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**Title: Metabolic Engineering of Cyanogenesis in the Tropical Root  
Crop, Cassava**

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**Abstract:**

Cassava roots are a major source of calories for subsistence farmers in sub-Saharan Africa. Cassava contains potentially toxic levels of cyanogenic glycosides (linamarin), however. We have engineered transgenic plants that either do not accumulate cyanogens in roots or have accelerated loss of cyanogens during processing. Plants that were specifically unable to synthesize cyanogens in leaves did not accumulate cyanogens in roots. We demonstrate that linamarin transport is essential for root growth, presumably associated with amino acid synthesis. Transgenic plants expressing elevated hydroxynitrile lyase (HNL) activity in roots were three times more efficient for cyanogen removal than wild-type plants.

**Key Words:** Cassava, Cyanogens, Linamarin, *Manihot esculenta*, hydroxynitrile lyase, cytochrome P450, acetone cyanohydrin, cyanide.

## **Introduction**

Many wild and domesticated crops produce secondary compounds that effectively reduce or deter herbivory by insects and animals. Some of these herbivore deterrents are relatively non-toxic, however, some herbivore deterrents are lethal if ingested. One of the more toxic classes of herbivore deterring compounds is the cyanogenic glycosides. The cyanogenic glycosides are a group of nitrile-containing plant secondary compounds that yield cyanide following their enzymatic breakdown (cyanogenesis). It is estimated that between 3,000 and 12,000 plant species produce cyanogenic glycosides (McMahon et al., 1995; Vetter, 2000). Many important crops are cyanogenic including, sorghum, almond, lima beans (non-domesticated) and white clover. The most agronomically important of the cyanogenic crops is the tropical root crop cassava (*Manihot esculenta*, Crantz). Over 153 million tons of cassava roots are harvested annually in the tropics, primarily for direct human consumption.

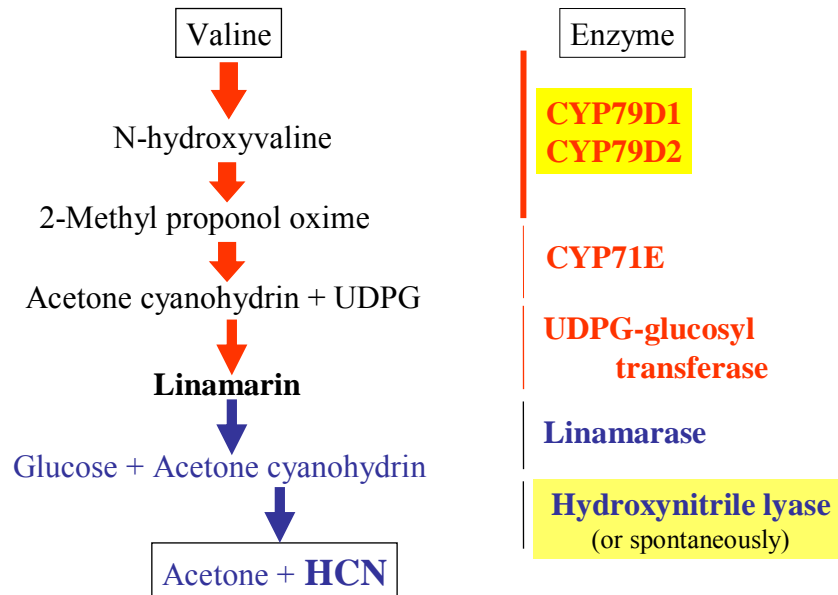
Cassava is widely cultivated in the tropics because it has many agronomic features that are ideal for subsistence farming on marginal lands in the tropics. It is drought tolerant, grows on nutritionally poor soils, its roots can remain in the soil for several years, and it produces large yields of starch; traits that provide enhanced food security. In addition, the presence of cyanogens in cassava has been shown to reduce herbivory as well as reduce crop theft in areas experiencing crop failures (Nweke et al., 2002).

Various human health disorders, however, have been associated with the consumption of residual cyanogens from poorly processed cassava. Chronic exposure to low levels of cyanogens from poorly processed cassava has been associated with hyperthyroidism and neurological disorders including tropical ataxic neuropathy (Tylleskar et al., 1992; Milingi et al., 1992). In addition, the consumption of unprocessed, highly cyanogenic varieties of cassava may result in a permanent paralysis of the legs (konzo). In Africa, the incidence of konzo is highest during crop failures or when less time is taken to process or remove cyanogens from cassava. Significantly, cyanide poisoning from poorly processed cassava is exacerbated by insufficient amounts of sulfur-containing amino acids (cysteine) in the diet (Tor-Agbidye, 1999). Reduced sulfur or sulfur-containing amino acids are required for the detoxification of cyanide by the enzymes rhodanese and  $\beta$ -cyanoalanine synthase. In the absence of sufficient cysteine, cyanide is converted to cyanate in the body. The brain is particularly sensitive to cyanate poisoning. The environmental or human conditions (e.g., drought and famine) that promote short-cut cassava processing practices often go hand-in-hand with reduced availability of protein and/or cysteine in the diet.

### **Cyanogen Synthesis in Cassava:**

Recently, the physiology and biochemistry of cyanogenesis in cassava has been elucidated (reviewed in McMahon et al., 1995; Andersen et al., 2000; Siritunga and Sayre 2004). The major cyanogen in cassava is linamarin (95%) which is synthesized from valine (Figure 1). Two cytochrome P450s (CYP79D1 and CYP79D2) catalyze the first-dedicated step in linamarin synthesis. The end product of the cytochrome P450 catalyzed reactions is the hydroxynitrile intermediate, acetone cyanohydrin (Andersen et al., 2000). Acetone cyanohydrin is subsequently glycosylated by a UDP-glucosyl transferase to yield the stable cyanogen, linamarin which is stored in the vacuoles (reviewed in McMahon et al., 1995).

## Target enzymes for regulating cyanogen **synthesis** and **turnover** in transgenic cassava



**Figure 1.** Linamarin synthesis and turnover pathway (Siritunga and Sayre, 2004).

### **Cyanogenesis in Cassava:**

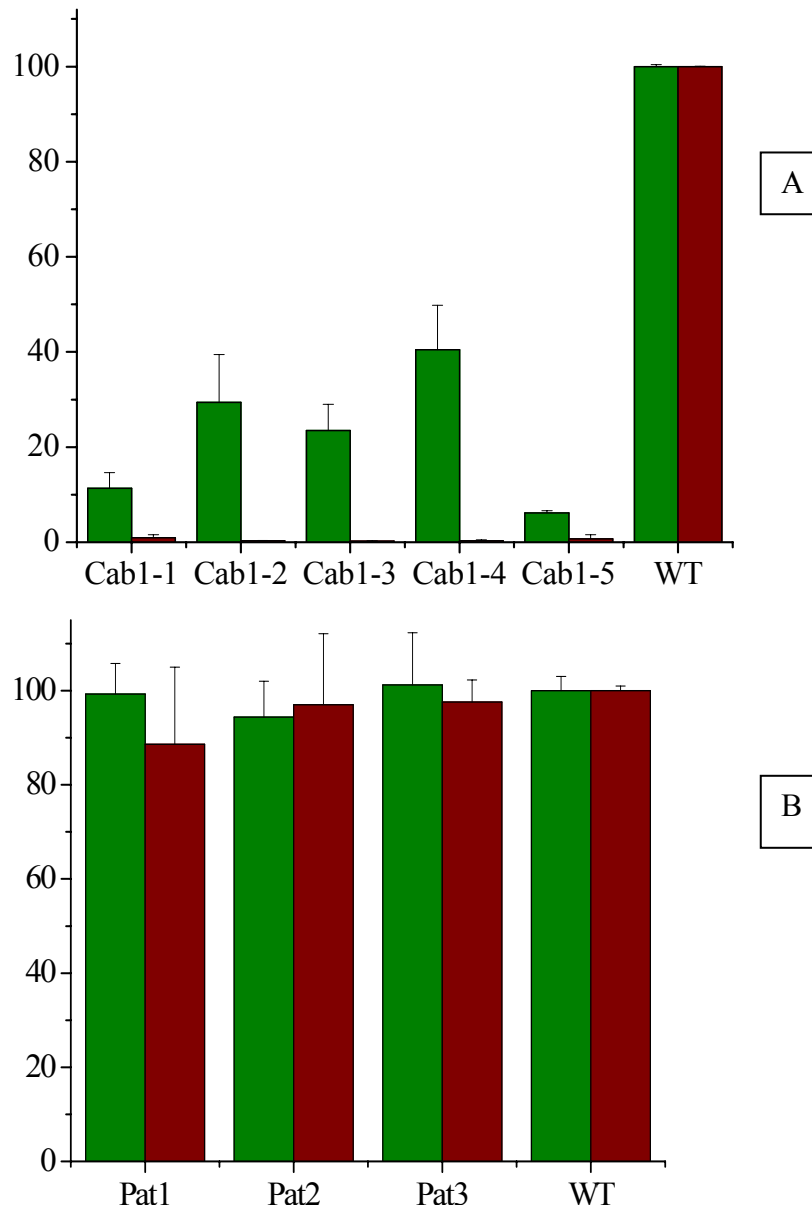
Cyanogenesis in cassava is initiated by tissue disruption. Rupture of the plant cell vacuole releases linamarin which is subsequently hydrolyzed by a cell-wall localized  $\beta$ -glycosidase known as linamarase (Mkpong et al., 1990). The products of linamarin hydrolysis are acetone cyanohydrin and glucose. Significantly, acetone cyanohydrin will spontaneously decompose to cyanide and acetone at pHs > 5.0 or temperatures > 35 °C. Acetone cyanohydrin can also be broken down enzymatically by hydroxynitrile lyase (HNL), however, HNL is present at very low levels in root tissues relative to leaf tissues (68X root levels) (White et al., 1998).

### **Transgenic Approaches to Reducing Cyanogens in Cassava Foods:**

We have generated transgenic cassava plants having potentially reduced cyanide toxicity using two different strategies, 1), inhibition of cyanogen production through suppression of the expression of the enzymes (CYP79D1 and CYP79D2) that catalyze the first-dedicated step in cyanogen synthesis (Figure 1) (Siritunga and Sayre 2003, 2004), and 2) enhancement of hydroxynitrile lyase (HNL) activity in roots to accelerate the turnover of cyanogens and cyanide volatilization during processing (Arias-Garzon and Sayre, 2000; Siritunga et al., 2004).

### **Inhibition of Linamarin Production in Transgenic Plants:**

In 2002, Dr. Birger Möller's lab identified the genes encoding the cytochrome P450s (CYP79D1 and CYP79D2) that catalyze the first-dedicated step in linamarin synthesis (Andersen et al., 2002). This discovery made it feasible to eliminate linamarin synthesis by suppression of CYP79D1 and CYP79D2 expression in transgenic plants (Siritunga and Sayre, 2003, 2004).

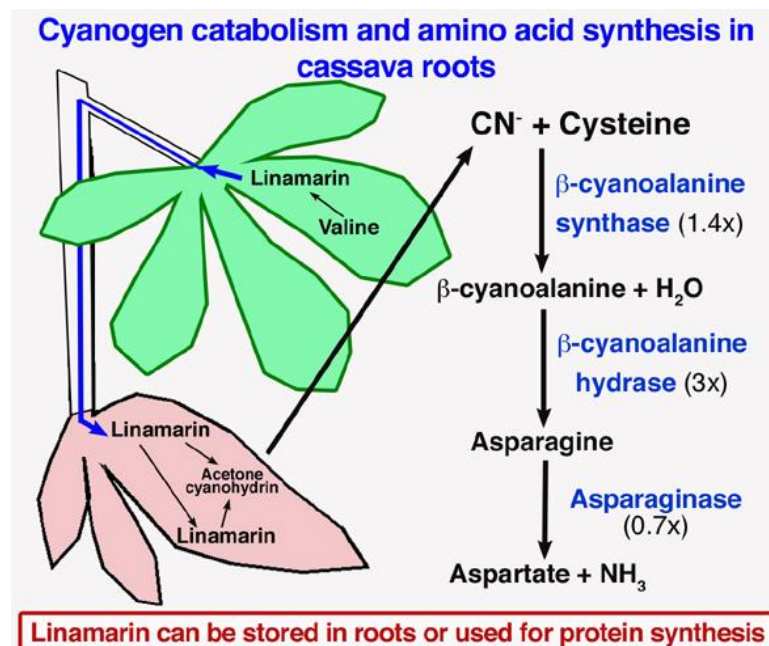


**Figure 2.** Linamarin content of leaves and roots from control, *cab1*-CYP79 (A) and *patatin*-CYP79 (B) cassava transformants. The maximum linamarin content (100%) in leaves (■) and roots (■) of wild-type plants was 80 and 3.4  $\mu\text{g/gdw}$ , respectively (Siritunga and Sayre 2003, 2004).

To down-regulate the expression of these genes in different tissues, two different organ-specific gene promoters, *cab1* (leaf) and *patatin* (root), were used to drive the anti-sense expression of the *CYP79D1* and *CYP79D2* genes. RT-PCR analyses of five independent *cab1*-CYP79D1/D2 antisense transformants constructs demonstrated a range of suppression of *CYP79D1* and *CYP79D2* transcript levels in leaves (Siritunga and Sayre, 2003). Concomitant with this reduction in leaf-specific *CYP79D1* and *CYP79D* gene expression was a reduction in leaf linamarin content (Figure 2A). *Cab1*-CYP79D1/D2 transgenic plants had leaf linamarin levels

ranging from 6% to 40% of wild-type levels. Surprisingly, Siritunga and Sayre (2003) observed a 99% reduction in root linamarin content in *cab1-CYP79D1/D2* antisense transformants even though root *CYP79D1* and *CYP79D2* transcript levels were unaltered relative to wild-type levels (Figure 3A). They also demonstrated that *patatin-CYP79* antisense plants, having no detectable *CYP79D1* or *CYP79D2* message in roots, had wild-type root linamarin levels (Figure 2B) (Siritunga and Sayre, 2004). These results indicated that nearly all the linamarin present in roots was synthesized in the leaves and transported to the roots.

Surprisingly, when *cab1-CYP79D1/D2* antisense plants were transferred from Murashige-Skoog (MS) media containing reduced nitrogen (ammonia) to soil, they all died. Earlier work had suggested that linamarin may play a role in nitrogen assimilation and transport in cassava. In 1969, Nartey demonstrated that cassava could efficiently assimilate cyanide into amino acids. In addition, Calatayud and Ru (1996) had shown that 60% of the reduced nitrogen in leaf phloem extracts was linamarin. As a result of these observations it was hypothesized that inhibition of linamarin transport from leaves to the roots may inhibit root amino acid and protein synthesis in the absence of an alternative source of reduced nitrogen. A simple experiment was designed to test this hypothesis. Root growth of wild-type, *cab1-CYP79D1/D2* and *patatin-CYP79D1/D2* antisense plants was compared when grown in MS media with or without reduced nitrogen. Normal root growth was observed in wild-type and *patatin-CYP79* antisense plants when grown in ammonia-free media but no root growth was observed in *cab1-CYP79* antisense plants grown in the absence of reduced nitrogen but an equivalent concentration of nitrate (Siritunga and Sayre, 2004). These results lead to a model for linamarin-derived nitrogen (CN) assimilation into amino acids by cassava roots (Figure 3). Siritunga and Sayre (2004) proposed that linamarin transported to the roots has two possible fates, a) storage in the vacuole, or b) assimilation into amino acids via cyanide generation from linamarin. In this latter process, cyanide would be assimilated into asparagine and aspartate for protein synthesis via the activity of  $\beta$ -cyanoalanine synthase and hydrazase (Figure 3). The relative linamarin flux between storage and amino acid metabolism could affect the linamarin storage capacity (toxicity) of the root.



**Figure 3.** Linamarin synthesis, transport, storage and assimilation into amino acids. Values in parentheses following enzyme are root/leaf enzyme activity ratios (figure drawn by C. Fauquet based on Siritunga and Sayre, 2004).

In summary, it was demonstrated that selective inhibition of linamarin synthesis in leaves resulted in the inability to accumulate linamarin in roots and an inability for the plant to grow in the absence of reduced nitrogen. The presence of high cyanide assimilatory enzyme activities in roots suggests that cyanide derived from linamarin may provide reduced nitrogen for amino acid synthesis. Alternative strategies for generating transgenic plants with reduced levels of linamarin in roots that do not inhibit protein synthesis in roots are currently being explored.

### **Differential Expression of Hydroxynitrile Lyase in Cassava:**

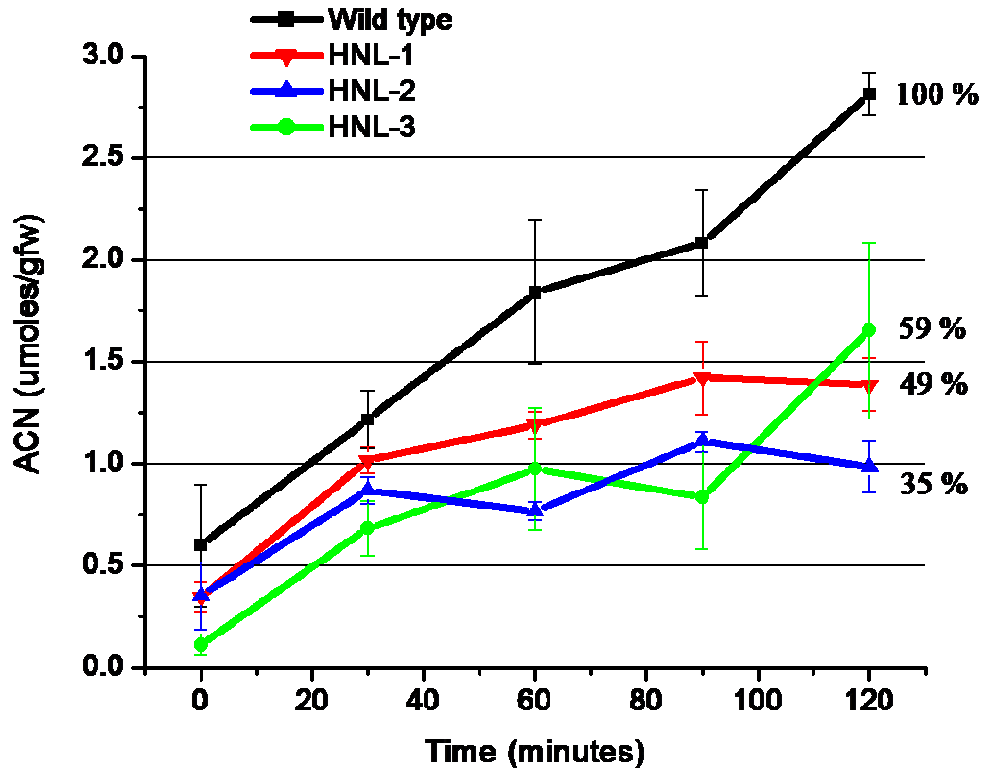
Until recently, it had been assumed that the only cyanogen present in poorly processed cassava foods was linamarin. In 1992, however, Dr. Hans Rossling and colleagues demonstrated that the major cyanogen present in poorly processed cassava roots was not linamarin but acetone cyanohydrin. This result was unexpected since it was assumed that acetone cyanohydrin would be eliminated from foods by spontaneous (high pH and/or temperature) or enzymatic breakdown (Tylleskar et al., 1992) and the product, cyanide, would be lost by volatilization (Tylleskar et al., 1992). To account for the presence of acetone cyanohydrin in cassava foods it was hypothesized that the low pH conditions that are often used during cassava processing (soaking and fermentation) reduce the rate of spontaneous acetone cyanohydrin decomposition. This hypothesis, however, did not address the issue of the apparent lack of acetone cyanohydrin turnover catalyzed by HNL.

In 1995, the Sayre lab characterized the distribution HNL in cassava roots, stems, and leaves (White and Sayre, 1995; White et al., 1998). Their objective was to determine the biochemical basis for the unexpectedly high levels of acetone cyanohydrin in processed cassava food products. They discovered that root HNL levels were < 5% (protein basis) of those present in leaves. Thus, it was apparent that the low HNL activity in roots could contribute to the high acetone cyanohydrin levels present in poorly processed cassava roots. This observation led to the development of transgenic strategies to facilitate cyanogen elimination from processed cassava foods by over-expression of HNL in roots (Siritunga et al., 2004).

Using an *Agrobacterium*-mediated transformation system the Sayre lab introduced a cassava cDNA encoding HNL into somatic embryos. Transgenic cassava plants which had integrated one or more copies of the HNL cDNA driven by the CaMV 35S promoter were verified by PCR and Southern blot analysis (Siritunga et al., 2004). HNL activity of root extracts obtained from *in vitro* transgenic plants ranged from 200 - 325  $\mu\text{mol CN/mg protein/hr}$  for three independent transformants. Roots of untransformed plants had an HNL activity of 25  $\mu\text{mol CN/mg protein/hr}$ . In contrast, the HNL activity of crude leaf extracts was 1.7  $\text{mmol CN/mg protein/hr}$ . Root HNL activities in transgenic plants approached 20% of that in leaves of wild-type plants.

The rate of acetone cyanohydrin turnover in roots of the best HNL transgenic plants was more than three times faster than for wild-type plants (Figure 4). As expected, acetone cyanohydrin turnover was stoichiometrically equivalent to the loss of cyanide from the processed root tissues.

These results demonstrated that the rate of cyanogen removal could be accelerated in plants over-expressing HNL. Significantly, plants over-expressing HNL had normal root linamarin and linamarase levels indicating that the protective benefits of cyanogens (herbivore or theft deterrence) should not be compromised in the transgenic plants (Sirtunga et al., 2004). These plants are ideally suited for subsistence farmers who rely on cyanogens to provide food security. Plants over-expressing HNL are currently in field trials.



**Figure 4.** Reduction of root acetone cyanohydrin levels in processed wild type and transgenic plants over-expressing HNL in roots. Analysis was performed on 8-month-old green house grown cassava (Sirtunga et al., 2004).

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### **Non-Technical Abstract:**

Cassava is a major source of calories for subsistence farmers throughout the tropics, particularly in sub-Saharan Africa. However, cassava contains potentially toxic levels of cyanogenic glycosides (linamarin) which yield cyanide when decomposed. Linamarin and the cyanogenic products derived from it can be efficiently removed from cassava foods by processing. When food supplies are limiting, however, processing procedures are often shortened resulting in potentially toxic food products. We report here our efforts to reduce the cyanogenic potential of cassava to provide safer food products. To achieve this goal two independent transgenic strategies were explored; 1) inhibition of linamarin synthesis, and 2) enzyme-mediated acceleration of cyanogen turnover and cyanide volatilization during processing. To achieve the first objective, the expression of the cytochrome P450 gene products (*CYP79D1* and *CYP79D2*) that catalyze the first-dedicated step in linamarin synthesis was specifically inhibited in leaves or roots. Specific inhibition of *CYP79D1* and *CYP79D2* gene expression in leaves resulted in a reduction of leaf linamarin steady-state levels to 6-40% of wild-type levels. Significantly, linamarin levels in roots of these transgenic plants were reduced by more than 99% relative to wild-type levels. In contrast, specific inhibition of *CYP79D1* and *CYP79D2* gene expression in roots had no effect on root or leaf linamarin levels. These results suggested that nearly all of the linamarin present in roots was synthesized in leaves and transported to the roots. Significantly, transgenic plants unable to accumulate linamarin in roots were unable to grow in the absence of reduced nitrogen. Since roots have elevated levels of cyanide assimilatory enzymes, that convert cyanide to amino acids, it was hypothesized that linamarin synthesized in leaves and transported to roots plays a primary role in reduced nitrogen metabolism in cassava roots. The second strategy to detoxify cassava foods was to accelerate cyanogenesis and cyanide volatilization during root processing. To accelerate cyanide volatilization we over-expressed the gene encoding hydroxynitrile lyase (HNL) in roots, which is strongly expressed only in leaves of wild-type plants. HNL catalyzes the conversion of acetone cyanohydrin (de-glycosylated linamarin) to cyanide. We observed a direct correspondence between the level of HNL expression in roots and reduction in acetone cyanohydrin levels in processed roots. We propose that cassava plants expressing HNL in roots will be more efficiently processed to remove cyanogens providing a safer food product for consumers.