REVIEW PAPER

Cyanogenesis in cassava and its molecular manipulation for crop improvement

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Abbreviations: CG, cyanogenic glycoside; CNp, cyanogenic potential; PPD, post-harvest physiological deterioration; ROS, reactive oxygen species

Abstract

While cassava is one of the most important staple crops worldwide, it has received the least investment per capita consumption of any of the major global crops. This is in part due to cassava being a crop of subsistence farmers that is grown in countries with limited resources for crop improvement. While its starchy roots are rich in calories, they are poor in protein and other essential nutrients. In addition, they contain potentially toxic levels of cyanogenic glycosides which must be reduced to safe levels before consumption. Furthermore, cyanogens compromise the shelf life of harvested roots due to cyanide-induced inhibition of mitochondrial respiration, and associated production of reactive oxygen species that accelerate root deterioration. Over the past two decades, the genetic, biochemical, and developmental factors that control cyanogen synthesis, transport, storage, and turnover have largely been elucidated. It is now apparent that cyanogens contribute substantially to whole-plant nitrogen metabolism and protein synthesis in roots. The essential role of cyanogens in root nitrogen metabolism, however, has confounded efforts to create acyanogenic varieties. This review proposes alternative molecular approaches that integrate accelerated cyanogen turnover with nitrogen reassimilation into root protein that may offer a solution to creating a safer, more nutritious cassava crop.

Keywords: Cassava, crop improvement, cyanide metabolism, cyanogenic glycosides, genetic improvements, linamarin, nitrogen allocation, post-harvest physiological deterioration.

Introduction

Among the world’s top 10 crops directly consumed by humans, only one, cassava (Manihot esculenta), requires processing before consumption to remove potentially toxic levels of anti-nutrients (Fig. 1A) (McMahon et al., 1995; Sayre et al., 2011). Linamarin, the dominant cyanogenic glycoside present in cassava, occurs in sufficient concentrations in high cyanogen varieties of cassava roots (Fig. 1B) to impair human health or potentially to be lethal (0.5–3.0 mg CN equivalents kg⁻¹ body weight) if not removed to safe levels (2–35 mg kg⁻¹ DW) [cyanogens include: cyanogenic glycoside (CG), its aglycone or hydroxynitrile derivative, and free cyanide] (Balagopalan et al., 1988; Sayre et al., 2011). Chronic exposure to even moderate levels of linamarin in cassava-based diets may result in: (i) hyperthyroidism, from the accumulation of thiocyanate, a cyanide detoxification
product that interferes with iodine metabolism; (ii) neurological disorders such as tropical ataxic neuropathy; and (iii) permanent paralysis of the legs resulting from acute exposure to nearly lethal cyanogen doses, a disease known as konzo in sub-Saharan Africa (Cliff et al., 2011; Kashala-Abotnes et al., 2019). It has been estimated that in Africa >100,000 people are afflicted with konzo each year. Since cassava’s original domestication in Brazil and later adoption as a major staple crop in sub-Saharan Africa (16th century) primarily as a food security crop, limited progress has been made to reduce cyanogen levels in cassava through traditional breeding approaches (Sayre et al., 2011). While there are clearly heritable differences in cyanogen content between low (<100 mg cyanide equivalents kg\(^{-1}\) FW) and high (as high as 500 mg cyanide equivalents kg\(^{-1}\) FW) cyanogen cassava varieties, no commercially viable cassava varieties have been developed to date that completely lack cyanogens in leaves or roots (Sayre et al., 2011).

The lack of progress towards realization of a cyanogen-free cassava cultivar is now more understandable given the greater knowledge of CG synthesis, transport, metabolism, and storage in cassava leaves, stems, and roots. Over the last 25 years, it has become apparent that the cyanogens of cassava not only function as anti-herbivory compounds but also play a central role in whole-plant nitrogen metabolism and transport (Zidenga et al., 2017). As will be discussed, cyanogens are synthesized in leaves, transported basipetally to roots, and play a critical role in providing reduced nitrogen for amino acid synthesis in roots (Siritunga and Sayre, 2003, 2004; Jorgensen et al., 2005; Narayanan et al., 2011; Leyva-Guerrero et al., 2012; Zidenga et al., 2017). In addition, cyanide liberated from roots following tissue damage during harvesting initiates the rapid onset of post-harvest physiological deterioration (PPD) through inhibition of mitochondrial electron transport and initiation of programmed cell death processes (Zidenga et al., 2012). If care is not taken to exclude oxygen from the roots immediately after harvesting, the roots will decay within 3 d to a state that is not usable for human consumption or commercial processing. The implications of PPD are that root harvests need to be limited to a quantity that can be immediately processed before PPD reaches a stage of severe damage. Additionally, PPD limits the ability to transport cassava roots over long distances to markets, reducing its potential to generate revenue for cassava farmers, merchants, and processors. As a result of these limitations, reduced PPD (i.e. longer post-harvest shelf life) has been recognized by subsistence farmers in sub-Saharan Africa as one of the major crop improvement traits for enhanced cassava food and economic security.

Early biochemical and later molecular studies demonstrated that the dominant CG in cassava is linamarin (>95%), with the remainder being lotaustralin (methyl linamarin) (McMahon et al., 1995). Linamarin is synthesized in leaves and possibly stems, and is transported via the stems to the roots where it is either stored in the vacuole or provides reduced nitrogen for amino acid synthesis. Significantly, roots do not synthesize linamarin. These observations have led to molecular strategies based on sink–source relationships to reduce root linamarin to safe levels by blocking the first dedicated step in linamarin synthesis: the cytochrome P450– (CYP71D1 and D2) catalyzed conversion
of valine into N-hydroxyvaline (Andersen et al., 2000; Siritunga and Sayre, 2003, 2004; Jørgensen et al., 2011). Based on anti-sense RNA-mediated suppression of CYP79D1/D2 gene expression in cassava leaves, it was demonstrated that it was possible to reduce linamarin accumulation in leaves. However, substantive reduction of linamarin synthesis in leaves resulted in transgenic plants that did not survive in soil without the addition of reduced nitrogen (ammonia) to replace nitrogen lost from linamarin metabolism in roots for amino acid and protein synthesis (Siritunga and Sayre, 2003, 2004). This was the first molecular evidence that linamarin served as a source of reduced nitrogen for roots, although earlier studies based on girdling stems and analysis of the relative nitrogen distribution in metabolites obtained from phloem exudates suggested that linamarin was the major source of reduced nitrogen for roots. As will be discussed below, later studies demonstrated that in contrast to leaves, cassava roots have robust cyanide assimilation pathway enzyme activities leading to the synthesis of asparagine and other amino acids for protein synthesis in roots (Narayanan et al., 2011; Leyva-Guerrero et al., 2012; Zidenga et al., 2017).

Subsequently, the focus on producing cyanogen-free roots using metabolic engineering strategies shifted towards reducing root linamarin storage sink strength and increasing root cyanogen assimilation (amino acid and protein synthesis) sink strength (Narayanan et al., 2011; Leyva-Guerrero et al., 2012; Zidenga et al., 2017). Overexpression of enzymes involved in cyanide release from linamarin or cyanide assimilation into amino acids and protein was shown to significantly increase root free amino acid levels and reduce root steady-state linamarin levels. These approaches indicate that there is competition for linamarin between amino acid synthesis and linamarin storage in the vacuole. This divergent competition for linamarin allocation for protein synthesis or storage for herbivore deterrence—presumably accounts for the lack of progress towards the selection or breeding of cassava varieties that completely lack linamarin stored in their roots. Metabolic pathways leading to linamarin storage in vacuoles compete with linamarin catabolism for protein synthesis. The potential implication of this bifurcation in linamarin metabolism is that if there was a major reduction in linamarin storage capacity, there would also need to be a corresponding increase in cyanide assimilation into amino acids and protein synthesis to avoid cyanide toxicity (Siritunga and Sayre, 2004).

Recent comparative genome-wide association studies (GWAS) between low and high cyanogen cassava cultivars indicated that the control of vacuolar linamarin content may be attributed in part (40%) to the differential expression of a tonoplast (vacuolar membrane) MATE (multidrug and toxic compound extrusion) protein that presumably transports linamarin into the vacuole, and an ATP-dependent plasma membrane proton pump presumably involved in linamarin transport throughout the plant (Ogbonna et al., 2020). As will be discussed, we propose a two-pronged genetic engineering strategy for reducing stored linamarin in cassava roots: by increasing linamarin-dependent amino acid and protein synthesis; and simultaneously reducing vacuolar sink strength by suppressing the activity of the putative MATE linamarin transporter. This approach may provide a solution to the question of how to generate a cassava cultivar that is safe to eat without processing, has an extended shelf life, and has increased root protein content.

In the following sections, we review the status of CG synthesis, cyanide generation from linamarin, linamarin transport to roots, root linamarin metabolism, storage, and protein synthesis, and the role of cyanide in driving PPD. Given this body of information, we propose molecular strategies to domesticate cassava for safer human nutrition and greater income generation. In addition, we note some of the unique pitfalls that need to be recognized when assessing cyanogen metabolism and the cyanogenic potential of cassava.

### Synthesis of cyanogens

**Enzymes involved in cyanogen synthesis, their properties, and regulation**

Cassava produces two CGs, linamarin and lotaustralin, whose amino acid precursors are valine and isoleucine, respectively. Linamarin and lotaustralin biosynthesis utilize the same cellular machinery; however, in cassava, linamarin is the predominant CG, with a ratio of ~95:5 linamarin to lotaustralin (Balagopalan et al., 1988). The linamarin/lotaustralin biosynthetic pathway consists of three distinct enzymatically regulated phases, which parallels the well-characterized metabolic pathway of the CG dhurrin in *Sorghum bicolor* (Bak et al., 1998; Jones et al., 1999). In the first enzymatically regulated step, valine or isoleucine is consecutively hydroxylated and dehydrated to sequentially form an N-hydroxyamino acid, an N,N-dihydroxyamino acid, and ultimately an E/Z oxime (Fig. 2). In the second enzyme-catalyzed step, the E,Z oxime is converted into a cyanohydrin by dehydration and C-hydroxylation. During the final step, the cyanohydrin is glycosylated by a UDP-glycosyltransferase to form the CG. The high degree of similarity in the biochemical pathways for cassava and *Sorghum* CGs indicates that CG biosynthesis is conserved in monocotyledonous and dicotyledonous plants.

In cassava, the first two stages of CG biosynthesis are catalyzed by multifunctional cytochrome P450 monoxygenases that are associated with the microsomal fraction, most probably the endoplasmic reticulum, although isolated vacuoles have been shown to synthesize linamarin (McMahon, 1997; Andersen et al., 2000) (Fig. 2). The first dedicated and rate-limiting step in linamarin synthesis is catalyzed by the homologs CYP79D1 and CYP79D2, which convert valine to a valine-oxime (Andersen et al., 2000). The genes CYP79D1 and CYP79D2 are 85% identical and share 54% sequence identity to their homolog from *Sorghum*, CYP79A1. While
both CYP79D1 and 2 are equally expressed, and both enzymes can use valine and isoleucine as substrates, the binding affinity is higher for valine than for isoleucine, which explains why linamarin is the predominant CG in cassava rather than lotaustralin. More recently, a second cytochrome P450, CYP71E7, has been identified as catalyzing the intermediate stage of linamarin/lotaustralin synthesis (Jørgensen et al., 2011). Like the isoenzymes CYP79D1 and CYP79D2, CYP71E7 binds multiple substrates and is localized to microsomes. CYP71E7 converts both valine- and isoleucine-derived oximes into their respective cyanohydrins. The final step in CG synthesis is the glycosylation of the cyanohydrin which is catalyzed by two paralog UDP-glucosyltransferases, UGT85K4 and UGT85K5 (Kannangara et al., 2011). These enzymes are presumably present in the cytosol based upon a lack of apparent organelar targeting domains. This presumptive localization is consistent with the cytoplasmic localization of the UDP-glucosyltransferase ortholog (UGT85B1) of *Sorghum* that is involved in dhurrin biosynthesis, as well as other plant UDP-glucosyltransferases (Jones et al., 1999; Ross et al., 2001).

In order to coordinate numerous cellular processes at the protein level, cells may assemble metabolons, temporary protein complexes that consist of functionally related enzymes involved in a common metabolic pathway (Srere, 1987; Zhang and Fernie, 2021). Metabolons efficiently channel substrates to products, and their transient nature creates an additional level of flexibility by which cells can adjust metabolism. While relatively few metabolons have been identified in plants, dhurrin biosynthesis in *Sorghum* utilizes a metabolon (Kannangara et al., 2011; Laursen et al., 2016). Laursen et al. (2016) proposed that dhurrin biosynthesis is regulated via metabolon assembly or disassembly, depending upon environmental stimuli which affect the negative charge of the membrane in which dhurrin’s cytochrome P450s are embedded. Because of the many parallels between the biochemical machinery of dhurrin and linamarin, it seems likely that enzymes responsible for cassava cyanogen synthesis are structurally organized in a similar fashion. Hydroxynitrile intermediates in linamarin and lotaustralin biosynthesis are labile, generating cyanide upon decomposition that is potentially harmful to the cellular machinery, thus structural organization of the linamarin synthesis machinery into metabolons may reduce spontaneous cyanide production. Jørgensen et al. (2011) suggested that the low substrate specificity of CYP71E7, which binds both aliphatic and aromatic oximes, is an indicator that the enzyme functions within a metabolon, where end-product production could be accelerated by substrate channeling.

There is considerable evidence that genes encoding proteins involved in cyanogen synthesis are structurally clustered. In plants, genes that encode proteins involved in biosynthesis of defense compounds are often organized into clusters of non-homologous genes (Chu et al., 2011; Takos and Rook, 2001).
The principal advantage of this type of gene organization is that it allows for more coordinated co-expression of genes functional in a shared metabolic pathway which could be coordinated via localized chromatin restructuring. It also increases the likelihood that favorable allele combinations are co-inherited. Presently, gene clusters for functionally related genes encoding CG synthesis proteins have been identified in *Sorghum*, *Lotus japonicus*, and cassava (*Takos et al.*, 2011). In cassava, this gene cluster is located on chromosome 12, and comprises both *CYP71E* paralogs, two UDP-glucosyltransferase genes, and one of the two cytochrome P450 genes from the *CYP79* family, *CYP79D2*. Aside from the presence of gene clusters involved in CG synthesis, there are no other similarities in the structure of these clusters in the three plant species, indicating that these gene assortments evolved independently. With >2500 taxa of known cyanogenic plants, it is likely that a similar organization of functionally related genes for cyanogen synthesis exists elsewhere. Functional clustering is not universal; for example, the cyanogenic tree *Eucalyptus cladocalyx* does not utilize this gene organization for its cyanogen synthesis proteins (*Mora-Poble et al.*, 2021).

**Storage and transport**

Cyanogenic glycoside content in cassava varies by cultivar, developmental stage, plant tissue, and environmental factors (including soil quality). To discuss regulation of cyanogenic potential (CNp), understanding how cyanogens accumulate and are transported is as important as identifying the genes involved in their synthesis. Cassava leaves and the peels of storage roots contain the highest concentrations of cyanogens, but the root cortex which is processed for food also contains substantial amounts of cyanogens which must be removed prior to consumption (*Balagopalan et al.*, 1988). Cassava cultivars are classified according to the bitterness or sweetness of the starchy roots, which approximately corresponds to the cyanogen content of the roots. Taste perception of cyanogens in humans has a genetic component, however, and so sweet versus bitter taste is not a universal indicator for cyanogen content. Regardless, ‘sweet’ cultivars generally contain <100 mg kg⁻¹ FW of cyanogens, while ‘bitter’ cultivars can contain up to 500 mg kg⁻¹ FW (*Ndubuisi and Chidiebere*, 2018).

In cassava, linamarin is synthesized primarily in the young leaves and apical meristem, and roots do not synthesize linamarin at rates that could account for their cyanogen content (*McMahon et al.*, 1995; *Siritunga and Sayre*, 2003, 2004). Thus, differential accumulation of cyanogens in the roots requires basipetal transport from the leaves and stems. Linamarin flux in leaves and roots is affected by several factors including source-sink balance, soil nitrogen availability, and transport controls (*Siritunga and Sayre*, 2003; *Jørgensen et al.*, 2005; *Ogbonna et al.*, 2020). *Siritunga and Sayre* (2003, 2004) demonstrated that in transgenic cassava plants where nearly all leaf expression of *CYP79D1/D2* was eliminated, root cyanogen content was also reduced to 1% of wild-type levels, even though roots expressed low levels of *CYP79D1/D2*. When *CYP79D1/D2* expression was inhibited only in roots, however, both leaves and roots accumulated linamarin at levels comparable with wild-type plants (Table 1). Subsequently, *Gomez et al.* (2021, Preprint) engineered *CYP79D1* and *CYP79D2* knockout plants using CRISPR/Cas9 technology. They demonstrated that expression of the *CYP79D2* gene accounted for the majority of leaf linamarin synthesis. Plants were recovered that could grow in soil supplemented with reduced nitrogen. However, no studies were carried out using nitrate as a nitrogen source to assess the contribution of linamarin to providing reduced nitrogen for the roots.

Several questions remain about exactly how linamarin is directed to cassava roots. In cassava, the enzymes which break down linamarin, linamarase and hydroxynitrile lyase, are present in leaf cell walls (*Mkpong et al.*, 1990; *White et al.*, 1998). Therefore, some strategy must be employed that will prevent linamarin catabolism and cellular damage from free hydrogen cyanide (HCN) potentially generated during apoplastic linamarin transport. One such mechanism would involve symplastic linamarin transport via phloem and laticifers. In support of this transport model, it has been shown that girdling cassava stems causes accumulation of linamarin to concentrations up to 75 times higher above the girdling site relative to below the girdling site (*Jørgensen et al.*, 2005). Other cyanogenic plants have evolved an alternative transport strategy that modifies the cyanogen into a metabolite that is not hydrolyzable by catalytic β-glucosidases. The Euphorb, *Hevea brasiliensis*, further glycosylates linamarin to form the cyanogenic diglycoside linustatin, which cannot be cleaved by linamarase (*Selmar et al.*, 1987). The presence of linustatin in cassava would support an apoplastic mode of linamarin transport to the roots. A few studies have identified trace quantities of linustatin in cassava phloem exudate or stem tissues, but this has not been generally confirmed. Furthermore, the low ratio of linustatin relative to free linamarin does not support linustatin as cassava’s principal cyanogen during transport (*Selmar*, 1993, 1994; *Píčmanová et al.*, 2015). Currently, the literature supports a symplastic mode of transport in which linamarin is unmodified. Imaging MALDI-TOF-MS has been used to identify the presence of potassium salts of linamarin and lotaustralin in cassava stem vascular tissues, stem laticifers, and the stem cortex. These are the same tissues in which the transcripts and corresponding enzymes required for linamarin synthesis have been found (*Kannangara et al.*, 2011; *Schmidt et al.*, 2018a). It is notable that linustatin was not detected in the phloem, which is consistent with a symplastic model for linamarin transport.

In cassava leaves, CG concentrations are modulated in part by transcriptional regulation of the genes which encode linamarin’s biosynthetic machinery, as well as other factors including nitrogen availability and environmental stimuli (see following section). Root CNp, however, is predominantly driven by cyanogen transport and partitioning, the regulation...
of which is not well understood. Recently, genome-wide association studies have provided evidence for genetic factors downstream of cyanogen biosynthesis that are important determinants of root CNp. Ogbonna et al. (2020) reported that the broad-sense heritability of root CNp was 0.82, and pinpointed two single nucleotide polymorphisms (SNPs) whose alleles consistently correlated with root CNp in low and high cyanogen varieties. It is interesting to note that neither of these SNPs was associated with genes involved in linamarin synthesis (on chromosome 12), but rather genes that were implicated in cyanogen transport. One SNP was located within a gene on chromosome 16 encoding a multidrug and toxic compound extrusion (MATE) protein (Manes.16G007900.1) and was close to a second MATE gene (Manes.16G007900.1). MATE transporters are a broad class of integral membrane transporters of secondary metabolites (Upadhyay et al., 2019). Both cassava MATE genes were expressed at relatively high levels in the root and shoot apical meristems, with differential expression between fibrous and storage roots. Interestingly, another MATE gene (Manes.12G129000.1) was mapped to a region ~325 kb from the linamarin synthesis gene cluster, suggesting but not confirming the role of this class of proteins in cassava cyanogen transport. Similarly, a MATE protein has been directly implicated in CG transport in Sorghum, and the cluster containing the genes which enable dhurrin biosynthesis also contains a MATE protein gene (SbMATE2). The SbMATE2 protein localizes to the vacuole membrane, the site of dhurrin sequestration in Sorghum leaves, and imports dhurrin and other CGs (Darbani et al., 2016). Ogbonna et al. (2020) also identified a second SNP region in the cassava genome associated with CG heritability located on chromosome 14. Candidate SNPs were identified within a region comprising genes encoding an integral membrane protein involved in

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<td>Very low</td>
<td>Inhibition of gene expression in leaves reduces root cyanogen levels by &gt;90%</td>
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<td>Inhibition of gene expression in leaves reduces root cyanogen levels by &gt;90%</td>
<td>Andersen et al. (2000); Siritunga and Sayre (2003, 2004); Jørgensen et al. (2005)</td>
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<td>UDP-glucosyl transferase</td>
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nitrite transport (Manes.14G074300) and a plasma membrane H+-ATPase (Manes.14G073900). This plasma membrane H+-ATPase activity is known to be coupled with MATE activity during citrate efflux under aluminum-mediated stress in several plant species (Zhang et al., 2017). More broadly, plasma membrane H+-ATPases maintain proton gradients by which molecules can be transported across the plasma membrane. Since linamarin has been detected in cassava stems as a potassium salt, it is possible that linamarin transport may be driven in part by proton gradients regulated by H+-ATPases and MATEs (Schmidt et al. 2018a).

There is also compelling evidence that cyanogenic glycoside transport may involve an NPF transport protein. NPF (nitrate transporter/peptide transporter) proteins are plant-specific transporters for a wide range of compounds, including nitrate, hormones, and secondary metabolites (Longo et al., 2018; Wen et al., 2020). Jørgensen et al. (2017) identified a cyanogenic glycoside transporter in cassava encoded by MeCGTR1, which was able to transport linamarin when expressed in Xenopus laevis oocytes. MeCGTR1 is homologous to GTR proteins which transport glucosinolates between tissues in Arabidopsis. Like other members of the NPF family, MeCGTR1 appears to be a proton symporter, creating a transmembrane charge during linamarin transport. Further characterization of MeCGTR1 expression and localization of the transporter protein will be helpful in evaluating its role in linamarin transport from leaf tissues.

Environmental effects on cyanogenic potential

Cyanogenic potential of cassava is heritable but plastic, and it is well documented that cyanogen content in cassava and other cyanogenic plants fluctuates as a result of environmental changes. Abiotic stresses including drought, salinity, and extreme temperature change plant physiological conditions, but can also alter DNA architecture and gene expression. Epigenetic modifications in the form of histone acetylation and DNA methylation alter chromatin structure, subsequently changing gene expression patterns and enabling plants to withstand adverse conditions (Hirayama and Shinozaki, 2010; Kim et al., 2010; Pandey et al., 2016). The close association between environmental cues and changes in CNp suggests that fluctuations in cyanogen levels may also be controlled epigenetically. In Sorghum, DNA methylation negatively regulates dhurrin synthesis during early seedling development and may also contribute to widespread phenotypic variation seen within adult plant lines (Rosati et al., 2019). Zhao et al. (2015) reported an up-regulation of histone deacetylases in drought-stressed cassava, indicating that chromatin remodeling can be activated as a result of certain types of environmental stress. Whether or how chromatin remodeling affects expression of genes involved in linamarin synthesis is not yet known.

While several studies have reported on changes to cassava’s gene expression profile from environmental changes, very few have examined the molecular control of CNp as a function of environmental cues. Molecular regulation of cyanogen production by light has been one of the best characterized responses to date. Changes in photoperiod are tightly associated with cyanogen content, most probably due to photosynthesis-driven alterations in fluxes of carbon and nitrogen pools in plant tissues. Burns et al. (2002) demonstrated that only under high light conditions will Eucalyptus cladocalyx plants allocate available nitrogen to cyanogens; light-limited plants will shift nitrogen allocation to photosynthesis components, particularly those associated with light-harvesting complexes. Diurnal light fluctuations also drive temporal patterns of cyanogen synthesis in plants. Kongswadworakul et al. (2009) reported diurnal fluctuations of linamarin levels in rubber tree (Hevea brasiliensis) and proposed that light may either promote protein degradation or down-regulate expression of cytochrome P450s involved in cyanogen synthesis. Diurnal fluxes are also apparent in the transcript levels of cassava genes involved in linamarin and lotaustralin synthesis; CYP79D1/D2, CYP71E7/11, and UGT85K5 transcripts increase overnight and decrease in the morning (Schmidt et al., 2018b). The promoter regions of these genes contain several transcription factor-binding motifs associated with light, abiotic stress, and developmental responses, with circadian regulation motifs most abundant in CYP79D1 and UGT85K5 promoters. The CYP71E11 promoter region includes three times as many ABAs- and dehydration stress factor-binding motifs compared with other linamarin synthesis genes, indicating that different environmental cues may activate different sets of linamarin synthesis genes.

Drought stress correlates with increased cyanogenic glycoside content in several cyanogenic plants including cassava and, because of its importance to agricultural regions often plagued by reduced water availability, the role of drought in cassava root quality has been extensively studied (Woodrow et al., 2002; Brown et al., 2016; Shehab and Guo, 2020). Cassava is drought tolerant, and the defensive chemistry of its tissues fluctuates with water availability. Under conditions of prolonged drought, cyanogen levels increase in low- and high-cyanogenic varieties; once water stress is reduced, root CNp will once again decrease (Bokanga et al., 1994; Vandegeer et al., 2013; Brown et al., 2016). A recent study of two cassava cultivars (Arg7 and SC124) under drought stress demonstrated differential linamarin accumulation patterns under identical drought conditions (Zhao et al., 2015). Leaves but not roots of SC124 had elevated levels of linamarin during drought, while roots but not leaves of cultivar Arg7 accumulated higher levels of linamarin. This study was augmented by tissue-specific proteomic analysis that identified several proteins that were differentially expressed during drought, and which included some of the proteins involved in linamarin metabolism. Roots of both drought-stressed cultivars displayed increased levels (4.08- to 4.21-fold) of CYP71E7 protein, which catalyzes the intermediate step in linamarin synthesis. Linamarin catalytic potential was also altered; leaf linamarase levels were down-regulated 20-fold, but only in...
cultivar SC124. Leaves of this same cultivar showed a 1.8-fold up-regulation of cyanohydrin UDP-glucosyltransferase (UGT85K4). This study indicates that linamarin accumulation during drought can be elevated in a cultivar-specific and tissue-specific manner, and that CNp could potentially be augmented by enhancing linamarin synthesis and/or decreasing linamarin catabolism. It should be noted that changes in production of the protein involved in the first dedicated step in linamarin synthesis (CYP79D1/D2) were not detected in this study, which is consistent with the general consensus that cassava roots do not make their own linamarin.

Globally, agricultural soil quality is negatively affected by increased salinity, but few studies exist which address the effects of salinity on cassava’s CNp, and nothing has yet been reported on any molecular responses to salinity stress. Gleadow et al. (2016) were able to demonstrate that linamarin accumulates in cassava depending upon salinity levels and in an age-specific manner. In this study, leaves from mature plants had reduced CNp under higher salinity stress whereas root CNp levels increased under moderate salinity conditions. These patterns of linamarin accumulation were opposite in younger plants, which accumulated more linamarin in leaves and none at all in roots. While there are no studies which have identified salinity stress-specific expression of cyanogen metabolism genes, a recent study showed that cassava up-regulates several A20/AN1-type zinc finger genes, a class of genes associated with abiotic stress. All members of this gene family (Metip1–Metip11) showed altered expression levels under high-salinity conditions and may be associated with negative regulation of plant hormones (Cheng et al., 2018).

The relationship between temperature and CNp in cyanogetic plants varies by species and is largely uncharacterized at the genetic level except in a few plants (Gleadow and Woodrow, 2000). Early studies examined the relationship between cold climates and the frequency of alleles (Li and Ac) associated with CNp in white clover (Trifolium repens) and suggested that allele combinations that support low CNp are advantageous in colder regions in order to reduce the risk of freeze-induced ‘HCN autotoxicity’ (Daday, 1965). More recent studies have not supported this theory, however, and the prevailing current theory—at least for white clover—is that maintaining CNp has trade-offs with reproductive fitness (Kooyers et al., 2018). Cold/freezing stresses do not seem to alter transcription of genes involved in cyanogenesis, although stress response genes involved in starch metabolism and scavengers of reactive oxygen species (ROS) increase (An et al., 2012). Large-scale transcriptome and proteome analyses of temperature-stressed cassava have demonstrated that a 24 h exposure to cold (4 °C) results in a myriad of differentially expressed long non-coding RNAs that are involved in a large network of responses including heat-shock protein synthesis, secondary metabolite synthesis, and hormone biosynthesis (Li et al., 2017a, b; Suksamran et al., 2020). Li et al. (2017a) demonstrated that cold triggers increased expression of genes encoding MYB transcription factors, as well as hormone response genes (particularly ethylene, auxin, and ABA). It is important to note, however, that these studies did not specifically indicate linamarin synthesis genes, although the ontology of differentially expressed genes did indicate enrichment for ‘biosynthesis of secondary metabolites’.

Global climate shifts are further endangering cassava agriculture and food safety, and the effects of both warmer temperatures and higher CO₂ levels have implications for cassava cultivation in areas where agricultural conditions are already less than ideal. While both drought and high temperatures usually threaten cassava cultivation simultaneously, most cassava studies have investigated effects of drought on CNp rather than heat, and currently no molecular studies have been published. It has been reported that leaves and roots of cassava plants grown at higher temperatures have lower CNp than plants grown at ambient temperatures, but these alterations in cyanogen pool sizes may be driven more by changes in nitrogen availability and carbon fixation, rather than by gene expression (Forbes et al., 2020). Cassava is relatively more heat tolerant compared with other crops, and its productivity does not seem to be greatly affected by high temperatures (Opabode et al., 2019). This heat tolerance may be related to the efficiency with which cassava scavenges ROS in leaf tissues. While cyanogenesis is linked to production of ROS, this relationship has only been characterized in harvested root tissues, rather than after heat stress (Zidenga et al., 2012; see also following section).

Cassava is often cultivated in soils that are low in nutrients, and the high energetic cost of cyanogen synthesis suggests that tissue cyanogen concentrations will fluctuate depending upon soil nitrogen availability. The importance of cyanogens as active contributors to cassava growth and development has been well demonstrated. Transgenic acyanogenic cassava lines have stunted root development, unless the medium is supplemented with nitrogen (Siritunga and Sayre, 2003). These plants also exhibit higher nitrate reductase activity than wild-type plants. A positive relationship between CNp and soil nitrogen has been reported in Soglow, but this same relationship does not seem to be in place in all cassava tissues (Burns et al., 2012; Shehlab and Guo, 2020). While increased nitrogen availability can result in elevated foliar cyanogen levels, cyanogen levels in leaves do not necessarily correlate with root cyanogen levels (Bokanga et al., 1994; Jørgensen et al., 2005). Burns et al. (2012) reported that cyanogen variation in cassava leaves and roots could not be explained by nitrogen levels alone in these same tissues. Brown et al. (2016) have suggested that differential partitioning of nitrogen to defense compounds may only take place when nitrogen is limiting, and that tissue nitrogen may be controlled independently of tissue CNp. It is clear that the interactions between environmental, metabolic, nutritional, and genetic factors that contribute to cyanogen content are complex and should be taken into account when developing cassava strains that have altered cyanogen capacity.
It is interesting to note that biotic (herbivore) stresses are not commonly associated with changes in CNp in cassava, although they have been reported in other cyanogenic plant species. It is possible that in cassava, biotic stresses that trigger herbivore resistance-related genes do not necessarily affect the same genes involved in chemical defenses. Transcriptome analysis of mealybug-resistant cassava cultivars indicated no increase in genes involved in CG synthesis, and reported a decrease in transcriptional levels of UDP-glycosyltransferases (Rauwane et al., 2018).

**Metabolism of cyanogens in roots**

**Assimilation of cyanogens**

Cyanogenic glycosides such as linamarin have been classified as ‘phytoanticipins’, compounds that are synthesized in healthy plants and which act as deterrents against damage by biotic agents (Morant et al., 2008). This is because CGs and their catabolic enzymes are compartmentalized in separate subcellular locations, and cyanogenesis will only occur in circumstances where enzymes and substrates are brought together, such as ruptured tissues. Mechanical damage initiates hydrolysis of linamarin by the generalized β-glucosidase, linamarase (EC 3.2.1.21), to produce acetone cyanohydrin, which can spontaneously decompose to yield cyanide and acetone (at pH >5.0 or temperatures >35 °C), or in the case of leaves can also be broken down by hydroxynitrile lyase (HNL) (EC 4.1.2.47). While linamarase is present in cassava leaves, stems, and roots, HNL is only present in leaves (Nambisan and Sundaresan, 1994; White et al., 1998, Siritunga et al., 2003).

The breakdown of CGs causes the release of HCN, which threatens mitochondrial electron transport and depletes reduced nitrogen reserves. Thus, cyanide reassimilation mechanisms may well be important to reduce poisoning of the cellular respiration machinery and associated programmed cell death responses, and to prevent a drain on nitrogen resources. Plants have evolved several mechanisms by which cyanide can be metabolized into non-toxic metabolites, but the most common mode involves β-cyanoalanine synthase (EC 4.4.1.9). This enzyme condenses cyanide with cysteine to form β-cyanoalanine, which is subsequently hydrolyzed to form asparagine by an enzyme that possesses both nitrilase and nitrile hydrase functions (EC 4.2.1.65) (Miller and Conn, 1980; Machingura et al., 2016). The nitrilase function results in the formation of asparagine, while nitrile hydrase forms aspartate and ammonium. Through these two reactions, cyanide is recycled into primary metabolites and supplements amino acid pools (Maruyama et al., 1997; Ebbs et al., 2010). β-Cyanoalanine synthase activity is present in all cassava plant tissues, but varies according to tissue and developmental stage, and presumably is related to the CNp of the tissue, due to ethylene-derived HCN production or CG catabolism (Machingura et al., 2016; Zidenga et al., 2017).

In addition to cyanide assimilation via β-cyanoalanine synthase, at least one other mechanism for cyanide detoxification has been identified. Rhodanese (cyanide:thiosulfate sulfurtransferase, EC 2.8.1.1) detoxifies cyanide by converting it to thiocyanate. Rhodanese is ubiquitous across the animal kingdom, and has also been identified in bacteria, fungi, and plants (Cipollone et al., 2007). Thiocyanate, however, is a metabolic dead-end in plants, and thus rhodanese activity in cassava would not be beneficial for nitrogen assimilation from cyanide. Rhodanese activity has been detected in cassava, but only at very low activity levels in leaves and not at all in roots, suggesting that linamarin breakdown and cyanide metabolism in roots occur primarily through β-cyanoalanine synthase (Zidenga et al., 2017). An alternative pathway to recycle nitrogen derived from CGs in plants including cassava has recently been proposed, which operates in parallel with the β-cyanoalanine synthase pathway (Pišmanová et al., 2015). In this model, the CG is glycosylated and then converted to an anitrile glucoside by the release of CO₂ and ammonia. This alternative pathway does not produce HCN, and ammonia is recycled into primary metabolic pathways. The putative metabolic intermediates in this alternative pathway have been identified via MS in cassava, *Sorghum*, and almond, but the relative steady-state levels of these metabolites were substantially lower than the dominant CGs. More recently, Bjarnholt et al. (2018) were able to present evidence for an acyanogenic recycling mechanism for *Sorghum’s* CG dhurrin. In this pathway, dhurrin is conjugated with glutathione and then is metabolized by a lambda class glutathione transferase (GSTL) to form p-hydroxyphenyl-acetonitride. A nitrilase further hydrolyzes this acetonitride molecule, producing ammonia that is then available for primary metabolism. GSTLs and nitrilases that might have specificity for linamarin—glutathione conjugates have not yet been identified in cassava, however, providing limited support for this pathway as a major means for cyanogen assimilation in cassava.

The importance of CGs in reduced nitrogen assimilation has also been supported by molecular studies. In transgenic cassava where linamarin synthesis was eliminated in the leaves, not only were the roots acyanogenic, but the plants were only able to grow well on media supplemented with reduced nitrogen (Siritunga and Sayre, 2004). Jørgensen et al. (2005) reported stunted development in acyanogenic cassava and indicated that plant height correlated with CNp of the plants. Since engineering acyanogenic cassava by reducing cyanogen synthesis negatively impacts crop development and yield, other approaches for modulating cyanogen levels have focused on altering root-specific linamarin catabolism. Overexpression of linamarase targeted to root vacuoles resulted in steady-state linamarin levels that were substantially reduced relative to the wild type (Leyva-Guerrero et al., 2012). Likewise, overexpression of HNL targeted to cell walls in roots also led to a substantial (>50%) reduction of root linamarin levels, as well as an increase in free amino acid and total protein levels.
(Siritunga et al., 2003; Narayanan et al., 2011; Leyva-Guerrero et al., 2012). This effect presumably was due to increased sink strength for cyanogen assimilation into proteins. Significantly, overexpression of HNL was originally justified to accelerate cyanogen detoxification of cassava roots during processing, since HNL is not present in wild-type roots (White et al., 1998; Siritunga et al., 2003; Narayanan et al., 2011). In fact, cyanogen detoxification was reduced from days to minutes in transgenic plants overexpressing HNL in root cell walls (Siritunga et al., 2003). Of all the amino acids that increased in roots overexpressing HNL, glycine, glutamine, aspartate, and asparagine increased the most compared with wild-type levels. These four amino acids are central to directing nitrogen fluxes in plants, and serve several essential functions: glutamine and glutamate are precursors for almost all amino acid biosynthetic pathways. Additionally, glutamine and glutamate are the immediate precursors to aspartate and asparagine, and all four amino acids serve as transportable nitrogen between source and sink in many plant species (Coruzzi, 2003). In support of the cyanogen–nitrogen assimilation model for cassava, recent GWAS analyses focused on determinants of Eucalyptus cladocalyx CNp indicated that the β-cyanoalanine synthase assimilation model was the dominant pathway for cyanide metabolism in this species (Mora-Poblete et al., 2021).

Other non-cyanogenic metabolites are also prominent in cassava’s nitrogen cycling and transport mechanisms. Obata et al. (2020) identified high levels of glutamine, putrescine, and nitrate in cassava stems, and high concentrations of arginine and ornithine in roots and sink leaves of nitrogen-fertilized cassava plants. In sink leaves, asparagine, serine, and glutamate were the dominant nitrogenous metabolites. Linamarin was also present at high levels, although not at the same levels as glutamine. Because glutamine and putrescine have high ratios of nitrogen to carbon, the authors postulated that these two molecules are the principal nitrogen storage forms in cassava. Preferential storage of nitrogen in amino acids which contain more than one nitrogen molecule may allow cassava to yield a productive crop even when grown in soils that are nitrogen deficient (El-Sharkawy, 2012).

While the importance of nitrogen in cassava root development is not disputed, an excess of nitrogen can result in reduced crop yields by creating an imbalance in photosynthetic production and incorporation (Gleadow et al., 2009). This imbalance can result in an increase in shoot growth over root growth, interference with stomatal conductance in the leaves, and reduced sucrose transport to the root (Cruz et al., 2003; Omondi et al., 2019; Obata et al., 2020). Indeed, cassava prioritizes nitrogen allocation to sinks in actively photosynthesizing leaves versus protein accumulation in roots. The interplay of C/N source–sink dynamics is further complicated by sensitivity of cassava’s photosynthetic apparatus to high CO₂ levels, which is atypical for C₃ plants. Total biomass and photosynthesis metrics have been shown to decrease under higher ambient CO₂ conditions, regardless of nitrogen abundance (Gleadow et al., 2009). Under high nitrogen/high CO₂ conditions, cassava allocates more nitrogen to cyanogens, but only in leaves.

### Metabolic engineering of cyanogens in roots

#### Role of cyanogens in root protein synthesis

As previously stated, initial efforts to reduce cyanogen accumulation in cassava resulted in stunted development in plants unless the plants were supplemented with ammonia, suggesting that cyanogens play a significant role in nitrogen metabolism in cassava (Siritunga and Sayre, 2003). These low-cyanogen plants, however, were essential in further studies to understand the metabolism of cyanogens and their impact in overall nitrogen metabolism and protein synthesis.

As a strategy to accelerate the turnover of linamarin and cyanide assimilation into amino acids in cassava roots, a vacuolar-targeted linamarase was expressed under the control of the root-specific promoter patatin to convert linamarin stored in the vacuole to acetone cyanohydrin (Leyva-Guerrero et al., 2012). This ‘push’ strategy resulted in a decrease in steady-state root linamarin levels of >50% and a 2.2-fold increase in total free amino acid levels in roots. Interestingly, this increase in root free amino acids was not accompanied by an increase in root total protein, suggesting that root protein sink strength may need to be increased to assimilate the additional free amino acids into root proteins. The leaves of these transgenic plants, however, had a 3-fold increase in total protein presumably due to export of root free amino acids to the stronger nitrogen sink, the leaves (Leyva-Guerrero et al., 2012).

To increase root nitrogen sink strength and to potentially accelerate cyanogen volatilization and detoxification, Narayanan et al. (2011) overexpressed HNL in cassava roots to accelerate conversion of acetone cyanohydrin into cyanide, resulting in a 50–75% reduction in root steady-state linamarin levels, an increase in free amino acid pools, and a 3-fold increase in total root protein in 7-month-old plants, suggesting that linamarin provided reduced nitrogen for protein synthesis, presumably via the β-cyanoalanine synthase pathway (Siritunga et al., 2003). Significantly, HNL has a well-balanced amino acid composition for human nutrition and is high temperature stable, making its expression in cassava roots compatible with a variety of food processing technologies that accelerate cyanogen detoxification (White et al., 1998; Siritunga et al., 2003; Narayanan et al., 2011).

Using a combination of biochemical assays and genetic engineering approaches, Zidenga et al. (2017) investigated the enzymes involved in detoxifying cyanide, namely rhodanese and β-cyanoalanine synthase, and the impact of overexpressing these enzymes on cyanide assimilation in transgenic plants. Enzyme assays in cassava roots and shoots indicated that only β-cyanoalanine synthase is responsible for assimilation of cyanide in cassava roots; while rhodanese
activity was detected in cassava shoots, no activity was detected in roots. Cyanide assimilation via β-cyanoalanine synthase and β-cyanoalanine hydrase/nitrilase 4 (NIT4) was found to be higher in cassava roots compared with shoots, consistent with anticipated elevated levels of cyanogens in roots compared with shoots. To investigate whether the β-cyanoalanine synthase pathway contributes significantly towards amino acid pool sizes from the metabolism of cyanogens, Zidenga et al. (2017) generated transgenic cassava plants expressing an Arabidopsis β-cyanoalanine synthase under the control of the tuber-specific patatin promoter and measured changes in free and total amino acids. Root free amino acid levels increased by up to 50% while root protein accumulation increased by 9% in plants overexpressing β-cyanoalanine synthase, suggesting a potential for redirecting cyanogen metabolism to enhanced protein nutrition. However, altered plant growth and morphology in the transgenic plants overexpressing these enzymes suggested a complex interaction between cyanide metabolism and hormonal regulation of plant growth.

In summary, enhancement of root cyanogen metabolism through the β-cyanoalanine-mediated cyanide assimilation pathway has been shown to increase free amino acids pools and, when coupled with increased protein synthesis sink strength in roots, can substantially reduce linamarin storage pool sizes and increase the nutritional quality of cassava roots.

Role of cyanogens in PPD

As previously discussed, cassava is one of the six most important crops in the world (along with wheat, rice, maize, potato, and barley) and one of the most important sources of calories. In addition to its role in providing food security, particularly in sub-Saharan Africa, cassava is also growing in importance as an industrial crop as a source of industrial starch (MunyiJka et al., 1997; Ihemere et al., 2008; Jansson et al., 2009) and as a biofuel feedstock (Dai et al., 2006; Drapcho et al., 2008). This growing importance has been limited, however, by the short shelf life of harvested cassava roots. Cassava undergoes rapid PPD, limiting the shelf life of untreated roots to 2–3 d (Booth, 1976; Wenham, 1995; Zidenga et al., 2012).

To reduce the effects of PPD, smallholder farmers often harvest cassava roots piecemeal to minimize storage constraints. However, this ‘in soil storage’ impacts quality while utilizing land that may be needed for other uses. Post-harvest processing techniques such as oxygen exclusion through waxing have been employed to mitigate the losses due to PPD. However, these are costly and generally not practical in smallholder settings. Effective control of PPD will require the development of cassava lines that have a longer shelf life. Breeding efforts for delaying PPD have been hampered by a number of factors, such as the large influence of environmental growth conditions and associated pre-harvest stress, as well as limited genetic variability (Wenham, 1995; Sanchez et al., 2005).

The biochemical events leading to PPD have been elucidated (Buschmann et al., 2000; Huang et al., 2001; Reilly et al., 2001; Iyer et al., 2010; Zidenga et al., 2012). PPD is initiated by mechanical damage, which typically occurs during harvesting. The damaged cassava storage root does not exhibit common wound repair responses observed in other plants, such as synthesis of suberin and lignin leading to the formation of a protective tissue layer (Beeching et al., 1994; Wenham, 1995). The formation of lignin in mechanically damaged cassava roots is poor under ambient conditions (Uritani, 1999). Instead, PPD is associated with changes in lipid composition as well as accumulation of secondary metabolites such as scopoletin (7-hydroxy-6-methoxy-2H-1-benzopyran-2-one), the oxidation of which gives the blue-black color characteristic of PPD (Gutierrez et al., 1995). Reilly et al. (2001) reported an oxidative burst 15 min after damage in cassava roots. Enhanced expression of genes involved in reactive oxygen metabolism including superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) was associated with PPD in cassava, but this differential gene expression occurs too late or is insufficient to significantly reduce the magnitude of the process (Liu et al., 2017).

To investigate if there was a link between cyanogenesis and the oxidative burst in wounded cassava roots, Zidenga et al. (2012) compared ROS accumulation in low and high cyanogen cassava, and reported that low cyanogen roots had significantly reduced levels of ROS accumulation. Biochemical complementation experiments in which cyanide was added to low cyanogen root sections at concentrations equivalent to wild-type levels confirmed the causal link between cyanogenesis and ROS accumulation. It was hypothesized that cyanide release during tissue damage inhibited mitochondrial electron transfer causing over-reduction of upstream electron transfer complexes and consequent elevated production of ROS. To verify that the source of ROS was mainly associated with mitochondrial respiratory cyanide inhibition, rather than, from example, other ROS-generating systems such as the plasma membrane NADPH oxidase, Zidenga et al. (2012) measured ROS accumulation in roots treated with the plasma membrane NADPH oxidase inhibitor, diphenyl iodonium chloride (DPI). The reduction of ROS accumulation by DPI treatment of only 20% suggested the main source of ROS to be mitochondrial in origin. As a strategy to reduce the cyanide-induced ROS accumulation and control PPD, Zidenga et al. (2012) overexpressed an Arabidopsis thaliana mitochondrial alternative oxidase (AtAOX), codon-optimized for cassava expression. The alternative oxidase (AOX) pathway branches at the ubiquinone pool, providing an alternative cyanide-insensitive route in the electron transport chain to reduce oxygen, facilitating the oxidation of over-reduced complexes I and III (known to generate ROS following cyanide inhibition of cytochrome c oxidase) (Maxwell et al., 1999). Roots of transgenic cassava plants overexpressing AOX had substantially reduced accumulation of ROS that was barely detectable in the base transgenic
lines. The transgenic lines exhibiting reduced ROS accumulation also had PPD delayed by up to 3 weeks under greenhouse growth conditions, and up to 10 d under field growth conditions (Zidenga et al., 2012).

Additional studies support the concept of ROS-induced PPD in cassava. Antioxidants that reduce ROS have been associated with delayed PPD. For example, high β-carotene content has been found to be associated with delayed PPD (Sanchez et al., 2005; Morante et al., 2010; Beyene et al., 2018). Overexpression of ROS-quenching enzymes such as SOD and CAT has also led to improvements in post-harvest shelf life (Xu et al., 2013). Melatonin (N-acetyl-5-methoxytryptamine) has also been explored for reducing cassava PPD, due to its ROS-scavenging properties (Hu et al., 2016, 2018; Ma et al., 2016). Exogenous application of melatonin was found to delay PPD, increase the activities of CAT and peroxidase, and decrease hydrogen peroxide (Hu et al., 2016). Genes involved in melatonin biosynthesis in cassava were identified and cloned (Wei et al., 2018), paving the way for transgenic approaches to a melatonin-mediated PPD delay in cassava.

Besides reducing ROS-induced damage, focus has also been put on the metabolites that accumulate during PPD. Using RNAi, Liu et al. (2017) reduced the expression of a key enzyme in scopoletin biosynthesis, feruloyl-CoA 6′-hydroxylase, in cassava, resulting in reduced accumulation of scopoletin and delayed PPD symptom development.

Conclusions

It is now evident that cyanogen synthesis, transport, and turnover in cassava plants is a highly integrated process involving the differential expression of multiple genes and enzymes in different tissues and organs of the cassava plant. Linamarin synthesis primarily occurs in leaves and stems, and not in roots. Linamarin is then either stored in the vacuoles of linamarin-synthesizing cells, presumably to serve as an anti-herbivory agent, or actively transported through the phloem to the roots. In the roots, there is an apparent competition for linamarin either for storage or for assimilation into asparagine and aspartate for protein synthesis. This model of whole-plant linamarin metabolism is supported by several observations including, (i) the near absence of linamarin in roots in which CYP79D1/D2 expression has been selectively inhibited in leaves; (ii) the presence of linamarin and not linustatin in the phloem of stems for cyanogen transport; (iii) the occurrence of high levels of cyanide assimilation enzyme activities in roots compared with leaves; and (iv) the observation that overexpression of genes facilitating cyanide turnover and assimilation in cassava roots results in increased root free amino acid pools, increased root protein levels, and reduced root linamarin storage levels. The metabolism of linamarin in roots, however, must presumably be tightly controlled to reduce the potential for cyanide poisoning. The absence of HNL expression in roots, which would accelerate acetone cyanohydrin turnover and cyanide generation, is consistent with the hypothesis that cyanogenesis in cassava roots must occur at a rate that can be accommodated by competing cyanide assimilation into amino acids, which is enhanced in roots. Interestingly, overexpression of HNL in cassava roots leads to increased protein as well as reduced linamarin storage, presumably by creating a sink for cyanide-based amino acid synthesis. In addition, expression of tonoplast-localized MATE proteins in cassava roots would presumably facilitate linamarin storage in vacuoles, helping to establish a balance between linamarin turnover and storage. These observations led to the prediction that engineering cassava roots to have reduced levels of MATE expression plus elevated protein expression (higher sink amino acid strength) would alter the dynamics between linamarin storage and turnover, leading to reduced linamarin levels and elevated protein. In addition, by simultaneously overexpressing HNL and blocking MATE protein activity, any residual cyanogens would be turned over more rapidly during root processing, creating a safer food product. The failure to achieve cyanogen-free roots through traditional breeding procedures is presumably due in part to the critical role of cyanogens in both protein synthesis and cyanide toxicity. Thus, a transgenic approach that simultaneously reduces linamarin storage and increases protein synthesis may be the most feasible strategy to achieve ‘cyanide-free’ cassava roots.

Besides playing a central role in nitrogen metabolism in cassava roots, linamarin also plays a central role in the rapid physiological deterioration that occurs in roots after harvesting. Biochemical assays and cyanide complementation studies in very low cyanogen-containing roots demonstrate that cyanide generated from linamarin during wounding results in the accumulation of ROS that initiate and drive PPD of cassava roots in a matter of days. These ROS were generated by cyanide inhibition of the mitochondrial cytochrome c oxidase. In contrast, transgenic plants expressing a cyanide-insensitive mitochondrial AOX had substantially reduced ROS production and long root shelf lives of >3 weeks.

A critical question to ask, however, is whether the elimination of cyanogen-based amino acid assimilatory metabolism from cassava and its replacement by another amino acid production, transport, and assimilatory pathway will have any unintended negative agronomic consequences. It is generally accepted that cassava was widely adopted as a staple crop in tropical regions outside of Central and South America, its site of origin, due to the food security it provides. Cassava potentially has a high crop index, is drought tolerant, and the roots can be banked in the soil for over a year. However, the cyanogenic traits of cassava may also enhance food security by reducing generalized herbivory. The complete elimination of cyanogenesis in cassava may necessitate the application of insecticides to control generalized insect feeders, and fencing of farms to exclude large herbivores, two practices that would have both environmental and financial consequences.
for subsistence and large-scale farmers. The potential to use recombinant and/or breeding strategies to reduce root cyanogen levels by redirecting cyanogens to root protein synthesis, while simultaneously reducing cyanide-dependent PPD, may outweigh the risks associated with the complete elimination of cyanogenesis, a natural defense system against herbivory. This is a challenge worthy of further techno-economic and environmental analysis.

In summary, cyanogens play a central role in cassava nitrogen metabolism and should not be perceived merely as secondary products in cassava plant metabolism. The associated cyanide toxicity makes the manipulation of cyanogen metabolism and reduction of root cyanogen toxicity challenging. Through a combined approach of channeling cyanogens away from storage and towards protein synthesis, it may be possible to finally reduce cassava root toxicity for the consumer.

Author contributions
JM and RS: conceptualization, investigation, writing—original draft, writing—review and editing, visualization. TZ: conceptualization, investigation, writing—original draft, writing—review and editing.

Conflict of interest
The authors do not have any conflicts of interest.

References


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