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ORIGINAL ARTICLE

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Generation of cyanogen-free transgenic cassava

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Abstract Cassava (*Manihot esculenta* Crantz.) is the major source of calories for subsistence farmers in sub-Saharan Africa. Cassava, however, contains potentially toxic levels of the cyanogenic glucoside, linamarin. The cyanogen content of cassava foods can be reduced to safe levels by maceration, soaking, rinsing and baking; however, short-cut processing techniques can yield toxic food products. 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 inhibition of *CYP79D1* and *CYP79D2* expression. Importantly, the linamarin content of roots also was reduced by 99% in transgenic plants having between 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.

Keywords *Manihot* · Cyanogenic glycoside · Cyanide · Linamarin · Cytochrome P450

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 on poor soils, and the roots (starch) can persist in soil for 1–2 years without decay. These agronomic attributes often allow cassava to provide a reliable source of food during periods of famine (Best and Hargrove 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; Hughes et al. 1994; White et al. 1994). 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) in 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 1974). 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 1991; Rosling et al. 1992; Tylleskar et al. 1992). Individuals with low protein and particularly cysteine intake in their diets are more susceptible to cyanide poisoning since the detoxification of cyanide to thiocyanate by rhodanese requires cysteine as a substrate.

To make cassava safe for human consumption, cassava roots and leaves must be processed to remove the cyanogens. Typically, processing involves tissue

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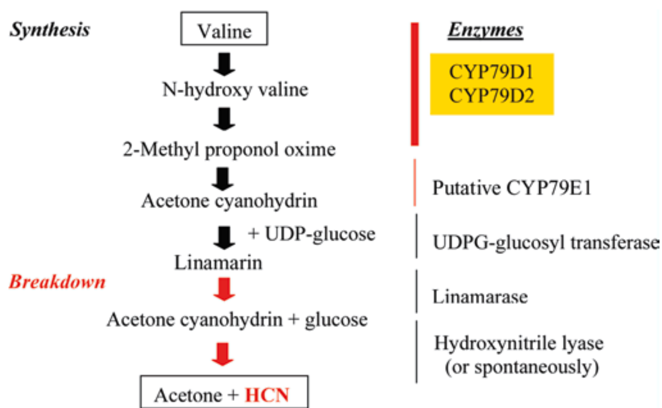

Fig. 1 Linamarin biosynthesis and breakdown pathway in cassava (*Manihot esculenta*). The CYP79D1 and CYP79D2 enzymes (cytochrome P450) convert both valine and isoleucine to its corresponding oxime for linamarin and lotaustralin production, respectively

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 (McMahon et al. 1995; Fig. 1). The cyanogenic product of linamarin deglycosylation, acetone cyanohydrin, is then decomposed to yield cyanide and acetone. This process may occur spontaneously, at pHs > 5.0, at elevated temperatures (> 35 °C), or enzymatically by hydroxynitrile lyase (HNL; McMahon et al. 1995), which 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 (Tylleskar et al. 1992).

For cassava to become a reliably safe and acceptable cash crop as well as to reduce the production of cyanide waste from cassava-processing plants there is a need for acyanogenic cassava cultivars. Recently, the genes encoding a small (CYP79D1 and CYP79D2) sub-family of cytochrome P450s that catalyze the first dedicated step in linamarin and lotaustralin synthesis were isolated (Anderssen et al. 2000). These two cassava cytochrome P450s are 85% identical and both share 54% identity to CYP79A1, a sorghum cytochrome P450 that catalyzes the first dedicated step in the synthesis of the cyanogenic glycoside dhurrin (Sibbesen et al. 1994; Kahn et al. 1997). We have expressed these genes in an antisense orientation in transgenic cassava 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. These results suggest that linamarin may be transported from leaves to roots in young plants. These acyanogenic cassava plants 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.

Materials and methods

Vector construction

The 5′ ends (650 bp each) of both *CYP79D1* and *CYP79D2* genes were cloned into the *Agrobacterium* binary vector, pBI121, in an antisense orientation. Each gene is flanked at the 5′ end by the leaf-specific Cab1 promoter and at the 3′ end by the nopaline synthase (nos) terminator (Bevan et al. 1983; Brusslan and Tobin, 1992). The T-DNA region also contains the *nptII* gene for kanamycin/paromomycin resistance. *Agrobacterium tumefaciens* strain LBA4404 (Life Technologies, Rockville, Md., USA) was transformed with the modified binary vector by electroporation and used to transform cassava (Arias-Garzon et al. 1994).

Plant material

Shoot apical meristems of cassava (*Manihot esculenta* Crantz.) cultivar MCol 2215, an important cultivar in northeast Colombia, were cultured on MS basal medium for production of in vitro plants (Murashige and Skoog 1962; Arias-Garzon and Sayre 1993). Apical leaves were placed on MS8 medium [MS basal medium supplemented with 2% (w/v) sucrose, 8 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 10 ml/l of 100× 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 a 12 h/day photoperiod (5 μmol photons m^{-2} s^{-1}) at 28 °C. Once embryos formed (21–28 days) they were transferred to germination medium [MS basal medium supplemented with 1 mg/l thiamine, 100 mg/l *myo*-inositol, 2% (w/v) sucrose, 0.01 mg/l 2,4-D, 1.0 mg/l N^6-benzylaminopurine (BAP) and 0.5 mg/l gibberellic acid (GA_3) at pH 5.7] to induce the development of cotyledonary leaves (21–28 days).

Cassava transformation

Germinated somatic embryos were used for transformation (Li et al. 1996). Two-day-old cultures of *Agrobacterium* containing the vectors described above were grown in YM liquid medium (Vincent 1985) 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 acetosyringone. Prior to co-cultivation the bacterial cultures were induced in MS basal medium containing 200 μM acetosyringone for 2–4 h at 28 °C. After 2 days of co-cultivation at 27 °C (in darkness) the tissue was transferred to medium 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-h photoperiod at 5–10 μmol photons m^{-2} s^{-1}. Four weeks after co-cultivation, clumps of somatic embryos were transferred to cassava germination 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 [4.3 g/l MS salts, 2% (w/v) sucrose, 0.04 mg/l BAP, 0.05 mg/l GA_3, 0.02 mg/l naphthalene-1-acetic acid (NAA), 1 mg/l thiamine, 100 mg/l *myo*-inositol, pH 5.7] lacking antibiotics for induction of root growth. Wild-type plants used for all biochemical experiments were regenerated from somatic embryos using the same protocols (minus antibiotic selection) used to regenerate transgenic plants.

Molecular analysis of transgenic plants

For PCR analysis, genomic DNA was isolated from 40 mg of 3- to 4-month-old in vitro-grown leaves from either wild-type plants or paromomycin-resistant putative transformants according

to Doyle et al. (1990). The presence of the *nptII* gene (not shown) and the transgenes was detected by PCR amplification. PCR reactions were performed in a total volume of 50 μl containing: 1× Vent buffer, 20–100 ng of leaf DNA, 0.1 mM each dNTP, 1 unit Vent polymerase, 0.4 μM each primer (IDT, Coralville, Iowa, USA). The transgenes were amplified with primers specific for the Cab1 promoter/*CYP79D1* junction (Cab1F1: CAATAC CAAACCCATTTCTTGGC) and *CYP79D1*/nos terminator junction (NOSAR1: ATCGCAAGACCGGCAACAGGATTC). The DNA was amplified using 10 touchdown PCR cycles of 30 s at 94 °C, 1 min at 69 °C–64 °C and 30 s 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 (GCCGACAGCACCCAGTTCAC and CCTGCCGTAAGTTTCACCTCACC). Southern blot analysis was performed following standard protocols (Sambrook et al. 1989). Total DNA was isolated from 3- to 4-month-old in vitro-grown plants, digested (10 μg DNA) with *Kpn1* and transferred to a membrane according to Soni and Murray (1994). *Kpn1* does not restrict the T-DNA region of the modified vector used in this study. Hybridization was conducted at 42 °C with *CYP79D1* probe (5′ end 650 bp) PCR-labeled with [^{32}P]dCTP. Hybridized membranes were washed once in 1×SSC buffer (0.15 M NaCl, 0.015 M sodium citrate) plus 0.1% SDS and twice in 0.1×SSC buffer plus 0.1% SDS at 50 °C (20 min/wash).

Total RNA was isolated from 100 mg of 3- to 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, Valencia, Calif., USA). Reverse-transcription–PCR (RT–PCR) was performed using 6.0 μg of total RNA according to the manufacturer's recommendations with the following modifications (RT–PCR kit from Invitrogen (Carlsbad, Calif.). The cDNA amplification was performed using 1×PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP each, 2.5 units Taq polymerase, 0.4 μM each primer, with 35 cycles of 30 s at 94 °C, 1 min at 56 °C and 30 s at 72 °C (Life Technologies). The primers used were specific for the 3′ end of *CYP79D1* (D1-F1: GCTAAAT CAACCAGAAATCCTGAAG and D1-R4: TGCAAGAGAAA CAAGATAACCCC) and *CYP79D2* (D2-F1: CTGATAAATCA ACCAGAACTTCTGGCA and D2-R5: CTAACAACTCACATT CATCCCTTCCC) genes. It should be noted that the 3′ primers are not complimentary to the antisense portions of transgenes introduced into cassava and thus should not amplify the T-DNA. First-strand cDNA synthesis yield 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 were included.

Linamarin quantification

The leaf and root linamarin contents were measured from three to five independently isolated 3- to 4-month-old in vitro wild-type or transgenic plants grown in tissue culture medium (same growth conditions as for PCR analyses). 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. GC–MS was performed on a 30-m-long Restek XTI-5 (5% diphenyl–95% dimethyl polysiloxane) capillary gas chromatography column coupled to a mass spectrometer, which was operated under a pressure control mode using pressures that gave flows near 1.0 ml/min. The GC oven temperature program was: 50 °C for 1 min after injection, ramp at 30 °C/min to 185 °C, ramp at 6 °C/min to 230 °C (linamarin elution), ramp at 12 °C/min to 300 °C (internal standard elution), and ramp at 40 °C/min to 360 °C for 3 min to clean the column. Each of the runs was normalized for the internal standard (phenyl β-D-glucoside) and linamarin is expressed both as absolute values and as a percentage of the quantity present in wild-type untransformed plants.

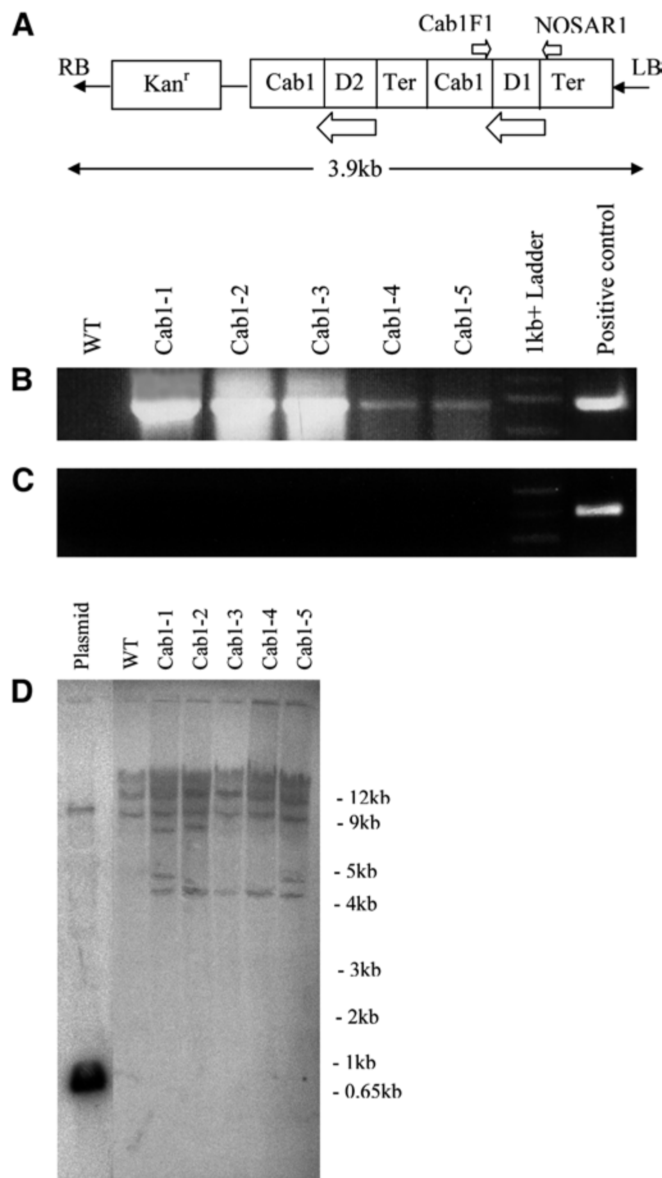

Fig. 2A–D Integration of the T-DNA cassette into cassava. **A, B** Amplification of a 700-bp T-DNA fragment with a primer pair (**A**) specific to the Cab1 promoter and the NOS terminator confirms the integration of the T-DNA (**B**). **C** The absence of *Agrobacterium* contamination was verified by the use of *VirG*-specific primers. **D** Southern blot analysis of the *CYP79D1* gene in wild-type and transgenic plants. The lane marked *Plasmid* is the modified binary vector restricted with *Hind*III and *Sst*I to release the 650-pb *CYP79D1* gene. Some uncut plasmid is detected as well. *WT* Wild type

Results

Transgenic cassava

To generate acyanogenic plants we used *Agrobacterium*-mediated T-DNA transformation 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 (Fig. 2). In addition to the

antisense *CYP79D1* and *CYP79D2* genes, the T-DNA included a bacterial *nptII* gene for antibiotic selection of transformants. It should be noted that the 5′ 650 bp of the *CYP79D1* and *CYP79D2* genes share no identity with other known cytochrome P450 genes other than each other; therefore, the generation of *CYP79D1* and *CYP79D2* antisense transcripts should not affect the expression of other cytochrome P450 genes.

Analysis of five paromomycin-resistant putative transformants (Cab1–1 through Cab1–5) representing a range of inhibition of *CYP79D1* and CYP79D2 expression is presented in this paper. Importantly, each putative transformant was obtained from a unique explant and not from a secondary somatic embryo. Similarly, 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 cannot be attributed to differences in plant culture. Furthermore, biochemical analyses of transgenic plants were carried out on plants that had been grown in the absence of paromomycin selection for at least 2 months.

To confirm the integration of the T-DNA the transformants were screened by PCR amplification of the *nptII* gene (results not shown) and the truncated *CYP79D1* gene. The DNA primers specific for the *CYP79D1* gene were designed to amplify the region between the Cab1 promoter/*CYP79D1* junction and the *CYP79D1*/NOS terminator junction (see Materials and methods; Fig. 2A). A diagnostic 700-bp *CYP79D1* fragment was amplified in each of the five transformants and its identity was confirmed by DNA sequence analysis (Fig. 2B). The absence of *Agrobacterium* contamination was verified by the use of *Agrobacterium VirG*-specific primers (Fig. 2C). 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. These results were further confirmed by Southern blot analysis. The five transformants described in detail here had between one and three independent T-DNA integration events (Fig. 2D). 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. These results suggest that there may be hot spots for DNA integration or the results may be fortuitous. Since Cab1–3 and Cab1–4 transgenic plants were obtained from independent transformation events they were not derived from identical somatic embryos.

The *CYP79D1* and *CYP79D2* transcript abundance in wild-type and transgenic plants was analyzed by RT–PCR amplification using primers specific for the 3′ ends of the *CYP79D1* and *CYP79D2* genes. These primers do not anneal to the 5′ portion of the genes used in the antisense construct and therefore would not directly amplify the T-DNA. To normalize the *CYP79D1* and *CYP79D2* RT–PCR products from separate plants the PCR products were normalized on the basis of the abundance of the cassava starch branching enzyme-II (SBE-II) RT–PCR product. 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 to 80% of the wild-type plant levels (Fig. 3).

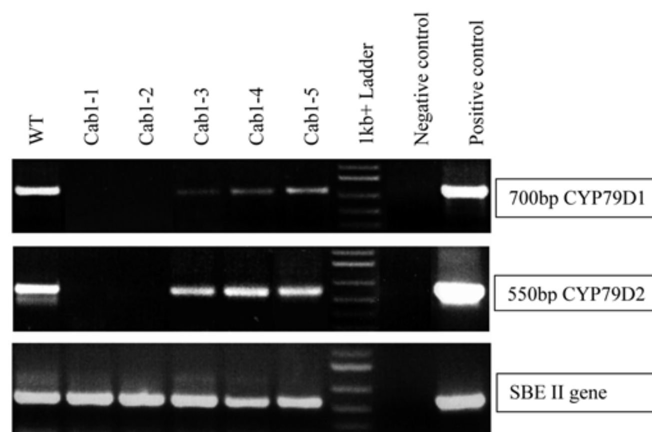

Fig. 3 RT–PCR amplification of the *CYP79D1* and *CYP79D2* transcripts from cassava leaves. Primers specific for the 3′ end of each gene were used (primer pairs D1-F1/D1-R4 and D2-F1/D2-R5 amplify *CYP79D1* and *CYP79D2*, respectively). RT–PCR amplifications of *CYP79D1*, *CYP79D2* and starch branching enzyme-II(*SBE-II*) transcripts were performed in parallel. *WT* Wild type

Reduction in linamarin content

The five transgenic cassava plants were then analyzed for their leaf linamarin content by GC–MS analysis. Commercial linamarin was derivatized and used as a standard (elution time of 13–14 min) 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 derivatization and extraction of linamarin. The SIM masses for phenyl β-D-glucoside were 189 and 393, respectively, and the elution time was 19–21 min. Transgenic plants used for the linamarin extractions and derivatizations were all approximately the same age (3- to 4-month-old in vitro plantlets). As shown in Fig. 4A, leaf-linamarin content was reduced by 60–94% (relative to wild type) in the five transgenic plants analyzed. Surprisingly, the linamarin content of roots of transgenic plants having reduced leaf linamarin levels was reduced to even a greater extent than in leaves. Root linamarin content was reduced by 99% in all transgenic plants analyzed, including plants having only a partial (60%) reduction in leaf linamarin content (Fig. 4B). The root linamarin content of in vitro-grown wild-type cassava was 3.5 ± 0.4 µmol/g dry weight (mean $\pm$ SD). This value is in agreement with root linamarin levels reported by Mkpong et al. (1990) (2.96 µmol/g dry

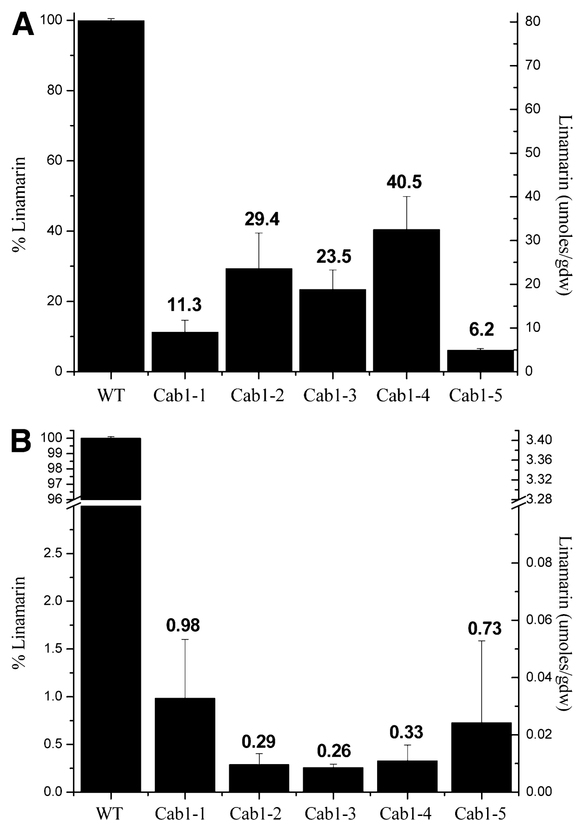

Fig. 4 GC–MS quantification of leaf (**A**) and root (**B**) linamarin contents in wild-type and Cab1 *CYP79D1*/*CYP79D2* antisense transformed cassava plants. Each sample was normalized on the basis of the internal standard (phenyl β-D-glucoside). Linamarin is expressed as a percentage of the quantity present in the wild-type untransformed plant (*WT*)

weight) and Wheatley et al. (1993) (0.4–2 µmol/g fresh weight) for mature field-grown cassava roots.

CYP79D1 and *CYP79D2* transcript levels in roots

One possible explanation for the low 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. To determine whether this was the case we quantified root *CYP79D1* and *CYP79D2* transcript abundance by RT–PCR. As shown in Fig. 5, there was no appreciable reduction in *CYP79D1* and *CYP79D2* transcript levels in 3- to 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.

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 (Anderssen et al. 2000). 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 the *CYP79D1* and *CYP79D2* antisense constructs were introduced into cassava plants under the control of the leaf-specific Cab1 promoter.

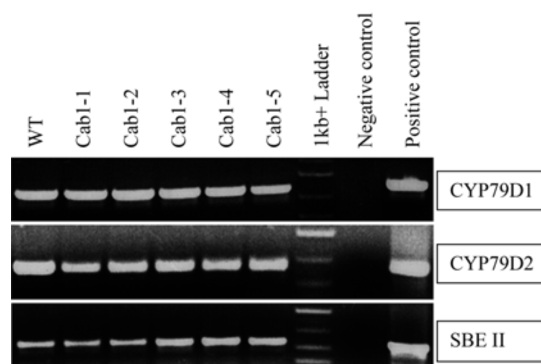

Fig. 5 RT–PCR analysis of *CYP79D1* and *CYP79D2* transcript levels in cassava roots. *CYP79D1* and CYP79B2 transcripts were amplified using primers specific for the 3′ end of the genes (Dl-F1/D1-R4 and D2-F1/D2-R5 primer pairs amplify *CYP79D1* and *CYP79D2*, respectively). *WT* Wild type

The five transformants with altered *CYP79D1* and *CYP79D2* transcripts had up to a 94% reduction in leaf linamarin content. Interestingly, for some transformants (Cab1–2 and Cab1–5) there was no correlation between the reduction in *CYP79D1*/*CYP79D2* transcript levels and linamarin content. This dichotomy may reflect additional metabolic flux controls that determine rates of linamarin synthesis, storage and export. A similar lack of direct correlation between transcript abundance and metabolic state has been observed for other transgenic plants in which a single gene has been targeted for silencing (Stitt and Sonnewald 1995). In addition, we view the results from the RT–PCR analyses of *CYP79D1* and *CYP79D2* transcript abundance as more qualitative (diagnostic of gene silencing) than quantitative.

Importantly, transformants that had between 94% and 60% reductions in their leaf linamarin content all had root linamarin contents that were less than 1% of wild-type levels. 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

between plant organs 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 (Selmar et al. 1988; Koch et al. 1992). At the sink site, linustatin is apparently metabolized by one of two mechanisms involving either a simultaneous or a 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 product, which is then deglycosylated to produce acetone cyanohydrin (Selmar 1993). It is noteworthy that linustatin has not been detected in cassava plants in quantities sufficient to facilitate cyanogen transport by the mechanism observed in *Hevea* (McMahon et al. 1995). However, Bediako et al. (1981) observed transport of ^{14}C-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 and Indira 1984).

It also is apparent, however, that cassava root protoplasts and microsomal fractions are capable of synthesizing linamarin (McMahon and Sayre 1994; Du et al. 1995). The contribution of root linamarin synthetic activity to the overall steady-state levels remains to be determined, particularly in young plants. These results suggest that the reduced root linamarin content of Cab1 *CYP79D1*/*CYP79D2* antisense plants having reduced leaf linamarin levels may be attributed to reduced transport of cyanogens to roots. If this explanation is correct, however, then there also must be a threshold leaf linamarin content required for linamarin transport to roots since plants having 40% of the wild-type leaf linamarin content have less than 1% of the wild-type root linamarin content. Currently, 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. Preliminary results indicate that root-specific inhibition of *CYP79D1* and *CYP79D2* expression has no effect on root linamarin levels, consistent with the hypothesis that root linamarin is largely transported from leaves to roots.

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 and Sayre 2000).

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