GENERATION OF CASSAVA WITH REDUCED LEVELS OF CYANOGENS THROUGH CRISPR/Cas9 TARGETED MUTAGENESIS OF THE CYTOCHROME P450 (CYP79D1) GENE

BICKO STEVE JUMA

MASTERS OF SCIENCE

(Biotechnology)

JOMO KENYATTA UNIVERSITY

OF

AGRICULTURE AND TECHNOLOGY

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Generation of Cassava with Reduced Levels of Cyanogens Through CRISPR/Cas9 Targeted Mutagenesis of the Cytochrome P450 (CYP79D1) Gene

Bicko Steve Juma

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature...Date…………………………………………

Bicko Steve Juma

This thesis has been submitted for examination with our approval as the university supervisors

Signature…………………………………Date……………………………………...

Dr. Cecilia Mweu, PhD

JKUAT, Kenya

Signature…………………………………Date………………………………………

Dr. Wilton Mwema Mbinda, PhD

Pwani University, Kenya

Signature…………………………………Date………………………………………

Dr. Mathew Piero, PhD

Kenyatta University, Kenya

DEDICATION

To my loving aunt, Norah Atieno Owino. “All that I am and all that I shall be, I owe it to my prayerful aunt.”

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LIST OF ACRONYMS AND ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
GA_3	Gibberellic acid
NAA	Naphthaleneacetic acid
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas9	CRISPR associated protein
gRNA	guide RNA
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription-polymerase chain reaction
Fwt	Fresh weight
bp	base-pair
PAM	protospacer adjacent motif
NHEJ	non-homologous end-joining
HDR	homology directed repair
DSBs	double-stranded breaks
INDELs	insertions and deletions

ABSTRACT

Cassava (*Manihot esculenta* Crantz) is the world's most significant food root crop, providing carbohydrates to millions of subsistence farmers in Sub-Saharan Africa. Cassava leaves and roots, on the other hand, contain toxic quantities of cyanogenic glycosides. Cyanide poisoning is produced by the body's conversion of leftover cyanogens to cyanide. Inadequately processed cassava consumption, in combination with protein-deficient diets, has been associated to neurological problems. Acyanogenic cassava cultivars are essential to make cassava a consistently safe and acceptable food and commercial crop. Traditional breeding efforts have previously failed due to the crop's extended life cycle, high heterozygozity of allopolyploid plants, lack of flowering and variable seed germination in some varieties, inbreeding depression, and time-consuming and labor-intensive nature. Modern biotechnology techniques are critical for overcoming these constraints and generating more nutritional, pest-resistant, economically viable, and safe cassava varieties. In recent years, sequence-directed nucleases have emerged as the most successful strategy for crop improvement via gene-specific genome editing. The CRISPR/Cas system, which uses sequence-specific nucleases to transmit genetic modifications into crops for improvement, is the most effective and successful genome-editing tool. To establish CRISPR/Cas9 capabilities in cassava, the cytochrome P450 gene (CYP79D1) was targeted in the TMS 60444 cultivar using a construct incorporating gRNA targeting a site inside MeCYP79D1 exon 3. Agrobacterium tumefaciens strain GV3101 was utilized to infect immature cassava leaf lobes with the CRISPR/Cas9 construct. A PCR assay was utilized to confirm the incorporation of the gRNA/Cas9 into the cassava seedlings. PCR amplicons from putative transgenic cassava were sequenced to detect areas with edits. RT-PCR was used to examine the amounts of Cas9-mRNA expression in transgenic cassava lines. A picrate test and high-performance liquid chromatography (HPLC) were employed to assess the cyanide levels in transgenic cassava. To analyze the agro-morphological properties of the transgenic cassava, plant height, leaf length, leaf breadth, number of leaves per plant, and stalk length of wild type and transgenic TMS 60444 cassava plantlets were assessed in the greenhouse. The pCRISPR/Cas9-MeCYP79D1 design triggered the deletion in cotyledon-stage somatic embryos regenerating on hygromycin selection media, and the plants regenerated after CRISPR/Cas9 reagent transformation into cassava cells via *Agrobacterium*-mediated transformation. Mutagenesis assays were performed on eight plants (1.78 percent). The Cas9 gene was expressed by all regenerated plants. The desired gene was expressed in all of the regenerated plants. A sequencing analysis demonstrated that 8/8 (100%) of the plants studied had a mutation at the MeCYP79D1 gene, with deletions and substitutions discovered. Cyanide levels in putative transgenic cassava leaves were measured and found to be up to sevenfold lower. Cassava cyanide was not totally eliminated by deleting CYP79D1. The findings show that CRISPR/Cas9-mediated cassava genome editing is both efficient and straightforward. The new study provides the framework for CYP79D1/D2 gene targeting in Kenya's farmer-favored cassava, as well as other cassava genome-editing technologies.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Cassava (*Manihot esculenta*, Crantz), a woody plant in the *Euphorbiaceae* family, is a root crop that originated in the Amazon basin of Latin America more than a century ago (Hussein, 2013), and was later distributed around the world by the Portuguese traders in the 16th century (Guira et al., 2017). It is now farmed in the vast majority (over 100) of tropical and subtropical countries, primarily for its carbohydrate-rich root tubers, and is consequently recognized as a good source of calories (Bull et al., 2018). After rice, wheat, and corn, the crop is the fourth most important calorie source in the tropics (Cruz et al., 2018). Cassava storage roots are consumed by about 800 million people every day, making it the world's sixth most abundant source of calories. Although the storage roots are the most commonly consumed, the leaves are also consumed in the majority of West African countries. The leaves are high in fiber, minerals, vitamins A and C, as well as protein (Saurabh et al., 2014). The storage roots can be dried, processed, and sold, generating income for farmers, the majority of whom are women (Tomlinson et al., 2018). The crop's leaves and tubers are utilized as animal feed in many nations worldwide (Afuape et al., 2013). Cassava storage roots are also used in the starch, ethanol, and textile sectors, and are thus regarded as vital (Neama et al., 2013).

The crop grows well on poor soils and can be harvested year-round, making it a viable crop in the face of unexpected climate change (Tomlinson et al., 2018). Cassava has become a popular crop in the tropics, particularly among African subsistence farmers struggling with drought and hunger (Jarvis et al., 2012). Planting sufficiently lignified cuttings in soil is the most common method of vegetative propagation. Cassava output has approximately tripled over the last four decades, and it is predicted to reach 300 million metric tons by 2025 (FAO, 2021). More than half of all crops are grown in Africa, one-third in Asia, and 14% in Latin America. Thousands of cassava varieties can now be

distributed throughout Africa, Asia, and South America's tropics and subtropics. Small-scale farmers in the tropics generally grow cassava on poor soils. These small-scale agricultural practices limit cassava production and consumption, resulting in challenges such as variable product quality, insecure supply, and an expensive marketing structure (Andersen et al., 2000). Furthermore, the harvest labor costs for perishable storage roots are substantial since they must be treated rapidly after harvest into a shelf-stable state. A combination of these challenges, including insect and disease infestation, has hampered global cassava production. Cassava mosaic disease and cassava brown streak disease are two viral diseases that can reduce yield capacity by up to 95% of overall production (Zárate-Chaves et al., 2021). Cassava storage roots can survive in the soil for up to a year before dissolving, but their shelf life after harvesting is restricted due to post-harvest physiological deterioration (PPD) (Djabou et al., 2017). Blue/black coloration of vascular parenchyma identifies this limitation, rendering the roots unpleasant and unmarketable (Zainuddin et al., 2018).

Cassava tissues contain potentially lethal quantities of the cyanogenic glycosides linamarin (95 percent total cyanogens) and lotaustralin (5 percent total cyanogens), which when digested by their respective enzymes release hydrogen cyanide (HCN) (Li et al., 2020). Cyanogenic glucosides are used as herbivore repellents, universal insect feeders, and human thievery prevention; cyanogens were recently proposed to play a role in reduced nitrogen transfer from leaves to roots (Islamiyat et al., 2018). Other than the seeds, all cassava tissues contain cyanogenic glucosides, with storage roots containing the least (10-500 mg CN equivalents/kg dry weight in various cassava varieties) and leaves containing the most (200-1,300 mg CN equivalents/kg dry weight in various cassava varieties) (Echeverry-Solarte et al., 2013). The amounts of these cyanogenic glycosides differ between cassava varieties. Cassava cyanogenesis is a complex and variable process that is controlled by a number of genes and proteins found in various tissues, the environment, and the plant's developmental stage (Tawanda et al., 2017).

Cyanogenic glycosides are secondary metabolites produced by plants as a consequence of ethylene production. They accumulate to a dry weight equivalent of roughly 200 mg/kg to 1300 mg/kg in various cassava types (Easson et al., 2021). When enzymatically hydrolyzed, these secondary metabolites produce cyanogen. Cyanogenic glycosides are generally stored in vacuoles, away from the enzymes like hydroxynitrile lyases that they are designed to inhibit (Imakumbili et al., 2019). Linamarin, for example, is kept in vacuoles, but its glucosidase, linamarase, is held in cell walls and laticifers (Cressey and Reeve, 2019). Any event that causes the cell wall to break allows the enzymes to interact with the substrate, resulting in the release of cyanogen. Cyanide poisoning occurs when these cyanogens enter the body, and cyanide concentrations of 0.5 to 3.5 mg/kg of body weight are regarded extremely dangerous to humans (Easson et al., 2021).

Because cyanogens are present in insufficiently processed cassava food items, people who eat cassava-based diets, especially those who are nutritionally deficient (poor to no protein intake increases the risk), may experience health hazards (Anorue et al., 2021). The severity of these disorders is determined on the degree and frequency of cyanogen exposure, as well as the dietary status of the consumer. (1) One of the health risks associated with a high cyanogen-containing diet is hyperthyroidism, which is caused by thiocyanate disruption in iodine metabolism (Bumoko et al., 2014), (2) tropical ataxic neuropathy, a neurological disease (Rivadeneyra-Domínguez and Rodríguez-Land, 2020), and (3) konzo, a quick and irreversible paralysis triggered by acute cyanide poisoning, which can result in death in some circumstances (Kashala-Abotnes et al., 2019). These diseases may occur gradually or rapidly, depending on the consumer's health and nutrition, as well as the amount of cyanogen consumed (Ndubuisi and Chidiebere, 2018). Because sulphur-containing amino acids are necessary for cyanide detoxification as thiocyanates, the amount of cysteine in the meal contributes to cyanide-induced disorder (Blagbrough et al., 2010).

Cassava cyanogenic glycoside levels have been lowered utilizing a range of strategies ranging from traditional to genetic engineering. Crushing or tissue maceration, boiling, sun-drying, baking and steaming, and fermentation are the most common traditional processes (Ani et al., 2019). Linamarin is liberated from the vacuole and deglycosylated by linamarase upon tissue rupture. Boiling is inefficient for removing cyanogen due to the high temperatures (Taleon et al., 2019). The amount of cyanide lost during baking and steaming is extremely low due to processing temperatures surpassing 100 °C and linamarin's resilience to neutral or weak acids. Because temperatures are frequently below 55 °C, the ideal temperature for linamarase activity and cyanogen breakdown, sun-drying is more effective than other traditional approaches (Airaodion et al., 2019). These methods are time-consuming, may lead to the generation of hazardous food products, and do not guarantee complete elimination of cyanogenic glycosides.

In an unbroken cassava plant, cyanogenic glycosides are continuously generated via a mechanism that utilizes amino acids, valine, and/or isoleucine as precursors (Hamza et al., 2020). The process is catalyzed by the 85% identical CYP79D1/D2 proteins. They are multifunctional enzymes that convert valine (95% of the time) or isoleucine (5% of the time, a low affinity substrate) into their oximes (Tawanda et al., 2017). The oxime is converted to the appropriate nitrile by a second cytochrome P450 (CYP71E). The final step in the creation of linamarin is the addition of glucose to acetone cyanohydrin, which is accomplished by a uridine 5'-diphospho- (UDP)-glucosyl transferase (Islamiyat et al., 2018).

Cassava plant development with lower cyanogenic glycoside levels has been examined utilizing traditional breeding strategies such as cross-pollination and sexual crossover. These strategies focus on combining appealing agronomic features with low cyanogenic potential, pest and disease resistance, and other desired qualities (Zhang et al., 2017). These methods have proven ineffectual since most subsistence farmers select high-

cyanogenic cassava cultivars. Taste preference, herbivory reduction, and theft avoidance are all reasons for this option (Naveena et al., 2021). Furthermore, because nitrogen transfer from the leaves to the roots is reduced, which is a substrate for the amino acid asparagine production in the roots, low cyanogenic cassava crops do not yield considerable tubers (Cressey and Reeve, 2019). Furthermore, conventional cassava breeding is severely limited due to its long-life cycle, high allopolyploid plant heterozygozity, poor flowering and seed set, inbreeding depression, and labor- and time-intensive nature (Ceballos et al., 2004). Because conventional approaches are ineffective, alternative technologies that are precise for the modification of the genes involved for cyanogenic glycoside production must be used (Behera and Ray, 2017).

Genome editing (GE) has changed scientific research by allowing scientists to precisely alter the genomes of living creatures. In recent years, GE techniques for editing simple and complex genomes have been studied (Vats et al., 2019). Zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs), and the recently established clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) system are all examples of gene editing technologies (Kamburova et al., 2017). ZFNs are DNA-cleaving proteins that can break DNA sequences anywhere within the molecule. TALENs cause double-stranded breaks (DSBs) in target sequences, which activate the DNA damage response mechanisms and modify the genome (Khan et al., 2017). Although ZFNs and TALENs have been widely used for GE in human, animal, and plant cells (since 2002 and 2011, respectively), there are still substantial restrictions that limit their efficiency (Samanta et al., 2016). The selectivity of ZFN is limited, and it commonly produces unintended mutations. Designing vectors for ZFNs and TALENs takes time and effort (Juma et al., 2021). As a consequence, the usage of CRISPR/Cas9, and later, a range of additional CRISPR/Cas variations, has gotten a lot of interest since 2013 (Doudna and Charpentier, 2014).

In comparison to ZFNs and TALENs, CRISPR/Cas is more effective, less expensive, and successful at multiplexing genome editing than any other known genome editing method. CRISPR/Cas9 technology has lately been used in a number of genome editing research, with applications as diverse as production enhancement, drought tolerance, disease resistance, and herbicide tolerance (Odipio et al., 2017; Hummel et al., 2018; Gomez et al., 2019; Veley et al., 2021). In this study, the CYP79D1 gene is being knock out using the CRISPR/Cas9 technique. This gene produces valine monooxygenase I, an enzyme that is specifically responsible for the initial step of cyanogenesis in cassava.

1.2 Problem statement

Despite the fact that cassava has many benefits as a food, feed, and raw material, cyanide poisoning is a risk due to the existence of the lethal cyanogenic glycosides, linamarin, and lotaustralin, which are released when a plant organ is damaged. If cassava is not adequately handled after harvesting, a number of medical issues may arise. Traditional approaches for reducing the amount of cyanogenic glycosides in finished cassava products in the past fell short of completely getting rid of the cyanogenic glycoside molecules (Siritunga and Sayre, 2003).

Conventional breeding techniques have also been explored to lessen the levels of these cyanogenic glycosides, but because to their sluggish and time-consuming nature, they have not been successful. RNAi, TALENs, and ZFNs are examples of biotechnological methods that have been used in the past to modify genes, but their expensive and time-consuming engineering makes them incompatible with the production of massive genome libraries. In these setups, two different DNA binding proteins surround a sequence of interest. These make it necessary to look for alternate methods to get rid of the cyanogens in cassava, which will lessen their harmfulness and increase food safety.

1.3 Justification

Genome editing, which allows for exact genome change, has been investigated as the most adaptable and successful strategy for agricultural genetic manipulation. Crops whose genomes have been altered using CRISPR/Cas9 technology, include rice (Macovei et al., 2018), tobacco (Gao et al., 2015), maize (Doll et al., 2019), wheat (Doll et al., 2019), *Arabidopsis* (Li et al., 2018), potato (Andersson et al., 2018), sorghum (Char et al., 2020). The CRISPR/Cas9 genome technique has proven to be the most efficient and can thus be utilized to reduce cyanogen levels in cassava landraces due to its ease of cloning and precision on potential off-targets. The CRISPR/Cas9 method was being used in this study to knock out the CYP79D1 gene in the model cassava genotype, TMS 60444, which encodes valine monooxygenase I.

1.4 Objectives of the study

1.4.1 General objective

To generate TMS 60444 cassava plantlets with reduced levels of cyanogenic glycosides through CRISPR/Cas9 controlled mutagenesis of the cytochrome P450 (CYP79D1) gene

1.4.2 Specific objectives

i. To develop TMS 60444 cassava plantlets harboring CRISPR/Cas9 constructs targeting CYP79D1 gene through *Agrobacterium*-mediated transformation
ii. To determine the expression levels of Cas9 mRNA in acyanogenic TMS 60444 cassava
iii. To evaluate the cyanide content in the transgenic TMS 60444 cassava genotype

1.5 Null hypotheses

i. It is not possible to develop cassava plantlets harboring CRISPR/Cas9 constructs targeting the cytochrome P450 (CYP79D1) gene through *Agrobacterium*-mediated transformation

ii. Cas9 mRNA cannot be differentially expressed in transgenic TMS 60444 cassava cultivars

iii. It is not possible to evaluate the cyanogens' content in transgenic TMS 60444 cassava plants

CHAPTER TWO

LITERATURE REVIEW

2.1 Biology and origin of cassava

Cassava (*Manihot esculenta*, Crantz) is a perennial herbaceous plant with palmate leaves and five to nine leaf lobes that can grow up to five meters tall. The crop is grown for its starch-containing roots, which are normally harvested six to nine months after planting, however it may take up to a year in some cases (Hussein, 2013). Cassava was first domesticated around 10,000 years ago in South American countries around the southern border of the Amazon Basin, with the first to be domesticated being *M. esculenta* subspecies *flabellifolia* and it was later introduced to other areas of the world by Europeans (Timaboot and Suthikarnnarunai, 2017). Cassava was later introduced to Africa in the 16th century by Portuguese traders from Brazil and farmed as a low-lying reserve crop (Jiang et al., 2019). It was introduced as a staple meal in the Democratic Republic of the Congo, where millet, banana, and yam were traditional staple meals, and was chosen because it provided a more reliable alternative supply of food during droughts, locust outbreaks, and famine seasons (Eni et al., 2021). Africa currently accounts for 35% of global cassava production (Balagopalan et al., 2017). Cassava is grown in over 40 African countries, with Nigeria, Tanzania, and Congo accounting for more than 70% of total output (Cock, 2017). Cassava is Kenya's second-most significant food root crop, growing on approximately 90,000 acres for an annual yield of roughly 540,000 tons. Despite its enormous potential, production is primarily concentrated in the coastal and western regions, with usage primarily limited to human consumption.

Cassava, as a drought-tolerant crop, has the potential to thrive in low-nutrient soils where other cereals cannot (de Oliveira et al., 2017). It can be grown in sandy soils and tolerates low *pH* (Zhu et al., 2020). Cassava yields provide a low-cost source of calories to roughly one billion people worldwide, particularly in third-world countries where chronic food scarcity and malnutrition are widespread. Roots (starch) can live in soil for 1-3 years before disintegrating (Bayata, 2019). In different West African locations, not just the roots

but also the leaves are high in vitamins, minerals, and proteins (Tao et al., 2019). Cassava tubers can be preserved in the field for multiple seasons as a back-up food source if other crops fail (Mapayi et al., 2013). It is also commonly utilized as animal feed and in the production of a variety of industrial goods (Bernard et al., 2020).

2.2 Economic importance of cassava (no information about Kenya)

2.2.1 Cassava as food

The starch content of cassava root makes up about 80% of its dry weight (Wongnoi et al., 2020). In Sub-Saharan Africa, cassava tubers make up nearly two-thirds of all direct human calorie consumption, whereas rice, wheat, and maize make up about one-third of all calorie consumption (Cock and Connor, 2021). It is a staple meal for nearly one billion people in 105 countries (Dada, 2016) and it is the fourth top most important carbohydrate-rich crops in the developing world (together with rice, sugarcane, and maize) (Manjunath et al., 2015).

Most regions of Kenya cultivate cassava primarily as a subsistence crop. The coastal, central, and western parts of the nation are where most farming is concentrated. It is the second-most significant edible root crop, behind the irish potato. The data that is currently available indicates a moderate but continuous rise in cassava output for food consumption. The two western and coastal regions are the principal cassava-growing areas among the three, contributing more than 80% of the nation's documented cassava output.

After being peeled, cassava can be eaten raw or utilized in a variety of culinary dishes. On the other hand, only cultivated cassavas low in cyanogenic glycosides are consumed raw. Before eating, cultivars with high levels of cyanogenic glycosides are treated and cooked. For many nations and areas, different initial procedures are required. Granules, flour, and chips are often produced using boiling, mashing, frying, and drying techniques (Ikuemonisan and Akinbola, 2021).

In some regions where cassava is grown, the leaves are eaten as a vegetable or as a sauce addition to the primary staple, which may or may not be cassava (Laya et al., 2020). Unlike the roots, cassava leaves are rich in minerals, riboflavin, ascorbic acid, methionine, and lysine in significant amounts (Udoh et al., 2017). As a result, in tropical areas, the leaves could be used as a supplement to disadvantaged people's starchy diets (Zoué et al., 2018).

2.2.2 Cassava as animal feed

Cassava, After peeled, it can be consumed raw or used to make a range of gourmet dishes. Cassava cultivars with low levels of cyanogenic glycosides, on the other hand, are consumed raw. Cultivars with high levels of cyanogenic glycosides are treated and boiled before consumption. Different beginning procedures are required for various locations and nations. Boiling, mashing, frying, and drying are common steps in the production of granules, flour, and chips (Trakulvichean et al., 2017). Cassava is crucial for animal nutrition since feed doesn't provide enough soluble carbohydrate energy. The shortage is severe in the tropics because feed crops are bulkier, coarser, and more unattractive than in temperate regions (Izah et al., 2017).

Cassava is a valuable carbohydrate source for animal diets because to its high amylase level. Fresh roots can be fed to the animals, but they should be boiled beforehand to lessen the risk of cyanide poisoning (Otache et al., 2017). According to Mathur and Koncz, (2009) replacing cereals with cassava by 50–100% has no effect on the amount and quality of milk produced by dairy cows. The roots, on the other hand, have a low protein content (0.7-1.3 percent fresh weight), which is offset by the use of protein supplements such as soya or microbiological methods (Otache et al., 2017). In many underdeveloped nations, especially during the dry season when alternative feed supplies are scarce, cassava leaves are utilized as animal feed (Rochana et al., 2018).

2.2.3 Industrial use of cassava

For industrial purposes, such as the manufacturing of sugars, acids, alcohols, and biodegradable polymers, cassava is primarily farmed in Asian nations. The starch from cassava has the ability to compete with maize, wheat, rice, and potato in the industrial sector. This is because cassava starch has distinct properties that make it suitable for a variety of applications in the food, textile, and paper industries. Previously, flour and starch from other crops had a monopoly in these sectors (Edhirej et al., 2017). Since cassava starch contains a lot of amylopectin, it makes pastes that are clear, don't gel, and have a low tendency to regress. Since cassava starch has the lowest gelatinization temperature when compared to maize, wheat, and potato starch, processing it uses less energy (Edhirej et al., 2017).

According to extensive research carried out by IITA and CIAT in the 1980s and 1990s, cassava flour can be used as a partial replacement for wheat in bread (up to 10-15%), biscuits, and snacks (40-100 percent). Food sweeteners include fructose syrup and fructose crystals made from cassava starch (Chisenga et al., 2019). In addition, cassava flour is used as a thickener, filler, binder, stabilizer, and texture enhancer in sauces, fast desserts, sausages, and processed meat. Because it contains little protein and fat, cassava flour has a neutral flavor and a white hue (Awoyale et al., 2016). Cassava starch is frequently used to make paper in Asian nations due to its inexpensive cost. Cassava is valued for its bright white color, low amount of dirt and fiber, and homogeneous lot size. Cassava starch is used in the textile industry for printing, finishing, and sizing. When cassava starch is saccharified, simple sugars are created. Sugar alcohols including sorbitol, mannitol, and maltol are used in a variety of commercial and therapeutic processes (Zhang et al., 2016). Dextrins, which are derivatives of starch, are found in a variety of products, including envelope gum, bottle labeling glue, postage stamp adhesive, cardboard boxes, and photographic mounting materials (Awoyale et al., 2017).

2.3 Production of cassava

Cassava is an energy source for over 250 million Africans, largely in Sub-Saharan Africa, and is mostly grown in tropical and subtropical nations (Mbanjo et al., 2021). In many African countries, it is renowned as the poor man's food because it can tolerate harsh climatic conditions such as high temperatures and infertile soils (Agre et al., 2017). Cassava farming has become popular in Africa, particularly in countries with rapidly rising populations, such as Malawi, where maize farming has been phased out as the primary food crop (Kante et al., 2020), this could be due to a combination of diminishing soil productivity and climate change. Cassava output has increased in recent years, owing to improved breeding research and agronomical approaches in the field of plant biotechnology (Parmar et al., 2017).

In Kenya, after irish potatoes, cassava is the second-most significant food root crop. It is mostly farmed in the western, coastal, and eastern portions of the nation (especially in arid and semi-arid areas) (Kidasi et al., 2021). It is mostly farmed in the regions for food and nutrition security, and any extra root tubers are sold to make money for the farm households. Compared to maize and wheat, the nation's staple crops, the production per unit area is great (FAOSTAT, 2018). Only the western and coastal regions yield more cassava than 80% of total cassava production in the country. Nonetheless, cassava is becoming a more important food and revenue crop in central Kenya. Kenya's western, coastal, and central regions produce 60%, 30%, and 10% of the country's total output, respectively. Small-scale, underprivileged farmers use the crop for subsistence, and it is recognized as a crucial crop for food security. In Kenya, the majority of growing regions only allow for the roasting and boiling of fresh roots for ingestion. Although it is thought of as a crop for food security, Kenya produces less cassava than it does maize, beans, and sorghum.

Cassava is used to make flour, feed, alcohol, starches for sizing paper and textiles, sweeteners, ready-to-eat foods, and biodegradable vegetables, among other things

(Ikuemonisan et al., 2020). These products are produced from a range of cassava sources, including fresh leaves and roots, as well as modified cassava starch. As one goes from the fresh form to the modified starch form, the degree of processing and technological circumstances tends to rise (Otekunrin and Sawicka, 2019).

2.4 Constraints to cassava production

2.4.1 Biotic stress

A range of biotic stressors limit cassava production and usage, resulting in productivity losses of up to 90% or crop failure (Campo et al., 2011). Cassava mosaic virus illness is the most significant biotic factor in cassava productivity (Zárate-Chaves et al., 2021) and cassava brown streak virus disease (Yadav et al., 2011); others include green mite, (Daniel et al., 2015), cassava mealy bug and cassava bacterial blight (Banito et al., 2010). Cassava mosaic disease (CMD) is a severe cassava disease, but diagnosing it is difficult (Banito et al., 2010). Cassava mosaic disease is transmitted by whitefly-transmitted begomoviruses, of which numerous species have been detected in African cassava-growing regions (Uke et al., 2021; Bull et al., 2009). Cassava Mosaic Disease causes yellow-green mosaics on the leaves, as well as leaf deformation, delayed growth, and decreased root size (Legg et al., 2011; Chikoti et al., 2020). Whiteflies also carry the Cassava Brown Streak Virus (CBSV), a serious disease that reduces root production and quality (Pennisi, 2010; Okoya et al., 2016). Cassava bacterial blight (CBB), caused by the bacterium *Xanothomonas axonopodis* pv. *Manihotis*, is another biotic constraint to cassava productivity (Xam). The bacterium is found in all cassava-growing locations. Cassava Bacterial Blight is projected to damage yields by 50% to 75%, depending on the climate (Ferris et al., 2020).

2.4.2 Abiotic stress

Cassava is a long-season crop that thrives in a wide range of Agro-ecological zones. This resilience makes it susceptible to abiotic stressors and contributes to its low average yield (Ou et al., 2018). Drought-prone areas, low fertility soils, and alkaline or acidic soils are the most common areas where the crop is grown (Ding et al., 2017). Despite the fact that

cassava is a long-season crop that can grow in a variety of Agro-ecological zones, this is a significant constraint on cassava productivity (Wu et al., 2018). Drought and salinity are common in many areas, and by 2050, it is expected that more than half of all arable lands will be extensively salinized (Ashraf and Wu, 2011). Water scarcity, in particular, is projected to remain a major abiotic factor influencing global crop yield. Salinity affects around 20% of the world's farmed area, resulting in a 50% decrease in agricultural productivity (Cruz et al., 2018).

Another distinct and significant impediment to cassava production, consumption, and commercialization is the limited shelf-life of cassava tubers due to rapid post-harvest physiological deterioration (Sánchez et al., 2006). This physiological and biochemical process begins 24 to 72 hours after harvest and is distinguished by an initial blue/black staining of the vascular parenchyma induced by reactive oxygen species oxidizing phenolic compounds (Djabou et al., 2017). The roots become unmarketable and unpleasant when the storage parenchyma discolors (Zainuddin et al., 2018). Crop losses, root quality, economic costs, marketability, consumer availability, and commercial procedures are all affected by postharvest physiological deterioration (Djabou et al., 2017). These postharvest problems can also be solved using the modern biotechnology techniques such as CRISPR system.

Cassava crops are also subjected to dangerous concentrations of cyanogenic glycosides, which are secondary plant metabolites. Linamarin and lotaustralin are two of the most frequent cyanogenic glycosides. When these cyanogenic glycosides are destroyed in the presence of their associated enzymes, cyanide poisoning occurs. The poisoning has been linked to a variety of problems and illnesses, ranging from mild cognitive impairment to konzo, a disease marked by rapid and irreversible leg paralysis.

2.4.3 Cyanogenic glycosides

Cassava tissues contain the cyanogenic glycosides linamarin (>90% total cyanogen) and lotaustralin (>10% total cyanogen), which permit the plant to produce hydrogen cyanide (HCN), through a process known as cyanogenesis (Ndubuisi and Chidiebere, 2018). Cyanogenic glucosides, which are routinely utilized to protect plants from herbivores, have recently been demonstrated to act as a translocable form of reduced nitrogen. Except for the seeds, all cassava tissue includes cyanogenic glucosides, with the leaves containing the most (5 g linamarin/kg fresh weight) and the roots containing the least (20-fold) (Jorgensen et al., 2011).

Cassava cyanogenesis is a complicated and dynamic process driven by the participation of several genes and proteins in various tissues, the environment, and the plant's developmental stage (Panghal et al., 2021). The first phase is the cyanogenic glycoside pathway, which involves the production of linamarin, from which two distinct pathways can emerge depending on whether the plant is wounded (Panter, 2018). During the production of cyanogenic glucosides, the enzymes CYP79D1/D2, CYP71E1, and UDP-glucosyl transferase convert valine to linamarin (or lotaustralin, whose precursor is isoleucine) (Pandey, 2019). Linamarin is maintained in the vacuole and does not interact with cell wall-bound enzymes linamarase and hydroxynitrile lyase (HNL), which are essential in the breakdown process, unless the integrity of the cell is disrupted, which is usually induced by a herbivore (Schmidt et al., 2018). Linamarase can then deglucosylate linamarin to generate acetone cyanohydrin, which HNL degrades to provide acetone and hydrogen cyanide. At pH more than 5.0 and temperatures greater than 35°C, HCN can be produced from acetone cyanohydrins (Echeverry-Solarte et al., 2013). Linamarin is primarily produced in plant leaves before being transported to the roots. As a result of the cell wall location of linamarin metabolizing enzymes, an apoplastic method of linamarin transportation has been postulated (Tawanda et al., 2017). Linustatin, a glycosylated version of linamarin found in other cyanogenic plants, is thought to be the translocable form (Cressey and Reeve, 2019).

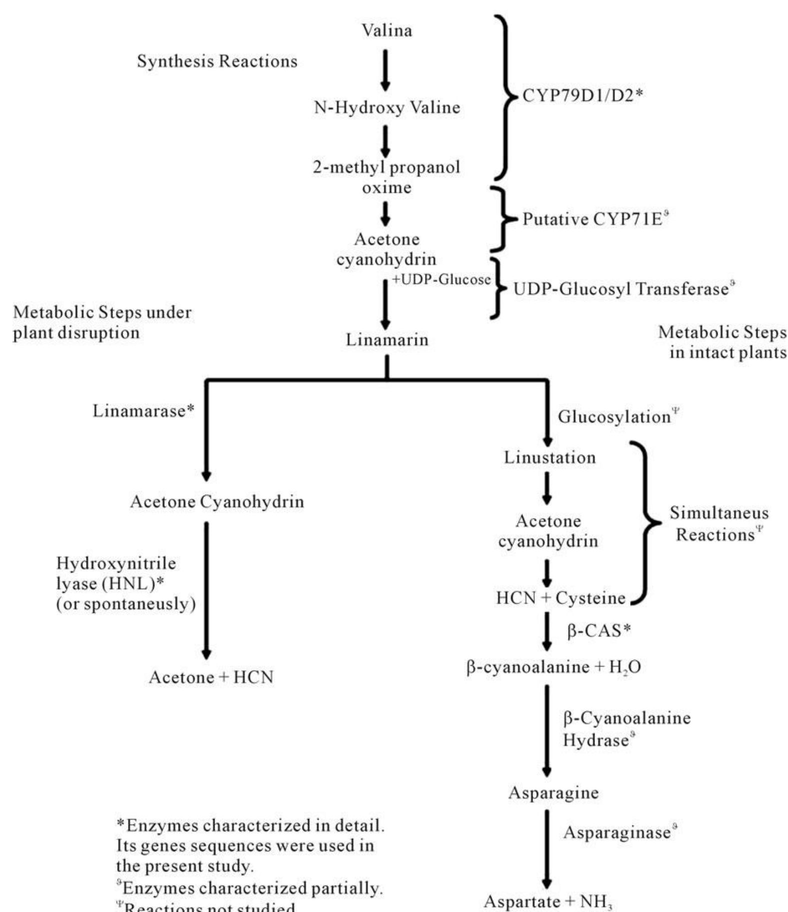

Figure 2.1: Cyanogenic glycoside metabolic pathways. Three main sets of reaction are illustrated: 1) the synthesis; 2) the metabolic steps under plant disruption and; 3) the metabolic steps in intact plants. (Echeverry-Solarte et al., 2013).

2.4.3.1 CYP79D1

Valine monooxygenase I gene is a cytochrome P450 (heme-thiolate) protein, consisting of 542 amino acids evidenced at protein level. The gene is involved in the manufacture of the cyanogenic glycosides linamarin and lotaustralin in cassava, catalyzing two consecutive hydroxylations, a crucial step in the biosynthesis of cyanogenic glycosides. It can work with either L-valine or L-leucine as a substrate (Andersen et al., 2000). These cyanogenic glycosides are often synthesized at the top of the plant and then delivered basipetally to the tubers (Jørgensen et al., 2005).

2.5 Conventional methods of cassava regeneration

Conventional breeding is still the most prevalent approach for cassava varietal creation worldwide, and it has had a good impact on the restrictions of cassava producers. However, conventional breeding for crop trait enhancement has historically been challenging, owing in part to heterozygous genetics, low seed yield, and seeds with erratic germination. Some do not flower at all, while others require specific circumstances to bloom (Ogero et al., 2012).

Cassava is propagated clonally from lignified stem cuttings. The plant begins to lignify once it has been seasoned, which takes about 5-8 months (Kidulile et al., 2018). A single plant can produce up to four hundred 50 cm long cuttings in a single year. Cuttings are frequently associated with viral diseases such as African mosaic disease and cassava brown streak disease, which result in damaged plants and lower agricultural yields. Cuttings have several drawbacks, including their high perishability (they dry up in a matter of days), high storage and transportation expenses, and material bulk (Legg et al., 2015).

2.6 Genetic engineering of cassava

Cassava genetic engineering took longer than other plants, and the cassava plant regeneration mechanism, based on somatic embryogenesis, friable embryo calli, and organogenesis of somatic embryo cotyledons, was not fully developed until the 1990s (Liu et al., 2011). *Agrobacterium*-mediated gene transfer and particle bombardment are the most common techniques for cassava genetic transformation. *Agrobacterium*-mediated transformation in cassava has been utilized to help mutate the CYP79D1/D2 gene, which is important for cyanogenesis (Siritunga and Sayre, 2003; Jørgensen et al., 2005).

Gene editing, which involves the addition, deletion, or substitution of DNA at a specific site in the genome of an organism or cell, can be accomplished with molecular scissors. There are two types of molecular scissors: designed nucleases and adeno-associated

recombinant virus (rAAV) (Juma et al., 2021; Pramanik et al., 2021). The designed nucleases function by altering genes to introduce double strand breaks (DSBs) where they are intended. This triggers the cell's own repair machinery, allowing for the formation of changes. Non-homologous end-joining (NHEJ) and homologous-directed repair (HR) can both accomplish this; however, NHEJ is prone to errors, leading in the implementation of INDELs. The most often used engineered nucleases are TALENs, ZFNs, and CRISPR (Nambiar et al., 2019).

Adeno-associated recombinant virus uses the homologous-directed repair mechanism to move donor DNA to cell nuclei and does not require any cleavage events, resulting in a reduced risk of non-target alterations. TALENs and ZFNs, for example, use DNA binding motifs to lead the same non-specific nucleases to break the genome at a specific location, with each domain recognizing a single nucleotide (Pattanayak et al., 2014; Pramanik et al., 2021). CRISPR/Cas9, an upgrade over previously used genome editing technologies, has gained application in the field of genome editing in plants due to its ease of use, efficiency, and ease of multiplexing (Juma et al., 2021).

Although genome alteration methods such as ZFNs and TALENs have been used to generate genome changes, the time and money required to build the system make them unsuitable for producing large-scale genome libraries (Heintze et al., 2013; Knott and Doudna, 2018). Two distinct DNA binding proteins flanked by interest sequences are used in such systems, each containing a C-terminal FokI nuclease module (Nekrasov et al., 2013). The designed nucleases function by altering genes to introduce double strand breaks (DSBs) where they are intended. This triggers the cell's own repair machinery, allowing for the formation of changes. Non-homologous end-joining (NHEJ) and homologous-directed repair (HR) can both accomplish this; however, NHEJ is prone to errors, leading in the implementation of INDELs. The most often used engineered nucleases are TALENs, ZFNs, and CRISPR (Mackelprang and Lemaux, 2020; Nekrasov et al., 2013). CRISPR/Cas9 technology has been applied in a variety of monocot and dicot

crop forms. Although CRISPR/Cas9 is not as advanced in terms of design and execution as other nuclease-based genome editing approaches, optimization entails considering DNA transport and tissue regeneration protocols for a specific species in order to attain precision and efficiency (Char et al., 2017; Kim and Kim, 2019).

2.6.1 CRISPR/Cas9 system

CRISPR/Cas was discovered in prokaryotes in 1987 while researching the *iap* enzyme responsible for alkaline phosphate isozyme conversion in *Escherichia coli* (Ishino et al., 2018). Other improvements in the CRISPR system have been reported since then, including the identification of the CRISPR-associated protein9 (Cas9) genes, which produce the Cas proteins (Jansen et al., 2002). Jennifer Duodna and Emmanuelle Charpentier, two scientists, created the CRISPR/Cas9 technology in 2013, revealing that CRISPR may be used to edit human genomes outside of the body (Jinek et al., 2012; Doudna and Charpentier, 2014).

When compared to ZFNs and TALENs, the CRISPR/Cas method for genome editing is faster, cheaper, and more effective (Wang et al., 2018). Because of these benefits, the adoption of this procedure is fast increasing (Knott and Doudna, 2018). Between the time of discovery and now, several CRISPR-associated gene cluster signatures have been revealed to be conserved and commonly next to repeat components, establishing the groundwork for the classification of CRISPR/Cas into two basic classes based on effector module design principles: There are two varieties: Part I and Part II (Makarova et al., 2015). These classes are further subdivided into six types, with type II being the most widely utilized in the class I CRISPR/Cas system (Rouillon et al., 2013). Much of the study is focused on the type II CRISPR/Cas9 system because it has significantly reduced the amount of Cas proteins (Yin et al., 2015).

CRISPR/Cas9 technology has rapidly become the most widely used gene editing tool (Zhang et al., 2019). This method operates in three stages: first, the spacer is acquired,

then the crRNA is processed, and last, the interference and targets are degraded (Juma et al., 2021). Cas9 and two non-coding CRISPR RNAs (tracrRNA and precursor CRISPR RNA) are the three main components of the CRISPR/Cas9 system (Walsh and Hochedlinger, 2013). Cas9 is a Streptococcus pyogenes endonuclease protein having an HNH nuclease domain in the middle that cuts the complementary strands of crRNA and a RuvC-like domain at the amino terminus that cleaves the opposite strand of DNA (Jinek et al., 2014). Cas9 is also involved in crRNA maturation. In the designed type II CRISPR/Cas9 system, two non-coding CRISPR RNAs are linked to form a synthetic dimer, a single guide RNA (sgRNA) (Niu et al., 2020). crRNA is essential for guiding the Cas9 enzyme's nucleolytic activity in order to degrade the target nucleic acid. (Hussain et al., 2018).

2.6.2 CRISPR/Cas9 activity

2.6.2.1 CRISPR/Cas9 delivery mechanisms

The two most essential mechanisms in plant genome editing are the delivery of editing reagents and the generation of editing events. Protoplast transfection, *Agrobacterium*-mediated transformation, and particle bombardment have all been employed to aid in reagent dispersion (Hui-Li et al., 2014), with particle bombardment and *Agrobacterium*-mediated transformation being the most widely used for producing stable edited plants (McFarlane et al., 2018).

2.6.2.2 CRISPR/Cas9 repair mechanism

CRISPR/Cas9 systems generate double-stranded breaks (DSBs) that are guided by a chimeric single guide RNA. Homonymy-directed repair (HDR) and non-homology-directed end-joining repair (NHER) are the two principal competing and partly overlapping DSB repair processes (NHEJ) (Khoury et al., 2018; Jayavaradhan et al., 2019).

NHEJ is an error-prone mechanism that inserts and/or deletes short DNA sequences at the DSB site, causing frameshift and non-sense changes. This approach has been mostly used in CRISPR/Cas9 genome editing technologies (Yan et al., 2018). The pathway is also known as the canonical homology-independent pathway since it just requires the alignment of one to a few complimentary bases to re-align two ends (Pardo et al., 2009). This process has been demonstrated before in maize and *Arabidopsis* (Palareti et al., 2016).

In contrast, homology directed repair is a conservative strategy that results in the exchange of genetic information between two homologous DNA sequences or the unidirectional conversion of genes (Juma et al., 2021). This method of transferring DNA segments demands higher sequence homology. It has a high degree of faithfulness and a low incidence rate (Jasin and Rothstein, 2013). This repair process happens throughout the S and G2 stages of the life cycle and requires homologous donor sequences for accurate insertion (Shimada, 1978). In order for the HDR process to work, NHEJ pathway enzymes such as DNA ligase IV must be inhibited (Schmidt et al., 2019).

2.6.3 CRISPR/Cas9 genome editing for cyanogenic cassava

With recent advances in genome editing using CRISPR/Cas9, the technology was required to knock out the gene responsible for the generation of cyanogenic glycosides in cassava. The genes CYP79D1 and CYP79D2, which catalyze the formation of the main cyanogens in cassava, can be knocked out individually or in combination using the approach (Sun et al., 2018). The ability to knock off the genes would allow scientists to examine unanswered problems for instance how drought-stressed plants upregulate cyanogen synthesis in leaves and roots, as well as the relationship between cyanogens and protein synthesis.

The development of cyanogen-free cassava will be a significant step forward in the general applicability of precision breeding to farmers' preferred cassava. The goal of this

strategy is to demonstrate that it is possible to change and examine a key feature, cyanogenic glycoside production, in this critical food security crop (Bart et al., 2017). In this study, the transgene-free CRISPR/Cas9 editing technique was employed to accurately knock out the CYP79D1 gene in cassava. We used *Agrobacterium*-mediated delivery, which has been shown to be efficient in several cassava varieties (Figure 2.2) (Juma et al., 2021).

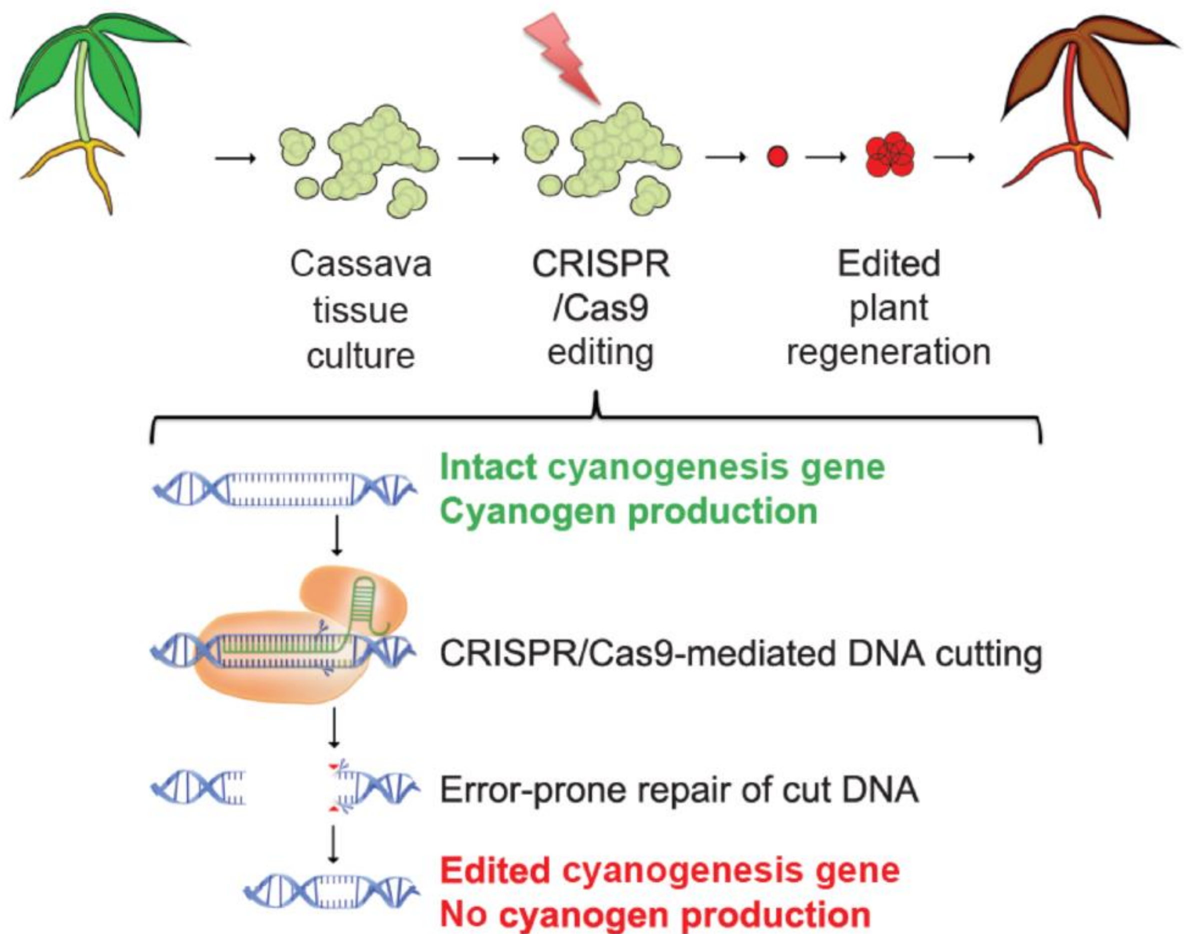

Figure 2.2: The process of engineering cassava plant which no longer produce cyanide using CRISPR/Cas9 genome editing method (Gomez et al., 2019)**.**

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant materials and *In vitro* micropropagation

The model cassava genotype (TMS 60444) was obtained from the International Livestock Research Institute's (ILRI) in vitro germplasm accessions in Nairobi, Kenya (Figure 3.1). Plantlets were grown on cassava micropropagation medium supplemented with Murashige and Skoog (MS) with vitamins (Murashige and Skoog, 1962) supplemented with 2% sucrose and 3 g/L gelrite (*pH* 5.8). The cultures were kept at 28 °C with a photoperiod of 16 hours of light and 8 hours of darkness. To keep the *in vitro* plants alive, sub-culturing was done every two weeks.

Figure 3.1: Maintenance of TMS 60444 cassava genotype in cassava micropropagation media: *In vitro* cultures of cassava at Kenyatta University – Plant Transformation Lab

3.2 Transformation of the TMS 60444 model cassava genotype

3.2.1 Designing and construction of guide RNA

Using BLAST, the reference cassava genome was searched for nucleotide sequences that matched the *Manihot esculenta* valine monooxygenase I gene in Phytozome 13 (v8.1) (https://phytozome-next.jgi.doe.gov/) (Bredeson et al., 2016). MeCYP79D1, a single copy

of the putative gene, was found and chosen. The web tool CRISPOR (http://crispor.tefor.net/) was used to generate a single guide RNA that targeted both ends of the cassava plant's MeCYP79D1 gene (Concordet and Haeussler, 2018). The NGG sequence necessary for *Streptococcus pyogenes* Cas9 was initially identified, preceded by 20 nucleotides above of the PAM for use as a spacer in the gRNA targeting MeCYP79D1 (Ding et al., 2016). A single gRNA was chosen from chromosome LG13 exon 2, with a G in its 5' end, to increase gRNA production in plant cells using the U6-U26 promoter. A complementary DNA oligonucleotide pair was produced (Sigma-Aldrich, USA) and hybridized with an empty vector digested with BsaI to generate a duplex with compatible ends.

In the empty vector construct, the coding sequence for SpCas9 from *S. pyogenes* was included, as well as a scaffold for a single gRNA with a BsaI restriction site. The resistance gene Hygromycin B (*Hph*) was inserted upstream of the sgRNA to assist in the identification of transgenic cells during the regeneration cycle. Cas9 was expressed through the 35S Cauliflower Mosaic Virus promoter, whereas gRNA was expressed through the U6-26 promoter. PCR and Sanger sequencing were used to establish the presence and stability of the integrated gRNA.

A ***MeCYP79D1* F**

……cgacagggtggtcggcaaagacaggcttgttcaagaatccgacatccccaaccttgactatgtcaaagcctgtgcaagagAAGCCTT
CAGGCTCCATCCAGTAGCACACTTCAATGTCCCTCATGTAGCCATGGAAGACACTGTCATTG
GTGATT**ACTTTATTCCAAAGGGCAGCTGG**GCAGTTCTCAGCCGCTATGGGCTCGGCAGGA
ACCCAAAGACATGGTCTGATCCTCTCAAGTACGATCCAGAAAGGCACATGAACGAGGGAG
AGGTGGTGCTCACTGAGCACGAGTTAAGGTTTGTGACTttcagcactggaagacgtggctgcgtagcttcgttgc
ttggaagctgcatgacgacgatgttgctggcgaggatgctgcagtgcttcacttggactccaccagccaatgtttc…… ***MeCYP79D1* R**

Construct	**gRNA sequence**
pCRISPR/Cas9-MeCYP79D1	ACTTTATTCCAAAGGGCAGC

B

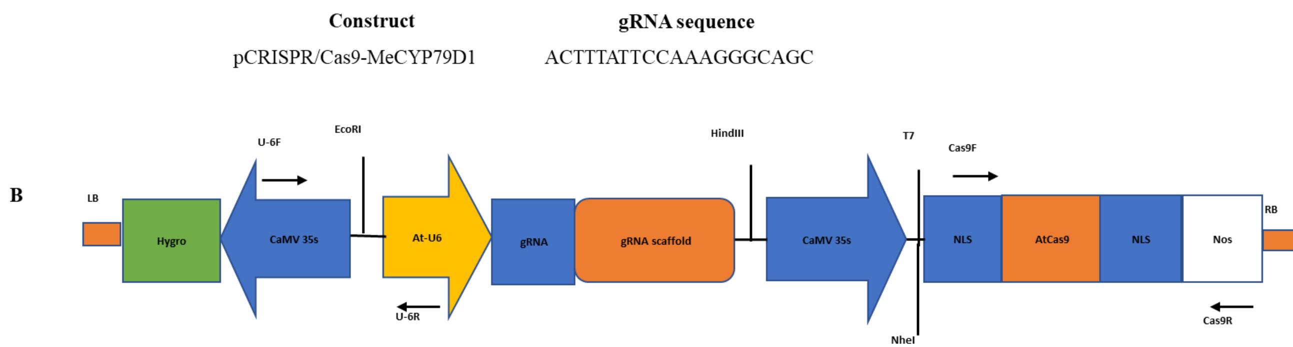

Figure 3.2: Schematic representation of the cassava MeCYP79D1 target gene, location of the gRNA and the CRISPR/Cas9 gene-editing construct. A: Schematic representation of target region showing the sequences and location of the 20 bp gRNA. gRNA is highlighted in green. Positions of forward (F) and reverse (R) primers flanking the target region in MeCYP79D1 are indicated with red arrows, respectively. B: The schematic of CRISPR/Cas9 binary vector, pCRISPR/Cas9-MeCYP79D1 used for stable *Agrobacterium*-mediated transformation of cassava.

3.2.2 *Agrobacterium tumefaciens* preparation and inoculation of the explant

By using the freeze and thaw procedure, the plasmid containing the Cas9 gene and gRNA was transformed in to *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium tumefaciens* strain GV3101 with the necessary help The Ti plasmid was cultured in 5 ml of (YEP) medium (Yeast extract 15 mg/L, peptone 10 mg/L, sodium chloride 5 mg/L, and bacterial agar 10 mg/L) overnight at 28 °C. To a 50 ml YEP medium in a 250 ml flask, exactly 2 ml of the overnight culture was added, and the mixture was vigorously agitated at 28 °C (250 rpm) until the culture reached an OD600 of 0.5 to 1.0. (growth for 3 – 4 hours). The culture was next placed on ice for an hour to chill it, and after that, the cell

suspension was centrifuged at 3000 g for 5 minutes at 4 °C. A supernatant was thrown out.

The cells were resuspended in (ice-cold) 0.15M NaCl for 15 minutes before being resuspended in 1 ml of 01.M CaCl2 solution for 1 hour. Aliquots of around 0.1 mL were then dispensed into pre-chilled Eppendorf test tubes. The cells were frozen in liquid nitrogen for 10 minutes after adding 2 to 4 µl (1 µg) of plasmid DNA. The cells were then thawed in test tubes for 5 minutes in a 37 °C water bath.

The tubes were filled with exactly 1 ml YEB medium and incubated for 3 hours at 28 °C with careful shaking before being centrifuged for 30 seconds in an Eppendorf centrifuge. The supernatant solution that resulted was discarded. The cells were then resuspended in 0.1 mL YEP media and disseminated on a YEP agar plate containing 100 mg/L kanamycin for plasmid selection and 100 mg/L gentamycin and 10 mg/L rifampicin for bacterial selection. For 2–3 days, the plates were incubated at 28 °C, during which time colonies appeared (Kámán-Tóth et al., 2018). The *A. tumefaciens* strain, GV3101 (carrying the CRISPR/Cas9 binary vector targeting the MeCYP79D1), utilized for transformation was grown on YEP medium supplemented with kanamycin 100 mg/L, gentamycin 100 mg/L and rifampicin 10 mg/L. For bacterial culture rejuvenation, the cultures were refreshed every two weeks. *Agrobacterium tumefaciens* strain GV3101 lacking the construct was produced and maintained in bacterial culture medium as a negative control.

Prior to infection of the immature cassava leaf lobes, a colony of *A. tumefaciens* GV3101 strains harbouring pCRISPR/Cas9-MeCYP79D1 was scrapped from the master plate containing the YEP medium, and suspended into a 50 mL falcon tube containing 25 ml of Luria Bertani Broth (LBB) with 100 mg/L kanamycin, 100 mg/L gentamycin and 10 mg/L rifampicin and cultured at 28 °C for 3 days in the dark. A thick colony of *Agrobacterium* was harvested and inoculated into 15 mL of LBB incorporated with the aforementioned antibiotics and grown overnight at 28 °C in dark. The cells were then harvested and

washed by re-suspending them in LBB without antibiotics in microcentrifuge. After the washing procedure, the cells were harvested and re-suspended in liquid infection medium then grown at 28 °C on a rotary shaker for 2 – 5 hours. The infection medium composed of liquid MS supplemented with 2% (w/v) sucrose and 200 µM acetosyringone. The bacterial growth was used adjusted to the required optical density OD_{600} 0.4 using a spectrophotometer at a wavelength of 260 nm, for infection of immature cassava leaf lobes.

3.2.3 Explant infection, co-cultivation and resting

Emerging leaf lobes from the *in vitro* grown cassava genotype, TMS 60444 were used as the target tissues for the transformation process. Leaf lobes from actively growing in vitro plants were cut with sterilized scalpel blades. The explant tissue was then treated with 25 µl of bacterial suspension of infection media and incubated in the dark for 5 minutes. Leaf explants were treated with bacterial suspension carrying empty vector served as a negative control. Excess infection medium was drained and placed with co-cultivation medium (MS basal salts supplemented with 2% (w/v) sucrose, B5 vitamins, 100 mg/L casein hydrolysate, 0.5 mg/L $CuSO_4$, and 10 mg/L 2,4-D supplemented with 200 M acetosyringone, 3g/L gelrite at pH 5.7). Wrapped in parafilm, the plates containing the explants in co-cultivation media were incubated for three days at 28 °C in the dark.

Explants were switched to resting medium (MS basic salts supplemented with 2% (w/v) sucrose, B5 vitamins, 100 mg/L casein hydrolysate, 0.5 mg/L CuSO4, and 10 mg/L 2,4-D supplemented with 250 mg/L timentin) for 2 days after 3 days of co-cultivation with *Agrobacterium tumefaciens*. To increase embryo development and visibility, CuSO4 (copper II sulphate) was used. A different batch of explants co-cultured with *A. tumefaciens* harboring an empty vector was placed in the resting media as a negative control.

3.2.4 Selection of putatively transformed calli

The live calli were transferred from the resting medium to the selection medium (MS basic salts supplemented with 2% (w/v) sucrose, B5 vitamins, 100 mg/L casein hydrolysate, 0.5 mg/L CuSO4, and 10 mg/L 2,4-D supplemented with hygromycin (10 mg/L) and timentin (15 mg/L) (Syombua et al., 2019). The proliferating callus was divided into smaller pieces after 2 weeks of culture to ensure good media contact before being introduced to a fresh second selection medium containing 20 mg/L hygromycin and 15 mg/L timentin. Timentin was added to the media to prevent *Agrobacterium* from returning, and hygromycin was added to eliminate escapes (non-transformed callus). The callus was retained in the selection medium for four weeks.

3.2.5 Maturation of somatic embryos

Calli were moved to MS medium supplemented with B5 vitamins, 2% sucrose, 1 mg/L NAA, 20 mg/L hygromycin, and 15 mg/L timentin at the age of eight weeks and cultured for up to four months. The cotyledon-stage embryos were maintained in somatic embryo maturation medium, consisting of MS medium with B5 vitamins, 2% sucrose, 1 mg/L BAP, 0.01 mg/L NAA, 0.5 mg/L GA3, 20 mg/L hygromycin, 15 mg/L timentin, 3 g/L gelrite, and a pH of 5.8. After four weeks, the proportion of cotyledonary embryos produced by each callus was counted.

3.2.6 Desiccation and germination of somatic embryo

Green cotyledonary embryos with distinct shoot and root apices were placed in glass bottles with 50 mL of hormone-free desiccation media to be adsorbent for phenolic components. MS salts, B5 vitamins, 2% sucrose, 0.8% activated charcoal, and 3 g/L gelrite were used to solidify the medium.

After 14 days, the germinated embryos were counted and placed to a cassava micropropagation medium enriched with 3 percent sucrose, 5 mg/l hygromycin, and 10

mg/l timentin and solidified with 3g/L gelrite (pH 5.8). This medium was kept at 28 °C under 16/8–hour cycles of light and darkness.

3.2.7 Hardening of *In vitro* regenerated cassava

In vitro-grown plants were transplanted to a greenhouse. To avoid root damage, putatively transformed cassava with distinct roots and shoots was removed carefully from the medium. The plants were then placed in miniature pots topped with peat moss and left in the glasshouse at room temperature for two weeks, wrapped in a plastic bag to manage humidity and temperature. A 50 mL mist of water was added with a hand sprayer. The plastic bags were gradually removed after two weeks to allow the plantlets to acclimatize to the glasshouse environment. The healthy plants were then transplanted into larger containers filled with a 50/50 mixture of peat moss and forest soil, and the plants were cultivated on an open bench in a greenhouse kept at 28 °C with both natural and artificial lighting, with a light/dark cycle of about 12 h/12 h. Following that, the seedlings were placed in soil-filled pots, and the watering frequency was reduced to 150 mL once per week.

3.2.8 Extraction of genomic DNA putative transgenic plants

For genomic DNA extraction, the first or second youngest healthy leaves of the *in vitro* plants were utilized. During the acclimatization stage, healthy leaf tissues were obtained from the in vitro regenerated plantlets. A 2-mL Eppendorf tube containing ceramic beads was filled with approximately 0.5 g of leaf tissue, which was vortexed to obtain a fine powder from. Using a modified cetyltrimethylammonium bromide (CTAB) technique, the genomic DNA was isolated as previously established by Doyle and Doyle, (1990). The 3X extraction buffer (20 g CTAB, 280 ml 5M NaCl and 100 ml 1M Tris HCl) was heated in a water bath to 65 °C. About 0.3 percent 2-β-mercaptoethanol was added to the buffer right just before use. Using a pre-chilled mortar and pestle, 50 mg of plant leaf samples were pounded into fine powder in liquid nitrogen. While the pulverized materials were

still in the mortar, 800 µL of warmed buffer was added and gently swirled with a pestle. After that, the mixture was transferred to a 2-ml microcentrifuge tube and incubated for one hour in a 65 °C water bath, with mild agitation every 20 minutes by pipetting up and down 20 times. After that, the mixture was allowed to cool to ambient temperature. After adding an equal volume of chloroform:isoamylalcohol (24:1), the mixture was inverted. The mixture was centrifuged for 15 minutes at 13000 rpm at room temperature. The upper aqueous phase was carefully transferred to a new 1.5-ml Eppendorf tube using a wide pipette. The tube was inverted to precipitate DNA after adding exactly 500 L of ice-cold 100 percent isopropyl alcohol. The mixture was then kept at -20 °C overnight. After an overnight incubation, the mixture was centrifuged for five minutes at 13000 rpm, and the supernatant was discarded. The tube containing the DNA pellets was then inverted onto tissue paper to completely drain the supernatant. The DNA pellets were then washed in 500 µL of 70% ethanol, inverted once to dissolve residuals, centrifuged at 13000 rpm for 5 minutes, and the ethanol was discarded from the tube. The tubes were then inverted on filter paper and allowed to dry at ambient temperature for 15 minutes. The DNA pellets were redissolved in 50 l of 1X Tris-EDTA (TE) buffer and incubated at 50 °C for an hour to ensure complete resuspension before being treated with RNase A to eliminate any RNA contamination and stored at -20 °C until needed. Negative control were DNA extracted from plants that had not undergone transformation.

The concentration of plant genomic DNA was determined by passing it over a 0.8 percent (w/v) agarose gel. Exactly 3 µl of extracted DNA were combined with 5 µl of loading dye and electrophoresed on 0.8 percent (w/v) agarose gel alongside 5 µl of 100 bp molecular marker. The gel was operated at 100 volts for 45 minutes before being visualized with a transilluminator under UV light. A Nanodrop spectrophotometer was used to assess the purity of the DNA.

3.2.9 Polymerase chain reaction analysis of putatively transgenic lines

Polymerase chain reaction (PCR) was used to verify incorporation of T-DNA from putatively edited plant lines, with 200 ng genomic DNA as the template and primers specific for the Cas9 gene and gRNA scaffold (Table 3.1). Prior to PCR amplification, the target gene was subjected to *In Silico PCR* to confirm the region of amplification. The presence of the Cas9 gene was confirmed by amplification with gene-specific primers resulting in a 900 bp amplicon. Each PCR reaction was performed in a 20 μL reaction mixture (total volume). The Cas9 gene was amplified using the following parameters: a five-minute initialization at 95 °C, 35 cycles of denaturation at 95 °C for ten seconds, annealing at 53 °C for thirty seconds, and extension at 72 °C for one minute, followed by a five-minute final extension at 72 °C. The target gene was verified by amplification using gene-specific primers (CYP79D1) - 350 bps. The PCR reaction was carried out under the following conditions: Initialization at 95 °C for 5 minutes, followed by denaturation for 10 seconds at 95 °C, annealing for 30 seconds at 53 °C, extension for 1 minute at 72 °C, and final extension for 5 minutes at 72 °C. A negative control and positive control (non-transformed plant and plasmid, respectively) containing 2 µl of DNA were run alongside the DNA from the putatively transformed plants.

A volume of 8 µL of amplified DNA fragments was mixed with 2 µL of the loading dye and electrophoresed at 100 volts for 45 minutes on a 1.2% agarose gel containing 5 µg/mL of SYBR Green dye. The gel was visualized under UV light and the molecular weight of the amplified DNA fragments was estimated using 100 bp plus molecular marker.

3.2.10 Mutant characterization by sequencing

Genomic DNA from putative mutant lines was used as template to amplify the MeCYP79D1 gene fragment by PCR. To characterize CRISPR/Cas9-driven changes, the amplicons were prepared using Exo SAP-IT (ThermoFischer Scientific) before being submitted to targeted Sanger sequencing with CYP79D1 F and CYP79D1 R primers. Bioedit software v7.2 was used to trim, edit to generate consensus sequence and align the

sequence results of putative mutant lines to the endogenous MeCYP79D1 gene of wildtype cassava plant. By dividing the total number of transgenic plants sequenced by the total number of mutant lines, the editing efficiencies were computed.

Table 3.1: Primers used to confirm T-DNA integration and the integrity of the CYP79D1 target sequence.

Primer	Forward sequence	Reverse sequence	Expected product size (bp)
CYP79D1	ACCAGGGCCTGAAGAAATCG	CCCAGCTGCCCTTTGGAATA	350
Cas9 gene	TGCAGACCTACAACCAGCTG	CCGTTCTTGGACTGGTCGAA	900

3.3 RNA expression analysis of the cassava progenies using RT-PCR

3.3.1 Total RNA extraction and purification

Transgene expression in the putative transgenic TMS 60444 cassava lines was examined using reverse transcriptase-PCR (RT-PCR). Total RNA was extracted from 30 mg of young leaves from in vitro putative transgenic lines and non-transgenic control plants using the Qiagen RNeasy Mini Kit (Hilden, Germany) (Appendix 4). To determine the amount and purity of RNA, a NanoDrop ND-2000 spectrophotometer (NanoDrop products, Wilmington, USA) was employed. To remove DNA contaminants, the extract was treated with 1 unit DNase for 15 minutes at room temperature (Invitrogen, Carlsbad, CA). To prevent the digestion of freshly generated cDNA, the DNase was deactivated according to the manufacturer's instructions.

3.3.2 cDNA synthesis and PCR amplification

The LunaScript™ RT SuperMix Kit was utilized to create first-strand cDNA from 1 g of total RNA, as directed by the manufacturer. The cDNA was amplified using 1X PCR buffer, 1.5 mM MgCl2, 0.1 mM dNTP, 2.5 units of Taq polymerase, and 0.4 M of each

Cas9 gene primer sequence (forward 5′-TGC AGA CCT ACA ACC AGC TG-3′ and reverse 5′-CCG TTC TTG GAC TGG TCG AA-3′). Actin-7 (ACT) amplification was utilized as an housekeeping gene to evaluate the efficacy of the generated cDNA. The primer sequences were: forward 5′-TGC AAT GTA TGT TGC CAT CCA GGC-3′ and reverse 5′ -TTA CAC CGT CAC CAG AAT CCA GCA-3′ - 300 bps. The RT-PCR outputs were run for 45 minutes on a 1% (w/v) agarose gel at 100 V and observed under UV illumination.

3.4 Linamarin quantification and total cyanide determination in leaves

3.4.1 Extraction of linamarin

Extraction of cyanogenic glycoside linamarin will be carried out according to (Sornyotha et al., 2007). 5 grams of cassava leaves initially homogenized for 14 seconds on low, then for 1 minute (2X) on high with 25 ml of chilled 0.25 M sulfuric acid. The homogenates were filtered with a filter cloth to eliminate any insoluble components. Before filtering, 40 mL of the aid was washed through the homogenizer jar. The filtrates were centrifuged at 4 °C at 10,000 rpm for 10 minutes. The crystal-clear supernatant liquid was collected and kept at -20 °C.

3.4.2 Detection and purification of linamarin in crude extract

The quantity of linamarin in acid extracts of cassava leaves was measured using high-performance liquid chromatography (HPLC). Methanol and water (25:75, v/v) were used to make the mobile phase. The experiment was performed on a C18 HPLC column at a flow rate of 1 mL/min, injection volume of 10 L, and column temperature of 40 °C. The detector was calibrated at 214 nm. Prior to usage, the gas bubbles from the mobile phase were eliminated. The standard was linamarin, a biochemical produced by A.G. Scientific in the United States. The stock solution containing 100 g/mL of linamarin standard was then prepared and stored at -20 °C until use.

The calibration plot, used to determine concentrations was prepared using standard linamarin (Sigma Aldrich, USA). From a stock solution of 100 g/mL linamarin, a dilution series with concentrations ranging from 10 µg/mL to 100 µg/mL (10, 20, 40, 60, 80, and 100) was created. The crude linamarin concentration was calculated by comparing the sample peak area to the standard peak area.

3.4.3 Determination of total cyanide in leaves

A picrate assay kit was used to determine the total cyanide concentration in the leaves of wild type cassava and the hygromycin-resistant transgenic TMS 60444 cassava lines (Bradbury et al., 1999). Cassava leaves that were still young were picked, quickly chopped with scissors, and crushed. The flat-bottomed vial containing the buffer discs was then covered with 100 mg of the ground leaves. Using the plastic pipette, add clean water (exactly 1.0 mL) and gently mix. Without letting it touch the liquid, yellow indicator paper was added right away, and the bottle's screw-capped lids were quickly fastened. There were three replications for each sample.

For the positive control, a buffer/enzyme paper disc and a regular pink paper disc were placed in the container, followed by 1 mL of fresh water. The bottle was tightly sealed with screw-capped lid after the yellow indicator paper was added. The bottle was filled with normal pink paper and 1 mL of water for the negative control, and the lid was tightened. For the next 24 hours, the bottles were left at room temperature.

After opening the bottles, the colors on the cassava cyanogen kit color chart were compared to the colors on the indicator sheets. The total cyanide level in leaves was calculated using a color chart and represented as parts per million (equivalent to mg HCN/Kg cassava fresh weight). The positive control offers a color on the label comparable to parts per million (ppm), whereas the negative control is zero. The indicator paper's plastic backing was carefully peeled off, and the paper was placed in a test tube with 5.0

mL of precisely measured distilled water. The test tubes were left at room temperature for about 30 minutes, with some gentle shaking in between. At 510 nm, the absorbance of the solutions was measured and the value of the negative control was subtracted. The overall cyanide level in ppm was calculated using the following equation:

$$\text{Total cyanide content (ppm)} = 396 \times \text{Absorbance}$$

3.5 Agronomic trait characterization

Under standard greenhouse circumstances, cassava plantlets of the wild type (control) and the transgenic TMS 60444 were produced. The agro-morphological parameters, including plant height, leaf length, leaf width, number of leaves per plant, and stalk length, were examined after four months in the greenhouse, with wild type cassava measurements and numbers serving as the control (Nadjiam et al., 2016).

3.6 Data management and statistical analysis

Three replicates of each trial were employed in the experiment's randomized sampling design. A replica was created using 150 leaf explants. For all quantitative data values (n = 150), Mean SEM was used. The frequency of somatic embryogenesis was determined using the proportion of explants grown in callus induction media to somatic embryos that produce calli. Calculating the proportion of PCR-positive events to all events that were examined allowed for the evaluation of transformation efficiency. Photographs of *in vitro* cultures and gels were used to demonstrate qualitative data. The unpaired student's t-test was used to analyze the agronomic traits, cyanide content estimation, and linamarin quantification data at a confidence level of 95% (p 0.05). The data was examined using GraphPad Prism 8.0.2, a statistical analysis program.

CHAPTER FOUR

RESULTS

4.1 *Agrobacterium*-mediated transformation of cassava

4.1.1 Design and construction of guide RNA

The CRISPOR online tool was used to design the guide RNA. A BLAST search for the reference gene using the A. thaliana CYP79D1 gene yielded the MeCYP79D1 gene in cassava. After obtaining the reference sequence from Phytozome v8.1, the sequence was placed into the CRISPOR input area, followed by the genome name, Manihot esculenta-phytozome v8.1, and the kind of PAM, 20 bp NGG-SpCas9, SpCas9-HF-1, eSpCas9 1.1, to be used for gRNA creation (Figure 4.1A). The CRISPOR output section supplied a variety of guide RNAs with scores ranging from the lowest probability of off-target to the greatest MIT specificity score and CFD specificity score (Figure 4.1B). A 20-bp single guide RNA with the highest on-target score, 5' - GAATCAAAATAGCTGAAATTA - 3', was created (Figure 4.1C). The gRNA sequence was produced (Complementary DNA Technology) and cloned in a binary vector (pCRISPR/Cas9-MeCYP79D1) including the Cas9 gene, the U6-U26 promoter, and the gRNA scaffold. The *Arabidopsis thaliana* promoter (AtU6-26) was employed to drive the gRNA that was used to target MeCYP79D1, with the gRNA ligated at the position indicated by the blue box, which was aided by the *HindIII* restriction site. Cauliflower mosaic virus (CaMV 35S) promoter causes Cas9 gene expression, which when paired with inserted gRNA results in alterations in the target region of the MeCYP79D1 gene (Figure 4.2). Because gRNA efficiency varies depending on the gRNA sequence, the Cas9/gRNA was sequenced before starting with the cassava transformation, demonstrating that the CRISPR construct was functional and successful. In this study, a single CRISPR/Cas9 plasmid construct, pCRISPR/Cas9-MeCYP79D1, was created with a *hptII* selectable marker that confers resistance to the antibiotic hygromycin (Figure 4.2).

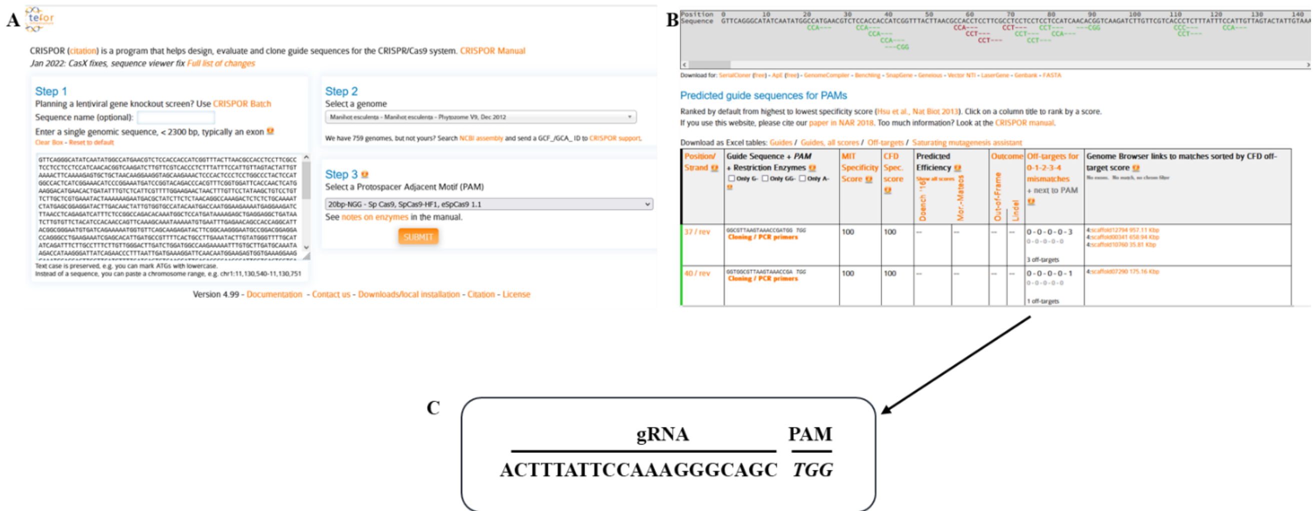

Figure 4.1: Schematic representation for designing of guide RNA using CRISPOR: A; the CRISPOR input section. B; the CRISPOR output section. C; the guide RNA.

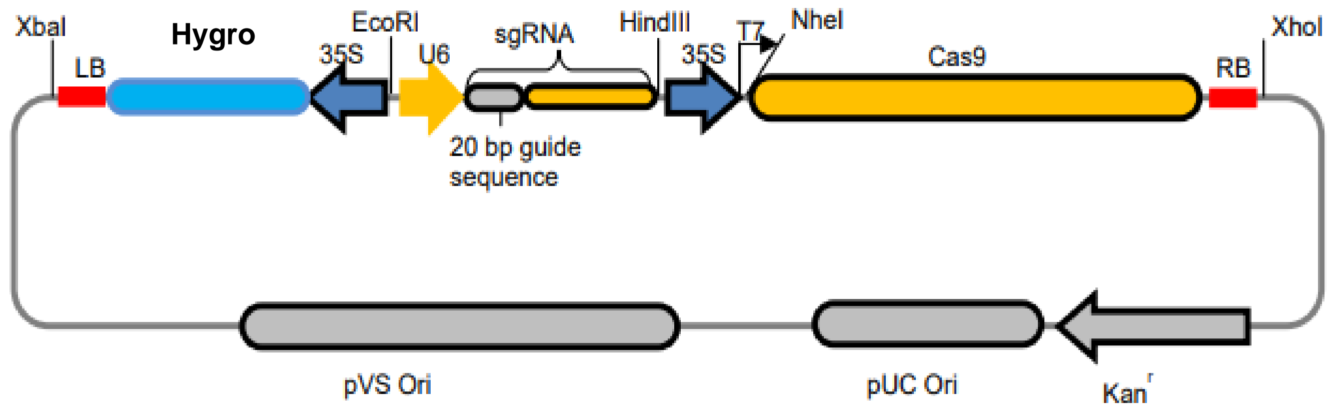

Figure 4.2: Schematic representation of the CRISPR/Cas9 binary vector used for stable *Agrobacterium*-mediated transformation of cassava. LB, left border; RB, right border.

4.1.2 Cas9/gRNA *Agrobacterium*-mediated transformation and regeneration of TMS 60444 cassava genotype

TMS 60444 transgenic cassava plants were transformed with *A. tumefaciens* GV3101 carrying the binary plasmid pCRISPR/Cas9-MeCYP79D1 (Table 4.1). In the greenhouse, eight distinct transgenic plant lines were grown and propagated (Figure 4.3). All of these transgenic plant lines were phenotypically indistinguishable from wildtype plants (Figure 4.3). Immature cassava leaf lobes were switched to selection-free media supplemented with 15 mg/l timentin for two days after infection to reduce *Agrobacterium* growth and recover from the shock caused by *A. tumefaciens*. At this stage, the calli began to grow (Figure 4.3B).

Cas9 activity was evaluated phenotypically by observing calli under hygromycin selection. Four weeks after infecting the leaf explants, several putative transgenic calli grew at the damaged regions and along the surface of the leaves under hygromycin selection (Figure 4.3B). While most calli were resistant to hygromycin, others necrotized, turned brown, and perished, and were isolated in each sub-culture (Figure 4.3C). Increasing the selection pressure from 10 mg/l hygromycin to 15 mg/l hygromycin caused quick necrosis and browning of more uninfected tissues during the second round of selection, enabling for early identification of tissues in which the T-DNA had been integrated (Figure 4.3B, C and D).

Embryos matured after about two months on BAP, NAA, and GA3 hormone media, and shoot-like structures appeared from regenerated calli (Figure 4.3D). The cotyledonary embryos of putatively changed plants were transplanted to hormone-free MS media with activated charcoal to limit moisture loss, which results in a high frequency of plant regeneration, eliminate phenolic chemicals, and establish a darker environment (Figure 4.3E). While only a few calli developed into somatic embryonic structures, an even smaller number of putative transgenic plants were created (Table 4.1). Calli that differentiated further developed shoots on medium and were switched to MS medium with

vitamins but no hygromycin for shoot formation (Table 4.1). After around two months, the shoots were transferred to cassava propagation media, and after one month, rooting was achieved (Figure 4.3F and G). Individual putative transgenic plants and negative control plants in culture bottles were grown for two weeks before being moved to the greenhouse (Figures 4.3H). The transformation frequency for pCRISPR/Cas9-MeCYP79D1 transgenes was 30.67%, with a regeneration efficiency of 1.78%. (Table 4.1).

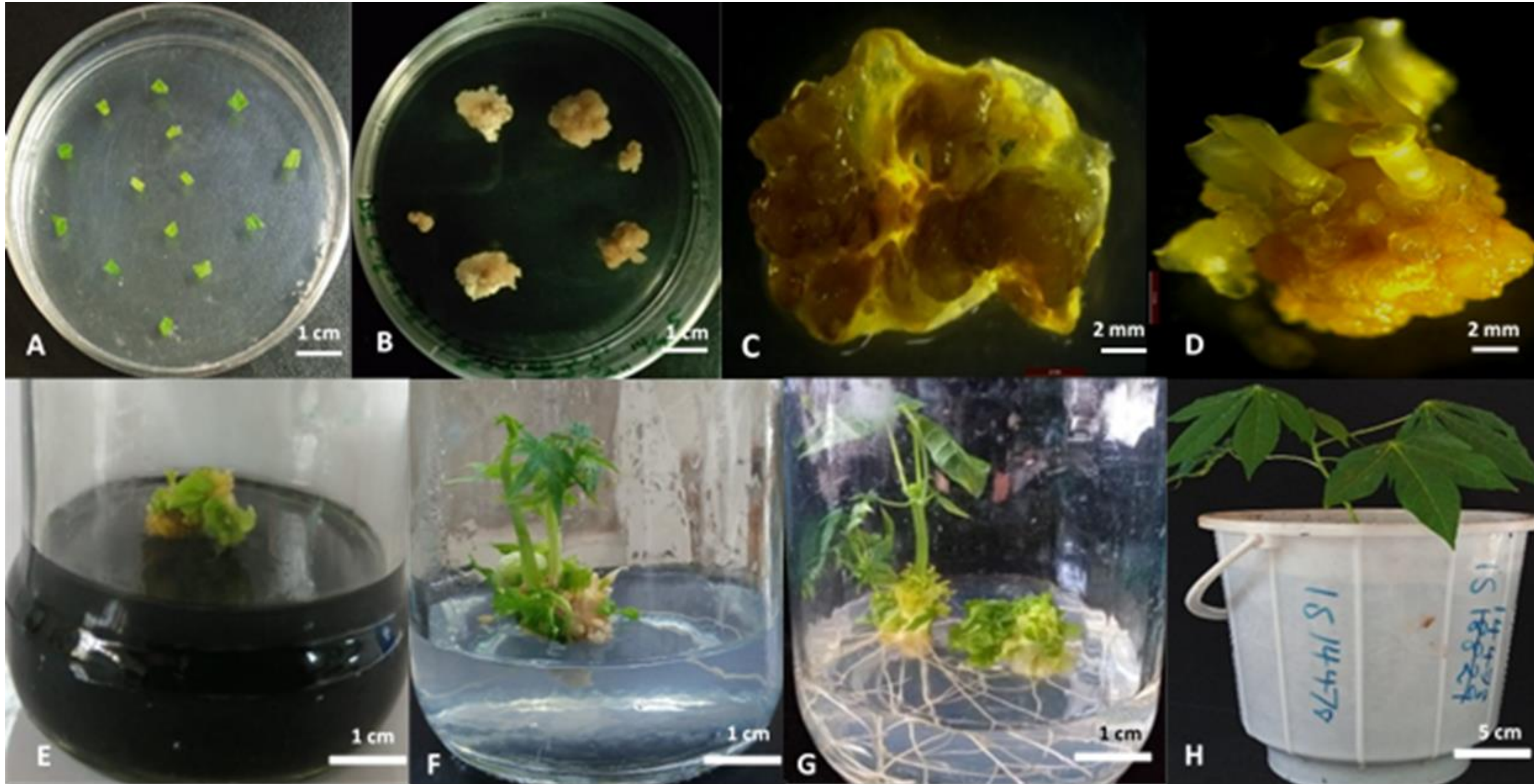

Figure 4.3: Ca9/gRNA Agrobacterium-mediated cassava transformation and shoot recovery. A: Immature leaf lobes co-cultured with Agrobacterium containing the construct; B: Agro-infected explants in callus induction media under selection; C: non-transformed leaf explant in selection medium; D: mature callus with cotyledonary embryos. E: Shoot desiccation (0.8%) in activated charcoal; F: Rooting in hormone-free MS medium. G: 12 days in rooting medium; H: In a glasshouse, putatively altered cassava hardened.

Table 4.1: Transformation, callus recovery and regeneration of TMS 60444 cassava

Experimental batch	No of explants	Live calli in selection media	Calli in germination media	Regenerated plants	Regeneration frequency (%)
Negative control	150	0	0	0	0
1	150	50	44	3	6.82
2	150	67	51	2	3.92
3	150	62	43	3	6.98
Total	450	179	138	8	5.91

Regeneration frequency = the number of shoots germinated divided by the total number of recovered calli events in germination media. Transformation frequency = the total number of recovered calli events on GM divided by the total number of infected explants. Regeneration efficiency = the total number of shoots regenerated divided by the total number of infected explants. The values represent the means and standard deviations of three different experiments.

4.1.3 PCR analysis for integration of T-DNA

PCR analysis was used to identify MeCYP79D1 and MeCas9 genes in TMS 60444 transgenic cassava plants that were regenerated (Table 3.1). The in-silico PCR analysis confirmed that the target gene was amplified using the chosen primers (Figure 4.4). While no PCR products were identified in the wildtype control, as shown in Figure 4.5, these transformants showed amplification products of 350 bp and 900 bp for the MeCYP79D1 and Cas9 genes, respectively. MeCYP79D1 gene amplification was detected in all eight putative transgenic plants and the non-transgenic wildtype cassava (Figure 4.5B). Amplification with the Cas9 gene was seen in all putative transgenic plants, but not in wildtype plants (Figure 4.5A).

```
>NC_035173.2:26984507+26984856 350bp ACCAGGGCCTGAAGAAATCG TCAATCATTAGCATGTAATT
ACCAGGGCCTGAAGAAATCGagcacattgatgccgttttcactgccttga
aatacttgtatggattttgcatatcagatttcttgcctttcttgttggga
cttgatctggatggccaagaaaaatttgtgcttgatgcaaataagaccat
aagggattatcagaaccctttaattgatgaaaggattcaacaatggaaga
gtggtgaaaggaaggaaatggaggacttgcttgatgttttcatcactctc
aaggattcagacggcaacccattgctcactcctgacgagatcaagaatca
aatagctgtaagatcactctcacttcttacAATTACATGCTAATGATTGA
```

Primer Melting Temperatures

```
Forward: 63.7 C accagggcctgaagaaatcg
Reverse: 48.2 C aattacatgctaatgattga
```

Figure 4.4: *In silico PCR* analysis of the targeted region in the CYP79D1 gene

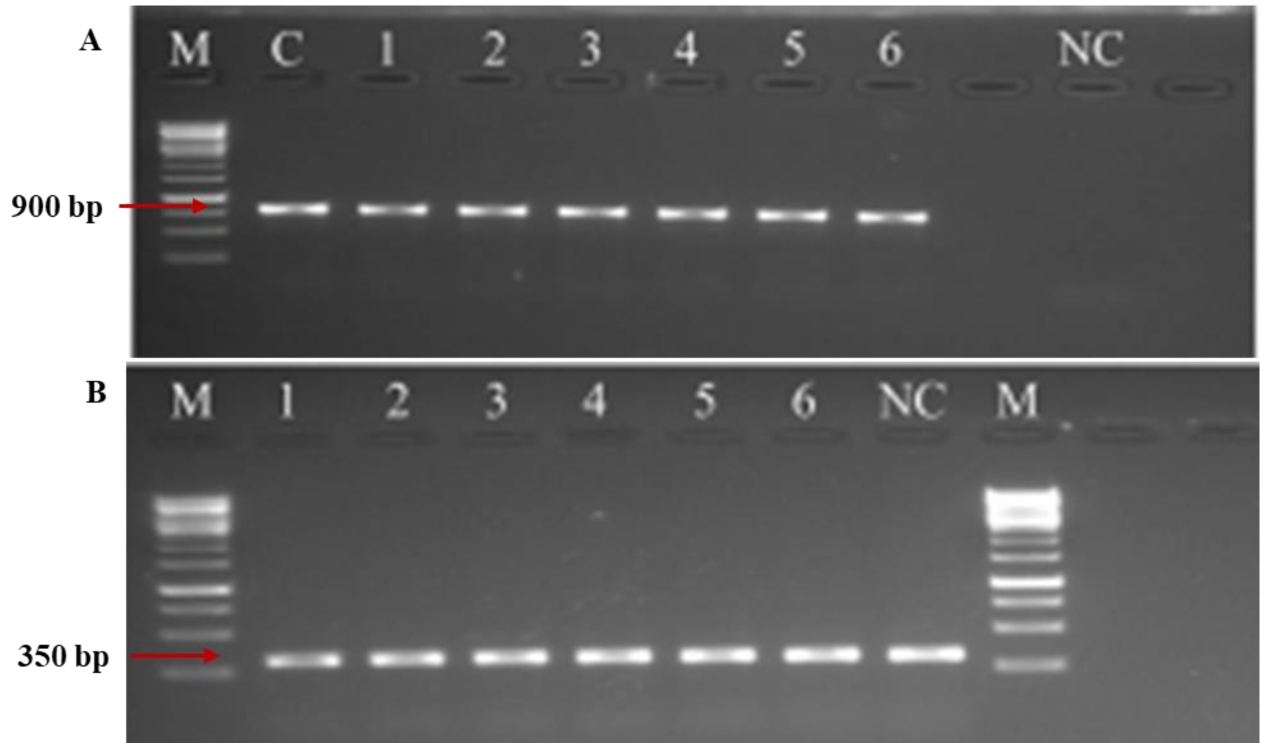

Figure 4.5: CRISPR/Cas9 T-DNA integration and MeCYP79D1 target sequence detection through PCR. A: Cas9 gene detection confirms the existence of CRISPR/Cas9. B: PCR amplification of the MeCYP79D1 target area (350 bp). M represents a molecular marker. (A) C represents plasmid DNA bearing the binary vector GV301, and NC represents a no template control. NC is the wild-type control in (B).

Only eight distinct transgenic plant lines were regenerated from 450 leaf explants all infected with A. tumefaciens strain GV3101 bearing the pCRISPR/Cas9-MeCYP79D1 construct, with a transformation efficiency of 1.78% (Table 4.2).

Table 4.2: Cassava transformation efficiency of TMS 60444

Experiment batch	No. of explants used	No. of putative transgenic plants	No. of PCR positive	Transformation efficiency (%)
Negative control	150	0	0	0
1	150	3	3	2.00
2	150	2	2	1.33
3	150	3	3	2.00
Total	**450**	8	8	1.78

*Transformation efficiency (%) calculated as the total number of PCR positive of the total number of the explants infected with *A. tumefaciens* GV3101 strain multiplied by 100. Experimental batches 1, 2 and 3, are independent experiments.

4.1.4 Detection of targeted mutations in putative transgenic lines

To determine the type of mutations acquired, targeted Sanger sequencing was done on the PCR amplicons of putative transgenic plants. After cutting and modifying the sequence chromatograms to obtain consensus sequences, the transgenic plant sequences were aligned using the endogenous valine monooxygenase I gene as the query sequence. All transgenic plants had MeCYP79D1 gene mutations. There were substitutions, insertions, and deletions (INDELs). The targeting efficacy of pCRISPR/Cas9-MeCYP79D1 was 100% (8/8) based on the mutations detected. The discovered mutations contained both INDELS and replacements (Figure 4.6). More deletions occurred than substitutions (Figure 4.6). According to the combined analysis, deletions were the most common form of modification (75% of all variations), with four base-pair deletions occurring three nucleotides upstream of the PAM area. These deletions were found in the target region of the relevant gene. One nucleotide alteration was reported, accounting for 25% of the total. The alterations were observed five nucleotides downstream of the PAM site, outside target location (Figure 4.6). TMS 60444 amplicon sequencing revealed no INDELs or alterations in the target gene (Figure 4.6).

MeCYP79D1, **TMS 60444**

PAM

WT GGAAGACACTGTCATTGGTGATTACTTTATTCCAAAGGGCAGC**TGG**GCAGTTCTCAGCCGCT

TMS1 GGAAGACACTGTCATTGGTGATTACTTTATTCCAAAG- - - -GC**TGG**GCAGTTCTCAGCCGCT

TMS2 GGAAGACACTGTCATTGGTGATTACTTTATTCCAAAG- - - -GC**TGG**GCAGTTCTCAGCCGCT

TMS3 GGAAGACACTGTCATTGGTGATTACTTTATTCCAAAG- - - -GC**TGG**GCAGTTCTCAGCCGCT

TMS4 GGAAGACACTGTCATTGGTGATTACTTTATTCCAAAG- - - -GC**TGG**GCAGTTCTCAGCCGCT

TMS5 GGAAGACACTGTCATTGGTGATTACTTTATTCCAAAG- - - -GC**TGG**GCAGTTCTCAGCCGCT

TMS6 GGAAGACACTGTCATTGGTGATTACTTTATTCCAAAG- - - -GC**TGG**GCAGTTCTCAGCCGCT

PAM

WT TCCAAAGGGCAGC**TGG**GCAGTTCTCAGCCGCTATGGGCTCGGCAGGAACCCAA

TMS7 TCCAAAGGGCAGC**TGG**GCAATTCTCAGCCGCTATGGGCTCGGCAGGAACCCAA

TMS8 TCCAAAGGGCAGC**TGG**GCAATTCTCAGCCGCTATGGGCTCGGCAGGAACCCAA

PAM

WT GGAAGACACTGTCATTGGTGATTACTTTATTCCAAAGGGCAGC**TGG**GCAGTTCTCAGCCGCT

SWT GGAAGACACTGTCATTGGTGATTACTTTATTCCAAAGGGCAGC**TGG**GCAGTTCTCAGCCGCT

Figure 4.5: Sequence-based detection of mutations induced by pCRISPR/Cas9-MeCYP79D1 vector in TMS 60444. WT; wildtype. SWT; sequenced wildtype TMS 60444: The sequences from each mutant plant are listed below the WT sequence (TMS1 - TMS8). The color yellow highlights deletions, while the color green highlights substitutions. In the wild-type (WT) reference sequence, the MeCYP79D1 target region is highlighted purple, with the protospacer adjacent motif (PAM) in bold.

4.2 Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Cas9 mRNA transcripts were detected in all 8 transgenic lines of the cultivar TMS 60444, but not in the wildtype line (Figure 4.7), proving Cas9 gene expression in transgenic cassava plants. The wildtype control yielded no PCR products, whereas the transformants yielded transcript amplification products for MeCas9 of 900 bp, which was commensurate with the expected size of amplified products (Figure 4.7A).

The actin7 was constitutively expressed in all mRNA transcripts target sequence in cDNA generated from both transgenic and wild-type plants using Cas9 amplification primers (Figure 4.7B).

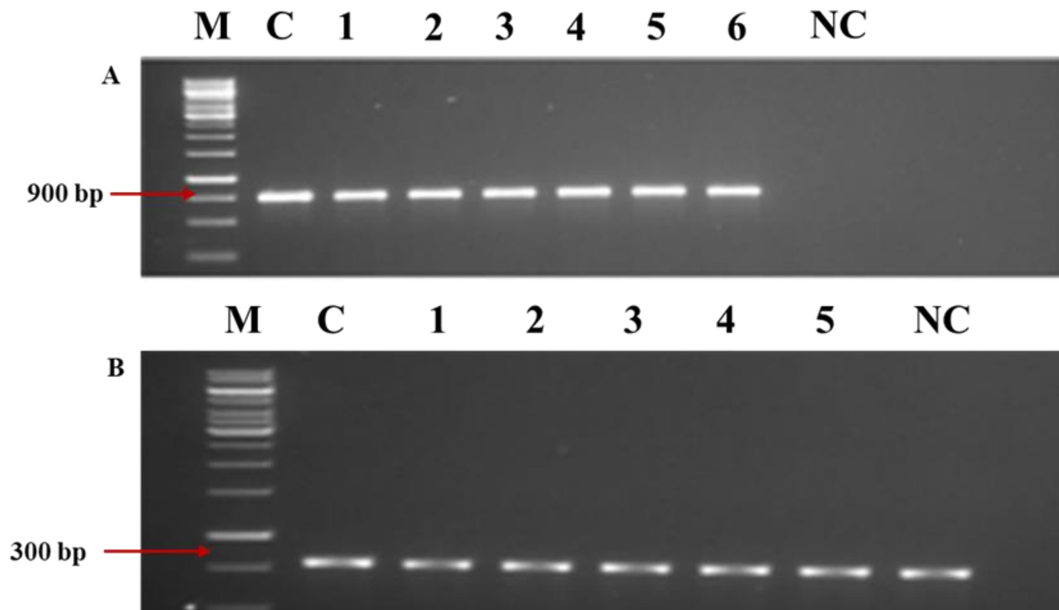

Figure 4.6: RT-PCR analysis of putative TMS 60444 cassava transgenic lines; A: RT-PCR analysis using Cas9 specific primers; B: PT-PCR analysis using ACT specific primers; C, positive control (Plasmid); NC, negative control (non-transformed regenerated plant); M is molecular marker.

4.3 Linamarin quantification and total cyanide determination

4.3.1 HPLC linamarin measurement from in vitro plantlets

The quantities of linamarin in the leaves of edited TMS 60444 plantlets were measured using high-performance liquid chromatography (HPLC), with age-matched wildtype plantlets acting as controls. The linear response graph curve was created using the peak regions from the HPLC analysis of the linamarin standard. The crude linamarin concentration was determined using the peak area values from the calibration curve (Figure 4.8), which were taken from the linamarin standard's HPLC reading. Linamarin concentrations were from 0.496 to 0.731g/kg fresh weight in transgenic crude extract, while non-transgenic values ranged from 2.405 to 3.143g/kg fresh weight (Table 4.3). In

comparison to transgenic lines, which had average linamarin levels of 0.650.03 g/kg fwt, non-transgenic cassava had high levels of 2.720.17 g/kg fwt (Figure 4.9: Table 4.4).

Table 4.3: Linamarin concentration of both transgenic and non-transgenic TMS 60444 cassava

Linamarin concentration (g/kg fresh weight)								
	Transgenic lines							
	TMS 1	**TMS 2**	**TMS 3**	**TMS 4**	**TMS 5**	**TMS 6**	**TMS 7**	**TMS 8**
Average	0.71±0.003	0.71±0.002	0.73±0.003	0.645±0.003	0.666±0.068	0.583±0.028	0.496±0.001	0.624±0.002
	Non-transgenic lines							
	TMS9	**TMS10**	**TMS11**	**TMS12**	**TMS13**	**TMS14**	**TMS15**	**TMS16**
Average	2.405±0.004	2.465±0.045	3.143±0.002	2.841±0.004	2.379±0.008	3.062±0.006	2.737±0.005	2.648±0.002

The results shown are for both mutant plants and wildtype cassava, and are represented as mean (± standard deviation of the mean).

Table 4.4: Concentrations ((g)/Kg cassava fresh weight) of linamarin from crude extracts of cassava leaf tissues using 0.25 M sulfuric acid

Plant	No of plants	Linamarin concentration	Df	P value	Summary
Transgenic	8	$0.65^{a} \pm 0.03$	14	<0.001	****
Non-transgenic	8	$2.71^{b} \pm 0.17$			

The stated results are for mutant plants and are given as mean (standard error). According to the student's t-test, the values in the same column designated by different superscripts differ considerably ($p<0.005$).

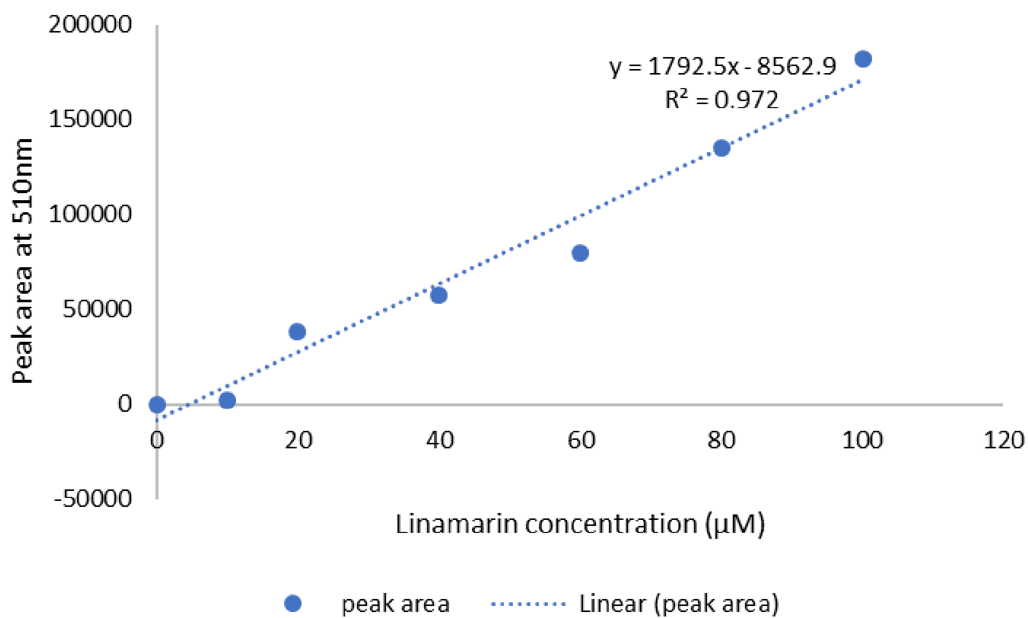

Figure 4.7: Linear response graph for the linamarin standard prepared using authentic Linamarin Sigma Aldrich

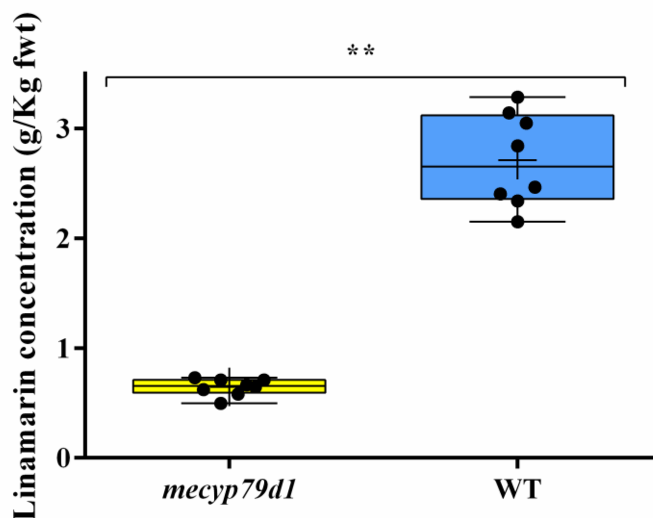

Figure 4.8: Total crude linamarin concentration of 0.25M sulfuric acid of freshly ground cassava leaves. Asterisks indicates statistically significant difference (p<0.05).**

4.3.2 Cyanide measurement from transgenic cassava plants

To determine the total cyanide concentration in the leaves, the picrate test was performed on both transgenic and wild type TMS 60444 cassava plantlets (Bradbury et al., 1999). Cyanide levels in the leaves of all transgenic cassava plants were considerably lower when the MeCYP79D1 gene was deleted ($p<0.05$) (Table 4.6). Cyanide levels in transgenic cassava ranged between 9.90 and 49.90 mg/kg fresh weight, while values in non-transgenic cassava were between 199.96 and 201.96 mg/kg fresh weight (Table 4.5). Non-transgenic plants had cyanide levels that surpassed 200 mg/kg fresh weight, with a mean of 201.0±0.99 mg/kg fwt. Cyanide levels averaged 17.44±2.482 mg/kg fwt across all transgenic genotypes (Figure 4.10).

Table 4.5: Total cyanide content (mg/kg fwt) in cassava leaves of both mutant and wildtype TMS 60444 cassava cultivars

Cyanide content in cassava leaves								
	Transgenic lines							
	TMS 1	TMS 2	TMS 3	TMS 4	TMS 5	TMS 6	TMS 7	TMS 8
Average	9.98±0.005	49.9±0.082	19.8±0.005	20.2±0.008	19.8±0.014	9.9±0.033	20.2±0.008	29.7±0.086
	Non-transgenic lines							
	TMS9	TMS10	TMS11	TMS12	TMS13	TMS14	TMS15	TMS16
Average	199.98±0.002	201.96±0.034	197.43±0.009	209.06±0.006	194.73±0.093	184.89±0.085	208.37±0.004	211.76±0.021

The results shown are for mutant plants and are represented as mean (± Standard deviation).

Table 4.6: The total cyanide content (mg/kg fwt) in leaves of both transgenic and non-transgenic cassava

Line	No of plants	Cyanide content in leaves	Df	P value	Significance *P*
Transgenic	8	17.44^{a} ±2.482	14	<0.001	***
Non-transgenic	8	201.0^{b} ±0.990			

The results displayed are for mutant plants and are reported as mean (standard error of the mean). By student's t-test, the values marked with various superscripts in the same row are substantially different (p<0.005).

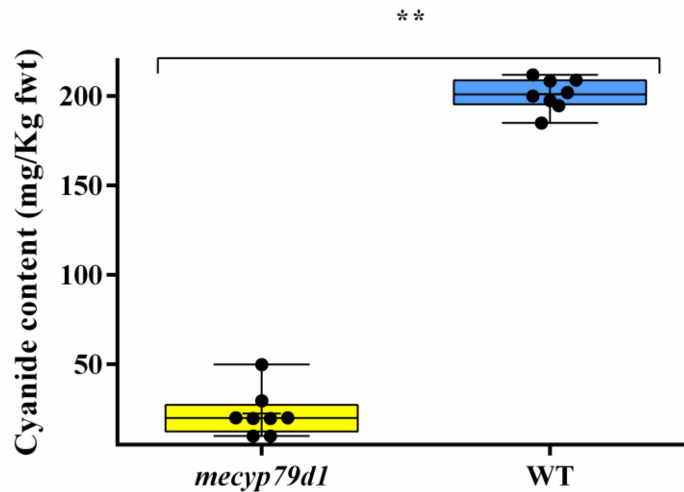

Figure 4.9: Total cyanide content (g/Kg fwt) extracted using picrate kit from fresh cassava leaves. Asterisks indicates statistically significant difference (*p<0.05).

4.3.3 Agronomic traits characterization

Plant height, leaf length, leaf breadth, petiole length, and the number of leaves per plant were measured in transgenic and wild type cassava to see if mutations in the MeCYP79D1 gene affected these agronomic characteristics (Figure 4.12). In both transgenic and non-transgenic plants, all of the agronomic features assessed were indistinguishable (Figure 4.12). Insects attacked transgenic cassava lines despite no visible variation in any of the examined agronomic metrics (Figure 4.11A and C). After being sprayed with insecticides to eliminate the insects, the transgenic plants grew well and had thinner stems than the non-transgenic lines (Figure 4.11B and D).

Figure 4.10: Agro-morphological traits of TMS 60444 cassava genotype in the greenhouse. A; 4 months old transgenic cassava B; 6 months old transgenic cassava. C; 4 months old non-transgenic cassava. D; 6 months old non-transgenic cassava.

The students' t-test performed on the data obtained from the agronomic traits at confidence level of 95% in GraphPad prism revealed that none of the transgenic cassava had significant difference from the wildtype counterparts under normal conditions in the green house with regards to the agronomic traits investigated (Table 4.7**).** The mean plant height (cm) in transgenic plants was not significantly different from that of the non-transgenic plants (Figure 4.11A). These findings were consistent with measurements of petiole length, leaf length-to-width ratio, and number of leaves per plant in both transgenic and wildtype cassava plants (Figure 4.11B, C and D).

Table 4.7: Comparison of the agronomic traits of transgenic TMS 60444 cassava plants

Cassava line	Plant height (cm)	Petiole length (cm)	Leaf length/leaf width (cm)	No of leaves per plant (cm)
Transgenic	40.00 [a] ± 2.31	5.69 [b] ± 0.38	3.30 [c] ± 0.08	6.37 [d] ± 0.38
Wild type	36.00 [a] ± 4.92	3.77 [b] ± 1.90	2.96 [c] ± 0.31	7.00 [d] ± 0.58

The results are shown for mutant plants and are represented as the mean ± SEM. The values marked with the same letters in the same column are non-significantly different ($p<0.05$, Student's t-test).

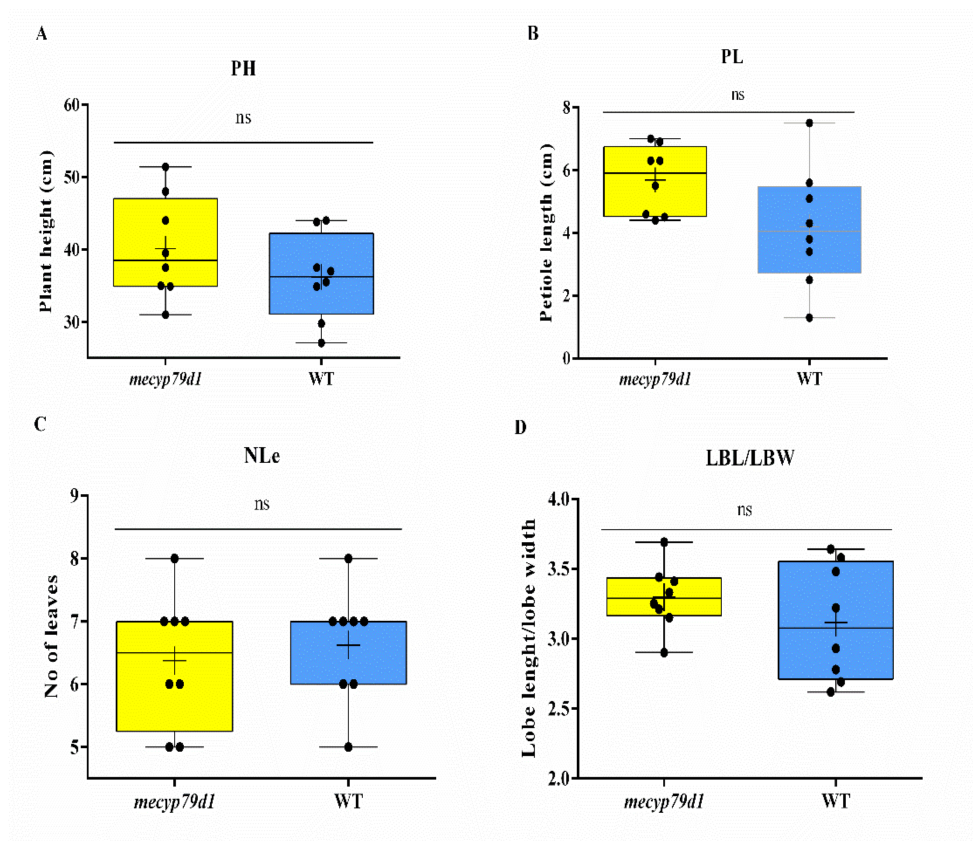

Figure 4.11: Agronomic traits for four months old cassava plants: A; Plant height B; Length of petiole C; The number of leaves per plant D; The ratio of lobe length to lobe width. Measurement in cm. The abbreviation (ns) indicates the values are not significantly different ($p<0.05$).

CHAPTER FIVE

DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

5.1 Discussion

5.1.1 *Agrobacterium* delivery of CRISPR/Cas9 system

Cassava is generally considered important stable food security owing to its ability to survive in infertile soils, unpredictable climatic conditions and ability to in the soil for up to 2 years without going bad (Tomlinson et al., 2018). The ability to change the plant genome to make mutant plants is critical for fundamental functional genomics and molecular breeding studies. Conventional plant breeding technologies rely on spontaneous or chemical, physical, and biological mutagenesis, which has a high rate of unpredictability and restrictions, making them unsuitable for large-scale genomic research and molecular design breeding (Fei et al., 2016). The advent of CRISPR/Cas9 gene editing technology in the last decade has allowed researchers to edit and knock off plant target genes quickly, easily, and conveniently, as well as generate mutants (Chen et al., 2019). Despite the fact that CRISPR/Cas9 genome editing technology has become one of the most potent tools for basic research and plant trait improvement, there have been few studies on its application for trait improvement and biotic stress resistance in tropical cassava (Juma et al., 2021). With efficient and successful cassava genome editing, new solutions for overcoming biotic and abiotic constraints on cassava production and post-harvest usage may emerge (Odipio et al., 2017).

The CRISPR/Cas9 technology was used to target the cassava CYP79D1 gene, which encodes for a key enzyme in biosynthesis. The CRISPR/Cas9 system was utilized to specifically target a portion of the CYP79D1 gene in cassava using a single guide RNA. An existing genetic transformation technology was used to insert T-DNA generated by the CRISPR/Cas system into embryogenic cells (Odipio et al., 2017; Syombua et al., 2021). By delivering CRISPR/Cas9 components primarily by cassava transformation mediated by *Agrobacterium tumefaciens* and antibiotic selection, eight distinct positive transgenic plantlets were generated. This is due to the low frequency of transgene

silencing in *Agrobacterium transfection*, which frequently results in straightforward gene insertion events of the transfer DNA (T-DNA) sequence in a binary plasmid (Wang et al., 2016). The pCRISPR/Cas9-MeCYP79D1 plasmid was delivered into leaf explants in the study using the *A. tumefaciens* strain GV3101 since it has a high transformation rate of up to 65% (Chetty et al., 2013). The study describes an *Agrobacterium*-mediated approach for transforming TMS 60444 plant cultivars with *A. tumefaciens* strain GV3101 carrying the pCRISPR/Cas9-MeCYP79D1 plasmid with *hptII* selectable marker to aid selection of transgenic cassava plants using hygromycin antibiotic.

Cassava regeneration frequency and transformation efficiency were both low in the current study, ranging from 5.91 to 6.82% and 1.33 to 2.0%, respectively. In the case of TMS 604444, Syombua et al. (2021) likewise demonstrated a similar transformation frequency of 0.5%. The observed low efficiency could be attributed to calli selection stress induced by the antibiotics hygromycin and timentin. Because hygromycin and timentin are both extremely toxic to cassava tissues, they were also used in the current study to identify altered tissues (Nyaboga et al., 2015). Additionally, the embryo's slow growth during the globular stage, as well as the transition from the torpedo to the cotyledonary stage, may be a contributing factor to the low regeneration rates and efficiency (Ochatt and Revilla, 2016).

As previously noted by Odipio et al. (2017), the source and type of explant is a critical element in somatic embryogenesis. The study describes a simple and efficient stable *Agrobacterium*-mediated transformation strategy for the immature leaf lobes of the TMS 60444 cassava cultivar, followed by transformed plants' recovery. The study's findings confirm that immature leaf lobes are excellent explants for cassava transformation. Previous study by Syombua et al., (2019) reported that leaf explants exhibited highest transient transformation (55 – 73%) and thus superior to embryogenic callus (24 – 52%) for cassava transformation. On the contrary, earlier research has extensively documented on the utilization of axillary bud and friable embryogenic calli (FEC) as explants for

transformation. The use of axillary for somatic embryo production is efficient, occurs quickly, and produces a high frequency of somatic embryos; nevertheless, axillary buds must be cultured to develop FECs, which are subsequently used for transformation (Nyaboga et al., 2015). FECs can take up to a year to be obtained using the FEC-based transformation (Utsumi et al., 2017). The increased transformation and embryogenic callus efficiency observed in leaf explants could be attributed to the leaf's flat shape, which ensures that a large surface area of the leaf is in touch with the medium (Syombua et al., 2019). Furthermore, because leaves are easily programmed to dedifferentiate into undifferentiated cells and are less lignified, they are more receptive to plant regulating hormones (Ubalua et al., 2018). The findings also suggest the use of hygromycin as an antibiotic for the selection of potentially altered plants, as well as timentin for preventing *Agrobacterium* bacterium growth throughout the transformation process. The use of hygromycin as a selective marker resulted in the creation of only plants with T-DNA incorporated into their genomes. This observation corroborates the results recorded by Utsumi et al. (2021), which showed that hygromycin antibiotics concentrations of 10 mg/l for first selection and 20 mg/l for second selection only resulted into generation of cassava plants with incorporated T-DNA.

The coordinated development and maturation of embryos formed from somatic cells, followed by their conversion into complete plants, is the basis for plant regeneration from somatic embryos. As indicated in the study's findings, these processes occur as embryos evolve from the pro-embryo stage to the globular, heart, torpedo, and cotyledonary phases. However, somatic cell division into embryos and subsequent plant regeneration are periodically impeded by embryo growth ending during the globular stage or during the transition from torpedo to cotyledonary stage. This study discovered that brief exposure of SEs to activated charcoal stopped the recalcitrance of embryo growth past the torpedo stage. Mathews et al. (1993) also reported that the desiccation of SEs before culture on cassava rooting medium enhances embryo conversion. During embryo desiccation in activated charcoal, *Agrobacterium* overgrowth was discovered, posing a considerable

barrier to the investigation. The activated charcoal absorbs not just phenolics but also antibiotics, such as timentin, which was employed to inhibit *Agrobacterium* growth and select the putative transgenic calli.

5.1.2 Molecular analysis *MeCYP79D1*-induced cassava mutants

The pCRISPR/Cas9-MeCYP79D1 transgene was successfully inserted into the cassava genome, according to PCR and RT-PCR data on putative transgenic lines of the TMS 60444 cultivar. The eight TMS 60444 pCRISPR/Cas9-MeCYP79D1 transgenic lines' RT-PCR assays demonstrated that the mRNA was expressed. The findings demonstrated that the genetic transformation strategy used in this work has a high replication potential for usage in refractory cassava genotypes. This CRISPR/Cas9 gene editing phenomena for the knockout of the MeCYP79D1 gene in cassava is consistent with the reported knockout of many other genes in cassava, including phytoene desaturase (Odipio et al., 2017), viral AC2 gene (Mehta et al., 2018), protein targeting to starch 1(PTST-1) and granule-bound starch synthase (GBSS) (Bull et al., 2018) , 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Hummel et al., 2018), multiple TFL 1-like floral repressor (Odipio et al., 2018), eukaryotic translation initiation factor 4E (elF4E) isoforms (Gomez et al., 2018), methylesterase 3 (MeSIII) (Li et al., 2020) and MeSWEET10a (Veley et al., 2021).

Transgenic and non-transgenic TMS 60444 cultivars were analyzed molecularly, with targeted sanger sequencing done on each plant line to detect mutations within the MeCYP79D1 target gene. The DNA sequence results showed that all eight of the examined regenerated plants (100%) exhibited INDELS and substitutions. Deletion happened 3 bp upstream from the PAM site in every line with an INDEL, and 4 bp deletions were recorded (Juma et al., 2022). The deletions seen here may have resulted from NHEJ repair after pCRISPR/Cas9-MeCYP79D1 integration, as was previously shown in cassava following CRISPR/Cas9-targeted modification of the PTST-1 and GBSS genes (Bull et al., 2018), MeSIII gene (Li et al., 2020) and elF4E isoforms (Gomez et al., 2018). The detection of a single nucleotide alteration downstream of the targeted

area implies that the MeCYP79D1-induced DSBs were repaired via activating the HDR pathway. In apple and cotton, the simultaneous activation of the HDR and NHEJ repair pathways was previously noted with the addition of CRISPR/Cas9 tools (Li et al., 2013: Gao et al., 2017). Taken together, the findings suggest that cassava uses the NHEJ mechanism to repair mutations generated by CRISPR/Cas9 rather than the HDR route. The NHEJ repair mechanism was found in the transgenic plants' sequenced genomic DNA because it is active throughout the cell cycle and does not require a template, as opposed to HDR, which is only active during the S and G2 phases of the cell cycle because it requires the sister chromatid as a template (Matsumoto et al., 2020).

The CRISPR/Cas9 system has evolved into a strong approach for precise targeted gene editing over the last ten years. It was used in this study to delete the MeCYP79D1 gene, and it efficiently induced mutations while being simple to use. The high efficiency of the target gene mutation can increase cassava plant gene function. However, traditional approaches used in the past have proven to be less effective and time-consuming (Heuberger, 2005; Lentz et al., 2018).

According to the findings of this study, the CRISPR/Cas9 gene-editing technique could be used to reduce cyanogen levels in cassava for farmers and consumers. However, the crop's ability to modify genes using CRISPR/Cas9 must be improved. Some of the exceptional efficiency described here is likely due to the combination of CRISPR/Cas9 and gRNA cassettes. The editing tools' ability to make modifications in the target region is enhanced by their continuous expression. While this approach is useful for basic research, it is not optimal for creating enhanced cassava germplasm that farmers will appreciate. This is possible by sexual crossing of transgenic cassava plants with farmer-favored cassava cultivars. As a result, the CRISPR/Cas9 system-incorporated T-DNA is eliminated, and the favorable features are transferred on to farmer-favored cassava cultivars. Despite sexual crossing can be employed to eliminate integrated T-DNA from cassava, it is not advised due to the crop's heterozygous nature and the resulting in-

breeding depression (Juma et al., 2022). As a result, it is critical to develop cassava genome-editing capabilities that are not reliant on CRISPR approaches. Direct distribution into totipotent cells and the use of ribonucleoprotein as an alternative to *Agrobacterium*-mediated transgenesis are two such approaches. Cpf1 and other Cas mutations are becoming more common.

5.1.3 Cyanogenic potential of *MeCYP79D1* edited plants

The amounts of linamarin in the leaves of transgenic cassava plants in the greenhouse were measured by HPLC, with age-matched wildtype plants serving as the positive control. Linamarin concentrations in transgenic plants were significantly lower than in wildtype plants ($p<0.05$). The wild type cassava had higher linamarin than the Food and Agriculture Organization's (FAO) allowed limit (10 g/kg fresh) (FAO, 2021). Due to the fact that just one gene, CYP79D1, encodes the enzyme required for catalysis of the first step (dedicated stage) of linamarin formation in cassava, the cyanogenic glycosides linamarin levels were not completely eliminated in transgenic plants (Gunasekera et al., 2018).

Two genes in the paleotetraploid plant cassava encode the CYP79D1/D2 enzyme. The genes CYP79D1 and CYP79D2 encode valine monooxygenase I and II, which are responsible for the initial phase of cyanogenic glycoside production in cassava (Bredeson et al., 2016). This pair of genes share 85% of their DNA. Only CYP79D1 was deleted, and the mean values of the genome edited lines were compared to the mean values of the age-matched wildtype accessions, yielding a three- to seven-fold drop in cyanide concentration. This is in line with prior research that employed RNAi technology to create CYP79D1 function defects in cassava, resulting in up to three times lower cyanide concentrations than cassava grown in the wild (Jørgensen et al., 2005; Piero, 2013; Siritunga and Sayre, 2003). When CRISPR/Cas9 was utilized to target CYP79D1 in *P. pastoris,* similar abnormalities were seen as reported by Jiang et al., (2021). According to the present study's findings, utilizing CRISPR/Cas9 gene editing to knock off the

CYP79D1 gene is considerably more successful and simpler than RNAi technology, which was previously used to target the same gene (Jørgensen et al., 2005; Piero, 2013; Siritunga and Sayre, 2003). The CRISPR/Cas9 technology makes it possible to knock out a gene in cassava with greater accuracy and speed without affecting other cassava features (Juma et al., 2022).

The cyanogenic values of known cassava varieties range from less than 10 mg/kg fwt to more than 500 mg/kg fwt, corresponding to classification as sweet or bitter cassava. The total amount of linamarin found in the cassava leaves of putative transgenic plants studied did not exceed the limit. According to the FAO, the safe cyanogenic level for eating is 10 mg/kg body weight (Cumbana et al., 2007; FAO, 2020). Linamarin quantitation was chosen for this work since prior research indicated it to be the most abundant metabolite among other cyanogenic glycosides. This is because linamarin is a precursor to a variety of cyanogenic glycosides, such as cyanohydrin and hydrogen cyanide. Even at different storage temperatures, it is more stable than other cyanogenic glycosides (Bolarinwa et al., 2016).

A picrate test was used to assess total cyanide levels in the leaves of wildtype and mutant TMS 60444 in addition to linamarin quantification (Bradbury et al., 1999). Four months after the plants were moved to the potted soil in the greenhouse, this test was carried out. Results from the determination of the cyanide content and linamarin concentration were equivalent. Transgenic plants showed up to seven times lower cyanide contents than their wildtype counterparts, which supports prior findings by Taylor et al., (2012) and Piero et al., (2013).

Moreover, with the severity of drought increasing due to climate change, the capacity to reduce cyanogenic glycoside levels in farmer-favored cassava cultivars to acceptable levels is critical (Ospina et al., 2021). As a result, in the future, this technique will serve as a springboard for the generation of farmer-favored cassava with little to no cyanogenic

glycosides. As a consequence, agriculture and preparation practices will very certainly be modified to accommodate these low levels. Farmers and consumers who grow acyanogenic or low cyanide cassava varieties would not be required to adjust their practices if environmental conditions favored cyanogenesis (Juma et al., 2022).

Reduced cyanogen concentration in cassava has the opportunity to assist both cassava farmers and consumers while also benefiting the environment. Cassava detoxification can take several days, however acyanogenic cassava may assist speed up the process and save time (Zhong et al., 2021). At an industrial scale, processing acyanogenic cassava would not release cyanide into wastewater, reducing wastewater treatment labor and costs while also reducing toxicity to local terrestrial and aquatic animals. Acyanogenic cassava variants may promote safety of food and consumer health. Excessive cyanide consumption combined with a protein-deficient diet can cause brain damage, including motor competence and cognitive function loss, as well as paralysis in severe cases. Acyanogenic cassava may be capable of preventing these severe diseases, allowing at-risk customers and potential consumers to focus on other matters.

5.1.4 Agro-morphological traits of MeCYP79D1 edited plants

Measurements of the plant height, the ratio of leaf lobe length to leaf lobe breadth, the petiole length, and the number of leaves per plant were used to analyze the agronomic features of both wildtype and transgenic cassava plantlets in the greenhouse environment. According to the results, the transgenic plants' investigated agro-morphological features could not be distinguished from those of wild-type plants (Figure 4.7), which was consistent with earlier findings by Veley et al. (2021).

Notwithstanding the fact that the vast majority of the investigated features in transgenic and non-transgenic plants were comparable, discrepancies were observed. These discrepancies could have been induced by environmental shock and/or herbivore predation. Herbivores, whiteflies, and aphids attacked transgenic plants' leaves and roots

but not non-transgenic plants (Brandt et al., 2020; Mikkelsen and Halkier, 2003). This could be due to a change in the linamarin biosynthesis pathway, which contributes to the plant's defense against herbivores by producing hydrogen cyanide when cyanogenic glycosides interact with their associated enzymes during herbivore attack. This is consistent with the findings published by Siritunga and Sayre, (2004), who reported that cyanogenic glycosides act as insect repellants in cassava. Additionally, the stems of the transgenic plants grew thinner than those of the non-transgenic plants. This characteristic results from a modification in the production of cyanogenic glycosides (Simonsen et al., 2017). Linamarin is utilized to biosynthesize the amino acid aspartate, which is then delivered to the roots of a healthy plant as a translocable form of reduced nitrogen. This encourages the growth of big roots and stems (Echeverry-Solarte et al., 2013; Simonsen et al., 2017).

5.2 Conclusions

CRISPR/Cas9 was used to investigate the potential functions of the CYP79D1 gene in the production of cyanogenic glycosides in cassava. The research advances our understanding of the CYP79D1 gene's critical function in the generation of cyanogenic glycosides, as well as the importance of the secondary metabolites that arise. T-DNA produced from the pCRISPR/Cas9-MeCYP79D1 construct can be used to grow cassava plantlets, according to the findings.

This study shows how cassava CYP79D1 gene knockouts are effective at reducing cyanogenic glycoside levels. Mecyp79d1 cassava plants exhibited lower amounts of cyanogenic glycosides, and were more susceptible to insect pests, and had slimmer stems than wild-type cassava plants. The results of the study demonstrate that cyanogenic glycosides are not completely eliminated by cassava CYP79D1 gene knockdown. The study conclusively shows that the bulk of the agronomic characteristics of cassava plants are unaffected by changing the production of cyanogenic glycosides.

Furthermore, it demonstrates that precisely altering the CYP79D1 gene and determining its function using CRISPR/Cas9 is doable. Thus, our findings could pave the way for greater CYP79 gene editing in cassava, improving plant biology and contributing in the resolution of food security issues.

6.2 Recommendations and Further studies

1. The CRISPR/Cas9 gene editing system established in this study should be replicated and optimized in other farmer-preferred cassava cultivars in Kenya. The use of other explants' such as stems, axillary bud and nodes for infection should also be explored.
2. Efforts should be made to achieve delivery of CRISPR/Cas9 editing constructs into the genome of cassava using nucleoproteins and nanoparticles.
3. Further analysis and evaluation, particularly in the field conditions, should be done to confirm the conformity of and genetic stability of acyanogenic cassava plants.
4. Stability of transgenes over several generations should be established.
5. Conventional breeding be carried out through sexual crossing to produce transgene-free cassava plants with knocked out CYP79D1 gene.

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APPENDICES

Appendix I: Hormone preparation

2, 4-dichlorophenoxyacetic acid (2, 4-D)

A stock solution of 10 mg/l of 2,4-D was prepared by weighing 0.5 g of 2,4-D (Duchefa company) dissolving it in 1ml of 70 % ethanol in a falcon tube and sufficient amount of sterile distilled water added to make 10 ml of stock solution. The solution was then filter sterilized using a syringe and 0.2 µm filter, aliquoted in 1.5 ml eppendorf tubes and stored at 4 °C.

α -Naphthaleneacetic acid (NAA)

A stock solution of 1 mg/l of NAA was prepared by weighing 0.05 g of NAA (Duchefa Company) which was dissolved in 1 ml of 1N sodium hydroxide (NaOH) and topped up to 50 ml with distilled water. The stock solution was aliquoted into sterile 1.5 ml eppendorf tubes and stored at 4 °C.

Benzyl aminopurine (BAP)

A 1 mg/l stock solution of Benzyl aminopurine (BAP) was prepared by weighing 0.05 g of BAP (Duchefa company) which was dissolved in 1 ml of 1N sodium hydroxide (NaOH) and topped up to 50 ml with distilled water. The stock solution was aliquoted into sterile 1.5 ml eppendorf tubes and stored at 4 °C.

Gibberellic acid (GA_3)

A stock solution of 0.5 mg/l GA_3 was prepared by weighing 0.025 g of GA_3 (Duchefa company) dissolved in 2 ml of 70 % ethanol, made up to 50 ml with distilled water and filter sterilized using a syringe and a 0.2 µm ministart filter (Sartorius company) into a sterile falcon tube before aliquoting into 1.5 ml sterile eppendorf tubes and stored at 4 °C.

Appendix II: Preparation of antibiotics

Kanamycin

A stock solution of kanamycin (100 mg/l) was prepared by weighing 1 g of kanamycin (Duchefa Company) that was dissolved in sterile distilled water to make 10 ml in falcon tube. The solution was filter sterilized using a syringe and 0.2 µm filter into a sterile falcon tube before aliquoting into sterile falcon tube before aliquoting into sterile 1.5ml eppendorf tubes and stored at 4 °C.

Acetosyringone (200 mM)

200 mM acetosyringone was prepared by dissolving 392 mg of 3’5’dimethoxy-4’-hydroxy-acetophenone in 1ml of 70 % EtOH. The volume was topped up to 10ml with dd H2O. The solution was then filter sterilized using a syringe and 0.2 µm filter into a sterile falcon tube before aliquoting into sterile 1.5 ml eppendorf tubes and stored 4 °C.

Gentamycin

A stock solution of 10 mg/l of gentamycin sulfate was prepared by weighing 10.8 g of gentamycin (Duchefa Company) that was dissolved in 10 ml of sterile distilled water in a falcon tube. The solution was filter sterilized using a syringe and 0.2 µm filter into a sterile falcon tube before aliquoting into sterile 1.5 ml eppendorf tubes and stored 4 °C.

Rifampicin

100 mg/l rifampicin was prepared by weighing 0.5 g of rifampicin (Duchefa Company) that was dissolved in 10 ml of 100% methanol to dissolve completely. The solution was filter sterilized using a syringe and 0.2 µm filter into a sterile falcon tube before aliquoting into sterile 1.5 ml eppendorf tubes and stored 4 °C.

Hygromycin

A stock solution of 10 mg/l of hygromycin B was prepared by dissolving 0.5 g of hygromycin (Duchefa Company) in 1 ml methanol. The volume was topped up to 10 ml by sterile distilled water. The solution was filter sterilized using a syringe and 0.2 µm filter into a sterile falcon tube before aliquoting into sterile 1.5 ml eppendorf tubes and stored 4 °C.

Timentin

A stock solution of 100 mg/l of timentin (ticarcillin disodium and potassium clavulanate) was prepared by weighing 1 g of timentin (Duchefa Company) that was dissolved in 10 ml of sterile distilled water. The solution was filter sterilized using a syringe and 0.2 µm filter into a sterile falcon tube before aliquoting into sterile 1.5 ml eppendorf tubes and stored 4 °C.

Appendix III: Media preparation and culture conditions

Micropropagation media

Micropropagation media was prepared containing MS salts with vitamins (Duchefa) Murashige and Skoog, 1962, with 3 % (w/v) sucrose and the pH adjusted to 5.8 before adding 0.3 % (w/v) Gelrite. The media was autoclaved at 121 °C for 20 minutes, cooled and about 50 ml poured in sterile culture bottles under aseptic conditions.

Selection media

Callus initiation media was prepared containing MS salts, Gamborg B5 vitamins, 100 mg/l myo-inositol, 0.2 % sucrose, 0.5 mg/l CuSO4, 50 mg/l casein hydrolysate supplemented with 2, 4-dichlorophenoxyacetic acid, 10 mg/l hygromycin, 15 mg/l timentin, and the pH adjusted to 5.8 before adding 0.3 % (w/v) Gelrite. The media was autoclaved at 121 °C for 20 minutes, cooled and about 25 ml poured in sterile 90×10 mm sterile Petri plates under aseptic conditions.

Maturation media

Maturation media was prepared containing MS salts, Gamborg B5 vitamins 0.2 % sucrose, 0.01 mg/l NAA (α Naphthaleneacetic acid), 1 mg/l BAP (6- Benzyl aminopurine), 10 mg/l hygromycin, 15 mg/l timentin and the pH adjusted to 5.8 before adding 0.3 % (w/v) Gelrite. The media was autoclaved at 121 °C for 20 minutes, cooled and 0.5 mg/l Gibberellic Acid (GA_3) was added. About 25 ml was poured in sterile test tubes under aseptic conditions.

Activated charcoal media

Activated charcoal media (0.8 %) was prepared containing 2 % sucrose, MS salts and B5 vitamins and the pH adjusted to 5.8 before adding 0.3 % (w/v) Gelrite. The media was autoclaved at 121 °C for 20 minutes, cooled and about 50 ml was poured in sterile culture bottles under aseptic conditions.

Yeast Extract Peptone

A litre of solid Yeast Extract Peptone (YEP) medium was prepared containing 10 g yeast extract, 10 g bacto peptone, 5 g NaCl and 15 g of bacto agar. The pH was adjusted to 7.0 and the media was autoclaved at 121 °C for 20 minutes. ultrafilter sterilized kanamycin 100 mg/l, gentamycin 100 mg/l and rifampicin 10 mg/l were added, cooled then about 25 ml was poured in sterile petriplates under aseptic conditions. Bacto agar was not added into liquid media.

Appendix IV: Buffer compositions and preparations

CTAB Buffer preparation

CTAB powder, Tris HCl, EDTA, NaCl

Weigh 20 g of CTAB and pour into a 1 L bottle. Add 100 ml of 1M Tris HCl (pH 8.0) followed by addition of 40 ml of 0.5M EDTA. Add 280 ml of 5M NaCl, and top up to 1L by addition of double distilled water.

1X Tris-EDTA

Weigh 15.759g of Tris Cl. Add 800 ml of distilled water, followed by addition of 2.92 g of EDTA to the solution. Add distilled water until the volume is 1L.

50X TAE Electrophoresis buffer

Contents and storage

Contents	**Amount**	**Storage**
50X TAE Electrophoresis Buffer	1 liter	15 °C to 25 °C

1X Buffer Composition

40 mM Tris, 20 mM acetic acid, 1 mM EDTA.

Applications

Nucleic acid agarose and polyacrylamide gel electrophoresis.

Agarose and polyacrylamide gel preparation.

Note

Buffer concentrate should be diluted to a working concentration of 1X before use.

For each electrophoresis fresh 1X buffer should be used.

RNeasy Mini Kit RNA extraction

A maximum of 100 mg of the leaf tissues were harvested and crushed in to fine powder in liquid nitrogen. Appropriate volume of Buffer RLT. The lysate was then centrifuged for 3 minutes at maximum speed and the supernatant carefully removed by pipetting. 1 volume of 70% ethanol was then added to the lysate, and mixed well by pipetting. 700 ul of the sample was transferred to a RNeasy Mini spin column placed in a 2 ml collection tube. The lid was closed and centrifuged for 15 s at >8000 x g. the flow-through was then discarded. 700 ul of Buffer RW1 was then added to RNeasy spin column, the lid closed and centrifuged for 15 s >8000 x g. the flow-through was then discarded. 500 ul of Buffer RPE was then added to the RNeasy spin column, lid closed and centrifuged for 15 s >8000 x g and the low-through discarded. The RNeasy spin column was placed in a new 1.5 ml collection tube and 50 ul of RNase-free water was added directly to the spin column membrane, and the lid closed and centrifuged for 1 min at >8000 x g to elute the RNA.

Linamarin extraction

Weigh 5 g of young cassava leaves and homogenize immediately with 25 ml of pre-chilled 0.25 M sulfuric acid for 14 seconds at low speed, followed by 1 minute (2X) at high speed in a blender. Filter the homogenates through a filter paper to remove insoluble materials. The homogenizer jar also rinsed with 40 ml of the acid and filtered the same way. Centrifuge the filtrates at 10000 revolutions per minute for 10 minutes, at 4 °C. collect the clear supernatant fluid and store at -20 °C until use.

Appendix V: Student's t-test results

Unpaired t test for cyanide content in leaves

Table Analyzed CyanideAbs

Column B Non-transgenic

vs. vs.

Column A Transgenic

Unpaired t test

P value< 0.0001

P value summary ****

Significantly different? (P < 0.05) Yes

One- or two-tailed P value? Two-tailed

t, df t=35.25 df=8

How big is the difference?

Mean ± SEM of column A 17.44 ± 2.482 N=8

Mean ± SEM of column B 201.0 ± 0.9900 N=2

Difference between means 183.5 ± 5.207

95% confidence interval 171.5 to 195.5

R square 0.9936

F test to compare variances

F,DFn, Dfd

P value

P value summary

Significantly different? (P < 0.05)

Unpaired student's t-test for linamarin concentration

Table Analyzed Linamarin concentration

Column B NoN-transgenic
vs. vs.
Column A Transgenic

Unpaired t test
P value < 0.0001
P value summary ****
Significantly different? (P < 0.05) Yes
One- or two-tailed P value? Two-tailed
t, df t=16.86 df=10

How big is the difference?
Mean ± SEM of column A 0.6461 ± 0.02780 N=8
Mean ± SEM of column B 2.714 ± 0.1726 N=4
Difference between means 2.067 ± 0.1226
95% confidence interval 1.794 to 2.341
R square 0.9660

F test to compare variances
F,DFn, Dfd 19.28, 3, 7
P value 0.0018
P value summary **
Significantly different? (P < 0.05) Yes

Unpaired student's t-test for the plant height

Table Analyzed Plant height

Column B Non-transgenic

vs. vs.

Column A Transgenic

Unpaired t test

P value 0.4444

P value summary ns

Significantly different? (P < 0.05) No

One- or two-tailed P value? Two-tailed

t, df t=0.7998 df=9

How big is the difference?

Mean ± SEM of column A 40.00 ± 2.307 N=8

Mean ± SEM of column B 36.20 ± 4.922 N=3

Difference between means -3.800 ± 4.751

95% confidence interval -14.55 to 6.948

R square 0.06635

F test to compare variances

F,DFn, Dfd 1.707, 2, 7

P value 0.4979

P value summary ns

Significantly different? (P < 0.05) No

Unpaired student's t-test for plant petiole length

Table Analyzed Petiole length

Column B Non-transgenic

vs. vs.

Column A Transgenic

Unpaired t test

P value 0.1537

P value summary ns

Significantly different? ($P < 0.05$) No

One- or two-tailed P value? Two-tailed

t, df t=1.558 df=9

How big is the difference?

Mean ± SEM of column A 5.688 ± 0.3833 N=8

Mean ± SEM of column B 3.767 ± 1.899 N=3

Difference between means -1.921 ± 1.233

95% confidence interval -4.710 to 0.8685

R square 0.2124

F test to compare variances

F,DFn, Dfd 9.199, 2, 7

P value 0.0220

P value summary *

Significantly different? ($P < 0.05$) Yes

Unpaired student's t-test for number of leaves per plant

Table Analyzed No of leaves per plant

Column B Non-transgenic

vs. vs.

Column A Transgenic

Unpaired t test

P value 0.4011

P value summary ns

Significantly different? ($P < 0.05$) No

One- or two-tailed P value? Two-tailed

t, df t=0.8813 df=9

How big is the difference?

Mean ± SEM of column A 6.375 ± 0.3750 N=8

Mean ± SEM of column B 7.000 ± 0.5774 N=3

Difference between means 0.6250 ± 0.7091

95% confidence interval -0.9792 to 2.229

R square 0.07945

F test to compare variances

F,DFn, Dfd 1.125, 7, 2

P value > 0.9999

P value summary ns

Significantly different? ($P < 0.05$) No

Unpaired student's t-test for the ratio of lobe length to lobe width

Table Analyzed Ratio of lobe length to lobe width

Column B Non-transgenic

vs. vs.

Column A Transgenic

Unpaired t test

P value 0.1631

P value summary ns

Significantly different? ($P < 0.05$) No

One- or two-tailed P value? Two-tailed

t, df t=1.519 df=9

How big is the difference?

Mean ± SEM of column A 3.298 ± 0.08213 N=8

Mean ± SEM of column B 2.963 ± 0.3090 N=3

Difference between means -0.3342 ± 0.2200

95% confidence interval -0.8319 to 0.1636

R square 0.2040

F test to compare variances

F,DFn, Dfd 5.308, 2, 7

P value 0.0791

P value summary ns

Significantly different? ($P < 0.05$) No