SPECIAL GUEST EDITOR SECTION

Transgenic Approaches for Cyanogen Reduction in Cassava

DIMUTH SIRITUNGA
University of Puerto Rico Mayaguez, Department of Biology, Mayaguez, Puerto Rico 00680
RICHARD SAYRE[1]
Ohio State University, Department of Plant Cellular and Molecular Biology, Columbus, OH 43210

For cassava to become a safe and acceptable crop, it is necessary to reduce the cyanogen levels in cassava foods. While this objective can be achieved by processing procedures, recent findings have shown that it is also possible to achieve it by suppression of cyanogen synthesis or by accelerating cyanogen turnover and volatilization. In 2003, cyanogen-free cultivars were generated by selective inhibition *CYP79D1/D2* gene expression. The CYP79D1/D2 enzymes catalyze the first-dedicated step in cyanogen synthesis. Tissue-specific inhibition of *CYP79D1/D2* expression in leaves lead to a 99% reduction in root cyanogen levels, indicating that the cyanogenic glycoside, linamarin, is synthesized in leaves and transported to roots. An alternative strategy to the reduce cyanogen content is to enhance cyanogen detoxification and cyanide volatilization during processing. This strategy has the advantage that cyanogen levels in unprocessed roots are not altered, potentially providing protection against herbivory and/or theft. To produce cultivars that promote rapid cyanide volatilization, hydroxynitrile lyase (HNL), which catalyzes the last step in cyanogenesis, was overexpressed in roots. Elevated HNL activity resulted in a 3-fold increase in the rate of cyanogen turnover. Importantly, the cyanogen content of the transformed and wild-type plants was identical, a potential benefit for farmers.

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. This woody perennial shrub serves as a basic staple for over 600 million people worldwide, most of whom live in developing regions of the world (1). Grown in over 60 countries in Africa, Asia, and Latin America, cassava is the fourth most important crop in the developing countries, surpassed only by maize, rice, and sugarcane as a source of calories (2). When cultivated under optimal conditions, cassava is one of the most efficient producers of edible carbohydrates among all of the world's major food crops. Between 1995 and 1997, the production of cassava was estimated to be 165.3 million metric tons/year, and it is projected to reach 290.8 million metric tons/year by 2020 (3). At 142 trillion kilocalories/year, cassava ranks first in edible energy production among major root and tuber crops and ranks fifth among all crops directly consumed by humans (3). Cassava is second only to sugarcane in caloric production/unit of land (3).

For a variety of reasons, cassava is the crop of choice for subsistence and small-scale farmers in the tropics. Most importantly, cassava provides food security. Cassava is able to withstand long periods of drought and can be cultivated in extremely poor, exhausted soils, thus making it suitable for growth in marginal areas unable to sustain many other crops. Cassava is also propagated vegetatively from stem cuttings; therefore, the farmers do not have to sacrifice part of the harvest (roots) for the next planting season. A flexible harvesting time, 8–24 months, makes cassava an excellent famine foodstuff as well. The roots can be banked in the soil for up to 3 years, and it is also amenable to partial harvest.

Cyanogens in Cassava

The cyanogenic glycosides, linamarin (95%) and lotaustralin (5%), are present in all parts of the plant with the exception of seeds (4–6). Cyanogenic glycosides have been shown to protect cassava from herbivory by animals and generalized insect feeders as well as from theft (7–10). Significantly, cyanogen levels in leaves [200–1300 mg cyanide (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 Food and Agriculture Organization of the United Nations. Therefore, cassava foods must be processed to remove cyanogens prior to consumption. The residual cyanogens, linamarin and acetone cyanohydrin (the deglycosylated form of linamarin), are the apparent source of CN toxicity to animals and can be converted to CN inside the body. For an adult human, consumption of 50–100 mg, or 2 mmol, hydrogen cyanide (HCN) equivalents within 24 h can completely block cellular respiration, leading

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[1] Author to whom correspondence should be addressed; e-mail: sayre.2@osu.edu

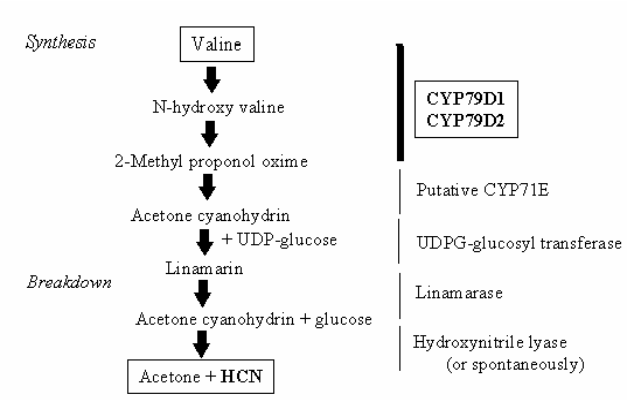

Figure 1. Linamarin biosynthesis and turnover pathway in cassava. UDP = Uridine diphosphate.

to death (11). Exposure to lower levels of CN can also cause a variety of symptoms, such as vomiting, nausea, palpitations, headaches, and impaired vision (12). The long-term ingestion of large quantities of poorly processed cassava has also been shown to be associated with chronic CN-associated health disorders in several areas of Africa (13, 14). During droughts, cassava-associated CN poisoning is aggravated by the lack of firewood, resulting in inadequate cooking and detoxification of cassava. CN intake from a cassava-dominated diet is also a contributing factor in 2 forms of nutritional neuropathies, tropical ataxic neuropathy described from Nigeria (15) and epidemic spastic paraparesis. (Konzo) descriibed from Mozambigue, Tanzania, and Zania (11) Furthermore, extensive studies in Zaire have established that goiter and cretinism due to iodine deficiency are aggravated by continuous dietary exposure to CN from insufficiently processed cassava. This effect is caused by high circulating levels of thiocyanate, the detoxification product of CN. Thiocyanate interferes with iodine intake in the thyroid gland (16).

Compartmentalization of the Synthesis and Breakdown of Cyanogens in Cassava

The first committed step in the synthesis of linamarin is the conversion of valine to 2-methylpropanol oxime and then to acetone cyanohydrin (Figure 1). Both of these steps are catalyzed by multifunctional cytochrome P450s. The initial *N*-hydroxylation of L-valine to *N*-hydroxyvaline is believed to be the rate-limiting step in linamarin synthesis (17, 18). Glycosylation of acetone cyanohydrin by indoxyl-uridine diphosphate glucose (UDPG)-glucosyltransferase yields linamarin (Figure 1). White et al. (19) showed that the conversion of valine to linamarin occurred in a nicotinamide adenine dinucleotide phosphate-dependent reaction in partially purified vacuoles, suggesting that the cytochrome P450s involved in the initial biosynthesis steps are localized in the tonoplast membrane or endoplasmic reticulum fractions and that the UDPG-glucosyltransferase, which glycosylates acetone cyanohydrin, is a vacuolar enzyme (20). More recently, Andersen et al. (18) isolated 2 full-length complementary deoxyribonucleic acid (cDNA) clones that encode cytochrome P450s catalyzing the conversion of valine to 2-methylpropanol oxime. These 2 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 synthesis pathway. The second cytochrome P450 has not been isolated from cassava. But the similarities between the linamarin synthesis pathway in cassava and the dhurrin synthesis pathway in sorghum lead to the belief that a cytochrome P450 closely related to sorghum CYP71E1 is involved the conversion of 2-methylpropanol oxime to acetone cyanohydrin in cassava (18, 21). The linamarin biosynthetic pathway is presumably localized on the endoplasmic reticulum or associated with the vacuolar membrane. Ultimately, linamarin is sequestered in vacuoles if not transported or metabolized directly (22).

The generation of CN from linamarin involves the initial deglycosylation of linamarin by linamarase followed by the cleavage of acetone cyanohydrin to produce CN and acetone (Figure 1). Linamarase is localized in the cassava cell wall and is also abundant in laticifers (23, 24). Therefore, release of CN occurs only after tissue damage when linamarin comes in contact with linamarase, as is the case during herbivore attack or during food processing (6, 8, 25). The production of CN from acetone cyanohydrin is catalyzed by hydroxynitrile lyase (HNL), which also is localized in the cell wall (26). Acetone cyanohydrin may also break down spontaneously at temperatures >35°C or pH values >5 (19, 24; Figure 1). The gene encoding HNL has been isolated, and the cassava protein shares 78% amino acid identity with rubber tree HNL sequence (19, 24). In leaves, linamarase and HNL have similar catalytic efficiencies and protein levels providing an efficient mechanism for cyanogenesis and food detoxification (26, 27). Cassava roots, however, have much lower linamarase activities than leaves and very little or no HNL activity, presumably accounting for the accumulation of potentially toxic levels of acetone cyanohydrin in poorly processed cassava roots (19, 26). More recently, Santana et al. (28) have reported the absence of linamarase messenger ribonucleic acid (mRNA) in the roots of 2 cassava cultivars (high and low cyanide). These results may suggest that root linamarase is not synthesized in roots but is transported to the roots through the laticifer system. Overall, the compartmentalization of linamarin in the vacuole and linamarase and HNL in separate sites prevents the formation of toxic CN in undamaged cells. The breakdown of the physical barriers between substrates and the enzymes following tissue damage initiates cyanogenesis (29).

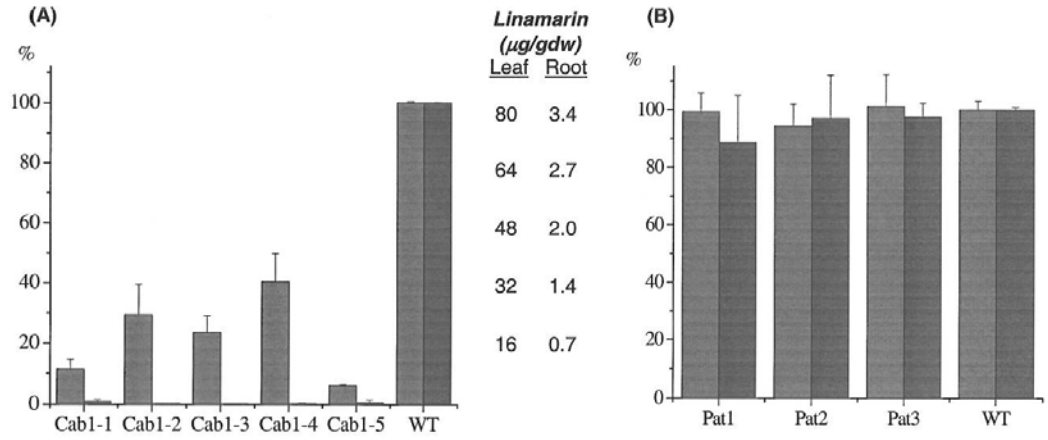

Figure 2. Linamarin content of transgenic and wild-type cassava plant in which the expression of the *CYP79D1/D2* genes has been selectively inhibited in the leaves (A) and roots (B) using *Arabidopsis* cab1 and potato patati promoters, respectively. gdw = Gram dry weight.

Strategies Employed for Cyanogen Reduction Using Genetic Manipulations

Two strategies have been explored to reduce the cyanogen toxicity of cassava food products: (*1*) inhibition of the expression of the *CYP79D1/D2* genes (22, 30, 31) and (*2*) overexpression of HNL in roots (32). The objective of the first strategy was to block linamarin synthesis and of the second to accelerate cyanogenesis, CN volatilization, and food detoxification during processing.

Generation of Acyanogenic Cassava

Although linamarin can potentially be synthesized in secondary roots of cassava (27, 33), the majority of the linamarin is apparently synthesized in the leaves and transported to the roots (22, 34–38). To determine the most effective strategy for reducing root linamarin content and to evaluate 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 (22, 30). To achieve this, the *CYP79D1/D2* genes that encode the enzymes catalyzing the first-dedicated step in linamarin synthesis were expressed in an antisense orientation driven either by the leaf- or root-specific *cab1* and *patatin* promoters, respectively (39–41). The transgenic plants were generated using Agrobacterium-mediated transformation of somatic embryos, and the integration of the transfer DNA (T-DNA) was confirmed by polymerase chain reaction (PCR) and Southern blot analysis (22, 30). Reverse-transcriptase-PCR analyses of CYP79D1/D2 transcript abundance in antisense plants indicated that CYP79D1/D2 transcript levels could be reduced to low or nondetectable levels in leaves in which the *cab1* promoter was used to drive the antisense expression of the *CYP79D1/D2* genes. In these plants, the CYP79D1/D2 transcript levels in roots were unaffected. In contrast, transgenic plants in which the antisense expression of the *CYP79D1/D2* genes was driven by the root-specific *patatin* promoter had very low or undetectable levels of CYP79D1/D2 transcripts in roots and only slightly reduced or unaffected CYP79D1/D2 transcript levels in leaves.

Quantitative analyses of the leaf and root linamarin content by gas chromatography/mass spectrometry using internal standards to account for linamarin turnover showed that the steady-state linamarin levels were unaltered in patatin-CYP79D1/D2 transformants (22). In comparison, the leaf linamarin content of Cab1-CYP79D1/D2 transformants was reduced between 60 and 94% (30). More importantly, the Cab1-CYP79D1/D2 transformants also exhibited a substantial reduction in root linamarin content, to <1% of wild-type levels (Figure 2; 30). 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 is associated with a reduction in root cyanogen levels.

Interestingly, cab1-CYP79D1/D2 antisense plants initially died when transferred to potting soil but had normal growth patterns when grown (in vitro) in Murashige and Skoog (MS) salts containing both nitrate (40 mM) and reduced nitrogen (20 mM NH_4Cl). When the same plants were grown on MS media in which the ammonia was replaced with nitrate (60 mM), they failed to produce strong roots (22, 42). 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 and served in part as a source of reduced nitrogen for root amino acid metabolism. Subsequently, independent cab1-CYP79D1/D2 antisense transgenic lines having <99.7% reduction in steady-state root linamarin levels have been shown to produce roots in soil, suggesting that other sources

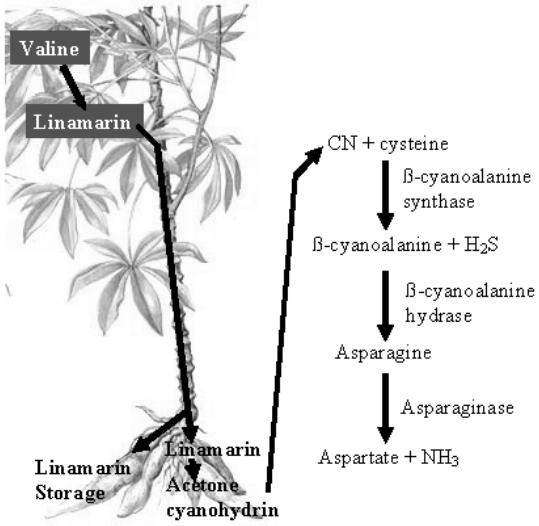

Figure 3. Proposed pathway for the transportation of linamarin from the leaves to root and the reassimilation of cyanide into asparagine.

of reduced nitrogen may compensate for reduced linamarin transport from leaves.

Recently, it has been proposed that mature cassava plants may use linamarin as a transportable source of reduced nitrogen for amino acid synthesis in roots (Figure 3). According to this model, linamarin is deglycosylated, presumably by a generalized β-glucosidase, generating CN. The CN is then assimilated along with cysteine via β-cyanoalanine synthase to produce β-cyanoalanine and sulfide. The β-cyanoalanine is then hydrated to form the amino acid asparagine. Deamination of asapargine generates aspartate and free ammonia, which can be reassimilated by the glutamine synthetase/glutamate synthase cycle (43, 44). Consistent with this model, it has been reported that the activities of β-cyanoalanine synthase and β-cyanoalanine hydrase are 3-fold greater in roots than in leaves (45, 46). In 1969, Nartey (47) reported that germinating cassava seedlings exposed to ^{14}CN incorporated 49% of the radioactive label into the amide carbon of asparagine and 6% into aspartate. In addition, some labeled carbon from the nitrile was found incorporated in glutamine and glutamate. As previously discussed, Cab1-4 transgenic plants having a 60% reduction in leaf linamarin steady-state levels required additional reduced nitrogen in the growth media to support normal root growth, suggesting that the flux of nitrogen through linamarin to root protein synthesis was limiting. In contrast, transgenic plants in which CYP79D1/D2 siRNA constructs driven by the CaMV 35S promoter were presumably transformed into plants were reported to have normal growth in soil (31). These plants also had reduced root linamarin levels, but no molecular evidence was provided indicating that the plants had integrated the transgenes or that the CYP79D1/D2 transcript levels had been altered in presumably transgenic plants. At the current time, the contribution of linamarin metabolism to root nitrogen balance remains unknown. There is sufficient evidence, however, to suggest that linamarin transport from leaves and its subsequent metabolism in roots plays a substantive role in root nitrogen metabolism.

Cyanogenesis and Postharvest Deterioration

One of the major constraints limiting the marketability of cassava is its rapid postharvest (24–72 h) physiological deterioration (PPD) following harvesting from the plant (48). The process of PPD has strong parallels to wound-induced oxidative stress and senescence responses. The initial events in PPD involve the formation of reactive oxygen species (ROS) followed by the induction of the expression of several enzymes involved in ROS metabolism, including superoxide dismutases, catalase, and various peroxidases (49). Sealing the root with wax or placement in an anaerobic environment substantially prolongs the shelf life of cassava, but these technologies are often beyond the reach of subsistence farmers. In nonphotosynthetic tissues, there are 2 major sources of ROS production, the mitochondrial electron transfer chain and the plasma membrane-bound nicotinamide adenine dinucleotide, reduced (NADH) oxidase. Unlike most root or tuber crops, cassava is distinguished by the presence of cyanogenic glycosides. Wounding of cassava roots will lead to the generation of CN. CN is a well-known inhibitor of the mitochondrial electron transport terminal electron donor, cytochrome C oxidase. Inhibition of cytochrome C oxidase by CN leads to overreduction of complexes I and III, which, in turn, can lead to the production of ROS (50).

We recently explored the possibility that cyanogenesis in wounded cassava roots induces ROS production and, in turn, PPD. Using young roots from wild-type and transgenic cassava plants lacking root linamarin, it was demonstrated that wounding induced copious ROS production in wild-type roots but virtually no ROS production in roots lacking linamarin (Siritunga et al., personal communication). In addition, we have observed that supplementation of root slices with cysteine, a substrate for β-cyanoalanine synthase, reduced the vascular streaking associated with PPD. Presumably, the addition of cysteine facilitated CN assimilation by β-cyanoalanine synthase, reducing the effective CN inhibition of cytochrome C oxidase and associated ROS production (T. Zidenga and R.T. Sayre, personal communication). These results suggest that cyanogens in cassava roots have multiple functions that impact the quality and safety of cassava foods.

Production of Safer Food Products While Retaining the Herbivore Deterrence Qualities of Cassava

An alternative strategy to produce safer cassava food products grew out of the observation that cassava roots have very low levels of HNL activity (26). Surprisingly, the most abundant cyanogen in poorly processed cassava may be

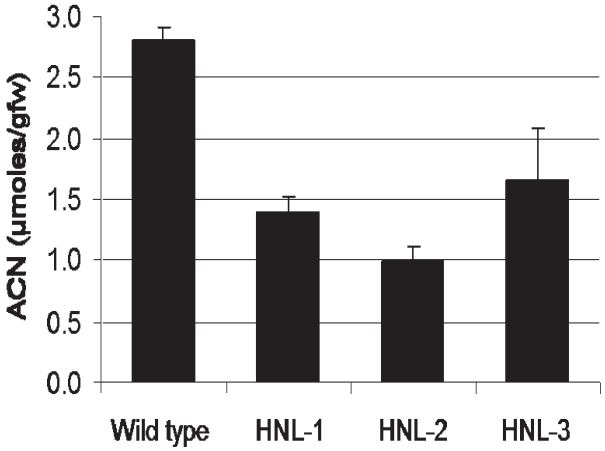

Figure 4. Acetone cyanohydrin (ACN) content of 8-month-old (greenhouse-grown) wild-type and HNL overexpressing transgenic roots 2 h posthomogenization. gdw = Gram dry weight.

acetone cyanohydrin, the substrate for HNL (13). It had been assumed that acetone cyanohydrin would not accumulate in poorly processed cassava roots due to spontaneous or enzymatic (HNL) decomposition releasing free CN. Since it was apparent that the lack of HNL in cassava roots could lead to the accumulation of acetone cyanohydrin, a possible effective strategy for cyanogen removal was to accelerate cyanogenesis and CN volatilization by overexpression of HNL in transgenic roots (32). To test this hypothesis, Siritunga et al. (32) generated transgenic cassava having increased root HNL activity (13-fold higher) relative to wild-type plants. Expression of HNL cDNA was driven by constitutive double 35S CaMV promoter, and transgenic cassava plants were generated via *Agrobacterium*-mediated transformation of germinated somatic embryos. Under experimental conditions (pH 5.0, 25°C) optimized to reduce the spontaneous decomposition of acetone cyanohydrin, it was observed that >80% of the linamarin was converted to acetone cyanohydrin within 2 h in well-homogenized cassava roots, and that virtually no free CN was released. In contrast, transgenic plants expressing 13-fold higher HNL activity had only 1/3 the level of acetone cyanohydrin of wild-type plants 2 h after homogenization associated with its breakdown to produce CN (32). The presence of elevated amounts of HNL enzyme in the transgenic roots resulted in an accelerated turnover of acetone cyanohydrins compared to untransformed wild-type plants (Figure 4). Importantly, the linamarin content and apparent linamarase activity of the transformed and untransformed plants were identical. Therefore, the observed differences in acetone cyanohydrin accumulation between wild-type and transgenic roots could not be attributed to strain-dependent differences in linamarin content or deglycosylation by linamarase. Notably, the relative reduction in acetone cyanohydrin content in processed roots paralleled the relative increase in root HNL activity between the different transgenic plant lines. Having unaltered levels of linamarin prior to harvesting and processing is a beneficial trait for subsistence farmers in Africa due to their generalist herbivore deterrent qualities.

Future Strategies for Transgenic Manipulation of Cyanide Turnover and Reassimilation Pathways

Based on an increasing appreciation of the dynamics of cyanogen metabolism in cassava roots, a number of new strategies are being tested for reducing the linamarin content of roots and the associated effects of CN generation from linamarin. One possible strategy to reduce root linamarin content is to divert symplastically loaded linamarin from storage in root vacuole towards assimilation into asparagine via CN production and assimilation into β-cyanoalanine via β-cyanoalanine synthase. While enhanced cytoplasmic cyanogenesis may accelerate ROS production and potentially initiate PPD, it is also possible that linamarin flux through the cytoplasm may occur at sufficiently low rates that increasing cytoplasmic rates of cyanogenesis may not adversely affect CN-dependent ROS production. To enhance the cytoplasmic turnover of linamarin, we have targeted linamarase to the cytoplasm and to the vacuole in transgenic plants. In addition, we are exploring overexpression of β-cyanoalanine synthase in root mitochondria to facilitate CN assimilation into free amino acids. Transgenic plants expressing these genes are currently being screened phenotypically to determine whether accelerated cytoplasmic metabolism of linamarin and its diversion from vacuolar storage towards β-cyanoalanine synthesis is an effective strategy for cyanogen reduction, increased amino acid synthesis, and reduced PPD.

Acknowledgments

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References

(1) Cock, J.H. (1982) *Science* **218**, 755–762
(2) Bradbury, J.H. (1988) *Asian Food J.* **4**, 3–13
(3) Scott, G.L., Best, R., Rosegrant, M., & Bokanga, M. (2002) *Roots and Tubers in the Global Food System: A Vision Statement to the Year 2020*, A copublication 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
(4) Conn, E.E. (1979) *Int. Rev. Biochem*. **27**, 21–43
(5) Conn, E. (1994) *Acta Hort*. **375**, 31–43
(6) Balagopalan, C., Padmaja, G., Nanda, S., & Morthy, S. (Eds) (1988) *Cassava in Food, Feed and Industry,* CRC Press, Boca Raton, FL
(7) Nahrstedt, A. (1985) *Plant Syst. Evol.* **150**, 35–47
(8) Bellotti, A., & Riss, L. (1994) *Acta Hort*. **375**, 141–151
(9) Bellotti, A., & Arias, B. (1992) in *Proceedings of the First International Scientific Meeting of the Cassava Biotechnology Network*, W.M. Roca and A.M. Thro (Eds),

Centro Internacional de Agricultura Tropical, Cali, Colombia, pp 406–409

(10) Nweke, F.I., Spencer, D., & Lynam, J.K. (2002) *The Cassava Transformation: Africa's Best Kept Secret*, Michigan State University Press, East Lansing, MI

(11) Rosling, H. (1993) *Lancet* **341**, 177–181

(12) Rosling, H. (1988) *Cassava Toxicity and Food Security*, United Nations International Children's Education Fund, Geneva, Switzerland

(13) Tylleskar, T., Banea, M., Bikangi, N., Cooke, R.D., Poulter, N.H., & Rosling, H. (1992) *Lancet* **339**, 208–211

(14) Mlingi, N., Poulter, N., & Rosling, H. (1992) *Nutr. Res.* **12**, 677–687

(15) Osuntokun, B.O. (1981) *World Rev. Nut. Diet* **36**, 141–173

(16) Bourdoux, P., Delange, F., Grard, M., Mafuta, M., Hanson, A., & Ermans, A.M. (1978) *J. Clin. Endicronol. Metab*. **46**, 613–621

(17) Koch, B., Nielsen, V.S., Halkier, B.A., Olsen, C.E., & Møller, B.L. (1992) *Arch. Biochem. Biophys*. **292**, 141–150

(18) Andersen, M., Bush, P., Svendsen, I., & Møller, B. (2000) *Biol. Chem*. **275**, 1966–1975

(19) White, W.L.B., McMahon, J.M., & Sayre, R.T. (1994) *Acta Hort*. **375**, 69–78

(20) Hughes, J., & Hughes, M.A. (1994) *DNA Seq.* **5**, 41–49

(21) Bak, S., Olsen, C.E., Halkier, B.A., & Møller, B.L. (2002) *Plant Physiol*. **123**, 1437–1448

(22) Siritunga, D., & Sayre, R. (2004) *Plant Mol. Biol.* **56**, 661–669

(23) Mkpong, O.E., Yan, H., Chism, G., & Sayre, R.T. (1990) *Plant Physiol*. **93**, 176–181

(24) Hughes, J., Carvahlo, F., & Hughes, M. (1994) *Arch. Biochem. Biophys*. **311**, 496–502

(25) Cock, J.H. (1985) *Cassava: New Potential for a Neglected Crop*, Westview Press, Boulder, CO

(26) White, W., Arias-Garzon, D., McMahon, J., & Sayre, R. (1998) *Plant Physiol*. **116**, 1219–1225

(27) McMahon, J., White, W., & Sayre, R. (1995) *J. Exp. Bot.* **46**, 731–741

(28) Santana, M.A., Vasquez, V., Matehus, J., & Aldao, R.R. (2002) *Plant Physiol.* **129**, 1686–1694

(29) Poulton, J. (1990) *Plant Physiol*. **94**, 401–405

(30) Siritunga, D., & Sayre, R. (2003) *Planta* **217**, 367–373

(31) Jørgensen, K., Bak, S., Busk, P.K., Sørensen, C., Olsen, C.E., Puonti-Kaerlas, J., & Møller, B.L. (2005) *Plant Physiol.* **139**, 363–374

(32) Siritunga, D., Arias-Garcon, D., White, W., & Sayre, R. (2004) *Plant Biotechnol. J.* **2**, 37–43

(33) Du, L., Bokanga, M., Møller, B., & Halkier, B.A. (1995) *Phytochem*istry **39**, 323–326

(34) Bediako, M., Tapper, B., & Pritchard, G. (1981) in *Proceedings of the First Triennial Root Crops Symposium of the International Society for Tropical Root Crops*, E.R. Terry, E.V. Doku, O.B. Arene, & N.M. Mahlungu (Eds), International Development Reasearch Center, Ottawa, Canada, pp 143–148

(35) Makame, M., Akoroda, M.O., & Hahn, S.K. (1987) *J. Agric. Sci. Cambridge* **109**, 605–608

(36) Koch, B., Nielsen, V., Halkier, B., Olsen, C., & Møller, B. (1992) *Arch. Biochem. Biophys.* **292**, 141–150

(37) Selmar, D., Lieberei, R., & Biehl, R. (1988) *Plant Physiol.* **86**, 711–716

(38) Selmar, D. (1994) *Acta Hort*. **357**, 68–81

(39) Brusslan, J., & Tobin, E. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 7791–7795

(40) Kim, S., Gregory, D., & Park, W. (1994) *Plant Mol. Biol*. **26**, 603–615

(41) Zourelidou, M., Torres-Zabala, M., Smith, C., & Bevan, M. (2002) *Plant J*. **30**, 489–497

(42) Siritunga, D. (2002) Ph.D. Thesis, The Ohio State University, Columbus, OH

(43) Lea, P.J., Robinson, S.A., & Stewart, G.R. (1990) in *The Biochemistry of Plants,* Vol. 16, B.J. Miflin & P.J. Lea (Eds), Academic Press, San Diego, CA, pp 121–159

(44) Lea, P.J., Blackwell, R.D., & Joy, K.W. (1992) in *Nitrogen Metabolism in Plants,* K. Mengel & D.H. Pillbeam (Eds), Claredon Press, Oxford, UK, pp 153–186

(45) Elias, M., Sudhakaran, P., & Nambisan, B. (1997) *Phytochemistry* **46**, 469–472

(46) Elias, M., Nambisan, B., & Sudhakaran, P. (1997) *Plant Sci*. **126**, 155–162

(47) Nartey, F. (1969) *Physologia Plantarum* **22**, 1085–1096

(48) Beeching, J.R., Han, Y., Gomez-Vasquez, R., Day, R.C., & Cooper, R.M. (1998) *Recent Adv. Phytochem.* **32**, 231–248

(49) Reilly, K., Gomez-Vasquez, R., Buschmann, H., Tohme, J., & Beeching, J.R. (2003) *Plant Mol. Biol*. **53**, 669–685

(50) Maxwell, D.P., Wang, Y., & McIntosh, L. (1999) *P. Natl. Acad. Sci. USA* **96**, 8271–8276