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Iron and protein biofortification of cassava: lessons learned

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Over two hundred and fifty million Africans rely on the starchy root crop cassava (Manihot esculenta) as their primary source of calories. Cassava roots, however, have the lowest protein:energy ratio of all the world's major staple crops. Furthermore, a typical cassava-based diet provides less than 10–20% of the required amounts of iron, zinc, vitamin A and vitamin E. The BioCassava Plus program employed modern biotechnologies to improve the health of Africans through development and delivery of novel cassava germplasm with increased nutrient levels. Here we describe the development of molecular strategies and their outcomes to meet minimum daily allowances for protein and iron in cassava based diets. We demonstrate that cyanogens play a central role in cassava nitrogen metabolism and that strategies employed to increase root protein levels result in reduced cyanogen levels in roots. We also demonstrate that enhancing root iron uptake has an impact on the expression of genes that regulate iron homeostasis in multiple tissues. These observations demonstrate the complex metabolic interactions involved in enhancing targeted nutrient levels in plants and identify potential new strategies for further enhancing nutrient levels in cassava.

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Introduction

Nearly two billion people worldwide are malnourished or consume insufficient nutrients for normal growth and metabolism. Malnutrition is directly responsible for approximately 35% of all global childhood (ages 0–5) deaths (2.5 million per year) and 11% of total global disease burden [1]. In addition, malnutrition contributes to the death of approximately 60% of children with diarrheal diseases, 50% with pneumonia, 45% of measles cases, and 55% of children with malaria [1]. Malnutrition is particularly prevalent in developing countries. In sub-Saharan Africa alone over half (51 million) of the disability-adjusted life years (DALYs) lost each year are attributable to malnutrition [1].

Globally, the major nutrient deficiencies contributing to malnutrition are vitamin A, zinc, and iron deficiency [2]. In some developing countries protein-energy malnutrition also accounts for a substantial number of deaths [3]. Malnutrition is frequently associated with food crises that impact subsistence farmers who rely primarily on one or a few staple crops for the bulk of their calories. If the foods consumed do not provide adequate amounts of essential nutrients and diet diversification is not sufficient to compensate for those deficiencies in the staple crops then the likelihood of malnutrition is increased, particularly for rapidly growing young children who are often less competitive for food during famines.

In sub-Saharan Africa, cassava is the second most important food crop in terms of total energy consumption and the first in land area planted [4]. Cassava is starchy root crop grown in many regions in sub-Saharan Africa due to food preference and the security it provides. It is more drought tolerant than maize, the largest source of calories in sub-Saharan Africa, and its roots can be banked in the soil for up to three years with little loss due to herbivory [5]. Thus, cassava provides increased food security relative to other major crops in Africa [6••].

A typical adult-sized cassava meal (500 g dry mass) provides sufficient calories (~1800) but is an inadequate source of iron, zinc, vitamin A, and protein [7–9]. A 500 g meal provides only 30% of the minimum daily requirement (MDR) for iron and zinc and 10% of the daily pro-vitamin A (β-carotene) requirement [8]. Cassava also has the lowest protein-to-carbohydrate ratio of the world's 10 major crops. In addition, cassava roots contain potentially toxic levels of cyanogenic glycosides (linamarin) that need to be removed by extensive food processing before consumption. During processing, linamarin is converted in a series of reactions to cyanide. Free cyanide is volatilized during processing creating a safe food product [10–12]. Long-term ingestion of poorly processed cassava, however, may result in the consumption of sufficient, non-volatile cyanogens (linamarin and

acetone cyanohydrin) to cause goiter, tropical ataxic neuropathy, or in acute cases (resulting from eating poorly processed roots obtained from high cyanogen varieties) permanent paralysis (i.e., Konzo) and/or death [12,13].

In 2005, the Bill and Melinda Gates Foundation established the BioCassava Plus (BC+) Program as part of the Grand Challenges in Global Health Program to address malnutrition in sub-Saharan Africa. The objectives of the BC+ program were to provide complete nutrition in a typical adult-sized cassava meal while incorporating several economic drivers to promote the adoption and acceptance of biofortified cassava [6••].

In this article, we specifically address the strategies used to biofortify cassava roots with iron and protein. The molecular strategies pursued and the associated outcomes provide insights into the unique biology of cassava and provide a model for future efforts to biofortify other orphan staple crops.

Iron biofortification

Iron deficiency is one of the major micronutrient deficiencies in the world especially in areas where people subsist largely on plant-based diets. Iron malnutrition is associated with increased vulnerability to infection, impaired growth, anorexia, and impaired cognitive function. It is estimated that over 1 billion people worldwide are affected by iron-deficient anemia [14]. In sub-Saharan African alone 50% of the population is thought to suffer from iron deficiency. In addition, as many as 32% of children under the age of five years may be disabled due to iron deficiency and as many as 16% of children die as a result of the effects of iron deficiency in sub-Saharan Africa [15].

Iron deficiency is further acerbated by the fact that in many crops consumed by subsistence farmers there is insufficient iron to meet the MDR in a typical sized meal [14]. In addition, due to its low solubility ferric iron is considered the third most limiting nutrient for plant growth [16•]. The low solubility of iron poses a great problem for plant nutrition as well since iron plays a major role in respiration, photosynthesis, the assimilation of sulfur and nitrogen, chlorophyll biosynthesis, and nitrogen fixation [17].

To biofortify plants with elevated iron levels, a clear molecular understanding of iron homeostasis including knowledge of the basic physiological processes of iron absorption, distribution, and storage from a variety of organisms would be ideal but is often lacking for many crops. Novel synthetic molecular approaches employing the targeted expression of unique iron uptake, distribution and storage proteins can potentially be exploited to enhance iron uptake in plants [18]. Significantly, cassava is particularly well suited for transgenic strategies to improve its nutritional qualities since it is clonally propagated [6••].

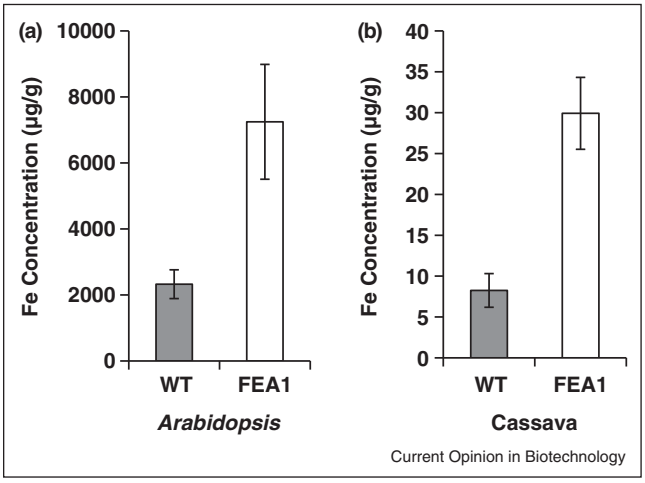
Recently, the novel algal iron assimilatory protein, FEA1, from *Chlamydomonas reinhardtii* was shown to increase selectively iron uptake in yeast and plants [19,20]. The FEA1 protein was shown to complement both yeast iron transporter (*ftr1*) as well as yeast iron endocytosis mutants, suggesting that the FEA1 protein may function as an iron transporter [21]. Based on protein structural predictions and cellular localization studies, it was hypothesized that the FEA1 protein undergoes a transition from a soluble periplasmic protein to a membrane protein following formation of a disulfide bond(s). Mutagenesis and sulfhydryl chemical reduction studies indicate that the formation of the disulfide bond is crucial for insertion of the protein into the membrane. Protein localization and iron transport studies have also shown that this structural transition is necessary for iron uptake. Additional studies have shown that the polar residues located in the predicted transmembrane spanning domains are crucial for FEA1 function presumably associated with the formation of a polar ion channel [21]. Finally, studies with yeast iron uptake mutants have demonstrated that the FEA1 protein specifically transfers ferrous iron in an ATP-dependent manner.

Significantly, the FEA1 protein does not increase sensitivity to toxic concentrations of potentially competing, non-ferrous metals nor facilitate their (e.g., cadmium) accumulation [22•] in yeast or plants. These results indicate that the FEA1 protein has a high specificity for iron consistent with the observation the FEA1 protein is overexpressed in cadmium stressed algae presumably to facilitate iron uptake [19].

This unique green algal iron assimilatory protein complements the *Arabidopsis* iron-transporter mutant, *irt1*, as well as enhances iron accumulation in FEA1-expressing wild-type plants grown at high pH (8.5). Under these conditions (high pH) the solubility of iron is substantially reduced relative to neutral or acidic pH values and growth is impaired in wild-type *Arabidopsis* [22•]. By contrast, transgenic plants expressing the FEA1 protein grown at high pH (8.5) had normal growth phenotypes and accumulated as much as eightfold more iron in their roots than wild-type plants. These results indicate that the FEA1 iron assimilatory protein functions well at high pH (Figure 1) [22•].

Expression of the FEA1 protein in cassava storage roots resulted in an increase in root iron levels from 10 to nearly 40 ppm (Figure 1). It is interesting to note that the expression of the FEA1 protein did not alter the accumulation of iron or zinc in non-root tissues. The leaves of FEA1 transgenic plants had normal iron and zinc levels consistent with the specific uptake and accumulation of

Figure 1

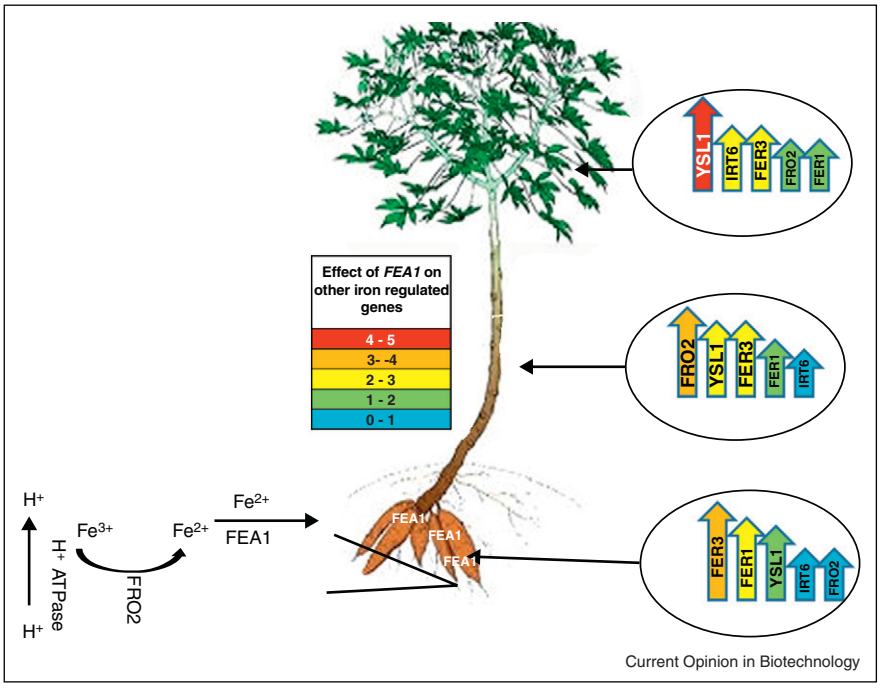


FEA1 expression increases iron levels in plants. **(a)** Wild-type and transgenic FEA1 *Arabidopsis* plants were grown hydroponically on 0.072 M Fe (Sprint 330) until day 48. Iron concentration of roots was measured by ICP-OES. Values are the average of triplicates. Error bars represent SE. **(b)** Wild-type and transgenic FEA1 cassava plants were grown for 6 months in greenhouse conditions. Iron concentration of roots was measured by ICP-OES. Values are average of triplicates. Error bars represent SE.

iron mediated by the FEA1 protein in roots [22•,23•]. To determine whether FEA1 expression impacted the expression of other genes involved in regulating iron homeostasis, we surveyed the expression of multiple genes involved in iron homeostasis in roots, stems and leaves of wild-type and FEA1 transgenic cassava. We observed that genes encoding the ferric chelate transporter, *MeYSL1*, and the iron storage proteins, *MeFer1*, *MeFER3* were up regulated in *FEA1* transgenic plants relative to wild type suggesting that these gene products play a role in iron translocation and homeostasis in cassava (Figure 2). By contrast, cassava ferric chelate reductase (*MeFRO2*) expression was found to be down regulated in roots of *FEA1* transgenic plants relative to wild-type plants (Figure 2). It is well known that ferric reductase activity in root hairs is down regulated in plants sufficient in iron [22•]. These results indicated that iron homeostasis in cassava is regulated partly at the transcriptional level and that engineering strategies that enhance iron accumulation in the root are compensated for in other portions of the plant by increased expression of genes involved in iron mobilization and storage.

Overall, these results demonstrate that the FEA1 iron assimilatory protein has ideal characteristics for the biofortification of crops and/or for the facilitated uptake of iron in plants grown in low iron, high pH soils, or soils contaminated with heavy metals.

Figure 2



The effect of root-specific FEA1 expression on the expression of cassava genes involved in iron homeostasis. Similar to other strategy II iron uptake plants, cassava roots reduce ferric iron to ferrous iron via ferric reductase. The FEA1 protein facilitates iron uptake. The impact of FEA1 expression on the expression of other genes involved in iron homeostasis in various tissues is presented as fold increases in mRNA abundance relative to wild-type plants.

Protein biofortification

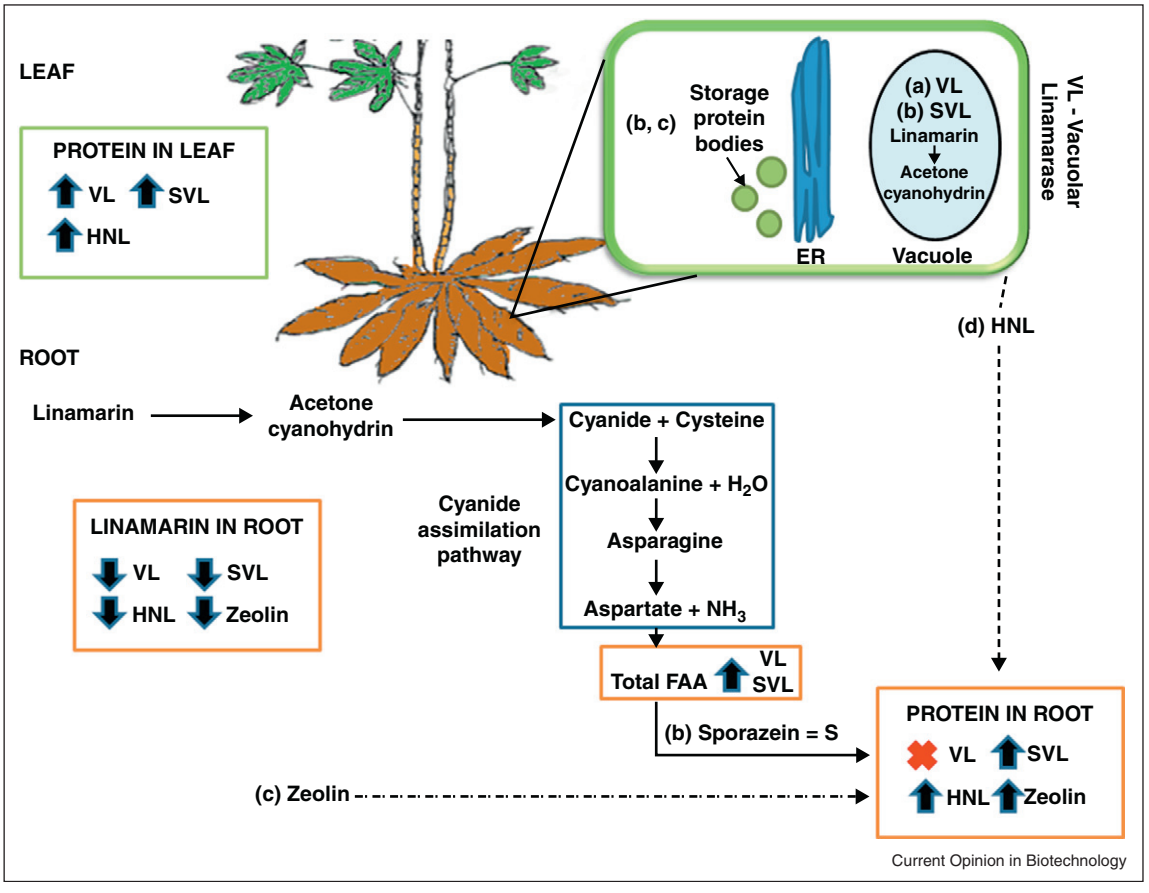
The protein content of cassava storage roots is among the lowest of all major crops [4]. Cassava roots typically have between 0.7 and 3% protein by dry weight [24,25]. This low protein content can be further exacerbated by the food processing methods used to remove cyanogens [26,27•]. As a result, subsistence on a cassava-based diet crop can result in protein-energy malnutrition (PEM) unless there are additional sources of protein in the diet [4,28,29].

One strategy to reduce PEM resulting from reliance on a cassava-based diet is to increase cassava root protein levels through targeted breeding or transgenic approaches. To address potential protein deficiency resulting from eating a cassava-based diet the BioCassava Plus program explored a variety of transgenic strategies to elevate root protein levels including (1) increasing root free amino acid pools for protein synthesis, (2) generating a strong nitrogen sink in roots by overexpressing storage proteins, and (3) a combination of both strategies.

Increasing free amino acid pool sizes

Previously, it has been demonstrated that increasing amino acid synthesis rates could increase free amino acid pools as well as total protein accumulation in plants [30]. In cassava roots, cyanogenic glycosides are a major source of reduced nitrogen for amino acid synthesis [6••,31,32•,33]. Inhibiting linamarin synthesis in roots has been shown to reduce root growth, a phenotype that could be rescued by adding ammonia to the growth media. Linamarin synthesized in leaves and transported to roots, is either stored in the vacuole or metabolized to provide reduced nitrogen for amino acid synthesis (Figure 3). During cyanogenesis, acetone cyanohydrin is generated by the deglycosylation of linamarin by linamarase. The

Figure 3



Four strategies for the enhancement of protein levels in cassava storage roots. **(a)** Expression of a vacuolar linamarase (VL lines). The expression of a vacuolar-targeted linamarase increased the deglycosylation of linamarin leading to an increased assimilation of cyanide into amino acids. **(b)** Co-expression of a vacuolar linamarase and the novel storage protein sporazein (SVL lines). The SVL lines, similar to the VL lines showed increased free amino acid levels, however, in difference to the VL lines the SVL lines showed a twofold increase in protein levels in the roots. **(c)** Expression of zeolin. The chimeric storage protein zeolin expressed under the control of the root-specific patatin promoter resulted in a fourfold increase in root storage proteins in older cassava roots. **(d)** Over expression of hydroxynitrile lyase (HNL lines). The overexpression of HNL in storage roots resulted in a twofold to threefold increase in protein content in younger cassava roots. The HNL enzyme is targeted to the apoplast.

cleavage of acetone cyanohydrin to release cyanide and acetone occurs either spontaneously at pH values >5, at high temperature >35°C, or catalytically by the enzyme hydroxynitrile lyase (HNL) [11]. Cyanide is then assimilated via the β-cyanolalanine synthase pathway leading to the production of asparagine, which in turn can be deaminated to generate aspartate and free ammonia [34–36]. Both of these compounds are building blocks for the synthesis of additional amino acids and subsequently protein. Significantly, the enzyme linamarase is found in laticifers and the apoplast of root cells while the apoplastic enzyme, HNL is expressed in leaves but not in roots [11]. Thus, it is presumed that generalized β-glucosidases present in the root cell cytoplasm slowly hydrolyze linamarin to produce acetone cyanohydrin and glucose for amino acid synthesis [37]. In addition, the slow spontaneous turnover of acetone cyanohydrin to cyanide at high pH allows for controlled cyanide assimilation by the β-cyanoalanine synthase pathway [11,38].

To accelerate the turnover of linamarin in the root cell cytoplasm and facilitate the production of amino acids two strategies were considered; a 'push' strategy in which linamarin stored in the vacuole is converted to acetone cyanohydrin by a vacuolar-targeted linamarase and a 'pull' strategy in which the low nitrogen/protein sink strength of the root was increased by overexpressing a root storage protein. The strong storage-root gene promoter patatin [31,39,40•,41•] was used to drive the expression of a vacuolar-targeted linamarase (VL plants). We observed that targeting linamarase to root vacuoles substantially reduced (≥50%) root steady state linamarin levels resulting in up to a 2.2-fold increase in total free amino acid levels in roots [38]. However, no increase in root protein levels was observed in VL transgenic plants. In contrast to roots, leaf protein levels were increased threefold in VL plants, consistent with the export of root free amino acids to the stronger nitrogen sink, the leaves [42,43,44•]. It is interesting to note that multiple attempts (>20) to target linamarase expression to the root cytoplasm (non-vacuole) failed. This is presumably due to the more rapid spontaneous turnover of acetone cyanohydrin at the higher pH values of the cytoplasm relative to the vacuole. The resulting elevated cyanide levels are presumably toxic. By contrast, cyanide production from the spontaneous turnover of acetone cyanohydrin generated in vacuoles is expected to be much slower due to the lower pH of the vacuole [11].

In summary the use of linamarin as a nitrogen source for the production of free amino acids and protein was restricted by the lack of a nitrogen sink or storage protein in roots. However, it was possible to observe increased free amino acid levels in the storage roots concurrently with decreased linamarin levels reiterating the relationship between the cyanogenic glucoside and overall nitrogen metabolism in cassava.

Overexpression of a storage protein in cassava roots

One of the first attempts to increase cassava protein levels involved the constitutive overexpression of an Artificial Storage Protein, ASP1 [45]. Transgenic plants expressing ASP1 in all tissues were observed to have a 30% increase in leaf protein levels, indicating again the strong nitrogen sink strength of leaves. However, the protein content of roots was not reported [46]. Subsequently, a recombinant storage protein, zeolin, was expressed exclusively in storage roots driven by the root-specific patatin promoter [40•]. Zeolin is a fusion between phaseolin, a storage protein from the common bean (*Phaseolus vulgaris*), and a region from the maize γ-zein protein that promotes protein body formation in the endoplasmic reticulum [47]. It was hypothesized that addition of the γ-zein protein stabilizing motif and the subsequent development of protein storage bodies would increase protein accumulation in cassava storage roots. Transgenic cassava lines expressing zeolin under the control of the patatin promoter were shown to accumulate up to fourfold more protein than wild-type storage roots [40•]. Interestingly, the root steady state linamarin levels were reduced by 50% in zeolin expressing plants consistent with the metabolism of linamarin to provide reduced nitrogen for protein synthesis. Unfortunately, some persons are allergic to phaseolin and so zeolin expressing cassava lines could not be developed for human consumption (SDAP, University of Texas medical Branch, fermi.utmb.edu/SDAP/index.html). Subsequently, the Fauquet lab developed a different chimeric protein consisting of sporamin and a truncated maize γ-zein sequence [47]. The novel protein was named sporazein and it was shown to form protein bodies in tobacco and cassava [48].

Overexpressing metabolic enzymes in cassava storage roots could increase protein accumulation as well as alter root metabolism in a targeted manner. Overexpression of the cassava enzyme HNL in roots is one such example. HNL catalyzes the degradation of acetone cyanohydrin generated by linamarase into cyanide. HNL is abundant in the apoplast of leaves but is not expressed in cassava storage roots [11]. As a result, in poorly processed cassava roots the major residual cyanogen is acetone cyanohydrin [13]. The consumption of cyanogens in poorly processed cassava foods may cause severe health problems including paralysis (Konzo) and even death [13,49,50]. Significantly, the overexpression of HNL in storage roots decreased acetone cyanohydrin content in processed cassava by as much as 80–90% in as little as 15 min after grinding, making it a safer food product [41•]. In addition, soluble protein levels in young roots expressing HNL under the control of the patatin promoter increased twofold to threefold relative to wild-type plants [41•]. Significantly, in contrast to zeolin, HNL is not known to be allergenic [51]. In addition, over half the amino acids in HNL are essential amino acids required for human nutrition. Overall, HNL expression in

Table 1

Summary of the cassava protein biofortification strategies

Strategy	Root FAA	Root protein	Root linamarin	Leaf FAA	Leaf protein	Leaf linamarin
Root-targeted expression of a vacuolar linamarase (VL lines)	220% increase	No difference	44% decrease	150% increase	300% increase	45% decrease
Root-targeted expression of the zeolin storage protein	Not reported	400% increase	55% decrease	Not reported	Not reported	50% decrease
Root-targeted expression of the hydroxynitrile lyase enzyme (HNL lines)	No change	300% increase	70% decrease	Not reported	300% increase	No change
Dual root-targeted expression of a vacuolar linamarase and the storage protein sporazein (SVL lines)	240% increase	200% increase	45% decrease	230% increase	300% increase	25% decrease

The different strategies utilized for the protein biofortification of cassava yielded different results. It is noted that the data were collected from storage roots of plants of different ages (VL and SVL lines = 4 months old, Zeolin lines = 7 months old, and HNL lines = 5 months old) grown under similar greenhouse conditions. In general protein levels increase in storage roots with age up to 12 months. Values reported for the different strategies were taken from references [38,40•,41•]. FAA: total free amino acids.

roots enhances both the quality and quality of root protein as well as facilitates the detoxification of cyanogens in cassava roots.

Combined push–pull strategy for protein accumulation

Given the previous successes to increase cassava root free amino acid levels (greater source strength) and storage protein levels (increased sink strength), we hypothesized that by combining increased source and sink strength transgenic strategies the greatest increases in root protein yield could be achieved. To test this hypothesis, we co-expressed a vacuolar-targeted linamarase, to increase root free amino acid levels, with the recombinant protein sporazein (increased sink strength). Both genes were driven by the strong storage-root-specific promoter, patatin. Previously, we had shown that root-specific expression of vacuolar-targeted linamarase resulted in a 2.2-fold increase in the free amino acid pool size in cassava storage roots, however, no increase in root protein levels was observed. Similar to the vacuolar-targeted linamarase transgenics, transgenic plants expressing both vacuolar-targeted linamarase and sporazein had greater than a twofold increase in root free amino acids but only a twofold increase in storage-root protein levels [38]. The protein increase observed in these transgenics (co-expressing a vacuolar-targeted linamarase and sporazein) was half that obtained by expression of zeolin alone [38,40•]. At present, it is unclear why there was reduced protein accumulation in the vacuolar linamarase plus sporazein expressing transgenic lines. It is evident that the free amino acid pool sizes are increased in the double transgenics, concomitant with a reduction in the linamarin pool size [38]. But, the additional root free amino acids were not utilized for increased sporazein accumulation. Significantly, the leaf free amino acid levels increased 1.3–2.2-fold [38]. Possible explanations for the reduced accumulation of sporazein in the double transgenics include limited co-expression of sporazein, reduced stability of sporazein relative to other storage proteins, and the greater nitrogen sink strength of leaves relative to roots expressing sporazein. In the latter case, root free amino acids would be expected to be preferentially mobilized to leaves as is evident from the increased free amino acid levels in the leaves of the double transgenics. The relative outcomes of the three different protein biofortification strategies are summarized in Table 1. It is important to note, however, that protein and free amino acids levels in roots and leaves of independent transgenic lines are compared at different ages of root maturity. In general, protein content in roots increases linearly with age.

Overall, attempts to biofortify both iron and protein in cassava roots revealed unexpected outcomes as well as new insights into the molecular control of iron and protein homeostasis in cassava. It was evident that the regulation of iron homeostasis in cassava was complex involving multiple levels of gene regulation in various tissues (Figure 2). In addition, attempts to elevate protein levels in roots had impacts on overall nitrogen allocation between leaves and roots reflecting the strong leaf sink strength for reduced nitrogen (Figure 3). It was also evident that linamarin plays a central role in reduced nitrogen mobilization in cassava plants. To our knowledge cassava is one of the few plants that utilizes cyanogens as mobile forms of reduced nitrogen throughout the life span of the plant [6••].

Future plans to enhance iron accumulation in cassava include co-expression of the FEA1 protein with ferric reductase. Ferric reductase activity was down regulated in FEA1-expressing cassava plants, presumably due to the accumulation of sufficient iron levels for general plant metabolism and its subsequent repression (Figure 2). By increasing ferrous iron levels concomitant with the expression of the FEA1 protein it is hypothesized that even greater levels of iron may be accumulated in cassava roots. Interestingly, co-expression of the FEA1 protein with the iron storage protein ferritin in cassava roots did not enhance iron accumulation (Uzoma Ihemere, unpublished results). As indicated in Figure 1, FEA1 expression

induced ferritin expression in roots. So overexpression of ferritin in transgenic plants is presumably adequate to accommodate the elevated iron levels in FEA1-expressing plants.

Enhancing protein accumulation in cassava roots is potentially more challenging. Increasing overall pools of reduced nitrogen (ammonium) in cassava plants may be one strategy to increase protein levels in all tissues. It is evident from transgenic experiments carried out to date that increasing reduced nitrogen supplies can result in increased protein levels in leaves. These results suggest that leaves have a greater potential to synthesize proteins than roots. Increasing total available reduced nitrogen for cyanogen, amino acid, and protein metabolism, coupled with overexpression of storage protein genes in roots may result in the greatest root protein yields.

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