Production of Safer Cassava Food Products having Reduced Cyanogen Levels

Dimuth Siritunga[1], Diana Arias-Garcon[2] and Richard Sayre[1],

[1]Department of Plant Biology, 318 W. 12th Avenue, The Ohio State University, Columbus, OH 43210 USA; [2]BASF Plant Science, RTP, NC 27709 USA

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INTRODUCTION

Cyanogenic glycosides are a group of secondary compounds that upon enzymatic breakdown yield cyanide. In many plants cyanogenic glycosides are thought to act as herbivore deterrents or as transportable forms of reduced nitrogen (2, 6, 10).

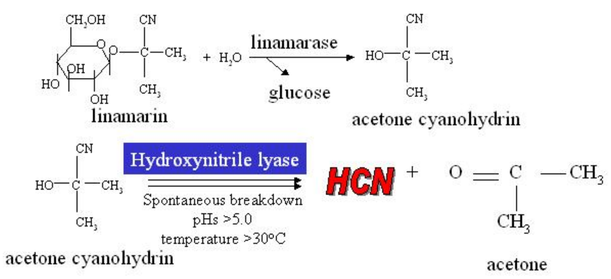
In humans, the ingestion of improperly processed cassava has been associated with chronic cyanide disorders such as hyperthyroidism, tropical ataxic neuropathy and konzo (7, 11).

Rupture of the vacuole initiates cyanogenesis in cassava by releasing linamarin that is hydrolyzed by linamarase, a cell wall β-glycosidase (6). The product, acetone cyanohydrin (ACN), spontaneously decomposes at pHs >5.0 or temperatures >35°C or is enzymatically broken down by hydroxynitrile lyase (HNL) to produce acetone and HCN (3, 13, 14).

It was thought that residual linamarin is the apparent source of cyanide toxicity until Tylleskar et al. (1992) demonstrated that the major cyanogen present in poorly processed cassava roots was ACN, not linamarin. The cloning and characterization of HNL led to the realization that HNL transcripts and protein are present at very low levels in cassava roots (14).

Thus, it is apparent that the high ACN levels present in processed cassava could be attributed to the absence of HNL in roots. The expression of HNL in roots of transgenic cassava plants may lead to safer food product by converting acetone cyanohydrin more efficiently to volatile cyanide during food processing. This transgenic strategy of expressing HNL in cassava root will not affect possible function of ACN as an anti-herbivory agent.

Figure 1: Cyanogenesis from linamarin

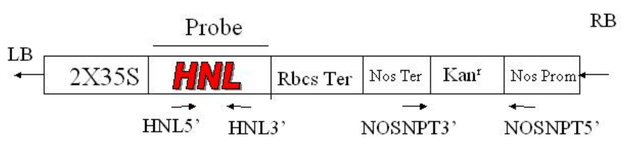


MATERIAL AND METHODS

Vector construction and transformation

The modified binary vector pKYLX-HNL was constructed for the expression of HNL gene in transgenic cassava (Figure 2). The vector was transformed into *Agrobacterium tumefaciens* strains LBA4404 by electroporation.

Figure 2: Modified binary vector pKYLX-HNL (13Kb) used for stable transformation of cassava



Cassava Transformation

Cassava plants were transformed by standard procedures (1)

Analysis for the transgenes

The presence of the *npt*II and the cDNA HNL genes were detected by PCR amplification using total genomic DNA. The absence of residual *Agrobacterium* was tested by using two primers that amplify the 400bp fragment of the *vir* zone G located outside of the T-DNA.

Integration of the transgenes was further confirmed by Southern blot analysis performed on total leaf DNA isolated from 8-month-old greenhouse grown plants (15). The DNA (10 µg) was digested with *XbaI* and hybridized at 42 °C with PCR-generated ^{32}P-labelled *HNL* cDNA probe following standard protocols (16).

Crude protein extractions, HNL assay, Western Blot

Leaf or root proteins were used for HNL assays using the following procedure: 20 µg of total protein in; 50mM sodium phosphate pH 5.0, 28mM acetone cyanohydrin (final volume 1ml) were incubated 30 minutes at 28 °C. 10µL 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, NJ).

5µg leaf-stem or root soluble protein were separated by SDS-PAGE and transferred to Immobilon-P membrane (4). Polyclonal primary antibodies were raised against purified HNL by the OSU Antibody Center (Columbus). Secondary antibodies (anti-goat) conjugated to alkaline phosphatase were used and detected by a colorimetric assay (4).

Root acetone cyanohydrin (ACN) assays

Eight month old green-house grown transformants were used for ACN assays. Root parenchyma (1g) was homogenized in 5mL of 0.1M sodium phosphate pH5 and incubated at 30°C for 0 to 120 minutes. After centrifugation the supernatant was subjected to two assays using the Spectroquant cyanide kit. The amount of cyanide was determined by adding 0.5mL of supernatant to 3.5mL of 0.1M sodium phosphate pH 5. The amount of ACN plus cyanide present was determined by adding 0.1mL of supernatant to 0.6 mL of 0.2M NaOH and 3.3 mL of 0.1mM sodium phosphate pH 5. The amount of ACN present in the HNL transformants is the difference in the values of the two assays.

RESULTS AND DISCUSSION

PCR analysis

PCR amplification of leaf genomic DNA of putative plants using primers for *nptII* gene and HNL cDNA resulted in the expected PCR products (Figure 3).

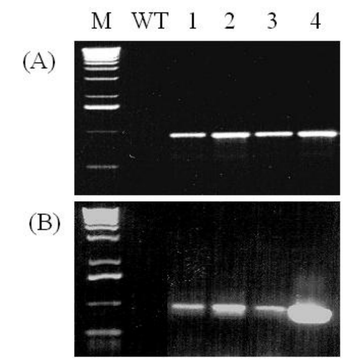
The endogenous HNL gene was not amplified from wild-type genomic DNA due to its large size (7kb).

The *Vir G* primers successfully amplified the *Vir G* gene from Agrobacterium but no PCR products were obtained from any of the cassava transformants (Figure 4). The *Vir* G gene is present in the Ti plasmid and is not transferred to the plant genome.

Figure 3: Amplification of 860bp nos promoter - *nptII* cassette fragment (A) and 800bp HNL cDNA from genomic DNA (B).

NOSNPT5' (5'CCGCCGATGACGCGGGACAAGCC3')
NOSNPT3' (5'GGTCCGCCACACCCAGCCGGCCA3')

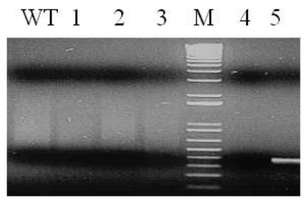
HNL5' (5'AAAGTCGACATGGTAACTGCACATTTTGTT3')
HNL3' (5'AAAGAATTCTCAAGCATATGCATCAGCCAC3')



M:1Kb ladder;
WT: untransformed
1: HNL-1 transformant
2: HNL-2 transformant
3: HNL-3 transformant
4: pKYLX-HNL vector

Figure 4: Amplification of 400bp fragment from *Vir* G gene

*Vir*G-F1 (5'GCCGACAGCACCCAGTTCAC3')
*Vir*G-R1(5'CCTGCCGTAAGTTTCACCTCACC3')

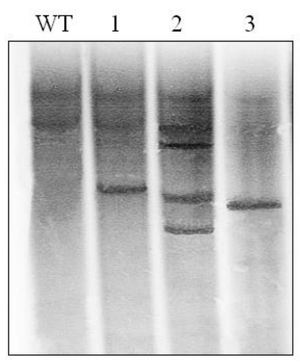


M:1Kb ladder;
WT: untransformed
1: HNL-1 transformant
2: HNL-2 transformant
3: HNL-3 transformant
M:1Kb ladder
4: Negative control
5: LBA4404

Southern blot analysis

Southern blot analysis performed on the HNL transgenic and WT plants show that 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 5).

Figure 5: Southern blot analysis shows the integration of the HNL gene into transgenic cassava

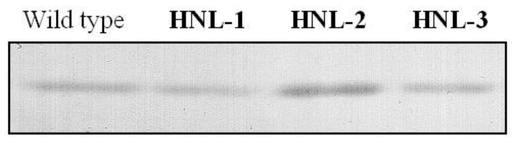


WT: untransformed
1: HNL-1 transformant
2: HNL-2 transformant
3: HNL-3 transformant

Western blot analysis

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 6).

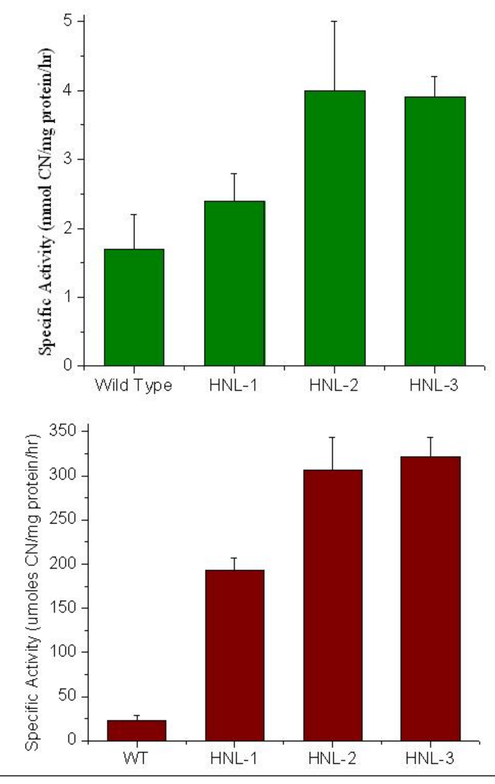
Figure 6: Immunoblot of HNL using 5 µg total protein cassava leaf tissue.



HNL enzymatic activity

Crude extracts from transformed plants had HNL activity increased up to two fold in leaves and 13 fold in roots on a protein basis compared to crude extracts from untransformed plants (Figure 7). It is noted that, spontaneous rates of acetone cyanohydrin decomposition were always subtracted from the enzyme catalyzed reactions.

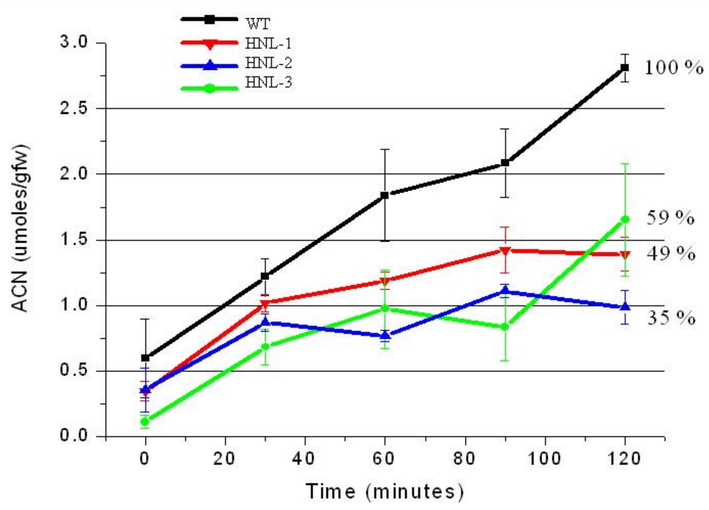
Figure 7: Leaf (green) and root (brown) HNL activities in wild type and transformed cassava



Root acetone cyanohydrin assays

Transformed roots had up to 65% less acetone cyanohydrin compared to untransformed roots after 2 hours incubation at 30°C (Figure 8). Transformants HNL-1, HNL-3 and HNL-2 had 41%, 51% and 65% less acetone cyanohydrin in the roots than untransformed cassava of the same age.

Figure 8: Reduction of root acetone cyanohydrin levels in processed transgenic plants. Analysis was performed on 8-month-old green house grown cassava.



CONCLUSION

Transgenic plants expressing the HNL gene in roots have linamarin and acetone cyanohydrin levels identical to untransformed cassava, thus, they have the same cyanogenic potential as wild type. In the past it was thought that all of the residual cyanogen present in cassava foods was in the form of linamarin. Recently, it has been demonstrated that the major cyanogen present in poorly processed cassava roots was indeed acetone cyanohydrin, not linamarin (11).

The presence of 13-fold higher levels of HNL in the root accelerates the 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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