Engineering Reduced Cyanogenic Glucoside Accumulation in Cassava Roots

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ABSTRACT

The cassava plant (*Manihot esculenta)* is a staple food crop across the globe, feeding many regions of Africa, Latin America, Asia, and the Pacific. Its edible starchy tuberous roots are major sources of carbohydrates, providing 25% of all calories for approximately 800 million people worldwide. The problem however, is that the roots of bitter cassava varieties contain cyanogenic glucosides (CGs). CGs play an important role in herbivory defense and environmental stress mediation, but can lead to cyanide poisoning, partial paralysis, and death in humans if consumed improperly. Previous research has identified the cyanogenic glucoside biosynthesis and transporter genes in cassava; additional studies have also successfully reduced CG levels through RNAi knockdown of the biosynthetic genes. In this study, I analyzed the relationship between cyanogenic glucosides and cassava crop yield and found a significant trend between high cyanogenic glucoside levels and higher yield. This further supports the existing notion that cyanogenic glucosides play an important role in maintaining the fitness of plants. Additionally, I produced knockouts of a systemic transporter of cyanogenic glucosides using CRISPR-Cas9. This resulted in an over-accumulation of CGs in source tissues, and potentially decreased amounts of CGs in roots; thus creating edited plants that are safer for consumption, without eliminating a key herbivory defense and physiological fitness mechanism. Through this experimental approach, we may gain additional knowledge regarding the cassava plant's physiology, and help eliminate the negative aspects associated with its consumption.

KEYWORDS

INTRODUCTION

Food security is one of the greatest challenges society faces. As the global population continues to grow rapidly, food producers are struggling to meet the increased demand for nutritious, affordable, and accessible food. Several factors limit the scale to which food production may be expanded. These factors include a decrease in arable land due to urbanization and environmental degradation, increased cost and potential shortages of fertilizers, and climate change (Burns et al. 2010). The cassava crop (*Manihot esculenta)* holds the possibility to provide a solution to these issues while playing an important role in combating food insecurity. Cassava is a major staple crop around the world, feeding approximately 800 million people, primarily in sub-Saharan Africa, as well as other regions of Africa, Asia, South America, and the Pacific. Its edible, starchy tuberous root serves as the third most important source of carbohydrates in the tropics (Wilson et al. 2017). This plant exhibits high productivity in poor soil and drought conditions, is easily propagated, and requires little cultivation. Furthermore, the tuberous roots may be harvested from the soil up to three years after maturity, safeguarding against social disruption, prolonged droughts, or other periods of stress and unrest (Lebot 2020). Therefore, the cassava crop can aid in increasing food production without requiring the use of large amounts of agricultural inputs such as water, pesticides, and fertilizers.

Nevertheless, there are issues that must be addressed if continued and increased reliance on cassava as a staple crop is to persist. The first issue is the tuber's rapid post-harvest deterioration following removal from the soil, which negatively impacts the marketability of the crop. Secondly, the roots are low in protein and some essential micro-nutrients, resulting in a lower nutritional value. Lastly, cassava contains cyanogenic glucosides (CGs), metabolites that release the harmful chemical gas hydrogen cyanide. Thus, many cultivars are toxic if not processed properly before consumption (Chauhan 2015). This study will focus on addressing this last limitation of cassava.

Cyanogenic glucosides are a crucial herbivory defense mechanism, protecting against pest and pathogen attack through the release of toxic cyanide. The cyanide is only released when needed, triggered by the CGs contact with an alpha-hydroxynitrile lyase enzyme. This typically occurs after the tissue is disrupted by the chewing of herbivores (Gleadow & Woodrow 2002). The CDC recommended safe level of CGs in food is 10 ppm, but cassava varieties can contain anywhere from 15 to 400 ppm. Consequently, the high toxicity related to the improper processing

of cassava can lead to a variety of health complications in humans, ranging from acute intoxication to chronic problems such as irreversible spastic paraparesis and konzo disease. Detoxification of cyanide in humans is possible using sulfur, so in areas where cassava is a main source of food in the human diet, adequate sulfur nutrition is also essential (Westley 1988). The required cassava processing methods are time consuming however, and it may take up to several weeks before the cassava is ready for consumption. The peel must be removed from the tubers, which are chopped and grated, then either sundried, soaked, or fermented. The dried cassava is pounded into flour, the fermented root pulp is roasted, and the starch extracted from the tuber can be fermented and dried again to make bread and biscuits (Balagopalan 2002). Another problem with these methods is that beneficial nutrients may be leached out or destroyed in the process, further lowering the nutritional value of the crop. Additionally, these methods require a significant supply of water, which is often limited in the cassava-growing regions of Africa, especially in drought periods (Montagnac 2009). The implementation of these methods is critical in order to effectively reduce the concentration of CGs. Thus, this tedious procedure, along with lack of dietary diversity and access to alternative foods and water, greatly hinders poor rural communities dependent on cassava from being able to limit their exposure to cyanogens.

In this study, I seek to genetically engineer cassava in order to overcome the limitations imposed by the presence of cyanogenic glucosides in tubers and create edited plants that are safer for consumption. CRISPR-Cas9 has recently emerged as a promising new genome engineering tool. It consists of a sequence-specific nuclease (Cas9) and a guide RNA (gRNA), which mediates the recognition of a target sequence and cleavage at that site by the nuclease, allowing for site-specific gene editing. CRISPR-Cas9 can induce loss-of-function mutations by creating double-stranded DNA breaks (DSB) at the defined locus, rendering the gene inoperative (Liu et al. 2016). Two possible approaches of eradicating cyanogenic glucosides from the cassava root are by targeting a transporter of CGs or eliminating the biosynthesis of CGs. In regards to the first approach, CGs are primarily synthesized in the shoot apex and then transported to the root, so by knocking-out the function of a transporter gene using CRISPR-Cas9, I expect the transport of CGs from the shoot apex to be blocked, thus reducing CG accumulation in the roots (Jørgensen et al. 2005). In regards to the second approach, previous work has used RNA interference to block the synthesis of the two enzymes responsible for cyanogenic glucoside synthesis, thus inhibiting the CG biosynthesis pathway and almost completely eliminating CG production and content

(Jørgensen et al. 2005). Completely eliminating CGs from the plant however, could greatly reduce herbivory defense and plant fitness. Thus, I plan to use CRISPR-Cas9 to enable a gene knockout that would eliminate the function of the primary CG transporter gene, resulting in cassava plants with low levels of cyanogenic glucosides in their roots.

My central research question is whether editing the cassava plant genome to contain decreased levels of cyanogenic glucosides in the roots will create plants that are safer for human consumption, and as a result, aid in improving food security and benefit populations in countries that are dependent on this crop for sustenance? I use three sub-questions to answer this central question: **1.** What is the relationship between cyanogenic glucosides and important fitness factors such as yield? **2.** How does the relationship between cyanogenic glucosides and yield differ spatially and temporally? **3.** Do cassava plant leaves exhibit increased amounts of CGs, while roots exhibit decreased CG amounts as a result of the CG transporter knock-out? I hypothesize that if CRISPR-Cas9 is used to knockout the function of a cyanogenic glucoside transporter gene in cassava, then there will be a decreased amount of CGs in the roots, and an over-accumulation of CGs in the source tissue, thus creating edited plants that are safer for consumption, without eliminating a key mechanism for improved physiological fitness. In order to address these questions, I will conduct a data analysis to elucidate any relationship between CGs and crop yield. I will also collect data from both wild-type and mutant cassava plants regarding CG content in the leaves and roots.

METHODS

Data analysis

To uncover the relationship between cyanogenic glucosides and cassava crop yield, I ran a data analysis using a large dataset of cassava varieties downloaded from Cassavabase. This data included measurements of HCN potential and fresh storage root weight, which are representative of cyanogenic glucoside levels and yield respectively. These measurements were collected from multiple locations throughout Nigeria. I created a bar graph of HCN potential vs. fresh storage root weight for this data, ran an ANOVA to observe any significant differences, and ran a Tukey HSD test to compare pairs within these differences in R (Version 4.0.3). To analyze spatial and temporal

differences within this data I selected two cities, Ibadan and Malam Maduri. Malam Maduri is in Northern Nigeria and Ibadan is in Southern Nigeria. I created bar graphs of HCN potential vs. fresh storage root weight for each city and ran an ANOVA and Tukey HSD test to determine statistical significance of the results. Lastly, I also selected data for three different years in each city, created the bar graphs again and ran the statistical tests to determine whether the relationship between CGs and yield was present from year to year. I selected these specific cities and years as they had the most data available.

gRNA & CRISPR/Cas9 binary construct design, cloning & assembly

In order to edit the CG transporter gene, I developed a CRISPR/Cas9 binary construct. I used Golden Gate Assembly to clone a CRISPR cassette with a functional Cas9 under a constitutive promoter (CaMV 35S) and two gRNAs into a pCAMBIA binary vector. I identified target sequences in the Me15G180400 gene of cassava using the online CRISPR-P software (Lei et al. 2014). I used this tool to select targets with predicted cut sites within exons and with minimal off-target potential. I performed BLAST analyses against the cassava genome for each gRNA target site including the PAM site (NGG) to check for specificity. To produce each gRNA, I carried out a PCR reaction with an oligonucleotide containing the gRNA sequence and used the pCAMBIA2300 plasmid as a template. I designed gRNA forward and reverse primers with overhangs compatible with the BsaI site. I used the Golden Gate (GG) cloning method to BsaI digest the pCAM vector and ligate with T4 DNA ligase in the gRNA. I verified the sequences of cloned CRISPR constructs via Sanger sequencing. The UC Berkeley Plant Genomics and Transformation Facility performed agrobacterium-mediated transformation to transform the vector into cassava friable embryogenic callus tissue.

Mutant verification, genotyping, & phenotyping

To genotype the mutant plants and verify that edits were present, I used Sanger sequencing. I collected 100 mg of leaf tissue from T0 edited cassava *in vitro* plantlets. I extracted the genomic DNA using the CTAB extraction procedure (Murray & Thompson 1980), and genotyped the edited plants for Cas9-induced mutagenesis via Sanger sequencing (Voytas 2013). I PCR amplified 100

ng of genomic DNA with primers encompassing the Cas9 target sites. I gel purified PCR amplicons on 1.5% agarose gel and purified with the QIAquick Gel Extraction Kit (Qiagen). The UC Berkeley DNA Sequencing Facility Sanger sequenced the amplicons for genomic-sequence analysis. I used SnapGene software (from GSL Biotech) and the Synthego ICE analysis tool to align the sequences to the wild-type sequence and discriminate INDEL polymorphisms. I used a Picrate Test Kit (Sigma-Aldrich) to quantify CG levels in the leaf tissue of both WT and mutant plants.

Off-target analysis

To search for potential off-target sites of the CRISPR/Cas9 system, I used the CRISPR-P software, with default settings, and successfully mapped the reads. I Sanger sequenced the PCR amplicons encompassing the off-target sequences in order to examine their effect.

Growth conditions

To grow the edited and wild-type plants, I first kept them in phytatrays containing gel media in the growth chamber. I then transplanted the plants to 1.5" pots containing SuperSoil mix and covered them with a dome to maintain humidity. I left the plants in the growth chamber for approximately three more weeks before moving them to the greenhouse. In the greenhouse, I repotted the plants into bigger 3" pots and encompassed the original SuperSoil mix with additional Hummert soil. I again covered the plants with a dome to maintain humidity. The plants were watered on a schedule by the greenhouse staff.

<div align="center">

RESULTS

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Relationship between CGs and plant fitness

I found a strong positive relationship between cyanogenic glucoside levels (HCN potential) and crop yield (fresh storage root weight). HCN potential was measured on a scale of 1-10. Low HCN potential was categorized as 0-3, medium was 3-6, and high HCN potential was 6-10. Data

analysis of cassava varieties in Nigeria revealed that the highest category of HCN potential, 6-10, had statistically higher yields compared to low and medium levels (Figure 1). Thus, there is a significant positive trend between cyanogenic glucoside levels and cassava crop yield.
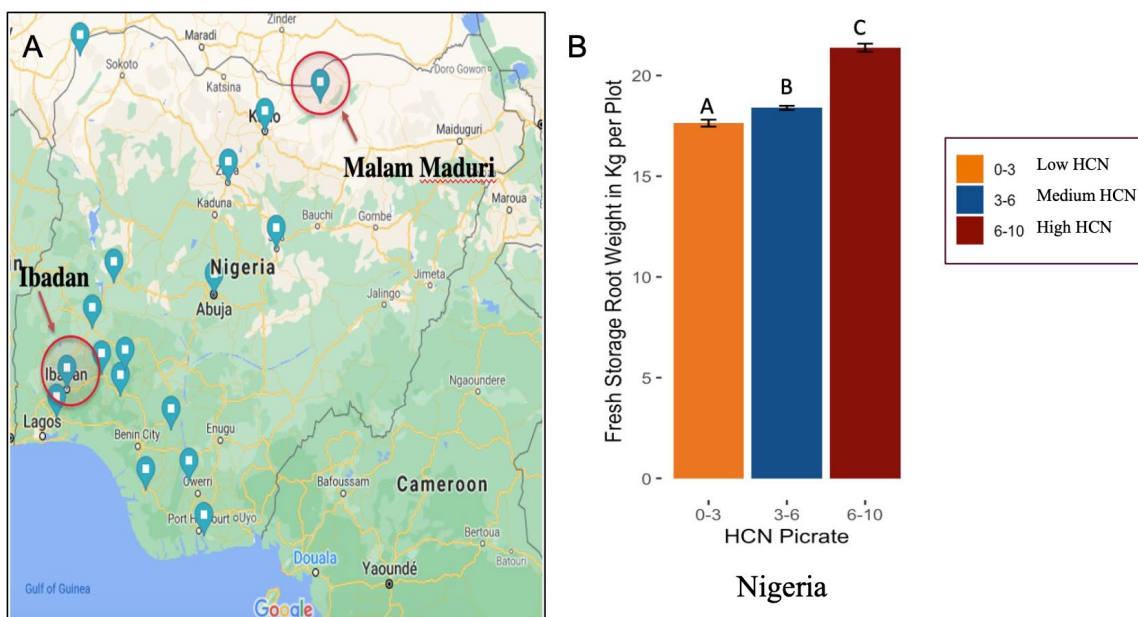

Figure 1. Relationship between cassava HCN potential and fresh storage root weight (kg/plot). (A) Map of locations in Nigeria where cassava data was taken, Malam Maduri and Ibadan are highlighted for further data analyses. (B) Bar graph of HCN potential vs. fresh storage root weight (kg/plot) for Nigeria as a whole (n = 32690). Data was analyzed at a significance level of $\alpha = 0.05$. Error bars are representative of one standard deviation. Letters A, B, and C above error bars denote statistically significant differences between categories.

Spatial and temporal differences

Overall, I found that the trend of high cyanogenic glucosides and higher yield held true within specific locations and years as well. The data for cities Malam Maduri and Ibadan as a whole showed significant increases in yield at higher cyanogenic glucoside levels (Figure 2A, 2E). Similarly, the data for Ibadan in 1998, 1999, and 2000 and Malam Maduri in 1999 all showed a significant positive relationship between cyanogenic glucoside levels and yield (Figure 2F-H, 2C). However, the data for Malam Maduri in 1997 revealed the reverse trend, with low and medium cyanogenic glucoside levels corresponding to higher yield (Figure 2B). Lastly, the data for Malam Maduri in 2000 exhibited no significant correlation between cyanogenic glucoside content and yield (Figure 2D). Altogether, the positive tendency between higher cyanogenic glucoside levels and higher yield is present spatially and temporally in most cases as well.
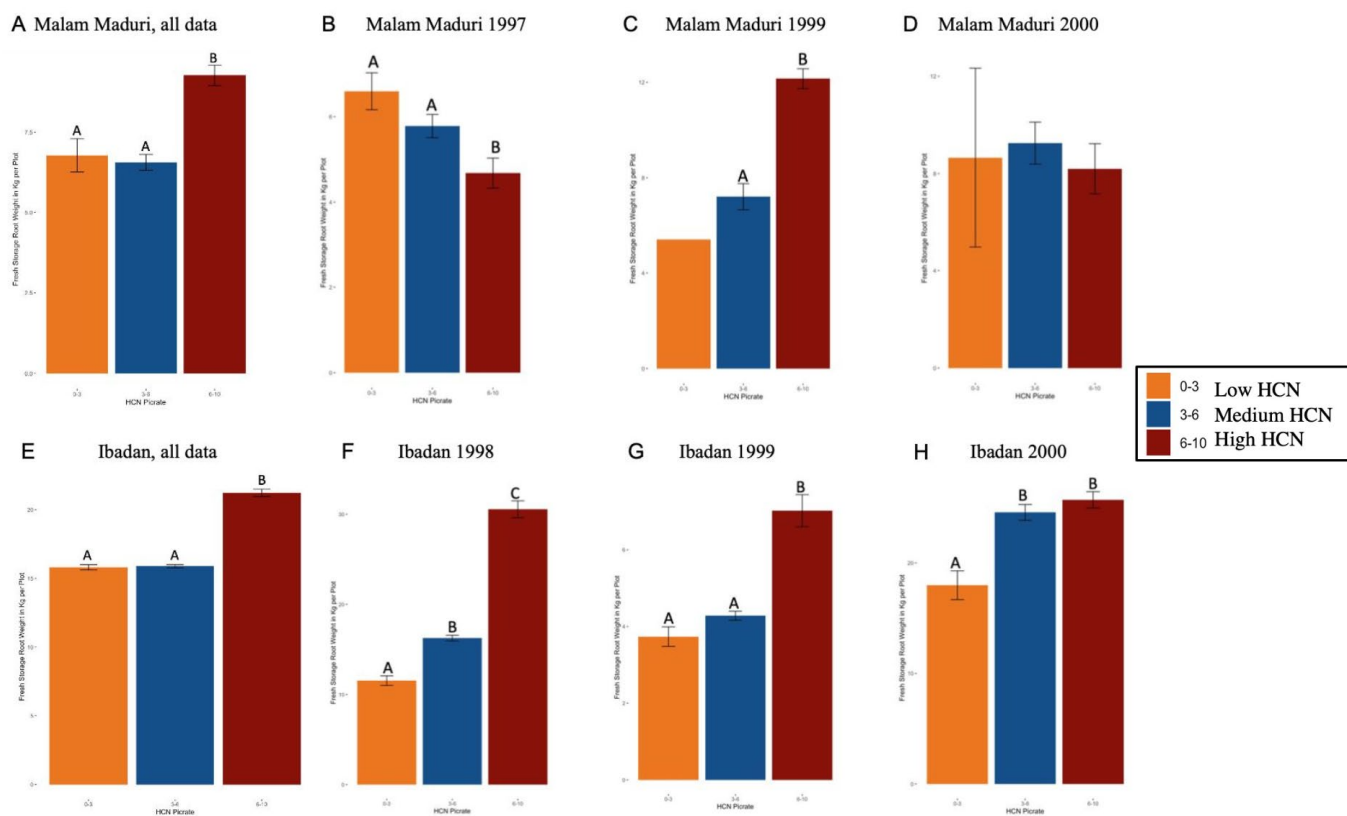
Figure 2. Data analysis of spatial and temporal differences. Bar graphs of HCN potential vs. fresh storage root weight (kg/plot) for (A) Malam Maduri as a whole (n = 1251). (B) Malam Maduri in 1997 (n = 605). (C) Malam Maduri in 1999 (n = 451). (D) Malam Maduri in 2000 (n = 195). (E) Ibadan as a whole (n = 25301). (F) Ibadan in 1998 (n = 4154). (G) Ibadan in 1999 (n = 2944). (H) Ibadan in 2000 (n = 980). Data was analyzed at a significance level of $\alpha = 0.05$. Error bars are representative of one standard deviation. Letters A, B, and C above error bars denote statistically significant differences between categories.

Quantification of CG amount in leaves

I found that CRISPR-Cas9 editing of the target CG transporter gene yielded one loss-of-function mutant plant. The mutation was a one base pair insertion right after the cut site, upstream of the PAM sequence (TGG), resulting in a homozygous mutant with a 99% insertion deletion rate (Figure 3). Furthermore, I found that cyanogenic glucoside content in leaves of mutant plants was greater than wild-type plants. Average wild-type cyanogenic glucoside levels were 27.92 ppm, with a standard deviation of 21 ppm. The mutant plant average cyanogenic glucoside levels were 112.1 ppm, with a standard deviation of 24 ppm (Figure 4). Whether the increase of cyanogenic glucosides in leaves of the mutant plant is significantly different from wild-type remains

inconclusive, as the sample size was too small for meaningful statistical analysis. Wild-type and mutant plants are comparable in physical appearance (Figure 5).
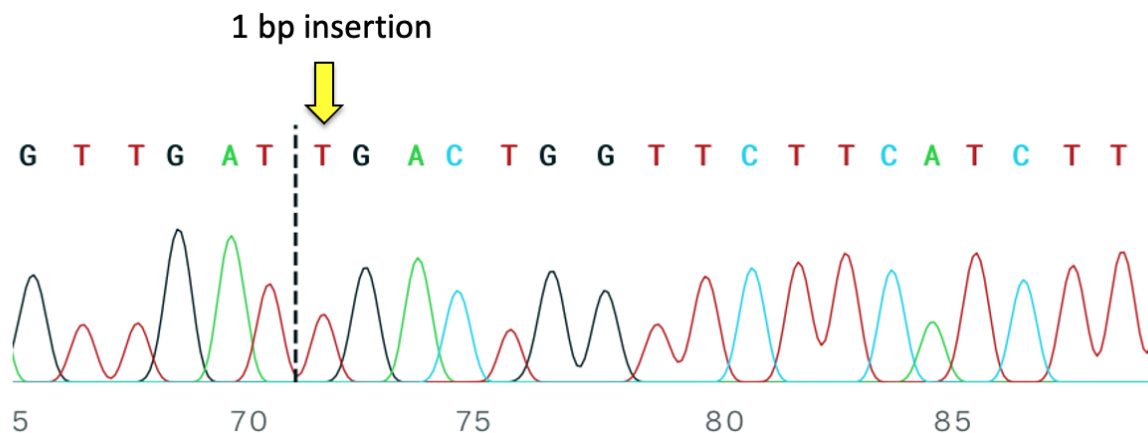

Figure 3. **Sanger sequencing data of the edited plant.** Vertical black dotted line indicates the cut site.
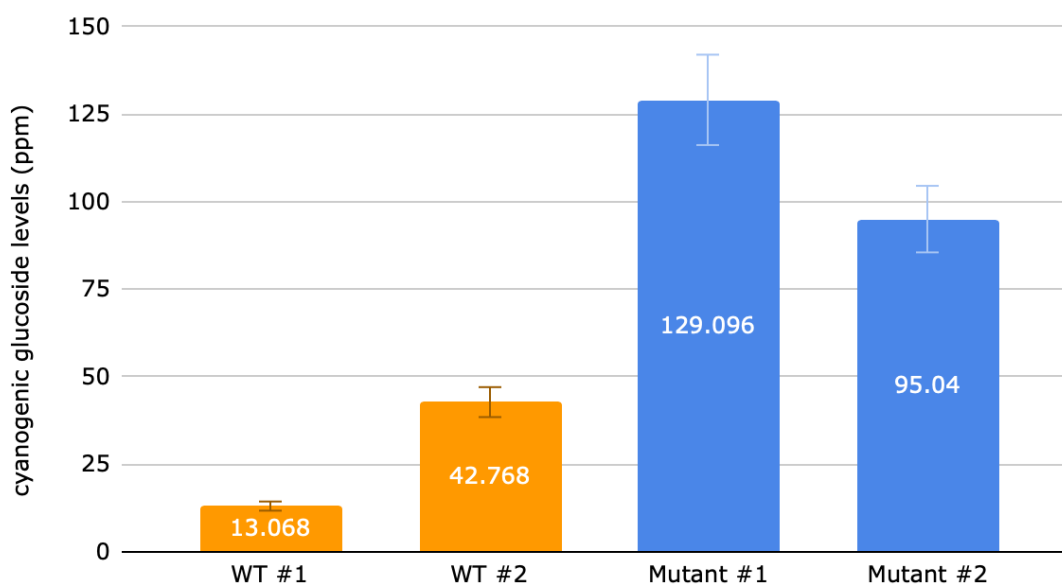

Figure 4. **Cyanogenic glucoside content (ppm) in leaf tissue.** Wild-type, tissue culture control plants are represented in orange (n = 2). Mutant, transformed plants are represented in blue (n = 2). CG levels are measured in the leaf tissue for each plant type.

Figure 5. **Phenotype of Mutant and Wild-type plants.** (A) Wild-type plants are labeled in blue. Mutant plants are labeled in purple. (B) From left to right: wild-type plant #2, mutant plant #1, and mutant plant #2.

DISCUSSION

The toxicity associated with cyanogenic glucosides in cassava is a major constraint to the increased reliance on this crop for combating global food insecurity. Cassava exhibits great robustness and resilience even in unfavorable environmental conditions. Thus, in the face of future global warming challenges, cassava may emerge as an integral component in expanding food production. However, we must first overcome the problem of its high toxicity to prevent health issues related to consumption such as cyanide poisoning and konzo disease. A potential solution is the CRISPR-Cas9 system, a powerful tool for plant genome editing and rapid-crop improvement. In this study, I found a significant positive relationship between cyanogenic glucoside content and crop yield. This further supports the existing notion that cyanogenic glucosides play an important physiological role in maintaining the fitness of plants. I also used CRISPR-Cas9 to target a cyanogenic glucoside transporter gene, *Me15G180400*, and eliminate its function. This approach enabled me to control tissue-specific cyanogenic glucoside accumulation and achieve increased cyanogenic glucoside levels in cassava leaf tissue, and likely reduced levels

in root tissue. As a whole, this research provides a mechanism to the creation of safer cassava varieties that may serve as valuable sources of sustenance and substantially benefit the world's increasing population.

The role of CGs in plant fitness

I found a strong positive relationship between cyanogenic glucoside levels and crop yield, suggesting that cassava plants benefit from the tradeoff between the amount of CGs present in the plant in relation to yield. Higher crop yield serves as an effective indicator of plant fitness thus, this result further substantiates the hypothesis that CGs play a critical role in herbivory defense and mediating environmental stress to maintain plant fitness. While the majority of the data exhibited this trend, two study sites did not. Malam Maduri in 1997 revealed the reverse trend, with low and medium cyanogenic glucoside levels corresponding to higher yield, and Malam Maduri in 2000 exhibited no significant correlation between cyanogenic glucoside content and yield. These outlying observations may be explained by the impact of different environmental conditions at the specified time and location. Future work could analyze precipitation data in Malam Maduri during 1997 and 2000 for example, to offer an explanation and establish any connection between rainfall, CG levels, and yield.

As a whole, my work provides additional evidence for the existing body of literature that demonstrates the necessity of CGs for improved plant physiology. A multitude of sources cite the advantages of cyanogenic glucosides in cassava to improve plant plasticity including establishment, robustness, and viability in response to environmental change (Gleadow and Moller 2014). Similar trade-offs arise in other plant species as well. Sorghum, lima bean, white clover, and eucalyptus have all exhibited an increase in cyanogenic glucosides concentrations during periods of drought and in response to herbivory attack (Gleadow and Moller 2014). Therefore, my approach illustrates how CRISPR-Cas9 may be leveraged to over-accumulate cyanogenic glucosides in cassava source tissue, where they are beneficial to the plant, and conceivably reduce cyanogenic glucosides in root tissue, where they are toxic to humans. Such an experimental design is unlikely to negatively affect plant fitness as high CG levels in leaves will continue to provide protection for the plant and maintain positively associated traits like yield. Consequently, editing the cassava plant genome in this way may create plants that are safer for human consumption and

as a result, aid in improving food security and benefit populations in countries that are dependent on the cassava crop for sustenance.

Implications of altered cyanogenic glucoside content

I found that cyanogenic glucoside content in leaves of edited plants was greater than wild-type levels. These results suggest that CRISPR-Cas9 editing of the primary transporter gene holds potential for engineering tissue-specific cyanogenic glucoside accumulation in cassava. Corresponding studies in *Arabidopsis thaliana* have yielded similar results. By using CRISPR-Cas9 to knock out a glucosinolate transporter, researchers were able to eliminate glucosinolates in seeds, while over-accumulating glucosinolates in leaves of arabidopsis (Eldin et al. 2012). Taken together, our studies provide evidence that such a targeted approach may be applied in many different species to engineer varying levels of cyanogenic glucosides. Moreover, this technique can likely be used to manipulate other pathways to control the allocation of defense compounds in a tissue-specific manner. For example, hydroxynitrile lyase (HNL) is an enzyme that catalyzes the conversion of acetone cyanohydrin to cyanide and acetone. Previous research has found that acetone cyanohydrin accumulates in cassava roots in the absence of HNL (White et al. 1998). By employing a similar method to the one described here but overexpressing HNL, rather than knocking out its function, CG content may be reduced via the reduction of acetone cyanohydrin (Bechoff et al. 2018). This pathway may serve as an alternative approach to reducing toxicity in cassava.

Cyanogenic glucoside content in wild-type plants was comparable to that found in the literature. Australia and New Zealand's food standards agency found that for bitter cassava varieties, cyanogen levels range from 15-400 ppm of hydrocyanic acid in roots on a fresh weight basis. Sweet cassava varieties typically contain only 15-50 ppm, consistent with the fact that bitter cassava varieties have higher cyanogenic glucoside levels than sweet varieties. Cassava leaves contain about ~10% more cyanide than roots. The average cyanogen levels I measured in bitter wild-type leaves were 27.92 ppm. Additionally, cyanogenic glucoside levels are generally higher in younger plants (Gleadow & Moller et al. 2014). I measured CG levels during early plant growth stages, which may have had an impact on my reported results. It is possible that final CG content will be lower once the plant has fully developed.

Australia and New Zealand's food standards agency also identified a correlation between higher CG ppm values, periods of drought, and higher yields. This is further supported by farmers who tend to prefer bitter varieties as they are less susceptible to pests, disease, and theft (Bechoff et al. 2018). Furthermore, a study investigating cyanogenic potential in cassava and its influence on a generalist insect herbivore *Cyrtomenus bergi* revealed that plants with reduced cyanogenic potential are more susceptible to attack by *C. bergi* (Riis et al. 2003). The crucial role of CGs in plant defense and environmental stress response necessitates that some baseline level is present to maintain plant fitness. Thus, by decreasing cyanogenic glucoside levels in roots while accumulating them in leaves, my approach allowed for the creation of plants that are safer for human consumption, without potentially compromising plant fitness.

Limitations and future directions

While the outcomes of this study prove significant, there are limitations. This study used a small sample size of mutant plants due to low selection and a high escape rate of the t-DNA insertions. Future studies can implement a longer, stronger selection protocol to generate a greater percentage of edited plants. Another limitation was time. Transformation of the cassava plants took 10 months, in addition to the several months necessary for the plantlets to grow and develop before I was able to measure and determine CG content in plant leaves. The roots require an additional growth period of 2-3 months before I may be able to determine CG content. Thus, next steps include measuring CG levels of the wild-type and mutant roots once they are fully developed. Future work should refine a more thorough and efficient transformation procedure that would decrease time required for transformation and allow for faster generation of mutant plants.

Additionally, there are several ways the results of this study could be expanded on through further research to promote a deeper understanding of cyanogenic glucosides in cassava. A crucial next step in this research is to conduct field testing and experiments of the edited lines to determine their success in real-world conditions. If these lines prove effective in the field, they may be distributed to farmers for large-scale use in cassava production. In addition, more data analyses may be run to further validate the role of CGs in plant fitness. For example, the correlations between CGs and drought tolerance, nitrogen allocation, and pathogen resistance should be assessed to demonstrate the value of cyanogenic glucosides. Moreover, running experimental

assays for covariates (e.g. drought tolerance, pest resistance, etc.) using plants with varying CG levels will contribute supplemental data that further supports CGs function as an herbivory defense mechanism and mediator of environmental stress.

Lastly, more research into the feedback system of CG biosynthesis and transporter genes may uncover potential sensing mechanisms for reduced cyanogenic glucoside levels and reveal additional knowledge about the specificities of transporters. It is possible for compensatory upregulation of the biosynthetic gene or an increase in root CG biosynthesis and alternative CG transporter functionality to occur, which increase CG production and transport to counteract the reduced levels due to elimination of the primary CG transporter function. The primary *Me15G180400* gene is a cyanogenic glucoside specific transporter that is strongly expressed in vascular tissue (Wilson et al. 2017), a positive indication that this gene is indeed facilitating transport of CGs to roots via the phloem. The *Me15G176100* and *Me14G074000* genes however, are also able to uptake and transport some degree of cyanogenic glucosides (Jørgensen et al. 2017). Thus, further studies quantifying the biosynthesis and alternative transporter genes' expression levels in both wild-type and mutant plants via qPCR should be conducted to determine whether compensatory upregulation is occurring. Continuing to explore and expand our understanding of cyanogenic glucosides in cassava will permit us to more accurately manipulate the cassava genome to meet the world's agricultural needs.

Broader implications

Through the genetic modifications described in this study, we seek to alleviate concerns surrounding food safety and the health of consumers in connection to cassava cyanogenic glucoside toxicity. Leveraging current gene-editing strategies provides an attractive means to engineering tissue-specific cyanogenic glucoside accumulation in cassava; thereby precluding the incidence of deleterious health effects related to consumption in humans, while preserving important herbivory defense and physiological fitness mechanisms of the plant. More fundamentally, this work allows us to gain a better understanding of the cassava plant's physiology and gives insight into the feedback occurring between CG biosynthesis and transporter genes. Implementation of these edited lines in the agricultural sector will require further research to ensure their viability and success, as well as the design of an equitable distribution policy among

populations. Furthermore, global commercialization of genome-edited products may face regulatory challenges and the stringency of these regulatory approaches greatly varies from country to country (Tripathi et al. 2020). So, while genome-editing has shown immense potential for crop improvement, there are still hurdles that must be overcome before we achieve widespread application. Overall, my approach presents an avenue to greatly enhancing the cassava crop to meet worldwide requirements for food, feed, and biofuel in a time of imminent environmental change and adaptation.

ACKNOWLEDGMENTS

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