Opportunities for Biofortification of Cassava for Sub-Saharan Africa: The BioCassava Plus Program

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Cassava is an important staple crop in sub-Saharan Africa. Between 1970 and 2007, its production and acreage tripled across Africa and quadrupled in Nigeria, the continent's and the world's largest producer (FAO, 2008). Sub-Saharan Africa produced over 117 million tons of fresh roots of cassava in 2008, of which no less than 95% was consumed as food; the starch provides >25% of dietary energy for an estimated 200 million Africans (Dorosh, 2007). Frequent consumers of cassava are at greater risk for malnutrition—especially deficiencies in vitamin-A, iron, and zinc—than consumers of other diets, particularly those that are cereal-based (Gegios *et al.*, 2010). A nutrition survey in cassava-consuming areas of Nigeria and Kenya revealed inadequate intake of vitamin A in 83% and 41% and inadequate iron intake in 43% and 78% of pre-school-aged children, respectively (Gegios *et al.*, 2010). Vitamin-A deficiency causes a loss of 964,000 disability-adjusted life years (DALYs[1]) in Nigeria and 161,000 DALYs in Kenya annually; iron deficiency causes loss of 596,000 DALYs in Nigeria and 103,000 DALYs in Kenya (Fielder, 2009).

[1]A measure of overall disease burden, expressed as the number of years lost due to ill-health, disability or early death.

Current efforts to combat micronutrient deficiencies in Africa include supplementation, through distribution of micronutrients to high-risk populations, addition to processed food and biofortification, *i.e.* the genetic improvement of nutrient content of crops via field-based breeding or genetic engineering. Supplementation requires tremendous effort to exceed 90% coverage and must be sustained for many years (Berti and Rowley 2001); few countries in Africa are able to run effective supplementation programs. Biofortification, on the other hand, can achieve 100% penetration and, although it requires a substantial initial investment in research and dissemination, it is self-sustaining. Cost per DALY saved for biofortification is 20% less compared to supplementation (Nestel et al., 2006).

BioCassava Plus (BC+) is a cassava-biofortification project at the Donald Danforth Center in St. Louis, MO, funded by the Bill and Melinda Gates Foundation. BC+ scientists are engineering cassava for increased accumulation of β-carotene, iron, and protein to provide minimum daily allowances of these essential nutrients as a means of ameliorating the burden of malnutrition that accompanies consumption of cassava as a staple food. Proof of concept for the enrichment of these nutrients has been demonstrated in the model cassava cultivar 60444, which, in greenhouse and confined field trials in Puerto Rico, contains up to 40 µg/g dry weight (DW) of β-carotene (provitamin A), 40 µg/g dry weight of iron, 10% protein storage roots, and reduced levels of anti-nutritional cyanogens.

Biofortification of Cassava

β-*carotene*

β-carotene enrichment of storage roots in cassava is conferred by two transgenes: the *Erwinia crtB* phytoene-synthase gene, and the *Arabidopsis* 1-deoxyxylulose-5-phosphate synthase (DXS) gene. The *crtB* transgene includes a 0.1-kb sequence for the plastid transit peptide for the Δ4-palmitoyl-acyl carrier protein desaturase from coriander. The phytoene synthase encoded by the *crtB* gene catalyzes the committed step in β-carotene synthesis using geranylgeranyl-diphosphate (GGDP) from the plastid isoprenoid pathway as its substrate. DXS catalyzes the first step in the plastid isoprenoid pathway. Increased expression of this enzyme is intended to enhance concentrations of GGDP for β-carotene synthesis and also to ensure sufficient amounts of GGDP to maintain vitamin-E production at or above wild-type levels. The *crtB* and *DXS* genes are each under the control of the *Solanum tuberosum* (potato) patatin promoter and flanked on their 3' ends by the 3' untranslated region (UTR) of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens*. The selectable marker is the neomycin phosphotransferase II (nptII) from *E. coli* under control of the cauliflower mosaic virus 35S (CaMV 35S) promoter (with 2x enhancer). The nptII gene is flanked on its 3' end by the CaMV 35S 3' UTR.

Plants expressing the *crtB* and *DXS* genes were initially evaluated in four-inch pots under greenhouse conditions. Amounts of total carotenoids ranged from 30 to 60 µg/g DW in storage roots of the top lines. By comparison, amounts of total carotenoids in storage roots of control 60444 plants in these and subsequent greenhouse and field studies ranged from 1.5 to 2.5 µg/g DW. Subsequent evaluations were conducted of storage roots of plants growing in soil beds in the greenhouse and in confined field trials in Puerto Rico.

Concentrations of total carotenoids in roots from greenhouse beds typically ranged from 30 to 45 µg/g DW. Concentrations of carotenoids from the confined field studies ranged from 30 to 40 µg/g DW. Concentrations of vitamin E were not significantly different between storage roots from the top β-carotene lines and non-transformed controls. In the transformed lines, the relative amounts of all-trans-β-carotene, the most nutritionally efficacious form of carotenoid provitamin A, were 85 to 90% of the total carotenoid content. Relative amounts of trans-β-carotene in non-transformed controls were only 50 to 60% of the total carotenoid content. In collaboration with partners in Africa, BC+ has confined field trials of the β-carotene-enriched GM events (*i.e.* transformed plant lines) ongoing in Nigeria and another trial is planned for fall 2010 in Kenya.

In addition to the consumer benefit of improved nutrient levels, BC+ β-carotene-rich GM events also have producer benefits in terms of extended shelf-life. Five of the events with the highest amounts of total carotenoids could be stored for up to 28 days after harvest, whereas the wild-type recorded up to 80% spoilage after 7 days. Reduced shelf-life of cassava roots, a result of post-harvest physiological deterioration (PPD), is a major limitation to marketing of fresh roots. PPD begins 24 hours after harvest and can render the roots unpalatable and unmarketable within 72 hours. Short shelf-life affects cassava value-added chains because it increases losses during processing and limits access to markets distant from production sites. Longer shelf-life was correlated with total carotenoid content ($r^2 = 0.80$) in the GM events, which is consistent with previous studies indicating that high levels of carotenoids in the roots (> 8 ppm fresh weight basis) delay PPD (Sanchez *et al.*, 2005).

Nutrient retention during food preparation was also evaluated in three transgenic lines expressing higher levels of β-carotene. In three common Nigerian food preparations—*gari*, *fufu* and boiled—retention of provitamin A ranged from 82% to 37%, equal to, or better than, that seen in the wild-type 60444 variety. Values for *in vitro* bioavailability of provitamin A, as measured by uptake into micelles of Caco human intestinal cells, were similar for the transgenic and wild-type sources, *i.e.* 21% and 23%, respectively.

Iron

Increased iron content was achieved by the expression of the *FEA1* gene, from *Chlamydomonas reinhardtii*, in cassava storage roots. The FEA1 protein is an iron-specific metal transporter. It transfers iron efficiently at very low concentrations or at high pHs at which iron is largely insoluble. It does not transport toxic heavy metals unlike all other known metal transporters in plants. As with the β-carotene trait, *FEA1* expression was driven by the patatin promoter and *nos* terminator. Seven GM events were tested in the greenhouse and in confined field trials in Puerto Rico; iron content in the GM events ranged from 30 to 40 µg/g DW in storage roots compared to 10 µg/g dry weight in the wild type. Real-time PCR analyses strongly suggest that the additional iron is stored as a ferritin complex in the transgenic plants.

The morphology, including branching and flowering, of transgenic plants was identical to that of wild-type plants in Puerto Rico.

Protein

Increased protein content in cassava was achieved by the expression of sporazein, a storage fusion protein consisting of a 180-bp fragment of β-zein from maize, the sporamin gene from sweet potato and a 506-bp fragment of β-zein. Sporazein is a nutritionally balanced protein of 49.6 kD in which the zein components drive accumulation of the product to form protein bodies within the endoplasmic reticulum. As with the other two traits, the patatin promoter and nos terminator drive sporazein accumulation in cassava storage roots.

Seven GM events expressing sporazein were tested in the greenhouse, in soil beds, and in confined field trials in Puerto Rico. Total protein content of storage roots harvested ranged from 9 to 11% DW across all seven transgenic lines studied. The morphology and growth habit of these transgenic plants have shown no significant differences to non-transgenic controls in all three locations tested to date. No changes in expression of trait accumulation have been observed over the 18 months during which these plants have been tested in the greenhouse and field. As protein bodies are water-insoluble, water soaking overnight at room temperature resulted in 94% retention of the total protein content of the cassava roots. Boiling for 30 min resulted in 95% retention of the total protein content.

It is well known from other plant systems that the direction of reduced nitrogen to the synthesis of new proteins may come at the expense of nitrogen allocation to essential proteins required for metabolism. Thus, elevating expression of storage proteins in cassava may impair the growth or biochemical properties of roots. To address this concern, we have over-expressed a vacuolar targeted linamarase, an enzyme that breaks down linamarine, a cyanogenic glucoside found in cassava leaves, stems, and roots. This has been shown to increase the pool sizes of free amino acids in cassava.

Product Development of Nutrient-Enriched Cassava for Africa

BC+ has achieved nutrient enrichment of cassava such that if a 5-year-old child consumes 100 g/day of roots from the β-carotene, iron, or protein-rich GM events, (s)he will obtain 100% of the minimum daily allowance (MDA) of these nutrients. BC+ has, therefore, embarked on the expression of genes for the aforementioned nutritional traits in farmer-preferred cassava varieties from Nigeria and Kenya, its two target countries. Genetic transformations at the Donald Danforth Plant Science Center (DDPSC) have successfully generated transgenic lines of Oko-Iyawo, the most popular Nigerian variety of cassava currently grown on 22 to 24% of total acreage (about 4 million hectares) in that country, and the Kenyan cultivar Serere.

Based on *ex-ante* impact studies for nutrient deficiency in Kenya and Nigeria, a β-carotene- and iron-enriched Oko-Iyawo for Nigeria, and a β-carotene-, iron- and protein-enriched, and virus-resistant, Serere for Kenya were selected as first products. Oko-Iyawo is resistant to cassava mosaic disease (CMD), which is of viral origin and is the principal production constraint of cassava in Africa, whereas Serere is susceptible to CMD and needs to be engineered for resistance. Product development is divided into several stages, namely:

- generation of transgenic events,
- greenhouse characterization and testing,
- field testing,
- selection of a lead event,
- food and environmental safety assessments in a regulatory field trial,
- biosafety approval for commercial release,
- on-farm trials, and finally
- variety release and dissemination.

Lead Event Selection

A commercial quality construct that is codon-optimized without extraneous genetic elements, will be created for the *crtB, DXS, FEAI* genes (β-carotene and iron traits) and transformed into friable embryogenic callus derived from Oko-Iyawo. Six hundred transgenic plants will be regenerated and screened for events that possess a single copy of the construct, no vector backbone sequences and good RNA expression. Events, an estimated 125, that meet these criteria will be planted in the greenhouse and roots evaluated for β-carotene and iron contents. Events that meet the set targets of 40 μg/g DW for the two traits will be transferred to confined trials in Puerto Rico and Nigeria for trait assessment under field conditions. This will be followed by two cycles of replicated confined field trials at three locations to select a lead event and a back-up for regulatory trials. A similar procedure will be followed for the Kenyan product, with the exception that the gene construct will contain sporazein and RNAi for CMD and cassava brown streak disease (CBSD), a viral disease of cassava that is specific to East Africa.

Biosafety Regulatory Activities

Cultivation and consumption of cassava expressing these genes will require the granting of approval from regulatory bodies in Nigeria, based upon food and environmental safety assessments of each novel gene/protein that confers the targets traits. A safety assessment is required for each event intended for commercial release. *DXS*, *crtB*, *FEA1*, sporazein, and *npt* II proteins will be purified, characterized, and assessed for potential toxicity in acute (single dose) oral gavage studies with mice. Allergenicity of the *DXS, crtB*, and *FEA1* proteins will be assessed in accordance with international guidelines. This is a 'weight of evidence' assessment that includes the source of the gene, documented dietary exposure to the protein, any amino-acid sequence homology to known allergens, and protein stability upon incubation in simulated gastric fluid.

The purified proteins will also be used to develop Western blot and enzyme-linked immunosorbent assays for detection of the proteins in plant material. As the *DXS* and *crtB* proteins are enzymes and will be present at higher levels than what are typical in cassava, regulatory authorities will likely require information on substrate specificity. Evidence from the peer-reviewed literature on known substrate specificity for these enzymes will be sought.

A key consideration of regulators is assessment of any unintended effects that result from elevated biosynthesis of metabolites in the carotenoid biosynthetic pathway. Unintended effects are traditionally addressed in the safety assessment by performing extensive nutrient-composition and agronomic performance analyses. Compositional analyses of starch quantity and quality, fatty acids, total and free amino acids, minerals, vitamins, cyanogenic glucosides and phytates will be conducted. Protocols for the compositional and agronomic regulatory field trials—a final field trial conducted on a lead event to generate agronomic performance, food and environmental safety information—will be in accordance with international guidelines.

Reaching End-Users

Agricultural development in Africa is replete with examples of well intended scientific advances that have had limited impact because they were not sustainably adopted by producers and consumers. Fortunately, there are also excellent examples of widespread successful adoption of new varieties. The strategy to reach end-users should avoid the mistakes of the former and build on the successes of the latter, while taking into account the special issues associated with transgenic varieties. Many failures have resulted from a narrow focus on preferred production characteristics. A critical aspect important for reaching end-users is seed production and distribution. In Africa, the vast majority of cassava stakes for planting is generated from current plants by the farmer or obtained from neighbors; the private sector has little interest in this crop. However, non-government organizations (NGOs) involved in development activities and some national agricultural systems now have good experience with dissemination of new varieties. BC+ will partner with organizations that have the best linkages to large numbers of farmers. Tissue culture and an inexpensive two-node multiplication system for rapid and massive propagation will be used to bulk up foundation seed for distribution to large NGOs and government agencies.

Cost-effective strategies for farmer adoption and consumer acceptance of β-carotene and iron-rich cassava entail a marketing and promotion plan. For example, *gari* is the most important food staple of the rural and urban poor and the *gari* market chains have extensive coverage; over 70% of cassava grown in Nigeria is used for *gari* production. A great advantage is that the deep yellow color of high-β-carotene and -iron cassava is similar to a yellow *gari* product, made by addition of palm oil, already accepted by consumers. Although *gari* processing tends to lead to a depletion of β-carotene and iron, levels of nutrient enrichment achieved in BC+ ensure that sufficient amounts of these nutrients remain to meet minimum daily allowances based on the average quantity consumed and bioavailability.

Anti-GMO NGOs may be expected to mount campaigns in opposition to dissemination and adoption of β-carotene- and iron-enriched cassava. Opposition can be overcome through demonstration of the benefits to consumers (improved nutritional quality) and to producers/processors (extended shelf life of storage roots). Other elements for countering such opposition include: adhering strictly to bio-safety protocols and regulatory requirements; being transparent by engaging key stakeholders in constant dialogue to communicate progress and building confidence that the process is being properly and