell damage directly caused by linamarin contained in cassava (*Manihot esculenta* Crantz). *Brit. J. Nutr.* 90: 467–472.
- Tylleskar, T., Cooke, R., Banea, M., Poulter, N., Bikangi, N. and Rosling, H. 1992. Cassava cyanogens and konzo, an

- upper motor neuron disease found in Africa. *Lancet* 339: 208–211.
- White, W., Arias-Garzon, D., McMahon, J. and Sayre, R.T. 1998. Cyanogenesis in cassava: the role of hydroxynitrile lyase in root cyanide production. *Plant Physiol.* 116: 1219–1225.
- White, W., McMahon, J. and Sayre, R. 1994. Regulation of cyanogenesis in cassava. *Acta Hort.* 375: 69–78.
- Zourelidou, M., Torres-Zabala, M., Smith, C. and Bevan, M. 2002. Storekeeper defines a new class of plant-specific DNA-binding proteins.