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

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Abstract

More than 250 million Africans rely on the starchy root crop cassava (*Manihot esculenta*) as their staple source of calories. A typical cassava-based diet, however, provides less than 30% of the minimum daily requirement for protein and only 10%–20% of that for iron, zinc, and vitamin A. The BioCassava Plus (BC+) program has employed modern biotechnologies intended to improve the health of Africans through the development and delivery of genetically engineered cassava with increased nutrient (zinc, iron, protein, and vitamin A) levels. Additional traits addressed by BioCassava Plus include increased shelf life, reductions in toxic cyanogenic glycosides to safe levels, and resistance to viral disease. The program also provides incentives for the adoption of biofortified cassava. Proof of concept was achieved for each of the target traits. Results from field trials in Puerto Rico, the first confined field trials in Nigeria to use genetically engineered organisms, and ex ante impact analyses support the efficacy of using transgenic strategies for the biofortification of cassava.

Contents

INTRODUCTION

Food insecurity or inadequate caloric intake is a major cause of death and morbidity in the world, particularly in developing countries (35, 104, 161). Chronic malnutrition, or insufficient intake of essential nutrients and vitamins, affects more than two billion people worldwide, contributing to considerable illness, disability, and mortality (15, 104). Collectively, nutrition-related deficiencies are responsible for approximately 35% of global child deaths (1–2.5 million per year) and 11% of the total global disease burden (104). Over half (51 million) of the global annualized loss in disability-adjusted life years (DALYs) is attributable to malnutrition in sub-Saharan Africa, including 761,000 deaths directly attributed to vitamin A, iron, and zinc deficiency alone (13) (**Table 1**). Significantly, populations most susceptible to malnutrition subsist almost exclusively on plant-based diets, with a heavy emphasis on a single major staple crop for most of their calories (94). Deficiencies in any essential mineral, vitamin, or protein in diets based largely on a single staple can have potentially damaging health effects if the diet is not supplemented with the deficient nutrients.

Until recently, the primary strategies for addressing malnutrition relied on the delivery of mineral and vitamin supplements either in foods or as pills (5). However, these strategies require substantial infrastructure and ongoing management to succeed. A more recent alternative to address malnutrition is the development of biofortified crops (108, 134, 153). Significantly, crop biofortification has the potential to be a sustainable, one-time solution. Traditional crop breeding and targeted biotechnology approaches have each proved efficacious (101, 105, 134). Transgenic approaches, particularly when applied to clonally propagated crops (e.g., cassava, banana), have the potential to accelerate product development and address genetic constraints that may impede traditional breeding approaches. To be successful, however, crop biofortification programs must develop integrated management practices by which molecular biologists, breeders, agronomists, nutritionists, educators, economists, farmers, and consumers are all engaged in product development and delivery. Here, we describe the objectives and strategies of the BioCassava Plus program to develop genetically biofortified cassava and future strategies to test and deliver nutritionally enhanced cassava for Africa.

CASSAVA IN AFRICA

In sub-Saharan Africa, cassava ranks as the second most important source of calories after maize (97). More than 117 million tons of fresh roots were produced in sub-Saharan Africa in 2008, ranking cassava number one in food crop production (32). Cassava (*Manihot esculenta*, Crantz) was first domesticated in what is now Brazil and brought to West Africa in the sixteenth century by Portuguese navigators (49, 98). The unique biology of cassava has significant impact on its cultivation and harvesting practices (reviewed in 55). Cassava is a shrub that grows to 1–4 m in height over the course of a year (**Figure 1**). Flowering, when it occurs, is monoecious, which accounts for high levels of genetic heterozygozity. Significantly, many cultivars are not known to flower. As a result, cassava is almost exclusively cultivated clonally by stem cuttings (**Figure 1*b***). The root is the main carbohydrate storage organ, and

Table 1 Disability-Adjusted Life Years (DALYs):[a] quantifying the global impact of malnutrition[b]

Thousands of Disability-Adjusted Life Years Lost				
Region	**Population**	**Vitamin A deficiency**	**Iron deficiency Anemia**	**Zinc deficiency**
DALYs				
East Asia and the Pacific	1,823	994	241	1,004
Eastern Europe and Central Asia	475	1	66	149
Latin America and the Caribbean	524	218	109	587
Middle East and North Africa	301	2,043	109	3,290
South Asia	1,378	4,761	704	8,510
Sub-Saharan Africa	674	13,552	596	14,094
High-income Countries	957	0	40	2
Total	**6,132**	**21,569**	**1,865**	**27,636**
Deaths				
East Asia and the Pacific	1,823	11	18	15
Eastern Europe and Central Asia	475	0	3	4
Latin America and the Caribbean	524	6	10	15
Middle East and North Africa	301	70	10	94
South Asia	1,378	157	66	252
Sub-Saharan Africa	674	383	21	400
High-income Countries	957	0	6	0
Total	**6,132**	**627**	**134**	**780**

[a]DALYs = deaths + years reduced lifespan + years lost to disability.
[b]Adapted from Caulfield et al. (15).

each plant may have multiple storage roots. The fully developed cassava storage root can be subdivided into the periderm (bark), peel, and parenchyma. The parenchyma is the edible portion of the root and consists mainly of starch (85% dry weight). The peel is made up of sclerenchyma, cortical parenchyma, and phloem and accounts for approximately 11%–20% of the root weight. Due to its high cyanogenic glycoside content, the peel is removed before processing the root cortex.

Relative to other crops, cassava has several agronomic traits that distinguish it as a food security crop that can be counted on to provide a source of nutrition during crop failures. Under the marginal conditions in which cassava is often grown, it produces more energy per unit area than most other crops and with limited human inputs. Cassava is unusually drought tolerant, and the presence of cyanogenic glycosides reduces crop losses attributed to generalized herbivores (28, 29, 88). In addition, the flexible harvest time, ability to store roots for long periods in the soil (up to 3 years), and the resilience of the crop to stress make cassava a major food security crop for subsistence farmers in sub-Saharan Africa (18, 116). It is the reliability of cassava harvests that is most important to cassava farmers.

Although cassava is highly regarded in many developing countries as a food security crop, it has a number of major liabilities. A typical adult-sized cassava meal (500 g) can provide adequate calories but is an insufficient source of iron, zinc, vitamin A, and protein (16, 32, 39) (**Table 2**). A meal of typical size meets only 30% of the minimum daily requirement (MDR) for iron and zinc and 10% of the daily provitamin A (β-carotene) requirement (32). Cassava also has the lowest protein-to-carbohydrate ratio of the world's 10 major crops. Uniquely, cassava contains potentially toxic levels of

Figure 1

Cassava (*Manihot esculenta*) cultivation. (*a*) Cassava shrub, (*b*) cassava roots and stakes, (*c*) cassava stem cuttings for propagation.

cyanogenic glycosides (linamarin) that must be removed through extensive food processing. During processing, linamarin is converted to cyanide, which is volatilized to create a safe food product (83, 154). Long-term ingestion of poorly processed cassava may result in consumption of sufficient cyanogens (linamarin and acetone cyanohydrin) to cause goiter, tropical ataxic neuropathy, or in acute cases, which typically occur during starvation, permanent paralysis (i.e., Konzo) and/or death (99, 140).

An additional constraint for cassava is its short shelf life. Within 72 hours after harvesting, the storage roots deteriorate and are inedible. It has been estimated that up to 26% (18 million tons) of all cassava produced in Africa is lost every year to postharvest physiological deterioration (PPD) (150). The most important constraint limiting cassava production, however, is viral disease. Cassava mosaic disease (CMD), a geminivirus endemic to all the cassava-growing regions of Africa, is the single greatest constraint to cassava production. Incidences of CMD infection can be as high as 100% of all plants in a given region, with average yield reductions of 30%–40%. New infections occur by white fly–mediated transfer, but the planting of infected stakes can also spread the disease. In sub-Saharan Africa, more than 30 million tons of fresh cassava roots are lost yearly owing to CMD (68, 69).

BIOCASSAVA PLUS

In 2005, the Bill and Melinda Gates Foundation specifically targeted the problem of global malnutrition through its Grand Challenges in Global Health (GCGH) Program. The

Table 2 Nutritional qualities of cassava foods (FAO). Cassava roots are a rich source of calories but do not provide complete nutrition

Cassava meal	Energy (kCal)	Protein (g)	Iron (mg)	Zinc (mg)	Vitamin E (mg)	Vitamin A (mg)
MDR (%)	~80	30	<30	<30	10	10
Boiled	740	5.5	2.0	2.0	1.0	5
Dry	1,775	10.5	4.0	4.0	1.0	15
Flour	1,710	7.5	4.0	3.0	1.0	0
Fresh	745	6.0	2.0	2.0	1.0	5
Roasted	1,360	10	2.5	3.0	1.0	5

Abbreviations: FAO, Food and Agricultural Organization; MDR, minimum daily requirement in a 500-g meal.

objective of Grand Challenge 9 (GC-9) of the GCGH program was to create nutritionally complete staple food crops for the most vulnerable populations of the world (22, 155). The BioCassava Plus (BC+) program was established as one of the four GC-9 projects to address malnutrition in sub-Saharan African populations. The objectives of the BC+ program were to provide complete nutrition in a typical adult-sized cassava meal (500 g) while incorporating economic drivers (reduced cyanogens, virus resistance, extended shelf life) for the adoption and acceptance of biofortified cassava.

To develop and deliver a biofortified cassava for Africa required the coordination of a diverse group of scientific and product development experts from around the world. The BC+ members included experts in metabolic engineering and transformation, enabling technologies, postharvest physiology, plant breeding and mapping, field trial assessment, biosafety, regulatory affairs and intellectual property, and human nutrition (see Supplemental **Figure 1**, follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**). In the following sections, we describe the research and development objectives, strategies, and accomplishments of the BC+ program completed during Phase I (2005–2010) of the program.

ENABLING TECHNOLOGIES

From its inception, BC+ focused on transgenic strategies to achieve its goals. This focus was driven by the need to develop products as rapidly as possible as well as to address constraints (iron and zinc biofortification, and cyanogenesis) that were difficult to handle using traditional breeding approaches.

In order to ensure production of comparable data across the collaborating research groups, early decisions were made to utilize the model variety 60444 as the germplasm for all transgenic plant production, and pCAMBIA-based plasmids (**http://www.cambia.org/daisy/cambia/585.html**) for integrating expression cassettes via *Agrobacterium*-mediated transformation. Previous evidence for efficacy of the Class II patatin promoter (54, 130) determined its use to selectively drive expression of transgenes coding for nutritional traits in storage roots.

Choosing cv. 60444 as the genetic background for proof-of-concept work in BC+ was justified from the available data. When transgenic work was initiated in 2005, reports of genetically modified plant production were confirmed in the cultivars MCol 22 (71, 61) and MCol 2215 (54, 55, 130, 131), in addition to 60444 (41, 121, 123, 158). However, recovery of transgenic 60444 plants had been achieved in three different laboratories (138), indicating the transferability of the transformation protocol developed for this variety. Originally collected from farmers' fields in Nigeria (85, 38), 60444 was also the only African germplasm for which transgenic capacity had been demonstrated, an important factor considering the African focus of BC+. Although no longer cultivated by

Supplemental Material

farmers owing to its susceptibility to African cassava mosaic disease, 60444 was known to be adapted to conditions in West Africa and was expected to perform well in confined field trials after transgenic modification.

Recovery of transgenic 60444 plants is achieved through the production of friable embryogenic callus (FEC) (136, 137), which is brought into contact with *Agrobacterium*, followed by selection on medium supplemented with an aminoglycoside antibiotic such as paramomycin (137) or hygromycin (8). Somatic tissues in the form of in vitro derived immature leaves are utilized as the starting material for the production of embryogenic structures, which are in turn converted to the pro-embryogenic FEC by sequential subculture on medium containing Gresshoff and Doy (GD) basal salts (45). After co-culture with *Agrobacterium* strain LBA4404, colonies of putatively transgenic FEC were recovered on antibiotic medium and regenerated to mature cotyledon stage embryos. Whole plants were recovered on MS media (86) supplemented with benzylaminopurine (BAP). Time from *Agrobacterium* coculture to regeneration of whole plants is 4.5–5 months with escape rates of 5% or less (8, 121).

The above transformation system has served to produce transgenic plants using more than 40 different Ti-plasmid-based constructs designed to address questions of nutritional enhancement for iron, zinc, β-carotene, and storage protein, and for the study of alternative promoters with potential efficacy for driving accumulation of these traits to xylem parenchyma tissues within cassava storage roots. With a goal of 25 independent events per construct, more than 1000 independent transgenic plant lines have been generated within proof-of-concept phase of BC+ for the nutritional traits alone. The need to supply plants on this scale has driven improvements in the protocol for production and analysis of 60444 and contributed to the development of a pipeline approach to transgenic plant production for cassava. As a result, recovery of large numbers of transgenic cassava plants for a given construct is now routine. Time to recovery of transgenic plants has been reduced by four weeks and the process evolved to become routine for the model variety 60444.

Production of transgenic 60444 served well the proof-of-concept phase of BC+, generating sufficient plants to screen transgene expression cassettes and test hypotheses for all the traits addressed in the project. However, to deliver products to farmers, the transgenically imparted nutritional traits must be integrated into genetic backgrounds of cassava favored by farmers in the target regions of Nigeria and Kenya. As for all the major crop species, morphogenic potential and transformability of cassava varies significantly between varieties. In the case of cassava, however, the option of integrating transgenes into amenable backgrounds followed by backcrossing with favored breeding stock to produce agronomically suitable cultivars is problematic. Therefore, the capacity to genetically transform a given cultivar for delivery to end-users needed to be developed empirically in each case. Special efforts have also been made to develop a cassava transformation protocol that can be adapted by African laboratories (8, 9). The improved protocol reduces bacterial and fungal contamination during tissue culture and facilitates regeneration of transgenic events through gradual antibiotic selection. Substantial reduction in the workload and complexity of the procedure will also help to establish the transformation technology in other laboratories. The protocol appeared particularly suitable for implementation in African laboratories, as recently reported in Tanzania (8).

Increasing Zinc Content

Cassava (60444) contains approximately 10 ppm zinc in the root cortex, well below the amount needed to meet the MDR (32). One goal of BC+ was to increase zinc content in the tuberous root by greater than fourfold by overexpressing vacuolar- and plasma membrane–localized zinc transporters (43, 62). Overexpression of the vacuole membrane localized *ZAT* gene had previously been shown to elevate zinc levels in plants and was used

to increase the tuberous root zinc content fourfold (E. Gaitan-Solis, N. Taylor, and D.P. Schachtman, unpublished data) through the targeted overexpression of the *Arabidopsis* ZAT transporter using the patatin promoter (130).

An *Arabidopsis* ZIP plasma membrane zinc transporter (43) was also overexpressed in cassava to increase tuberous root zinc content. Cassava tuberous root zinc concentrations were enhanced two- to tenfold; however, leaf zinc concentrations were reduced and the leaves of the transgenic plants appeared zinc deficient (E. Gaitan-Solis, N. Taylor, and D.P. Schachtman, unpublished data). The higher zinc concentrations in roots and lower concentrations in leaves in cassava due to overexpression of a ZIP transporter were similar to what was observed when *OsZIP4* or *OsZIP5* was overexpressed in rice (57, 67). Overexpression of zinc transporters has been successful in altering the zinc content of multiple tissues in several plant species. However, the reduced zinc concentrations in cassava and rice leaves and decreased grain yield in rice caused by transporter overexpression highlight the need to further refine this approach.

Increasing Iron Content and Bioavailability

Iron is one of the most abundant elements in Earth's crust, but it is considered the third most limiting nutrient for plant growth owing to its low solubility (60). Iron concentrations (10 ppm) in cassava storage roots, however, are insufficient to meet the MDR. BC+ pursued several strategies for increasing iron in cassava roots, including increasing iron uptake using an iron-specific assimilatory protein (FEA1) from *Chlamydomonas reinhardtii*; overexpression of the iron storage protein, ferritin; and a combination of both strategies (87, 117). All three strategies effectively gave similar results. We focus here on a description of the results obtained using the FEA1 iron assimilatory protein because it is an iron-specific assimilatory protein and is operational in high-pH soils that limit iron uptake (87). Expression of the *FEA1* gene in wild-type *Arabidopsis* was shown to increase root iron content eightfold (87). A codon-optimized *FEA1* gene was expressed in cassava roots under control of the patatin promoter. The iron content of young (2 months old), wild-type fibrous roots was approximately 800 ppm, but by the time the roots had fully expanded the iron content had been reduced nearly 100-fold (56). This progressive decrease in iron content with age was presumably a result of tuberization (starch accumulation) and a reduction in iron uptake associated with the loss of root hairs in storage roots. The root iron content of the best performing transgenic lines was 42 ppm at the 6-month stage in Puerto Rico. Importantly, no secondary phenotypic effects were observed in greenhouse- or field-grown plants. Preliminary analysis of the expression levels of genes involved in iron homeostasis indicated that ferritin expression increased sixfold in roots of transgenic plants expressing the *FEA1* gene. As cassava contains virtually no detectable phytic acid, it is expected that ferritin-associated iron will be very bioavailable (N. Taylor, unpublished data).

Enhancing Protein Content in Cassava Roots and Its Relationship to Root Cyanogen Levels

Cassava roots contain on average 1%–2% protein by dry weight, substantially less than maize. A 500-g cassava meal provides only 30% of the daily protein requirement. To address protein deficiency in cassava, a variety of transgenic strategies were attempted, including creating a strong root protein sink for storage proteins and increasing root free-amino-acid pool sizes to support elevated root protein accumulation.

It has been demonstrated that cyanogens provide reduced nitrogen substrates for amino acid synthesis in cassava roots (90, 130, 131). Inhibition of linamarin synthesis in leaves results in up to a 99% reduction in root linamarin content and a suppression of root growth (63) (**Figure 2**). The partitioning of linamarin between storage in root vacuoles and assimilation into amino acids represents a potential control

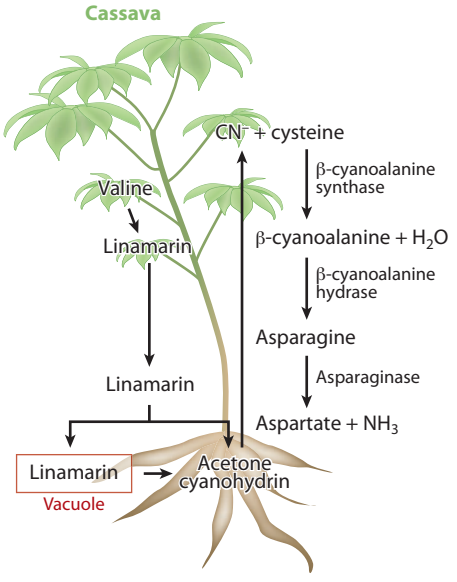

Figure 2

Linamarin metabolism in cassava. Linamarin is synthesized in leaves. Some fraction of the linamarin is transported symplastically to roots, where it is either stored in the vacuole (*red box*) or metabolized to provide reduced nitrogen (nitrile) for amino acid synthesis (72).

point for regulating amino acid pool size and presumably root protein levels. It was hypothesized by the Sayre lab that accelerating the conversion of linamarin to acetone cyanohydrin and cyanide would elevate root amino acid pools through the assimilation of cyanide via β-cyanoalanine synthase (**Figure 2**). Targeting linamarase to the vacuole using a barley vacuolar targeting sequence resulted in an average 60% increase in total free amino acids in roots as well as a 25% decrease in leaves (70). Although there was no increase in root total protein levels in transgenic plants, there was a 16% increase in leaf protein levels, suggesting that the leaves are a sink for reduced nitrogen.

Creating a strong protein sink in roots was by itself sufficient to elevate total root protein. Cassava hydroxynitrile lyase (HNL) is the enzyme that catalyzes the conversion of acetone cyanohydrin to cyanide. Because this enzyme is localized in the apoplastic space around leaf cells, it could presumably be induced to accumulate there by overexpression without turning over. Significantly, HNL is not expressed in roots. Thus, in wild-type plants, acetone cyanohydrin, the substrate for HNL, can accumulate in poorly processed roots (129, 140, 154, 155). Once ingested, acetone cyanohydrin spontaneously decomposes to produce cyanide. Transgenic lines were produced expressing a patatin-driven HNL construct that had a threefold increase in total root protein and, importantly, an 80% reduction in root linamarin levels. Leaf linamarin levels were unaffected, thus continuing to provide protection against generalized herbivores. These results indicate that creating a strong root protein sink effectively reduces the steady-state root cyanogen pool size, which is consistent with the metabolism of cyanogens for root protein synthesis (131). Notably, overexpressing HNL in roots also accelerates (0.5 versus 24 h) the conversion of acetone cyanohydrin to cyanide (volatile) during processing, further reducing the potential for cyanide poisoning from cassava foods (131).

Because little was known about the biological factors regulating the accumulation of storage proteins in cassava roots, alternative approaches for designing, expressing, and targeting storage proteins to increase root protein accumulation were explored. One of the earliest attempts to accumulate proteins in roots was the expression of artificial storage proteins and their accumulation in the cytoplasm. Cassava plants were transformed with an artificial storage protein gene, *ASP1*, designed to be rich in essential amino acids (158). The transgene was expressed in leaves and roots under control of the CaMV 35S promoter. Total protein content of in vitro leaves was found not to differ from the nontransgenic plants; however, proline and serine levels were significantly elevated, whereas asparagine, alanine, and methionine were depressed compared with controls (158). These results suggested that the amino acid composition of storage proteins could impact the overall amino acid composition in plant tissues. No analyses were carried out on root protein levels.

Note Added in Proof

Erratum

Figure 3 and one paragraph on page 259 described the production and accumulation of protein bodies in transgenic cassava plants expressing a chimeric protein named zeolin. Figure 3 and the paragraph refer to a publication in *PLoS ONE* (Abhary M, Siritunga D, Stevens G, Taylor NJ, Fauquet CM (2011) Transgenic Biofortification of the Starchy Staple Cassava (*Manihot esculenta*) Generates a novel Sink for Protein. *PLoS ONE* 6(1): e16256). The authors of the *PLoS ONE* publication have been unable to confirm the presence of the zeolin gene within the transgenic cassava plants. This raised concerns about the validity of the results reported in the article, which resulted in retraction (http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0016256). Consequently Figure 3 and the paragraph on page 259 in the *Annu. Rev. Plant Biol.* 2011. 62:251-272 are also retracted.

The authors apologize to the readers and wish to point out that other data and publications discussed in the *Annu. Rev. Plant Biol.* 2011. 62:251-272 article are not affected by the retraction of the *PLoS ONE* publication cited above.

Similar to results with HNL-overexpressing roots, transgenic roots accumulating zeolin also had substantial (55% of wild type) reductions in linamarin content, consistent with the role of cyanogenic glycosides in root protein synthesis (131). Importantly, more recent protein fusion constructs designed to form protein storage bodies in cassava roots reduce the likelihood of introducing food allergens associated with potentially allergenic domains present in phaseolin.

Enhancing Provitamin A Content

Carotenoids are essential components of the human diet. This diverse class of molecules function as provitamin A or precursors of vitamin A. Vitamin A, or retinol, plays critical roles in a number of aspects of human health, including vision, bone development, reproduction, and immune function (9, 31, 43). Of the different forms of carotenoids, all-*trans*-β-carotene is considered to be the most nutritionally important form because it is most readily converted to retinol by the body (32, 44). Deficiencies in vitamin A are manifested as night blindness and increased susceptibility to infections (35, 44). Major portions of the population in the cassava production area in sub-Saharan Africa are plagued by deficiencies in vitamin A, particularly in children (2).

A number of carotenoid-rich cultivars with elevated levels of different carotenoid forms have been described and may provide a solution to vitamin A deficiency in sub-Saharan Africa (53, 91, 92, 139). In this regard, a number of yellow-flesh cultivars with increased concentrations of all-*trans*-β-carotene have been reported and are currently being explored for use in Nigeria for nutritional biofortification (139).

A biotechnological approach for provitamin A biofortification has been pursued as a component of BC+ to introduce this trait into farmer-preferred varieties and to combine it with traits such as virus resistance and enhanced iron content. The goal of combining or "stacking" several unrelated traits into a single cultivar is currently difficult or unattainable by

breeding. The target for a biotechnological-based strategy for provitamin A biofortification is all-*trans*-β-carotene, which is produced by the plastid localized methyl-erythritol phosphate (MEP) isoprenoid pathway. From research done in other species, enhancement of β-carotene can be achieved by directing and enhancing flux into the carotenoid biosynthetic pathway or by downregulating the turnover of β-carotene. The most widely cited examples of the former approach are those used for the generation of Golden Rice and Golden Rice 2 (101, 157). In these examples, increased concentrations of β-carotene in rice were achieved by endosperm-specific coexpression of phytoene synthase and bacterial phytoene desaturase (*crtI*) transgenes. Phytoene synthase catalyzes the condensation of two molecules of geranylgeranyl diphosphate to form phytoene. This is the committed step in carotenoid synthesis, and expression of the phtyoene synthase and desaturase transgenes under the control of strong promoters effectively increases the flux of isoprenoid precursors to the synthesis of β-carotene and other carotenoid species. In addition to these studies, strong promoter-mediated expression of phytoene synthase genes from plants and bacteria (e.g., *crtB*) have been used to enhance carotenoid accumulation in a number of species and organs, including canola seeds (127), potato tubers (23, 24), and tomato fruits (37).

In addition to enhancing flux into carotenoid synthesis, increased carotenoid accumulation can be achieved by downregulation of carotenoid catabolism or turnover. In addition to serving as a nutritional source of provitamin A, β-carotene is also a precursor of ABA via a catabolic pathway involving hydroxylation and chain cleavage reactions (124). With regard to nutritional enhancement, allelic variation in the maize β-carotene hydroxylase genes has been attributed, for example, to natural differences in β-carotene concentrations in maize (143, 156). In addition, antisense suppression of β-carotene hydroxylases has been shown to enhance β-carotene concentrations in potato tubers (24).

BC+ has focused primarily on enhancement of provitamin A content of cassava storage roots by enhancing flux into carotenoid biosynthesis. Two strategies were undertaken (132). The first was the expression of the bacterial phytoene synthase gene *crtB* under control of the storage-root specific promoter for the potato patatin gene (132). A plastid transit peptide sequence was linked to crtB for proper subcellular localization of phytoene synthase. The second strategy was the co-expression of codon-optimized *crtB* (with plastid transit peptide coding sequence) and the *Arabidopsis* 1-deoxyxylulose-5-phosphate synthase (DXS) genes, placed individually under control of patatin promoters. DXS was used in the second strategy to increase total flux in the plastid isoprenoid pathway for enhanced production of the geranylgeranyl diphosphate (GGDP), the substrate of phytoene synthase. Because GGDP is also a precursor of the hydrophobic side chain of vitamin E tocochromanols, it was hypothesized that DXS upregulation would result in increased carotenoid production without impact on vitamin E concentrations in storage roots. Enhanced expression of DXS has been previously used in transgenic approaches to increase carotenoid levels in *Arabidopsis* leaves (31), tomato fruits (30), and *Escherichia coli* (81).

Stable *Agrobacterium*-mediated transformation of cassava (cv 60444) was achieved with constructs for the two strategies outlined above (132). Transgenic expression of phytoene synthase alone in cassava storage roots yielded increases in total carotenoid concentrations of 10- to 20-fold relative to amounts in roots from nontransformed controls. In these engineered roots, concentrations as high as 25 $\mu g\ g^{-1}$ dry weight (DW) were detected. By comparison, carotenoid concentrations were 1 to 2.5 $\mu g\ g^{-1}$ DW in storage roots from nontransformed plants. These measurements were obtained from storage roots at approximately six to eight weeks of age that were harvested from plants grown under greenhouse conditions in small pots with compacted root systems. Using the second strategy of co-expression of phytoene synthase and DXS

Figure 4

Transgenic cassava accumulate high levels of provitamin A. Plants overexpressing DXS and *crtb* (*right*) have elevated β-carotene relative to wild-type roots (*left*) (132).

transgenes, carotenoid concentrations in roots of similar age were 15- to 30-fold higher than those in storage roots from nontransformed plants, reaching concentrations >50 μg/g DW (**Figure 4**). In the highest carotenoid-producing roots, all-*trans*-β-carotene accounted for 85% to 90% of the total carotenoid content of storage roots, with minor amounts of lutein, 9-*cis*-β-carotene, and 13-*cis*-β-carotene. These studies from greenhouse-grown plants indicated that approaches involving the enhancement of flux into carotenoid biosynthesis are viable methods for provitamin A biofortification in cassava. Of the two strategies tested, co-expression of phytoene synthase and DXS was more effective at increasing carotenoid concentrations than expression of phytoene synthase alone. It is notable that in contrast to the Golden Rice studies (101, 157), the transgenic expression of the bacterial phytoene desaturase gene *crt*I was not necessary for increased production of β-carotene.

Levels of β-carotene were maintained in roots of engineered plants in subsequent field testing in Puerto Rico, indicating the effectiveness of this strategy for biofortification of cassava storage roots under not only greenhouse but also field conditions. Notably, carotenoid concentrations in roots from the top transgenic events were similar to those obtained in Golden Rice 2 (101). An additional finding from these studies was that vitamin E concentrations in roots co-expressing phytoene synthase and DXS transgenes were largely unaffected by enhanced carotenoid accumulation, despite the fact that these pathways have a common intermediate.

Postharvest physiological deterioration. Cassava storage roots suffer from a rapid deterioration upon harvest that can render them unpalatable and unmarketable within 24–72 h after harvest (59, 93, 150). Postharvest physiological deterioration (PPD) is a major constraint to the development and exploitation of cassava as a crop, food, and commodity. A recent *ex ante* impact assessment estimates that extending the shelf life of cassava to several weeks would reduce financial losses by $2.9 billion in Nigeria alone over a 20-year period (118).

The onset of PPD is associated with an increase in respiration (142, 50, 141), changes in lipid composition (64), synthesis of ethylene (50), accumulation of secondary metabolites from the phenylpropanoid pathway, and increases in many enzyme activities, including PAL and chalcone synthase, glucanase, chitinase, proteinase inhibitors, HRGPs, invertase, catalase, dehydrogenase, peroxidase, and polyphenol oxidase (113, 135). Wounding of cassava roots enhances respiration rates within the first day, which is followed by primary physiological deterioration (141, 142). The phytohormone ethylene is produced in cassava roots within 6 h of wounding (106, 50), with higher ethylene production in susceptible roots. Solomos & Laties (133) suggested the activation of an alternative cyanide insensitive respiratory pathway by ethylene. This pathway is known to exist in cassava tissues (102), and it is proposed that this pathway may lead to the formation of peroxides (16). However, preharvest pruning, a practice known to delay PPD, did not prevent ethylene accumulation (50), thus raising questions about the role of ethylene in the onset of cassava root PPD.

The phenolic compounds associated with the development of physiological deterioration have been identified and include scopoletin, scopolin, esculin, proanthocyanidins, (+)-catechin, and (+)-gallocatechin (115, 151, 152). Wheatley & Schwabe (152) showed

the crucial role that scopoletin plays in the development of physiological deterioration of cassava root. They showed that when applied to freshly cut roots, scopoletin was able to produce intense and rapid discoloration of the tissue. It was shown that (*a*) pruning of the aerial portions of the plant 2–3 weeks before harvest and (*b*) curing (i.e., storage under high-humidity conditions to facilitate wound healing response and reduce access to oxygen) can dramatically reduce the susceptibility of the root to PPD (7, 74, 78, 147). But application of scopoletin (1000 mg dm^{-3}) to freshly cut roots from plants pruned 1, 2, or 3 weeks before harvest resulted in intense deterioration of the treated tissues regardless of the length of pruning-to-harvest interval (152). Fluorimetric assays of scopoletin revealed the presence of greater than 170 $\mu g\, g^{-1}$ DW within proximal root sections 24 h after harvest (152). Scopolin and esculin have also been detected within 10 h after wounding (11).

Enzymes involved in the phenylpropanoid pathway, including PAL and 4-coumarate: CoA ligase, have been shown to be differentially regulated upon wounding (72). The expression of PAL activity closely follows the progress of cassava root PPD, and peaks after approximately 2 days of wounding (115). Peroxidases at the cassava root wound surface act upon the phenolic compounds generated by PAL (114, 115). Apparently, accumulation of the enzyme occurs by de novo synthesis and is induced after a 24 h lag period. Experiments utilizing cycloheximide to inhibit protein synthesis (142) and other studies (6) have shown that PPD is an active process involving gene expression and protein synthesis.

Several of the ketones and other volatiles detected in cassava root (33) have been previously detected in other plants; however, the formation of 2-propanone and 2-butanone is presumably due to either the spontaneous or enzyme (HNL in leaves and stems only) catalyzed decomposition of acetone cyanohydrin in cassava (65). HNL uses the cyanogenic glycosides 2-propanone cyanohydrin and 2-butanone cyanohydrin, and becomes active upon mechanical chewing or fungal infestation, thus leading to HNL release of hydrogen cyanide (HCN) and a ketone depending on the substrate (125). Using RNA fingerprinting analyses, Huang et al. (52) showed that transcripts involved in important biochemical and physiological processes, notably oxygen stress and carbohydrate and protein metabolism, are involved in PPD (52, 19). In an attempt to identify the entire subset of genes that are differentially regulated in cassava during PPD, Reilly et al. (109) carried out a large-scale cDNA microarray analysis of the cassava root transcriptome. They found 72 differentially regulated ESTs, of which 63 were upregulated and 9 were downregulated. Many of the upregulated PPD-specific ESTs were predicted to play roles in cell wall repair, reactive oxygen species (ROS) generation and turnover, programmed cell death, ion/water/metabolite transport, signal transduction, stress response and metabolism, and protein synthesis (109).

ROS have been shown to increase very early during PPD (110–112) and evidence for the involvement of ROS and associated turnover enzymes during PPD is accumulating (16, 109). Several lines of evidence suggest that there is a controlled production of ROS in plant defense, especially in response to wounding and pathogen attack. Reilly et al. (109) reported a rapid oxidative burst within 15 min of harvest, signaling the start of PPD, predominantly due to a rapid production of superoxide and hydrogen peroxide (110). Several roles have been attributed to the accumulated ROS species, among which cell wall repair and remodeling, induction of defense-related genes, signal transduction, and triggering host cell death are significant. Using sequentially sectioned cassava roots, it was found that superoxide dismutase (SOD), catalase, and peroxidase were predominantly expressed in regions closer to the wound site (58).

Aldehydes have been detected from harvested root by Iyer et al. (58) These emissions are known to be derived from fatty acid oxidation, and were previously detected in cassava leaves. The commonly known pathway in fruits and vegetables leading to C6 aldehyde and

alcohol formation is derived from fatty acids via lipoxygense (LOX) and alcohol dehydrogenase (ADH) (48, 122, 149). The increase in aldehydes is associated with senescing plant tissues (128). Given the detection of aldehydes and only trace quantities of alcohols at 3 h after root detachment (58), the ADH pathway is seemingly operating during cassava root PPD.

Based on the overwhelming evidence that an oxidative burst was associated with the onset of PPD, two different strategies were developed to reduce PPD: prevention of ROS production and scavenging ROS. As discussed above, one of the unique physiological attributes of cassava roots is the presence of cyanogenic glycosides, which are the substrate for CN generation upon cellular disruption. Cyanide blocks cytochrome C oxidase, leading to the overreduction of complex I and III, resulting in the generation of ROS. It was observed in transgenic, low-cyanogen (1% of wild-type) plants that ROS production was related to the level of cyanogens (130, 16). Upon tissue damage, transgenic, low-cyanogen (1% linamarin of wild type) plants produced substantially less ROS than wild-type plants. Significantly, the reduced ROS production in low-cyanogen plants was complemented by adding cyanide back to the roots.

This data suggested that PPD could be controlled by reducing cyanide-dependent ROS production and accumulation. Overexpression of the cyanide-insensitive alternative oxidase (AOX) in transgenic tobacco had been shown to decrease ROS accumulation generated by the respiratory chain electron transport (82). Overexpression of *Arabidopsis* AOX in transgenic cassava roots resulted in substantially reduced ROS accumulation and delayed the onset of PPD by as much as three weeks, enough time for the shipping or processing operations necessary after harvesting the crop (**Figure 5**).

The second strategy to reduce PPD was to quench ROS production by overexpression of ROS-metabolizing enzymes (e.g., catalase, SOD, ascorbate peroxidase) or by the overaccumulation of anti-oxidants, such as β-carotene. It was also previously observed that cassava varieties with elevated β-carotene content had extended shelf life (40, 132). Indeed, the shelf life of transgenic plants with elevated β-carotene (40 ppm) content was extended to four weeks. Overall, these results suggest that ROS production from cyanide-poisoned mitochondria initiate PPD and that reduction in ROS accumulation will extend the shelf life of harvested cassava roots.

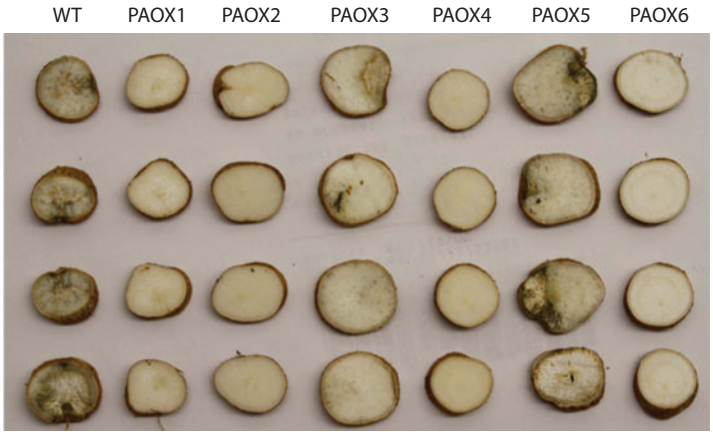

Figure 5

Reduced postharvest deterioration in cassava roots overexpressing a mitochondrial alternative oxidase from *Arabidopsis*. Root slices were made in roots harvested 21 days earlier. Independent transgenic lines expressing a patatin–alternative oxidase construct are labeled PAOX1–6. Abbreviation: WT, wild type.

Cassava mosaic disease. As a result of its vegetative propagation, cassava is particularly vulnerable to viral infections. CMD and cassava brown streak disease (CBSD) are the two most prominent cassava diseases in Africa. CMD is caused by cassava mosaic geminivirus, which is transmitted by whiteflies [*Bemisia tabaci* (3, 69, 103)], and was the primary focus of BC+.

Cassava geminiviruses have two components, DNA-A and DNA-B, which encode a total of eight viral proteins. DNA-A encodes six proteins involved in replication, transcription, and encapsidation, whereas DNA-B encodes two proteins required for virus movement (46, 47). In the last two decades, various approaches have been tested in plants to engineer geminivirus resistance (reviewed in 144). Strategies tested were mostly based on interference in key

viral protein functions such as the Rep-associated protein, which is required for replication. The discovery that gene silencing participates in plant defense against viruses (149) opened new routes to engineer virus resistance via RNA interference (RNAi) pathways. Pooggin and colleagues (107) had previously shown that transient expression of hairpin double-stranded (ds) RNAs homologous to the noncoding intergenic region of *Vigna mungo* yellow mosaic virus could enhance plant recovery from viral infection. Stable transgenic cassava lines overexpressing hairpin dsRNAs homologous to the noncoding intergenic region of African cassava mosaic virus (ACMV) were produced. The cassava lines remained susceptible to ACMV infection, but they had an enhanced recovery phenotype compared with wild-type plants (146). ACMV replication appeared to be strongly impeded in leaf disks of transgenic cassava lines. Due to its key role in viral replication, the viral replication-associated protein (Rep) appeared as an obvious target for silencing (159). Constitutive expression of artificial hairpin dsRNAs homologous to the Rep coding sequence in transgenic cassava demonstrated that an ACMV-susceptible cultivar could become immune to ACMV infection (145). Virus resistance levels correlated with the load of hairpin-derived small RNAs. These and other RNAi transgenics are undergoing field trials in Uganda and will soon be in trials in Kenya as well.

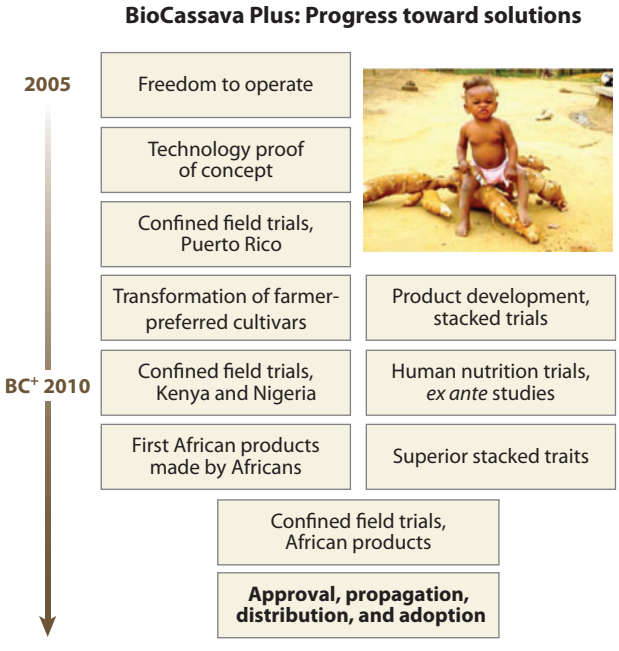

Figure 6

The BioCassava Plus (BC+) roadmap for product development and delivery.

Confined field trials in Puerto Rico and Africa. To properly gauge the engineered trait's performance under natural environmental conditions, the BC+ program conducted confined field trials (CFTs), first at the University of Puerto Rico, Mayaguez, and then at the National Root Crops Research Institute in Nigeria. By 2010, 110 independent transgenic lines (>3,800 plants) had been tested in Puerto Rico, including genetically enhanced cassava biofortified with β-carotene, iron, zinc, and protein and cassava lines with low cyanogens, increased shelf life and virus resistance. In addition to the trait of interest, plant architecture, root yield, morphology, above-ground yield, and cyanogen content were monitored. In 2010, the National Root Crops Research Institute (NRCRI) in Umudike conducted the first ever transgenic CFT in Nigeria. Four transgenic cassava lines with the highest β-carotene content were chosen from the best performing lines from Puerto Rico. In addition to analyses of β-carotene content and yield, plant responses to CMD, cassava bacterial blight, and cassava anthracnose disease are being observed.

Complementing the field trial work in Nigeria were extensive human nutrition surveys conducted in Nigeria and Kenya. These studies included surveys of cassava and other food production, consumption and market analyses in targeted villages, as well as analyses of the nutritional content of commonly grown cassava varieties. Nutritional surveys were conducted for children under the age of 5 to assess health and vitamin, mineral, and protein deficiencies. These data were used for *ex ante* economic and health impact analysis studies (35, 95). Potential net benefits from cassava

biofortification with vitamin A alone were estimated to be $1.2–1.4 billion in Nigeria and $76–81 million in Kenya compared with $1.4–1.6 billion in Nigeria and $105–110 million in Kenya for enhancement with both vitamin A and iron. Costs per DALY saved are estimated at $4–5 for Nigeria and $56–77 for Kenya, which compare favorably with costs for alternative methods such as food fortification and supplementation (95) and are substantially lower than the average annual income (approximately $600–700 year^{-1}) in the target countries.

FUTURE DIRECTIONS

To date, BC+ is the most ambitious plant metabolic engineering program targeted to the biofortification of food crops in Africa. The next phase of the BC+ program focuses on bringing products to the farmer and consumer (**Figure 6**) and includes stacking traits in farmer-preferred cultivars, testing those products in the field, ensuring their safety and stability, and educating consumers. Not to be overlooked is the likelihood of adoption and acceptance of BC+ products by farmers and consumers. Phase II objectives are as critical as were the development of high-performance transgenic plants in Phase I. The long-term vision of BC+ is to build a cassava product development and delivery platform in Africa to be managed by African partners to meet local needs (**Figure 6**). Toward that objective, substantial infrastructure development has occurred at NRCRI in Umudike, Nigeria, and at the Kenyan Agricultural Research Institute (KARI) in Kakamega, Kenya. NRCRI and KARI have also successfully applied for biosafety permits to conduct CFTs, among the first awarded in their respective countries. BC+ has also trained seven young scientists from NRCRI and KARI who are to develop the next generation of biofortified cassava in farmer-preferred cassava with multiple stacked traits. These plants are to be provided royalty-free at or below local propagation costs to farmers in need of improved crops for food, nutrition, and commerce. The development of African biotechnology will undoubtedly be one of the long-term legacies of BC+.

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The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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