Induction of early flowering in cassava (*Manihot esculenta*) and *Nicotiana benthamiana* by overexpression of cassava *FLOWERING LOCUS T (MeFT1)*

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Declaration

This research project is my original work and has not been presented for a degree in any other university for examination.

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AtFT	*Arabidopsis thaliana* Flowering locus T
BLAST	Basic local alignment search tool
CAM	Cassava axillary media
CaMV35S	Cauliflower Mosaic Virus 35S promoter
CBM	Cassava basal media
CIM	Callus induction media
CMV	Cassava mosaic virus
CSPD	Chloro-3'-methoxyspiro[adamantane-4,4'-dioxetane]-3'-yl)phenyl] dihydrogen phosphate
Ct	Cycle threshold
CTAB	Cetyltrimethylammonium bromide
DPI	Days-post infiltration
EACMV	East African Cassava Mosaic Virus
FEC	Friable embryogenic callus
FT	Flowering locus T
GD	Greshoff and Doy media
HPT	Hygromycin phosphotransferase
IITA	International Institute of Tropical Agriculture
LB	Luria broth
MeFT1	*Manihot esculenta* Flowering locus T 1
MES	2-(N-morpholino) ethanesulfonic acid
MS	Murashige and Skoog media

NoST	Nopaline synthase terminator
OES	Organized embryogenic structures
PCR	Polymerase chain reaction
POI	Point of infiltration
Rep A	Replication initiator protein A
RPM	Revolutions per minute
RT-PCR	Reverse transcriptase- polymerase chain reaction
TFL1	Terminal flowering locus 1
VIF	Virus induced flowering
VIGS	Virus-induced gene silencing

Abstract

Flowering is a prerequisite in conventional plant breeding. In cassava, delayed and non-synchronous flowering makes conventional breeding difficult. This study aimed at accelerating flowering by overexpressing the endogenous cassava (*Manihot esculenta*) Flowering Locus T variant 1 (*MeFT1*) in cassava and *Nicotiana benthamiana*. The *MeFT1* gene was overexpressed through 1) agroinfiltration into *N. benthamiana* and four cassava cultivars using the Cassava Mosaic Virus (CMV)-based vectors, and 2) constitutive expression under the 35S promoter in the same cassava cultivars and *N. benthamiana* to serve as positive controls. Molecular characterization of the regenerated and agro-infiltrated plants was conducted using polymerase chain reaction (PCR), Southern blot hybridization, and reverse-transcription PCR (RT-PCR). Flowering characteristics, including flowering time and the number of flowers, were evaluated for the infiltrated *N. benthamiana* plants. Southern blot analysis confirmed successful viral delivery in both *N. benthamiana* and cassava and replication at the point of infiltration (POI) in *N. benthamiana*. Viral systemic movement was only observed in *N. benthamiana* and not in cassava. The agro-infiltrated *N. benthamiana* plants flowered earlier and had more branches and flowers than their controls. For the positive controls, the regenerated *N. benthamiana* plants tested positive for the transformed gene and flowered early at two weeks post-transfer to rooting media. Recalcitrance to transformation was also observed in the four cassava cultivars used in the study. Combined, the findings from this study report the use of the East African Cassava Mosaic Virus (EACMV) virus vector in gene delivery into *N. benthamiana* for functional trait characterization. Since flowering was not observed in the infiltrated and regenerated cassava plants despite the successful delivery of the vector, further studies on how to improve the vector structure and infiltration procedures are necessary to achieve a successful flowering induction in cassava.

CHAPTER ONE

Introduction

1.1 Background of the study

Cassava (*Manihot esculenta* Crantz) is not only considered an important food source but also considered the fourth most substantial industrial crop worldwide (FAOSTAT, 2017). Cassava serves as a staple food to approximately 800 million individuals worldwide (FAOSTAT, 2017). Though it is a perennial crop, cassava is widely grown as an annual crop in the tropical and subtropical regions of the world. Most cassava cultivars are characterized by long reproductive cycles (more than 10 months) associated with late and scarce flowering, a trait that is undesirable for crop improvement (Adeyemo *et al*., 2017). Conventional cassava breeding, which mainly relies on sexual reproduction, has generally been successful in generating cassava varieties with improved agronomic traits. This breeding approach, is however faced with various inherent cassava challenges including: i) delayed and non-synchronized flowering in the different breeding lines ii) heterozygosity of the cassava genome, iii) low fertility associated with pollens, iv) low seed production, and v) self-incompatibility which slow down cassava improvement efforts (Ceballos *et al*., 2015). Efforts to overcome the shortcomings limiting the success of conventional cassava breeding are being tested and include early flowering induction (Adeyemo *et al*., 2017; Odipio *et al*., 2020).

Flowering in cassava is tightly linked to the lateral branching of the stem. Floral nodes are usually formed at each branching point thus resulting in a direct association between the branch and flower numbers. That is, the higher the branching, the more the flowers are developed (Lentz *et al*., 2018b). Flowering in angiosperms is regulated by multiple genes that induce or suppress the universal flowering signal called florigen. The role of florigen in flowering induction has been

vastly studied in various plants, including the model plant *Arabidopsis thaliana* (Tsuji and Taoka, 2014). Four major pathways are associated with flowering in plants, namely autonomous, aging, photoperiod, vernalization, and gibberellin pathways. The autonomous pathway promotes flowering by repressing the flowering repressor, FLOWERING LOCUS C (FLC) (Cheng et al., 2017). Similarly, vernalization activates flowering by suppressing FLC in response to prolonged exposure to lower temperatures (Glover, 2007). Meanwhile, the photoperiod pathway induces flowering depending on the day length. Long-day plants require approximately 14 to 16 hours of daylight to induce flowering genes, while short-day plants require less than 12 hours. However, day-neutral plants express genes regardless of the daylight hours they are exposed to (Tsuji and Taoka, 2014). Gibberellin pathway independently promotes flowering by activating LEAFY (LFY), SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1), and FLOWERING LOCUS T (FT) (Bao et al., 2020) Flowering Locus T (*FT*), a florigen gene, exhibits a critical function in the flowering initiation process of various crops. *FT* expression is photoperiod dependent; therefore, flowering plants sense seasonal changes and determine when to express the gene. When the flowering conditions are favorable, the *FT* gene is released from the companion cells in the leaves and translocated via the phloem to the shoot apical meristem where it induces the transition from vegetative to reproductive stage (Odipio *et al*., 2020).

Genetic engineering offers versatile crop improvement approaches which when combined with the conventional breeding methods, could transform the field of plant breeding. Through such techniques, the selected gene can be efficiently inserted into plants via suitable vectors *(*Ayre *et al*., 2020; McGarry *et al*., 2016). Viral-based vectors are regarded as the most suitable vectors for transient transformation because of their ability to move systemically within the plant. This allows for the inserted gene to be systemically expressed (McGarry *et al*., 2016). Consequently,

geminivirus-derived vectors have become efficient yet simple tools for the transfer of useful traits into plants (Lozano-Durán, 2016). Geminiviruses exhibit various desirable properties which make them amenable to genetic engineering techniques. These include: i) having DNA as their genetic material; ii) replication that requires only a single viral protein (Rep/RepA) and iii) the ability of their genomic DNA to accumulate to higher levels (Li *et al*., 2019). Most of the geminiviruses are bipartite with two genomes, DNA A and DNA B (Figure 1), making them more useful in genetic engineering. On the sense strand, DNA A encodes a coat protein (V1) and a movement protein (V2), and four replication-associated proteins (AC1, AC2, AC3, and AC4) on the complementary strand. In contrast, DNA B encodes a movement protein (MP) and a nuclear shuttle protein (NSP) on the sense and complementary strands, respectively. Both DNA A and B have a common region containing a bidirectional promoter and an origin of replication necessary for gene expression (Figure 1). Since, geminiviruses can replicate within the plant without integrating into the plant's genome, they make an efficient transient expression system (Tennant and Fermin, 2015). Accordingly, Cassava mosaic virus (CMV), a common geminivirus that infects cassava across the tropical and subtropical, presents as a good viral vector candidate for efficient transient cassava transformation (Tennant and Fermin, 2015).

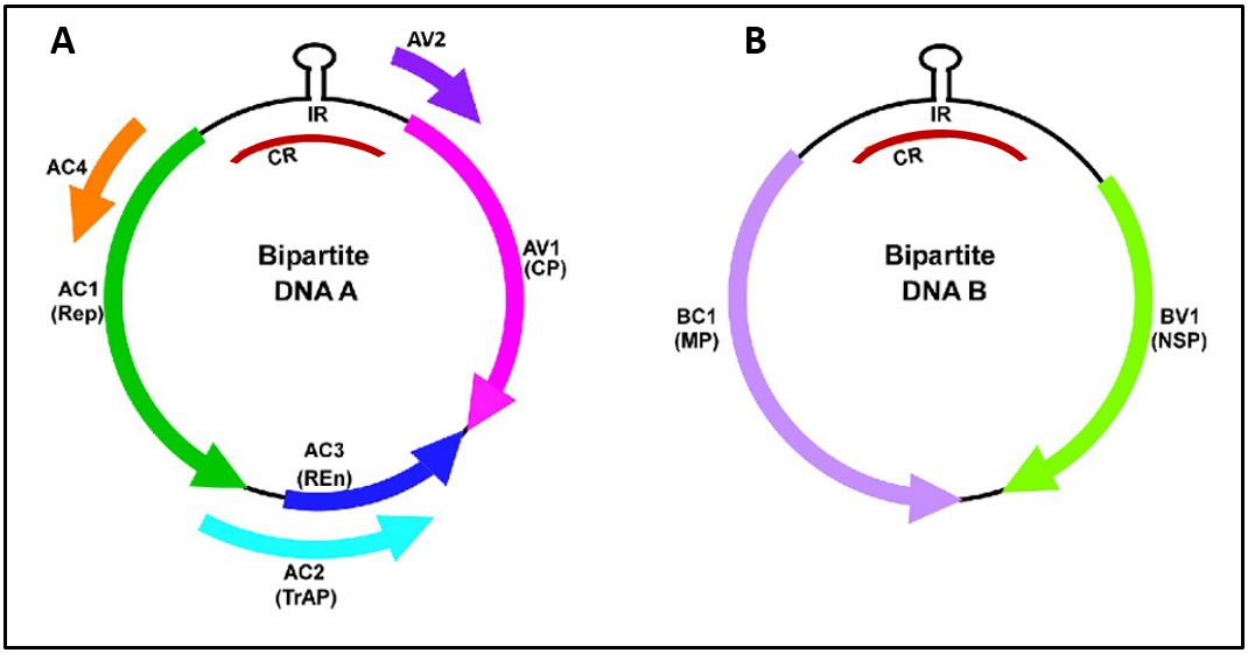

Figure 1: Bipartite genome of geminiviruses. (A) DNA A of the Geminivirus. AV1: Coat; AV2: Pre-coat protein; CR: Common region; IR: intergenic region; Rep A: Replication A protein AV1: Coat protein; AV2: Pre-coat protein; AC1: Replication-associated protein (Rep), AC2: Transcriptional activator protein (TrAP), AC3: Replication enhancer protein (REn), and AC4: RNA-silencing suppressor. (B) DNA B of the Geminivirus. CR: Common region; MP: Movement protein; NSP: Nuclear shuttle protein (Veluthambi and Sunitha, 2021).

Overexpression of the *FT* gene has been reported to promote early and prolonged flowering in various crops including cassava. According to a study by Adeyemo *et al*. (2017), overexpressing an exogenous *Arabidopsis thaliana FT* gene *(AtFT*) hastened flower initiation in cassava by inducing associated fork-type branching and lateral branching. Increased flower proliferation with an extended flowering duration was also recorded. Moreover, Tanaka *et al*. (2014) also highlighted that exogenous overexpression of *FT* gene in apple plants resulted in increased and perpetual flowering. Endogenous overexpression of the FT gene also exhibited a similar flowering effect in different plants. Pasriga *et al*. (2019) demonstrated that very early flowering could be induced in

rice by overexpressing the rice flowering locus T1 (*RFT1*). Similarly, Odipio *et al*. (2020) reported early flowering in cassava by overexpressing the cassava flowering locus T gene, *MeFT1*. Given the efficacy, overexpression of the *FT* gene has therefore been stipulated to be a significant strategy that could accelerate the flowering process in crop breeding programs.

Nicotiana benthamiana, a species within the tobacco group and indigenous to Australia, is the gold-standard model plant in plant virology studies due to its high vulnerability to multiple pathogens including viruses (Bally *et al*., 2018). Moreover, *N. benthamiana* exhibits a high amenability to genetic transformation with high regeneration efficiency. Its large leaves have enabled its application in transient protein expression assays using either *Agrobacterium*-mediated infiltration or engineered viral vectors (Davis *et al*., 2020). The recent technique for suppressing gene expression, virus-induced gene silencing (VIGS), has also been widely applied on *N. benthamiana* (Shi *et al*., 2021). Senthil-Kumar and Mysore (2014) utilized tobacco rattle virus (TRV) for post-transcriptional silencing in *N. benthamiana* through agro-infiltration. Several proteins, including Human IFNγ (Jiang *et al*., 2019), HDA19 recombinant protein (Ma *et al*., 2019; Jamshidnia *et al*., 2021), bioactive recombinant reteplase (Ma *et al*., 2019), wheat puroindoline (Niknejad *et al*., 2016), and glycosylated SARS-CoV-2 antigens (Ruocco and Strasser, 2022), have also been transiently expressed in *N. benthamiana*. The transient gene expression analyses involving *N. benthamiana* are faster and easier to perform. For example, the (TRV)-based virus-induced gene silencing (VIGS) takes only four weeks to implement (Shi *et al*., 2021) and the recombinant proteins produced via transient expression can be obtained within 1-2 weeks post-transformation (Ma *et al*., 2019). Therefore, this study used *N. benthamiana* for the proof-of-concept for both transient and stable transformation assays owing to the recalcitrance of cassava.

1.2 Statement of the problem

Most cassava cultivars are characterized by delayed and non-synchronous flowering patterns, resulting in long reproductive cycles. This further impedes breeding processes, such as intra-cultivar cross breeding. In addition, delayed flowering compromises the productivity and reproduction process of cassava. Various studies succeeded in inducing early flowering in cassava through the continuous overexpression of the *FT* gene. However, continuous overexpression of the flowering gene causes precocious flowering in plants. This could interfere with the apical growth dominance, due to increased lateral branching which eventually could result in stunted growth. Therefore, there is a need to develop an inducible synchronous flowering system that can be prompted when needed, to speed up the reproductive process in cassava.

1.3 Justification of the study

Despite being the mainstay of cassava improvement, conventional cassava breeding is delayed by non-synchronous, reduced or no flowering especially in cultivars with desirable traits. Biotechnological techniques offer powerful tools for plant breeding as they enable plant breeders to develop crop cultivars with desirable agronomic traits in more efficient ways (McGarry *et al*., 2016). Unlike genotype-dependent approaches of flowering induction such as grafting, virus-induced flowering is a more potent approach that allows for the efficient transformation of a wide spectrum of cultivars. It also ensures systemic expression of the delivered gene. Furthermore, the desired gene can be transiently introduced into the plant genome without being integrated in the germ cells. The approach also reduces the risk of somaclonal variations commonly associated with tissue culture procedures for generating transformable explants in cassava. A systemic overexpression of *Arabidopsis thaliana FT* gene was achieved in strawberry plants using a viral

based vector with a strong inducible promoter, which induced early flowering in the transformed plants (Li *et al*., 2019). Cassava is an important food crop and *N. benthamiana* is a model plant widely used in transient expression assays.

1.4 Objectives

1.4.1 General objective

To develop an early flowering induction system in cassava and *N. benthamiana* based on over expression of endogenous cassava *FT* gene.

1.4.2 Specific objectives

1. To produce friable embryogenic callus (FEC) of the four cassava cultivars, TMS 60444, TME 419, KBH2016B/521, and KBH2016B/185.
2. To establish a flowering induction approach by constitutive over-expression of*MeFT1* in cassava and *N. benthamiana.*
3. To develop a virus-induced flowering approach in cassava and *N. benthamiana* by overexpressing the MeFT1 gene in *Cassava mosaic virus* (CMV).

1.5 Significance of the study

Achieving controlled induction of synchronized inflorescence in cassava will pave the way for accelerated breeding. It will also render conventional breeding techniques such as cross breeding and mass selection much more feasible for breeders. As a result, enhanced cassava genetic traits will be obtained since the *FT* gene not only promotes early flowering in plants, but also regulates other developmental changes in plants such as increased pod formation and seed set (Li *et al*., 2015).

CHAPTER TWO

Literature review

2.1 Origin, distribution and biology of cassava

Cassava (*Manihot esculnta* Crantz) originated from Brazil, South America, with its first domestication being approximately 10, 000 years ago in central Brazil, and was brought to Africa through the Congo basin by the Portuguese in the 16th century (Allem, 2002). Today, cassava is grown in various tropical and subtropical areas worldwide, with the cultivation area being around 26 million hectares, of which Africa accounts for 66%, followed by Asia (22%) and Latin America (12%). Africa accounted for 61% of the 278 million metric tons of worldwide cassava production reported in 2018 (FAOSTAT, 2020). Cassava is grown in 17 Asian countries, among which Indonesia and Thailand represent the largest cassava growing areas, with 1.2 and 1.38 million hectares, respectively. Brazil has the largest cassava growing area (1.4 million hectares) in Latin America, followed by Colombia (0.2 million hectares) (FAOSTAT, 2020). Nigeria leads in cassava production in Africa, having a cultivation area of 3.6 million hectares, followed by Democratic Republic of Congo (2.1 million hectares) and Angola (1.2 million hectares). The leading cassava producer in East Africa is Tanzania, with a production area of 0.8 million hectares. Kenya is ranked 9th in cassava production in East Africa and 35th globally, with the growing area of 0.08 million hectares (FAOSTAT, 2020).

Cassava belongs to the *Manihot* genus containing more than 100 species, among which *Manihot esculenta* Crantz is the only one cultivated commercially (Hillocks et al. 2002). Cassava is monoecious with both female and male flowers on the same plant. The inflorescence is typically formed at branching point, but occasionally form in leaf axils on the upper parts of the plant. The female flowers are usually located on the lower part of the inflorescence are often fewer than the

male flowers, which are located on the upper parts of the inflorescence. Female flowers open about 2 weeks earlier than male flowers in the same inflorescence; however, female and female flowers on different branches but same plant can open concurrently (Anikwe & Ikenganyia, 2018). Cassava leaves are lobed and alternate with palmeted veins and the fruits are globular, ovoid or trilocular capsule measuring 1-1.5 cm in diameter, with six prominent longitudinal ridges. The seeds are ovoid-ellipsoidal measuring 6 mm wide, 100 mm long and 4 mm thick, a dark brown and gray colouration. Mature cassava plants generally assume two form; the erect type (with or without branches at the top) and the spreading type (Hillocks *et al*. 2002).

2.2 Significance of cassava

Cassava serves as a basic food to approximately 800 million individuals in the tropical regions, especially sub-saharan Africa (Waisundara, 2018), where it is the 3rd leading essential source of carbohydrates after maize and rice (Guira *et al*., 2017). Cassava tubers contain 80% of starch, making the crop the 2nd leading starch source worldwide (Karlström *et al*., 2016). Cassava tubers can be consumed in various forms, including raw, boiled, roasted, fried, or fermented (Gezahegn and Bazie, 2021; Frediansyah, 2018). Some of the most common cassava-based foods in Africa include Garri, Fufu, Abacha, Tapioca, and Ugali (Ngoualem and Ndjouenkeu, 2023). Cassava leaves also contain important nutrients, such as fibers, proteins, and vitamins (Boukhers *et al*., 2022; Alamu *et al*. 2021; Latif and Müller, 2015). In addition to the nutritional values, cassava leaves also exhibit medicinal values. For example, vitamin C and flavonoids contained in cassava leaves has antioxidant properties which can protect against cardiovascular diseases. Vitamin C also exhibits antiviral activities and can protect viral infections (Boukhers *et al*., 2022).

In South Asia, cassava is widely used as an industrial crop to produce animal feeds and biofuels such as bioethanol (Wangpor *et al*., 2017; Krajang *et al*., 2021; Nuwamanya *et al*., 2012). The growing demand of biofuels has made cassava a cash crop in countries such India, China, Vietnam, and Cambodia (Adeleye *et al*., 2020). Cassava-based animal feeds are composed of cassava tuber peels, leaves, unpeeled tubers, or peeled tubers (Fanelli *et al*., 2023). In China, about 40% of cassava production is used as source of animal feeds. For example, in 2001, 32.2% of total cassava yield was as animal feed in Yunnan province (Li *et al*., 2017a). Moreover, cassava peels reportedly increased egg production of the layers by 27% (Morgan and Choct, 2016).

2.3 Conventional breeding of cassava

Plant breeding is regarded as a versatile tool with the potential to promote global food security. It facilitates rapid development of plant varieties exhibiting desirable agronomic characteristics such as resistance to pests and diseases, tolerance to extreme environmental conditions, reduced time to maturity and increased yield (Parry and Hawkesford, 2012). The establishment of the Mendelian laws of genetics transformed the field of plant breeding. Scientists and breeders employed the knowledge of genetics for proper and enhanced breeding (Parry and Hawkesford, 2012). In agriculture, flowering forms the basic prerequisite for breeding of sexually propagated crops and it has recently received a lot of attention from plant breeders and scientists (Jung *et al*., 2017). Flowering, particularly in cassava, is faced with a lot of challenges attributed to its extreme genetic diversity and environmental conditions (Adeyemo *et al*., 2017). Conventional breeding techniques used for genetic improvement of crops characterized with clonal propagation, such as cassava, mostly focuses on the development of inbred lines. Self-pollination either by hand or insect vectors have been widely employed to develop partial inbred lines in cassava *(*Ceballos *et al*., 2015). Another approach that has been utilized for inbred lines development is the double haploid

technology where initial haploids are induced followed by subsequent chromosomal duplication to obtain a suitable flowering cultivar. For example, Lentini *et al*., (2020) developed double haploids in cassava using non-pollinated ovules. These convention techniques are, however, time consuming, costly and require a lot of space (Ceballos *et al*., 2015). Figure 2 illustrates a typical conventional breeding cycle in cassava.

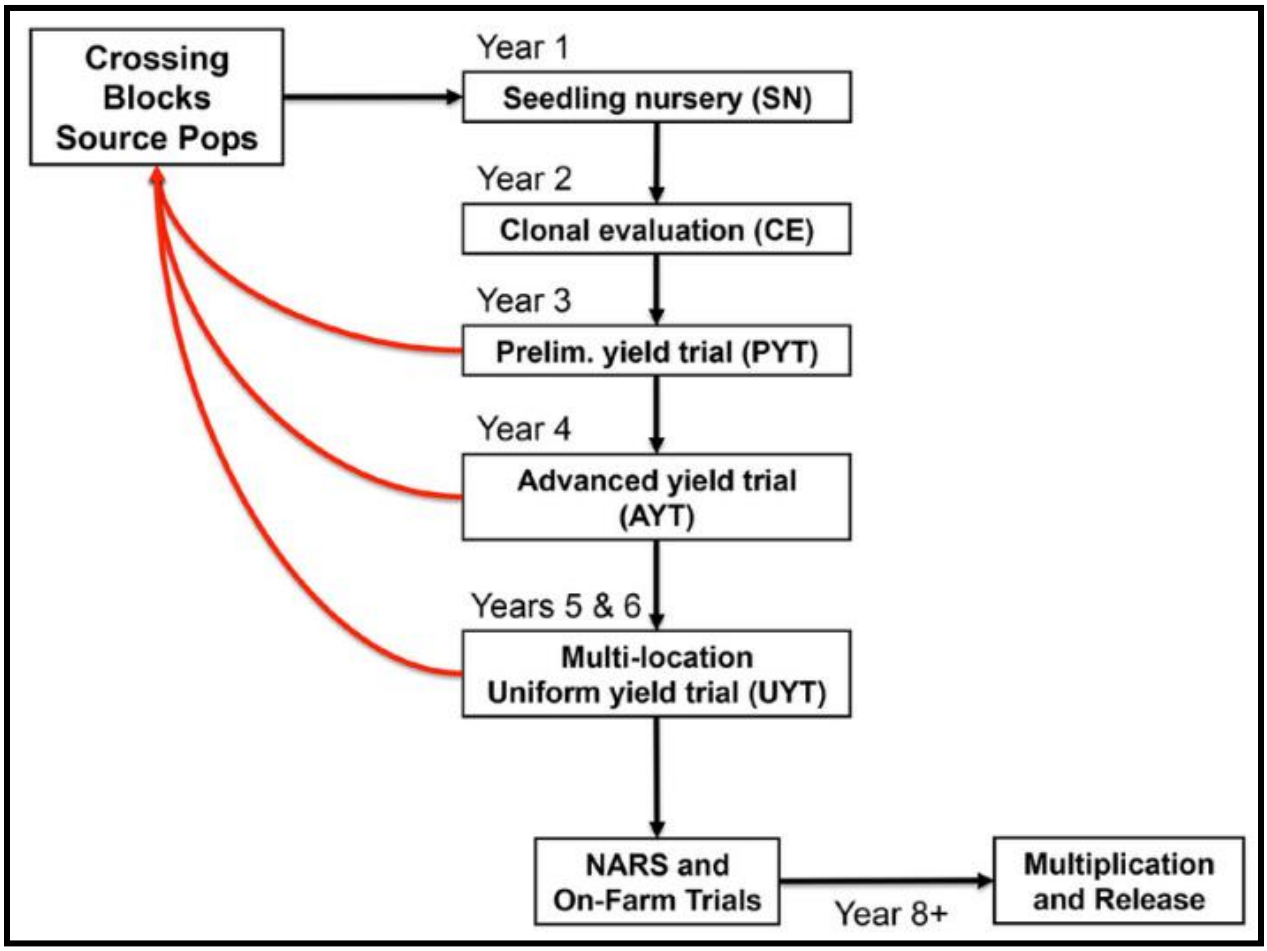

Figure 2: Conventional breeding cycle in cassava (Wolfe *et al*., 2017).

2.4 Challenges of cassava conventional breeding

Cassava breeding is limited by various factors, including long reproduction cycles, heterozygosity, low-seed rate, and delayed non-synchronous flowering (Oluwasanya *et al*., 2021; Silva Souza *et al*., 2018; Ibrahim *et al*., 2020). Cassava is extremely heterozygous making it difficult to select

parental lines with desirable agronomic values for breeding (Mansfeld *et al*., 2021; Qi *et al*., 2022). Such heterozygous parental lines result in genetically diverse progenies which need to be selected multiple times to obtain those with desirable traits (Bart and Taylor, 2017). Additionally, very little is known about the inheritance of the traits with agronomic values (Amelework and Bairu, 2022). Lack of synchronized flowering is a major factor hindering cassava breeding because some cultivars flower early (about four months after planting) and some flower late (more than ten months after planting) (Bandeira *et al*., 2021), making cross-breeding difficult. Moreover, the non-synchronous flowering also reduces the seed production efficiency, resulting in low-seed set rate (Pineda *et al*., 2020).

2.5 Flowering induction in cassava

Availability of the knowledge about the genes regulating flower initiation and development in model plants such as *Arabidopsis thaliana*, coupled with further identification of homologous genes in other plants has paved way for the various possibilities of flower induction in cassava (Adeyemo *et al*., 2017). The Flowering locus T gene, *FT*, has been reported to tightly regulate floral induction and is highly conserved across all angiosperms (Tsuji and Taoka, 2014). Accordingly, constitutive over-expression of the *FT* gene reportedly mediates early flowering in cassava and other plants (Adeyemo *et al*., 2017; Odipio *et al*., 2020; Velázquez *et al*., 2016).

2.5.1 Use of hormones in induction of flowering in cassava

Several growth hormones such as paclobutrazol and cytokinins have been shown to induce in vitro flowering in cassava (Premkumar *et al*., 2011; Medina *et al*., 2012). Medina *et al*. (2012) showed that applying paclobutrazol on the leaves induced flowering in cassava by suppressing the vegetative growth of the cassava plants. In a related study, Hyde *et al*. (2019) also showed that silver thiosulfate (STS), which inhibits the activity of ethylene in plants, increased the number of

flowers produced and flowering duration in cassava crops. Similarly, other studies have demonstrated that the use of hormones induced flowering in other plants as well. Premkumar *et al*. (2011), in an *in vitro* study, demonstrated that cytokinins not only promoted shoot regeneration but also flower development in *Scoparia dulcis*. Gibberellins were shown to correspondingly enhance flowering in *Chrysanthemums* plants grown under short and long photoperiods (Dong *et al*., 2017). Despite their flowering induction abilities, usage of such growth hormones for floral induction is not reliable because minute changes in their concentration could result into undesired effects and their efficiency is also highly dependent on plant species (Dong *et al*., 2017; Hyde *et al*., 2019; Premkumar *et al*., 2011).

2.5.2 Use of genetic engineering for flowering induction in cassava

Introduction of robust genetic engineering techniques greatly revolutionized genetic improvement of clonally propagated crops. Genome editing techniques, such as the CRISPR/Cas9, have been used to stably transform plants through targeted mutagenesis (Zhang *et al*., 2018). Soyk *et al*. 2016 reported that the CRISPR/Cas9-mediated mutation of the flowering repressor SELF-PRUNING 5G (SP5G) accelerated flowering in tomato plants. Similarly, Han *et al*. (2019) conducted *Agrobacterium*-mediated CRISPR/Cas9 mutagenesis of the maturity *E1* gene in soybeans. The result was early flowering of the soybean mutants. The genes are usually stably integrated in the plants' genome but a number of studies have reported potential harm to human health and the environment (Kawall *et al*., 2020; Ghimire *et al*., 2023). Additionally, incorporation of these stable transformation techniques into the actual breeding process is very tiring and time-consuming. Plants without well-established regeneration systems makes the development of stable transgenes even more difficult (Ahmad and Mukhtar, 2017).

2.6 Origin, distribution and biology of *N. benthamiana*

Named after the English botanist George Bentham, *N. benthamiana* is a Solanaceae species native to northern Australia, where it grows in seasonally arid-like habitats such as rocky hills and cliffs, which are inhospitable to other *Nicotiana* species (Chase *et al*., 2022). Although *N. benthamiana* has wide distribution in the tropical and subtropical regions of Australia (Figure 3), the origins of the strain commonly used for research (the laboratory strain; LAB) have been reported to be the extreme environment of the Granites Gold Mine located in Tanami desert (Pierroz, 2022). *N. benthamiana* evolved to attain early rapid growth and production of high amount of small seeds to increase the chances of surviving the extreme environment. This evolution also contributed to the loss of the *RNA-dependent RNA polymerase 1* (*Rdr1*) gene conferring resistance to various pathogens, including viruses (Bally *et al*., 2015). This explains why *N. benthamiana* is susceptible to various pathogens, an attribute that has made it a model plant of choice for plant-pathogen interaction studies.

N. benthamiana is an allotetraploid with 19 chromosomes and classified under *Nicotiana* Suaveolentes section. *N. benthamiana* has been reported to have resulted from a hybridization between the maternal progenitor from section Noctiflorae, and paternal progenitor from section Sylvestres (Schiavinato *et al*., 2021). The genome of *N. benthamiana* is about 3.1 Gb in size (Kourelis *et al*., 2019). *N. benthamiana* plants grow up to 1.5 m in height, with the lower-half of the stem having large branches (up to 20 cm long). Basal leaves are petiolate measuirng 2.0–5.0 cm long, while the upper leaves are sessile (Chase *et al*., 2022). The flowers are white and the seeds are dark-brown (56–100 µm wide and 500–805 µm long). However, very little has been reported on the reproductive biology of the Australian Nicotiana species, including *N. benthamiana* (Wylie and Li, 2022).

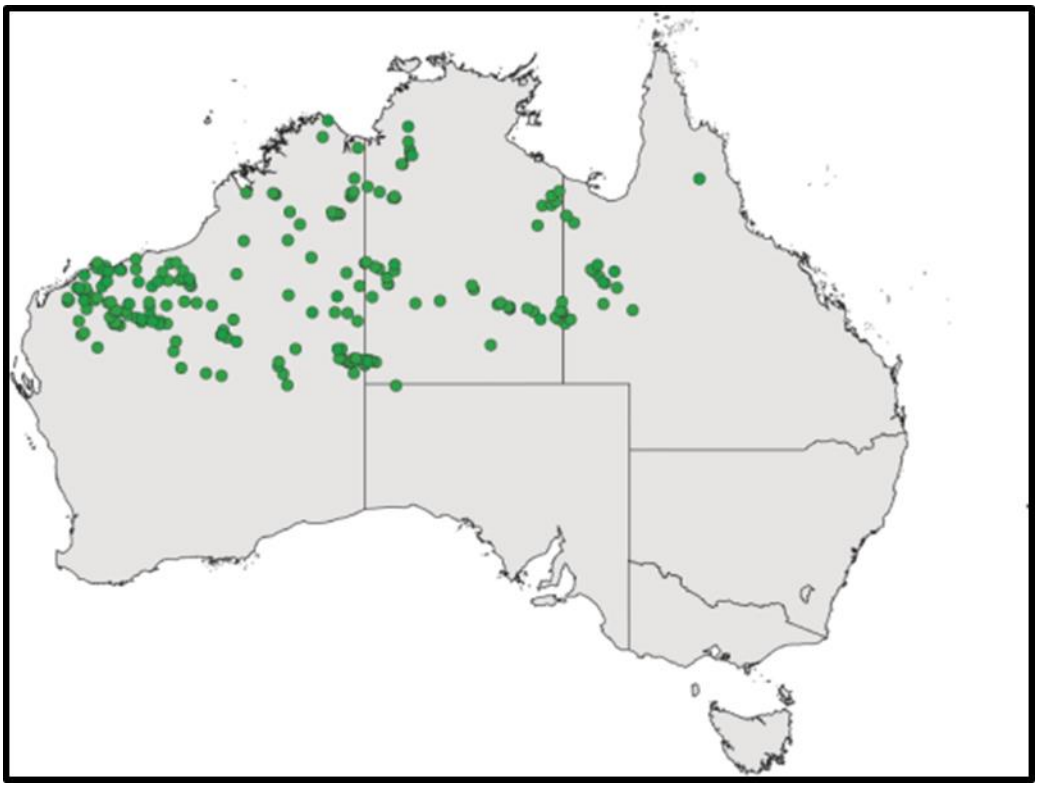

Figure 3: A map showing the distribution of *Nicotiana benthamiana* in the tropical and subtropical parts of Australia (Chase *et al*., 2022).

2.7 *N. benthamiana* as an efficient system for transient assays

N. benthamiana is a preferred model plant for transient expression assays because of its fast growth rate and amenability, allowing for high-throughput screening of the expressed gene (Debler *et al*. 2021). The most common method used for *N. benthamiana* transient expression assays is *Agrobacterium*-mediated infiltration which involves introducing the gene of interest into young leaves (2-3 weeks old plants) (Stephenson *et al*., 2018). The indispensable role of *N. benthamiana* as the plant-of-choice for transient assays has been reported by many studies in various thematic areas of plant research, including plant-pathogen interaction, protein localization, RNA interference, protein interaction, and generation of recombinant proteins (Baró *et al*., 2022; Rolland, 2018; Li *et al*., 2017b; Soni *et al*., 2022; Wilbers *et al*., 2016).

Raffaello and Asiegbu (2017) utilized *N. benthamiana* to demonstrate that *Heterobasidion annosum*, a basidiomycete that commonly infects conifers of the temperate forests, induces cell death via small secreted proteins. The study reported that transiently expressing small secreted proteins of *H. annosum* (HaSSPs) in *N. benthamiana* caused rapid death of cells two days after infiltration. The proteins also activated the genes associated with defense responses in the infiltrated *N. benthamiana*, indicating host responses to *H. annosum* infection. Additionally, transient expression of RXLR53 effector of *Plasmopara viticola*, the oomycete responsible for grapevine downy mildew, suppressed the induction of host-immunity-related gene in *N. benthamiana* (Liu *et al*., 2021). This suggested that *P. viticola* secretes RXLR53 effector to suppress the immunity of the host. The RNA interference mechanisms of plant pathogens have also been studied in *N. benthamiana*. Wang *et al*. (2022) infected *N. benthamiana* with *Alternaria longipes*, the main pathogen responsible for tobacco brown spot, and reported that the pathogen induced the expression of 175 novel miRNAs in *N. benthamiana*. These miRNAs suppressed the expression of *A. longipes* gene in *N. benthamaina* through RNA interference.

Several recombinant proteins have also been transiently expressed for mass production in *N. benthamiana*. Maharjan and Choe (2019) produced a complex-glycosylated hemagglutinin of Canine influenza virus in the *N. benthamiana* leaves which could aid in rapid influenza vaccine production. Prado *et al*. (2019) reported a mass production of a biologically active α-amylase inhibitor for controlling cotton boll weevil. The recombinant SARS-CoV-2-related proteins have also been transiently expressed in *N. benthamiana* for vaccine production (Diego-Martin *et al*., 2020; Ceballo *et al*., 2022; Jirarojwattana *et al*., 2023; Song *et al*., 2022; Rattanapisit *et al*., 2020; Siriwattananon *et al*., 2021). Large-scale production of cocaine hydrolase (CocH3)-based product

used to treat overdose or addiction was reported using *N. benthamiana* as the bioreactor (Wang *et al*., 2016).

2.8 Flowering induction in *N. benthamiana*

The functions of flowering time genes such as APETALA have been evaluated in *N. benthamiana*. Mlotshwa et al., (2006) studied the effects of overexpressing *Arabidopsis* APETALA 2 (AP2) and microRNA 172 (miR172) genes on the floral pattern of *N. benthamiana*. The study showed that the *Arabidopsis* AP2 (wild type) induced floral defects in *N. benthamiana* floral pattern, with transgenic *N. benthamiana* exhibiting flowers with delayed petal emergence, smaller petals and stunted-bulging carpels. However, the heterologous overexpression of *Arabidopsis* miR172 induced early flowering in *N. benthamiana*. Overexpression of the Oil Palm Knotted-like 1(OPKN1) gene delayed flowering and reduced the number of lowers in *N. benthamiana* (Adly *et al*., 2015). Similarly, Yuan *et al*. (2021) reported that overexpressing the first flower node gene of *Capsicum annuum* (CaFFN) induced notable delayed flowering in *N. benthamiana*. Suppressing NbTM8, the Tomato MADS 8 (TM8) homolog, () and NbSVP (SHORT VEGETATIVE PHASE) genes (which are repressors of floral initiation) via VIGS accelerated flowering in *N. benthamiana* (Coenen *et al*., 2018). Fourquin & Ferrándiz (2012) characterized the functions of AGAMOUS (AG) family members in *N. benthamiana* and found that *N. benthamiana* AG (NbAG) was expressed in the carpel and stamen during flower development, and had similar spatio-temporal distribution to the Arabidopsis homolog. This suggested the involvement of C-function genes in *N. benthamiana* flowering. Thus, the present study aimed to overexpress a flowering-related gene from cassava, *Manihot esculenta Flowering Locus T 1* (*MeFT1*), in *N. benthamiana* to induce early flowering.

2.9 Pathways associated with the *FT* flowering gene

Four pathways, namely photoperiod, autonomous, vernalization, and gibberellin pathways, have been shown to be associated with flowering in plants. In the photoperiod pathway, light signals trigger a series of molecular events that activate the circadian clock-related genes at different times of the day (Helliwell *et al*., 2022). *CONSTANS* (*CO*) is among the most studied genes regulating photoperiodic flowering. Its expression is activated in the leaves after the perception of the long day light signals and promotes flowering by activating the *FT* and *SOC1* genes (Yang *et al*., 2014). Different plants require different photoperiod for flowering induction, and are thus classified as long-day, short-day or day-neural plants. Long-day plants require prolonged duration of day light for flowering induction, while the short-day ones require long dark periods. Day-neutral plants flower regardless of the photoperiod (Nakamichi, 2014). Examples of long-day plants include *A. thaliana*, wheat, lettuce, and spinach, while short-day ones include rice, soybean, cotton, and amaranth. Day-neutral ones include tomatoes, potatoes and eggplants (Nakamichi, 2014). The autonomous pathway involve several factors that downregulate the expression of the flowering repressor, *FLC*. These factors include several genes such as *FLOWERING LOCUS Y* (*FY*), *FLOWERING LOCUS CA* (*FCA*), *FLOWERING LOCUS PA* (*FPA*), *FLOWERING LOCUS KH DOMAIN* (*FLK*), *FLOWERING LOCUS VE* (*FVE*), *LUMINIDEPENDENS* (*LD*), and *FLOWERING LOCUS D* (*FLD*) (Cheng *et al*., 2017). These genes mainly suppress *FLC* via RNA-based post-transcriptional and epigenetic modifications. *FPA* and *FCA* have been shown to limit the expression of *FLC* by promoting polyadenation of the long non-coding RNAs encoding the antisense sequence of the FLC locus (Wang *et al*., 2014). Similar to the autonomous pathway, vernalization promotes flowering by repressing the *FLC* gene. FLC, a MADS-box transcription factor, directly inhibits the expression of *FT* and its expression must be suppressed before *FT* is

activated. Therefore, vernalization and autonomous pathways converge upstream of *FT* to repress the activity of FLC. Vernalization down-regulates the expression FLC within two weeks of exposure to lower temperatures after which the gene is epigeneticatlly silenced by the activity of plant-homeodomain zinc-finger (PHD)-PRC2 complex (Song *et al*., 2013). Gibberellin promotes flowering by activating *LFY*, *SOC1*, and *FT* genes. Dong *et al*. (2017) reported that gibberellin upregulated *LFY* and *SOC1* in *Chrysanthemums* and induced flowering under both long-day and short-day conditions. Gibberellin also promotes the activation of *FT* to stimulate flowering under long-day conditions. A previous study showed that GA-deficient mutant plants had lower *FT* expression, but exogenous treatment with gibberellin dramatically increased the *FT* expression in the plants (Porri *et al*., 2012). A schematic representation of the four pathways associated with flowering in plants is shown in Figure 3.

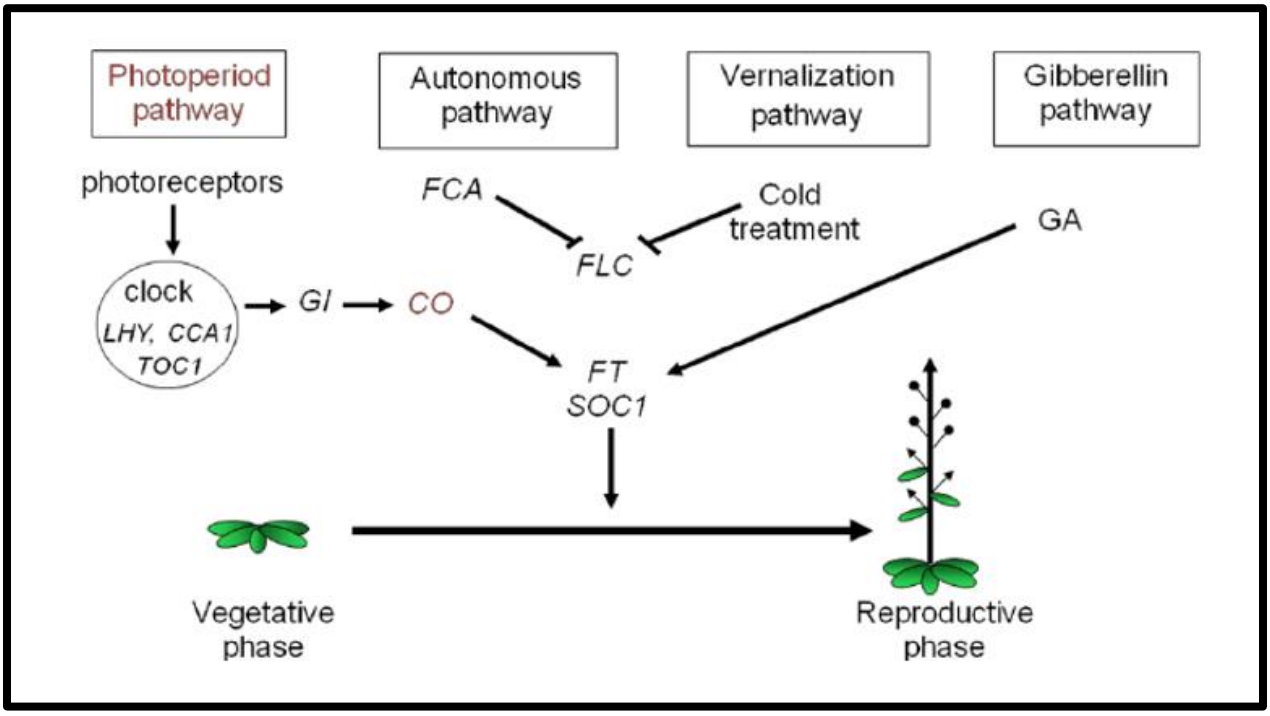

Figure 4: A schematic representation of the four major pathways that promote flowering in plants by inducing the transition from the vegetative to the reproductive phase (Kumar *et al*., 2011).

2.10 Virus-induced flowering

Constitutive over-expression of transgenes is not a suitable method for floral induction in plants since it results in precocious flowering (McGarry *et al*., 2016). Virus-induced flowering (VIF) is a preferable form of flowering activation since it presents a controllable and efficient floral induction system. Transformation processes for the virus induced flowering are much simpler as compared to those applied in standard transformation. The risk of somaclonal variation is also minimal as the plants do not need to go through tissue culture. Viral vectors have been utilized to deliver *FT* genes delivery to various plants. An accelerated flowering of young citrus plants was achieved overexpressing the citrus *FT* gene delivered through the citrus leaf blotch virus (Velázquez *et al*., 2016). Li *et al*. (2019) demonstrated an efficient delivery of the *Arabidopsis thaliana FT* (*AtFT*) gene in strawberries via the deconstructed apple latent spherical virus (ALSV). The strawberry seedlings infected with the vector were reported to flower within two months after the infiltration, resulting in fruits containing viable seeds. Additionally, McGarry *et al*. (2016) indicated that ectopically overexpressing the *AtFT* gene via the deconstructed Cotton leaf crumple virus (dCLCrV)-based vector not only promoted flowering but also enhanced lateral shoot outgrowth in cotton. In cassava, over-expression of the endogenous FT homolog, *MeFT1*, under the tight regulation of Cassava vein mosaic virus (CsVMV) promoter resulted in accelerated flowering and increased branching (Odipio *et al*., 2020). However, constitutive over-expression of transgenes results in precocious flowering. Also, the transformation and regeneration techniques, associated with constitutive gene over-expression, are prone to somaclonal variations. This therefore necessitates the incorporation of alternative inducible flowering systems such as transient gene expression systems using virus-based vectors. The virus-mediated expression system by-passes the regeneration step making the expression of exogenous genes fast and convenient

(McGarry *et al*., 2016). Geminiviruses have been successfully used in transient gene expression assays in plants. For instance, Yamamoto *et al*. (2018) demonstrated that Bean yellow dwarf virus (BeYDV)-derived vector dramatically enhanced the expressed levels of green fluorescent protein in *N. benthamiana*. Yin *et al*. (2015) on the other hand, used the Cabbage Leaf Curl virus (CaLCuV)-based VIGS in *N. benthamiana*. The vector delivered guide RNA (gRNA) targeting phytoene desaturase 3 (*NbPDS3*) and isoprenoid synthesis H (*NbIspH*) genes in transgenic *N. benthamiana* plants expressing Cas9. The resulting photo-bleached phenotype of the plants confirmed the efficiency of geminivirus-based gene expression.

Viral vector systems also serve as superior tools for rapid suppression of targeted genes in plants thus down-regulating their expression (Flachowsky *et al*., 2012). Yamagishi *et al*. (2016) highlighted that apple latent spherical virus vector successfully inhibited the terminal flowering 1 (*TFL1*), a flowering repressor gene, yielding apple and pear plants with hastened flowering. Furthermore, Freiman *et al*. (2011) also reported that repression of the *TFL1* gene mediated floral induction in pear (*Pyrus communis* L.). In general, flowering induction in plants involves activating the *FT* gene or inactivating the *TFL1* gene.

CHAPTER THREE

Materials and Methods

3.1 Target gene selection

Arabidopsis thaliana FT (AT1G65480) gene was obtained from the GenBank and blasted against the cassava genome in the NCBI (National Center for Biotechnology Information) database through a BLAST search to identify the cassava FT homolog. The first hit (XM_021775680.2) was designated *MeFT1* after it was identified to be an *FT* gene (Odipio *et al*., 2020). A pairwise alignment was conducted with the Bioedit software (https://bioedit.software.informer.com/7.2/) to determine the similarity between the *MeFT1* and *AtFT* genes.

3.2 Construction of Cassava Mosaic Virus-based vectors carrying FT gene

3.2.1 Construction of the vector for stable transformation

The *MeFT1* gene was cloned into pLSU-4 plasmid containing the 35S Cauliflower Mosaic Virus (CaMV) promoter and nopaline synthase terminator from *Agrobacterium tumefaciens*. Briefly, *MeFT1* was amplified with forward primer MeFT1-KpnI-F and reverse primer MeFT1-XbaI-R to create KpnI and XbaI restriction sites. pLSU-4 vector containing *AtFT* gene under the 35S promoter and NOS terminator, obtained from the International Institute of Tropical Agriculture (IITA), Nairobi, was digested using restriction enzymes KpnI and XbaI to remove the *AtFT* gene. The amplified *MeFT1* gene was then cleaved with KpnI and XbaI restriction enzymes and ligated to the digested pLSU-4 plasmid. The ligation reaction mixture (10 µl) contained 1µl of T4 ligation buffer (T4 DNA Ligase Buffer (Invitrogen™), 1 µl (50ng/µl) of the plasmid, 1 µl of T4 ligase (Invitrogen™), and 6 µl of nuclease-free water. After a brief vortexing, the mixture was incubated for 5 min at room temperature. Figure 4 shows the construction process of pLSU-4 MeFT1 vector. The pLSU-4 MeFT1 vector was transformed into electrocompetent *Escherichia coli* cells via the

heat shock method (Appendix 9). The *E.coli* cells containing the transformed plasmid were then validated using colony PCR and inserted into *A. tumefaciens* stain AGL1 via electroporation (Appendix 11). Empty pLSU-4 plasmid without the gene of interest was also inserted into *A. tumefaciens* AGL1 cells and all constructs were sequenced to ensure that there were no mutations.

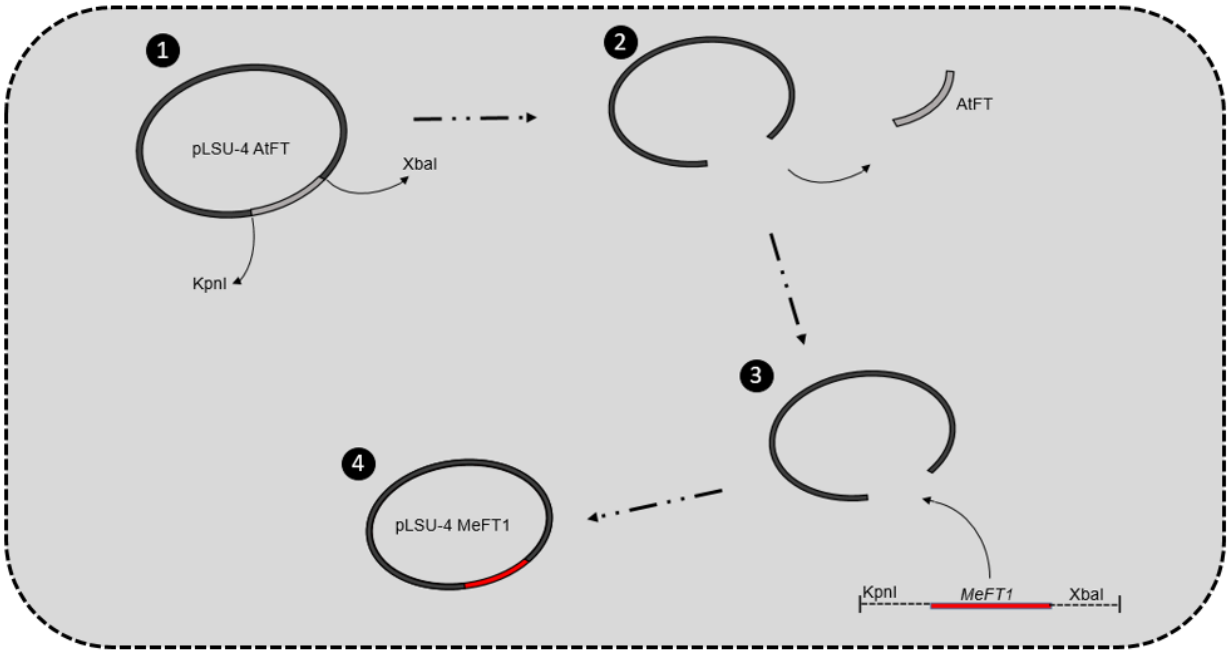

Figure 5: Construction of pLSU-4 vector. 1. Digestion of pLSU-4 AtFT with KpnI and XbaI restriction enzymes to (2) remove the Arabidopsis thaliana FT (AtFT) gene. 3. Cloning the MeFT1 gene containing KpnI and XbaI restriction sites into the pLSU-4 plasmid to form (4) pLSU-4 MeFT1 vector.

3.2.2 Construction of the vector for transient transformation

For the transient transformation, cassava mosaic virus-based vectors were developed by replacing of the pre-coat or coat proteins in the EACMV A genome with the gene of interest while retaining genes essential for viral replication. The A and B genome were then separately cloned into pLSU-1 plasmids under control of the viral promoter. For the A genome, 0.5 mer of the A genome digested with BamHI and SphI was cloned into the pLSU-2 (Lee *et al.*, 2012) digested with BamHI

and SphI restrictions enzymes, forming a plasmid vector denoted pLSU-1 0.5 mer A. The truncated AV1 (trAV1_1_1595_S and trAV2_1275_2045RC) and AV2 (trAV2_1_1400_S and trAV2_1275_2045RC) fragments were fused into 1-mer fragments using Q5 HF polymerase (NEB, UK). The fragments (20 ng each) were amplified under these PCR conditions: 30 sec of initial denaturation (98 ℃), 35 cycles of 10 sec of denaturation (98 ℃), 20 sec of annealing (62 ℃), and of 2.5 min extension (72 ℃). No primers were used for the amplification. The obtained amplicons were digested with BamHI restriction enzyme and inserted into the pLSU-1 0.5 mer A cleaved with BamHI and Eco53kI restriction enzymes to form pLSU-1 1.5 mer ΔV1 and ΔV2 plasmids. For the pLSU-1 1.5 mer ΔV1, the V2 gene was substituted with a multiple cloning site (MCS), while the V1 gene was replaced with a MCS for the pLSU-1 1.5 mer ΔV2. *MeFT1* cleaved with KpnI and XbaI was then inserted into the multiple cloning sites in the pLSU-1 1.5 mer ΔV1 and ΔV2 plasmids. Figure 5 illustrates the construction of the truncated AV1 and AV2 pLSU-1 plasmids. Similarly, 0.5 mer of the B genome was cleaved with BamHI and SphI restriction enzymes and inserted into pLSU-2 cleaved with BamHI and SphI restrictions enzymes to form a plasmid vector denoted pLSU-1 0.5 mer B. The CMVB1-1400_S and CMVB1200-2797RC fragments of the B genome were fused to form a 1-mer fragment using PCR, as described for the truncated fragments of the A genome. Thereafter, the amplicon was digested with BamHI and cloned into pLSU-1 0.5 mer B digested with BamHI and ScaI to form pLSU-1 1.5 mer B. Like pLSU-4 plasmids, the heat shock method was used to transform the pLSU-1 plasmids containing the A and B genomes of the EACMV were transformed into electrocompetent *E. coli* cells. The *E.coli* cells containing the transformed plasmids were then validated using colony PCR and inserted into *A. tumefaciens* AGL1 cells via electroporation. Empty pLSU-1 plasmids without the

gene of interest were also transformed into the *Agrobacterium* cells and the constructs were sequenced to confirm that there were no mutations.

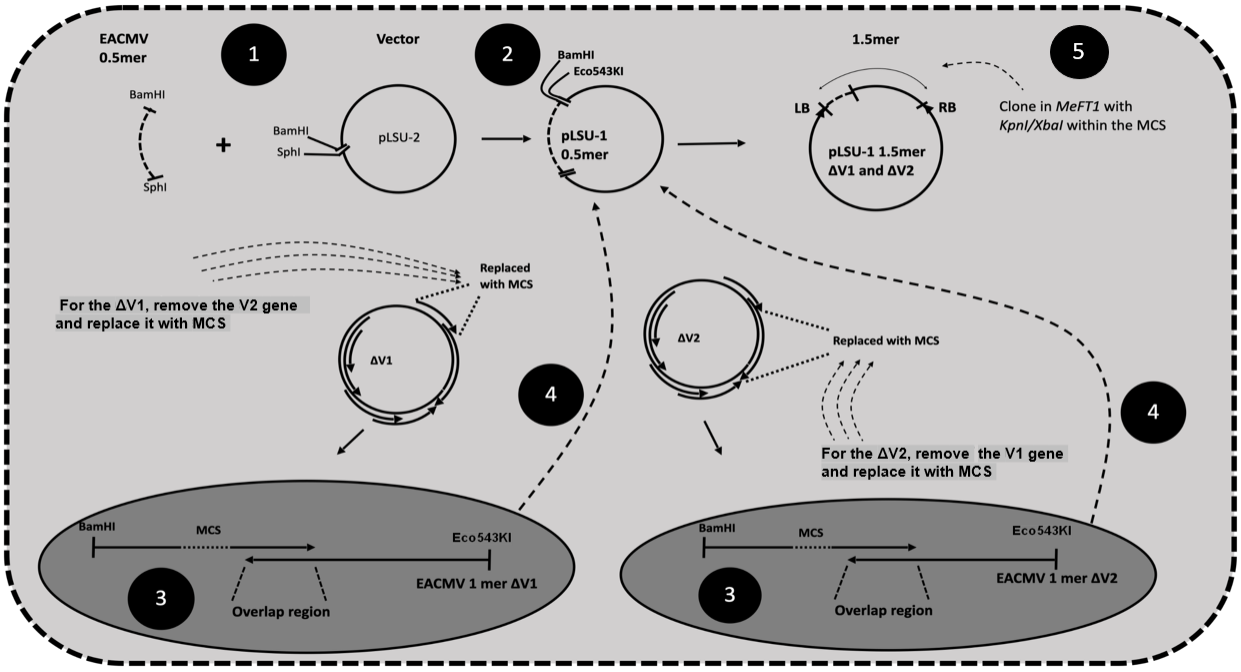

Figure 6: Construction of the truncated (Δ) V1 and V2 vectors of the East Africa Cassava Mosaic Virus (EACMV) A genome; 1. Digestion of the 0.5 mer of the EACMV A genome using BamHI and SphI restriction enzymes. 2. Cloning of the 0.5 mer A into the pLSU-2 plasmid, denoted pLSU_1 0.5 mer A. 3. Fusion of the ΔV1 and ΔV2 fragments into 1-mer fragments. 4. Cloning of the 1-mer ΔV1 and ΔV2 fragments into the pLSU-1 0.5 mer to form pLSU-1 1.5 mer ΔV1 and ΔV2 plasmids. 5. Cloning of MeFT1 into multiple cloning sites of the pLSU-1 1.5 mer ΔV1 and ΔV2 plasmids.

3.3 Plant materials

Four cassava cultivars (Table 1) were obtained from the *in vitro* cultures at IITA, Nairobi. The plants were micropropagated on the cassava basal medium (CBM) (MS including vitamins (4.4 g/L), 2% (w/v) sucrose and 2mM CuSO4) using the standard protocols (Feyisa, 2021). *N.*

benthamiana seeds obtained from the Kenya Plant Health Inspectorate Service (KEPHIS) were planted in pots (11 cm height and 7 cm diameter) which contained peat moss and vermiculite (at 1:1 ratio) and grown at 26 ℃ in a controlled environment under a 16/8 hour of light/dark photoperiods.

Table 1. The different cassava cultivars and their flowering time (data obtained from farmers in Tanzania).

Cultivar	**Flowering Time**
TMS 60444	Not known
KBH2016B/185	Early and profuse flowering
KBH2016B/521	Late flowering
TME 419	Late flowering

3.4 Production of friable embryogenic callus (FEC)

The nodal explants of *in vitro* cassava plantlets (4 weeks old) were used for the generation of friable embryogenic calluses (FECs) (Nyaboga *et al*., 2015). Briefly, the explants were segmented into pieces measuring approximately 2 cm and were cultured horizontally in the Petri plates containing cassava axillary medium (CAM) (CuSO4 (2 uM), sucrose (0j.2%), MS including vitamins (4.4 g), BAP (10 mg/L), and noble agar (0.8%)). The plates were incubated at 26°C in the dark for 4 days. Thereafter, sterile needles were used to excise the enlarged axillary buds under light microscope, and the excised axillary buds were cultured on callus induction medium (CIM) (sucrose (0.2%), MS including vitamins (4.4 g), BAP (10 mg/L, biotin (0.2mg/L), and noble agar (0.8%)). The plates were incubated at 26°C in the dark for 2 weeks, after which the formed organized embryogenic structures (OES) were transferred into Gresshoff and Doy (GD) medium

with vitamins, supplied with picloram (12 mg/L), sucrose (2%), and noble agar (0.8%). The plates were incubated at 26°C in the dark for the first two cycles (one cycle had two weeks, and the OES were moved into fresh GD medium after every 14 days). For the subsequent cycles, the OES were cultured under a 16/8 hour photoperiod until the FECs were formed. The generated FECs were then subjected to stable transformation mediated by *Agrobacterium* (Nyaboga *et al*., 2015). The FECs generation steps are highlighted in Figure 6.

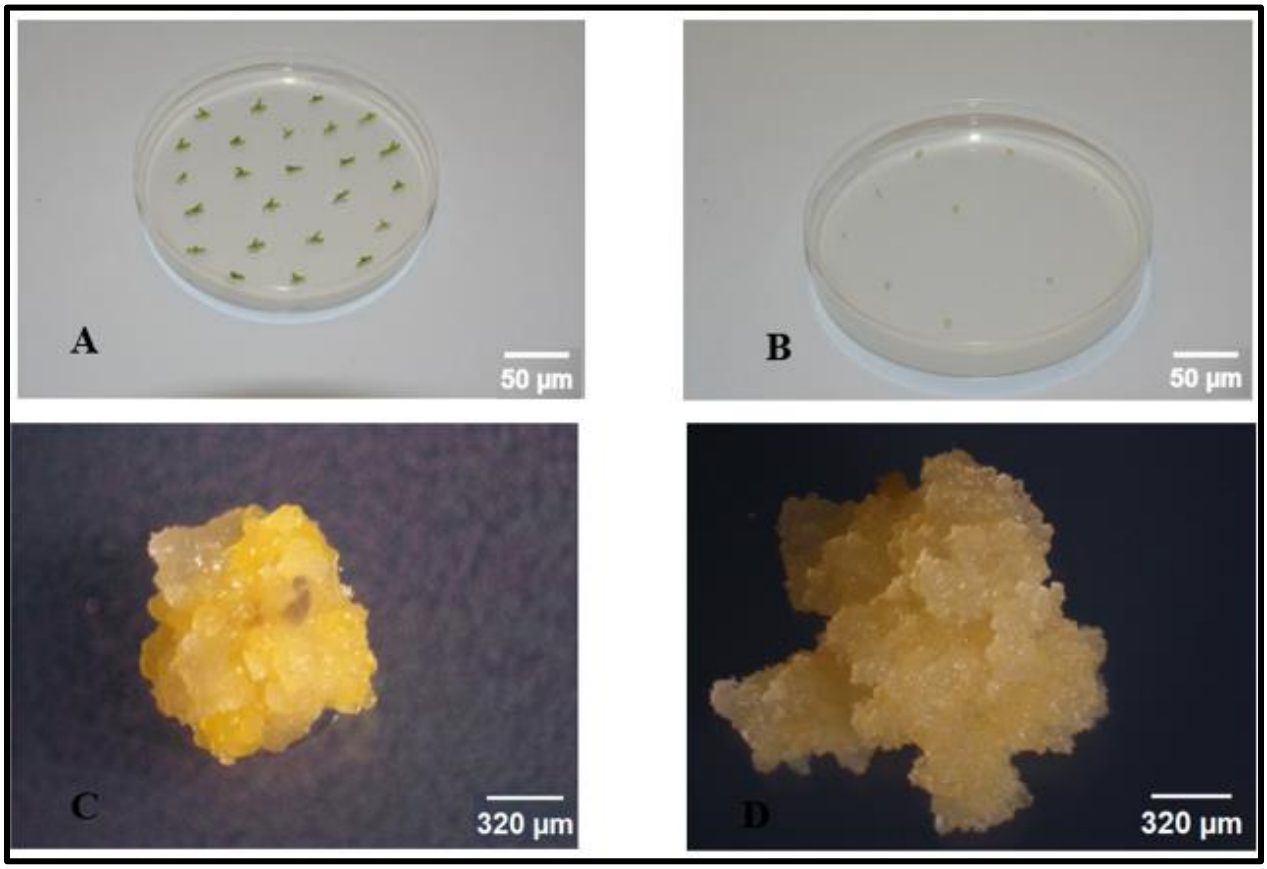

Figure 7: Production of the friable embrogenic callus (FEC). (A) The nodal explants placed horizontally on the cassava axillary medium (CAM) for the friable embryogenic callus (FEC) initiation. (B) Enlarged axillary buds on the callus induction medium (CIM). (C) Organized embryogenic structures (OES) formed from the axillary buds. (D) Friable embryogenic calluses (FECs) formed from the OES. Scale (A,B): 120 pixels/µm; (C,D): 4 pixels/µm.

3.5 *Agrobacterium*-mediated stable transformation and regeneration of transgenic plants

Agrobacterium tumefaciens strain AGL1, containing the cassava mosaic virus-based FT (CMV-FT) vector, was used to transform cassava FECs and *N. benthamiana* leaves. Briefly, 3µl of *A. tumefaciens* cultures, were cultured in LB broth (3 ml) supplemented with Rifampicin (25 mg/L) and Kanamycin (50 mg/L), and incubated for 48 hours at 28 ℃. Rifampicin and Kanamycin were used for selection because *A. tumefaciens* strain AGL1 has a gene responsible for Rifampicin resistance and the plasmid transformed into the *Agrobacterium* cells contained Kanamycin resistance gene. The cultures were then inoculated into fresh LB broth (50 ml) supplied with Rifampicin (25 mg/L) and Kanamycin (50 mg/L), and incubated at 28 ℃ for 24 hours. The concentration of the cells was determined by measuring their optical density (OD) at 600 nm and standardized to 0.8. Thereafter, the cultures were subjected to a 15 minutes centrifugation at 4000 g and the pellets were reconstituted in the same volume of fresh GD liquid containing 200 mM acetosyringone. For cassava, the FECs were gently disintegrated into smaller clumps using one ml cut pipette tips and placed into GD liquid containing the *Agrobacterium* cells (Nyaboga *et al*., 2015; Utsumi *et al*., 2022). Young leaves of four-week old *N. benthamiana* plants were subjected to a 1 minute of 70% ethanol sterilization and cleansed thrice in sterile distilled water. The leaves were then subjected to a 3 minutes disinfection with 3.5% hypochlorite, rinsed 5 times in sterile water and dry-blotted in sterile paper towels. Thereafter, the leaves were cut into smaller pieces (approximately 2 cm) and transferred into 50 ml falcon tubes containing the agro-cultures (Ghaderi *et al*., 2017). The FECs and *N. benthamiana* leaves were gently shaken at 45 revolutions per minute (rpm) on a shaker (SSM4 Mini See-Saw Rocker Shaker SSM4/120V/60 120Volt, Cole Parmer Stuart) for 1 hour at room temperature for transformation. After that, excess *Agrobacterium* cells were removed from the transformed leaves and FECs by blot-drying using sterile paper towels.

The transformed *N. benthamiana* leaves were placed on MS3 (Murashige and Skoog with BAP and sucrose) plates supplemented with acetosyringone (200 mM), while the transformed FECs were transferred onto sterile mesh membranes on MSNB (MS salts with vitamins (4.4g/L), BAP (1 mg/L), NAA (0.1 mg/L), and 3 % (w/v) sucrose) plates containing the same concentration of acetosyringone. All plates were then kept in the dark for 3 days at room temperature to allow for co-cultivation. Thereafter, the co-cultivated FECs and *N. benthamiana* leaves were transferred into MSNB and MS3 media supplemented with cefotaxime (450mg/ml), and were incubated in the dark at room temperature for 7 days to eliminate excess *Agrobacterium* cells. After that, the FECs and *N. benthamiana* leaves were moved into MSNB and MS3 media containing hygromycin (5mg/ml) and cefotaxime (450mg/ml) (Tripathi *et al*., 2014), and the plates were kept at 26 ℃ under a 16/8 hour of light/dark photoperiod for two weeks. The FECs and *N. benthamiana* leaves were moved into fresh selective media after every 14 days (two weeks) until the cotyledons (for cassava cells) and shoots (for *N. benthamiana*) started forming. The cassava cotyledons were transferred into (cassava shoot elongation media) (CEM) (MS salts with vitamins (4.4g/L), 2mM CuSO4, BAP (4 mg/L), and 2 % (w/v) sucrose) and refreshed after every two weeks in the same media until shoots started forming. The regenerated cassava shoots were transferred into CBM containing hygromycin (5mg/ml) for root formation. For *N. benthamiana*, the regenerated shoots were transferred into MS3 media containing iron sulphate ($FeSO_4$) for root development. The rooted cassava and *N. benthamiana* plantlets were then analyzed to verify the presence of the transformed gene. Figures 7 and 8 show the transformation steps of cassava FECs and *N. benthamiana* leaves, respectively.

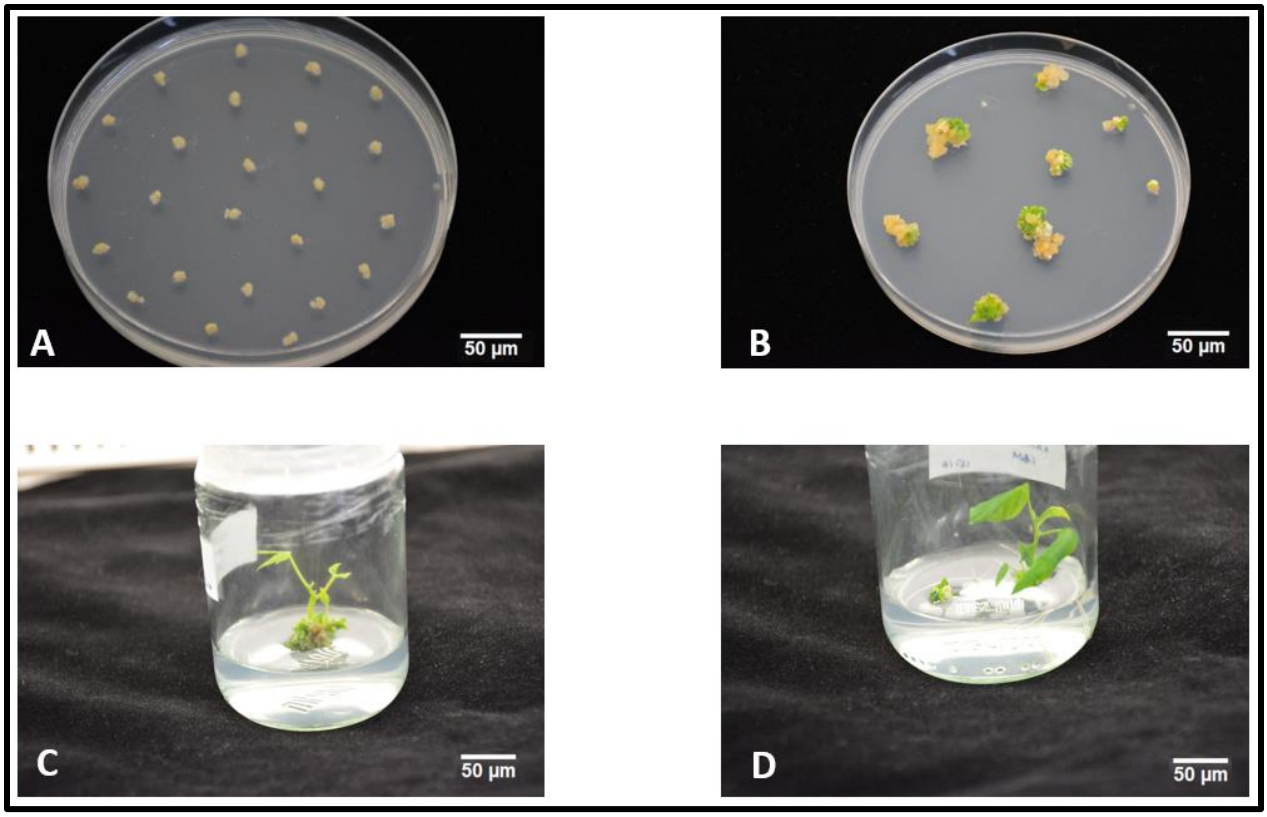

Figure 8: Transformation and regeneration of cassava. (A) Transformed friable embryogenic calluses (FECs) in the selection media. (B) Transformed FECs producing cotyledons (the green structures). (C) Cassava shoots regenerating from the cotyledons. (D) Rooted cassava plantlet. Scale: 120 pixels/µm.

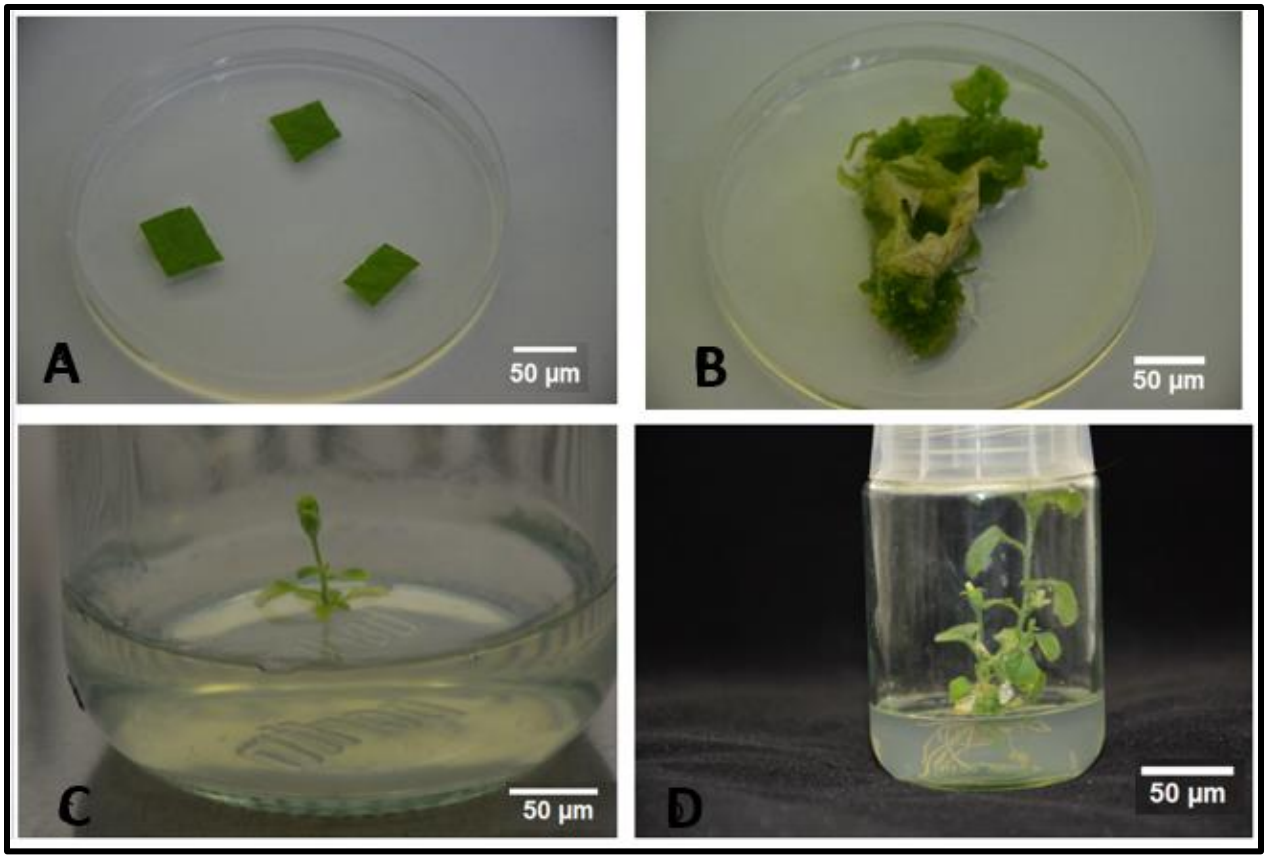

Figure 9: Transformation and regeneration of *Nicotiana benthamiana*. (A) Transformed *N. benthamiana* leaves in selection media. (B) Callus formation from the transformed leaves. (C) Regenerated *N. benthamiana* shoot. (D) Rooted *N. benthamiana* plantlet. Scale: 120 pixels/µm.

3.6 *Agrobacterium*-mediated transient transformation of cassava and *N. benthamiana*

Agrobacterium solution containing the CMV-FT constructs were used to infiltrate the cassava (nine weeks old) and *N. benthamiana* (four weeks old) plants. The transformation culture was prepared as described in section 3.4; however, bacterial cells were reconstituted in an infiltration buffer (10 mM MgCl2, 10 mM MES and 200 mM acetosyringone) instead of GD liquid. Cassava plants were transiently transformed based on a previous procedure by Lentz *et al*. (2018b), with slight adjustments. Briefly, *Agrobacterium* suspension was introduced into the leaves (the third-most open leaf) and at 3 sites adjacent to the axillary meristems. Surface cuts were also made with

the same syringe in the lower stem sections of the infiltrated cassava plants to prompt the release of *Agrobacterium* T-DNA. The protocol by Zhang *et al*. (2020) was used for *N. benthamiana* transient transformation. The point of infiltration (POI) and systemic (third-most open leaf) leaves were sampled at four days and eight days post-infiltration (DPI), respectively, from the infiltrated plants and stored at -80 ℃ for further analysis.

3.7 Molecular characterisation of regenerated and agro-infiltrated transgenic plants

3.7.1 PCR Analysis of the stably transformed plants

Genomic DNA isolation from the regenerated cassava and *N. benthamiana* plants was conducted with the cetyltrimethylammonium bromide (CTAB) method (Osena *et al*. 2017), with slight modifications. Briefly, the liquid nitrogen-frozen cassava and *N. benthamiana* leaves were ground on a tissue lyser (TissueLyser II, QIAGEN) at 15 Hz for 30 sec (for *N. benthamiana*) and 25 Hz for 45 sec (for cassava). The extraction buffer (Appendix 12) (600 µl) was mixed with the ground samples by vortexing and the mixture was heated at 65 ℃ for 30 min. Thereafter, the mixture was cooled for 10 min at room temperature, followed by the addition of 600 µl of chloroform: isoamyl (24:1). After inverting the tubes (five times) to mix the samples and centrifuging the samples at 14000 g for 10 min, the supernatant was transferred into sterile Eppendorf tubes (1.5 ml) and treated with 4 µl of RNase (Thermofisher). The mixture was incubated at 37 ℃ for 30 min. After that, chloroform: isoamyl (24:1) (600 µl) was mixed with the samples and were spun at 14000 g for 10 min. After transferring the supernatant into clean Eppendorf tubes (1.5 ml), the samples were precipitated overnight with 600 µl of ice-cold isopropanol at -20 ℃. The samples were washed with 70% ethanol by a 10 min centrifugation at 14000 g, after which the supernatant was disposed. The DNA pellets were allowed to air-dry at room temperature and then eluted in 30 µl

of deionized water. A nanodrop spectrophotometer (Thermo Fisher Scientific™ NanoDrop™ 2000/2000c Spectrophotometers) was used to measure the DNA quality and quantity, and the samples were analyzed for the transformed *FT* gene by PCR using Taq polymerase on a thermal cycler (T100™ Thermal Cycler, Bio-Rad). The PCR conditions were: 4 mins of initial denaturation (94 ℃), 35 cycles of 30 sec of denaturation (94 ℃), 45 sec of annealing (62 ℃), and 1 min of extension (72 ℃), with 7 min of final extension (72 ℃). Gel electrophoresis of the PCR amplicons was conducted using1.2% agarose gel at 80V for 90 min. Primers used for the PCR analysis are shown in Table 2.

Table 2. Primer sequences for the PCR analysis of the stably transformed plants.

Primer	**Sequence**
pLU 2 5’ Fwd	TTCTAAAAGTGTTCTAAGCGGGC
MeFT-qPCR-Tqn-R	GTCTCCTTCCACCGGAGCCACT

3.7.2 PCR analysis of the transiently transformed plants

For the infiltrated plants, PCR analysis was conducted to check for the systemic movement of the viral vector. DNA isolation from the systemic cassava and *N. benthamiana* leaves was conducted based on the method in section 3.6.1. PCR was conducted using primers targeting the viral vector backbone, with the same reaction conditions as in section 3.6.1. Gel electrophoresis of the PCR products was conducted using 1.2% agarose at 80V for 90 min. The PCR primers are shown in Table 3.

Table 3. Primer sequences for the PCR analysis of the stably transformed plants.

Primer	Sequence
AC2 1124-1154 F	AGGCGCAGTGATGAGTTCCCCT
AC2-Seq2 R	AGGACGGGGAAGACGATGTGGG

3.7.3 Southern blot analysis of the transiently transformed plants

Southern blot analysis (Akram *et al*., 2022) was conducted for the POI leaf samples of the infiltrated cassava and *N. benthamiana* plants to determine the replication of the viral vector. Briefly, the DNA samples were extracted as described in section 3.6.1, normalised to 2 µg (final concentration), and loaded onto the 0.8% agarose gel (Appendix 14). A DIG-labeled marker was prepared (as described in appendix 5) and loaded together with the samples on the gel. The gel electrophoresis was performed at 80V for 5 hours on a Bio-rad gel electrophoresis system (Wide Mini-Sub Cell GT Cell) and the UV gel imaging machine (inGenius, SYNGENE) was used to capture the gel image. Thereafter, the gel was depurinated in 0.25M HCl (depurination buffer; Appendix 3) for 15 min with gentle shaking, rinsed with distilled water and submerged into the denaturation buffer (87.75 g/L of NaCl and 20 g/L of NaOH) for 30 min. The gel was rinsed twice with distilled water and transferred into the neutralization buffer (60.57 g/L of Tris, 87.75 g/L of NaCl and 0.3724 g/L of EDTA; pH 7.5) for 30 min, after which the gel was equilibrated in 20X sodium citrate dihydrate (SSC) solution (88.23 g/L of SSC and 175.32 g/L of NaCl) for 10 min. The gel was placed upside-down on the blotting apparatus assembled in a casting tray containing 20X SSC. The blotting membrane was then placed on the gel and a 15-20 cm stack of dry paper towels (with the same size measurements as the membrane) were added on top. A 500 g weight was then placed on the paper towels to enhance the capillary transfer, which proceeded for 24 hrs. After the transfer, the well positions in the gel were highlighted on the membrane using a pencil.

The membrane was separated from the gel and the gel was discarded. The membrane was then placed in a UV crosslinker (Hoefer UVC500 Ultraviolet Crosslinker, Amersham Biosciences) for DNA fixation at 120X 1000V. Pre-hybridization was conducted by placing the membrane in a hybridization bottle containing 14 ml of pre-warmed (at 42 ℃) DIG Easy Hybridization buffer (Roche DIG Easy Hyb™) without any probe, followed by incubation at 42 ℃ in the hybridization chamber (Stuart SI30H Hybridization Shaking Incubator, UK) at a speed of 60 rpm for 2 hrs. Thereafter, the pre-hybridization buffer was replaced with the hybridization buffer containing a PCR-labeled probe (Appendix 4), followed by an overnight incubation under the same conditions. The probe size was 23 bp and was binding to the AC1 (replication-associated protein) region of the viral vector backbone. After hybridization, the blot was rinsed twice using 200 ml of low stringency buffer (2X SSC and 0.1% SDS) for 5 min with gentle shaking. The blot was then transferred into 200 ml of preheated high stringency buffer (0.5X SSC and 0.1% SDS) and incubated at 65°C in the hybridization chamber for 15 min. This step was repeated twice. Thereafter, the blot was normalized in 20 ml of washing buffer (maleic acid + 0.3% tween 20) for 5 min and soaked in 60 ml of the blocking solution (Appendix 3) (without the antibodies) for 30 min with gentle shaking. The blocking solution was discarded and the blot was soaked in fresh 40 ml of the blocking solution containing the antibodies for 30 min with gentle shaking at 30 rpm. The blot was then rinsed twice with the washing buffer for 30 min with gentle shaking at 60 rpm, followed by a 5 min soaking in 20 ml of the detection buffer. The blot was placed on a saren wrap with the DNA side up and treated with a CSPD (chloro-3'-methoxyspiro[adamantane-4,4'-dioxetane]-3'-yl)phenyl] dihydrogen phosphate) chemiluminescent (Roche CDP-Star®) for 5 min, after which the saren wrap was sealed and excess CSPD bloated out. The blot was then viewed

and photographed using a chemiluminescence machine (Azure 600 Imaging System, Azure Biosystems, US).

3.7.4 RNA Extraction and cDNA synthesis for the transiently transformed *N. benthamiana* and flowering wild-type cassava plants

Isolation of total RNA from the systemic leaf sampes of the infiltrated *N. benthamiana* and flowering wild-type cassava plants was performed using a modified CTAB method. The flowering wild-type cassava plants were collected from the field in KALRO, Kandara. Briefly, *N. benthamiana* leaf samples were ground at 15 Hz for 30 sec, while cassava leaves were ground at 25 for 45 sec using a genogrinder. Pre-heated CTAB buffer (600 µl) was mixed with the samples by vortexing and the mixture was heated at 65 ℃ for 15 min. The samples were then chilled on ice for 10 min, followed by the addition of chloroform isoamyl (600 µl) and a 10 min centrifugation at 4 ℃ at a speed of 14000 g. After transferring into clean centrifuge tubes (1.5 ml), the supernatant was treated with 2 µl of DNase1 (Thermo Fisher Scientific) at 37 ℃ for 30 min. Thereafter, 600 µl of chloroform isoamyl was mixed with the samples, and then the mixture was separated by a 10 min centrifugation at 4 ℃ at a speed of 14000 g. The supernatant was transferred into clean centrifuge tubes and the samples were precipitated overnight in 600 µl of isopropanol. The samples were subjected to 70% ethanol washing and then eluted in 30 µl of deionised water. After that, 200 µl of trizol reagent (TRIzol™ Reagent, Thermo Fisher Scientific) was mixed with samples, followed by the addition of 600 µl of chloroform. The samples were vortexed and spun at 4 ℃ for 10 min at a speed of 14000 g and the supernatant was precipitated in isopropanol for 2 hrs at -20 ℃. Finally, the samples were washed with 70% of ethanol and eluted in 30 µl of deionised water. All steps were conducted on ice whenever possible. For cDNA synthesis, RNA samples

were subjected to genomic DNA removal using DNase1 (Thermo Fisher Scientific) and the activity of RNase was inhibited using RNase inhibitor (Thermo Fisher Scientific). Briefly, 1 µl of the 10X reaction buffer (Lucigen, Wisconsin, USA), 1 µl of DNase1, 0.125 µl of RNase inhibitor, and an appropriate amount of nuclease free water were mixed with 1 µg of the RNA sample to make up a 20 µl reaction mix. The reaction mix was subjected to a 30 min incubation at 37 ℃ and the process was terminated by adding 1 µl of 50mM EDTA (Thermo Fisher Scientific), followed by a 10 min incubation at 65 ℃. The RNA obtained was then used as a template for reverse transcription, conducted with the Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific). Briefly, 14 µl (700 ng) of the DNase 1-treated RNA samples were mixed with 4 µl of the 5X reaction mix (Thermo Fisher Scientific) and 2 µl of the Maxima enzyme mix (Thermo Fisher Scientfic) to constitute a 20 µl reaction volume. After a brief vortexing, the mixture was heated at 25 ℃ for 10 min, and then at 50 ℃ for 15 min. Thereafter, the reaction was terminated by a 5 min heating at 85 ℃.

3.7.5 Quantitative reverse transcription PCR (RT-PCR)

The synthesized cDNA served as the template for the RT-PCR analysis. Flowering wild-type cassava samples were used as the positive control, while the non-infiltrated *N. benthamiana* samples served as the negative control. The infiltrated *N. benthamiana* samples were the treatment samples. The internal control gene for the cassava samples was *Me26S*, while that of *N. benthamiana* samples was protein phosphatase 2 subunit A (*NbPP2A*). Three different molecular probes, MeFT1, NbPP2A and Me26S probes, were synthesized by Macrogen, Inc., South Korea, for the qPCR analysis. The probe sequences are shown in Table 2. The 5’ end of the probes was tagged with a fluorescein (FAM) with an absorption/emission at 495/520 nm, while the 3' end had a Black Hole Quencher 1 (BHQ1) tag. The reaction mix contained the PCR master mix (2X Luna®

Universal Probe qPCR Master Mix) (5µl), primers (0.5 µl each; 10 pmol/ul), probe (0.2 µl; 10 pmol/ul), nuclease free water (2.8 µl), and cDNA template (1 µl). The reaction was conducted on a QuantStudio qPCR machine (Applied Biosystems™ QuantStudio5-qPCR System) and the reaction conditions were: 10 min of initialization (94 ℃), followed with 40 cycles of 30 sec of denaturation (94 ℃), 30 sec of annealing (60 ℃), and 30 sec of extension (72 ℃). The experiment was conducted in triplicates. The probes used for the analysis are shown in Table 4. The primers used and the target fragments are shown in Table 5.

Table 4: The sequences of the probes used for the qPCR analysis.

Probe Name	**Sequence**	**Target gene**
MeFT	TGCCAATCAAGCACTTCAGTCTATTG	*Manihot esculenta Flowering Locus T1* (*MeFT1*)
Me26S	CAATGGACGACCCTGCCTC	*Manihot esculenta* 26S proteasome
NbPP2A	TGCCAATCAAGCACTTCAGTCTATTG	*Nicotiana benthamiana Phosphatase 2 subunit A* (*NbPP2A*)

Table 5. Primer sets and their target fragment sizes for the qPCR analysis.

Primer Name	**Sequence**	**Target Fragment**
MeFTqPCR Tqn-F	TATGCACCTGGGTGGCGTCAGA	125 bp
MeFTqPCR Tqn-R	GTCTCCTTCCACCGGAGCCACT	
Me26S qPCR-F	AATGCGCTCCTACAACAAGC	101 bp
Me26S qPCR-R	GATCATCCGTAGCAGCCTCT	
NbPP2A F	GACCCTGATGTTGATGTTCGCT	124 bp
NbPP2A R	GAGGGATTTGAAGAGAGATTTC	

3.8 Measuring the flowering parameters of the transiently transformed *N. benthamiana*

The flowering time, number of flowers, and number of branches were obtained for the infiltrated and non-infiltrated *N. benthamiana* plants.

3.9 Data analysis

Microsoft Excel 2013 (Microsoft Corporation, 2018) was used to generate the bar graphs for the flowering parameters collected from the infiltrated *N. benthamiana* plants.

CHAPTER FOUR

Results

4.1 Gene selection, construct design and sequencing

Cassava genome database in NCBI (*M. esculenta*_v8 reference Annotation Release 101) was searched via BLAST using *A. thaliana FT* (*AtFT*) and 11 hits were obtained. The first hit was designated MeFT1 (Figure 9A) and its amino acid sequences were aligned with those of the AtFT to identify the conserved regions (Figure 9C).

Figure 10: Identification of the *MeFT1* gene and construction of the expression vectors. (A) The first hit designated *MeFT1* and (B) the distribution of the top 11 hits on the query sequence. Pairwise alignment of the *AtFT* and *MeFT1* amino acids showing the conserved regions. (C) Alignment of the *MeFT1* gene with the *AtFT*. The colored amino acids represent conserved regions.

The *MeFT1* was cloned into pLSU-4 (Figure 10) and pLSU-1 (Figure 11) plasmids and the constructs were sequenced. The sequencing results showed that the constructs had no mutations.

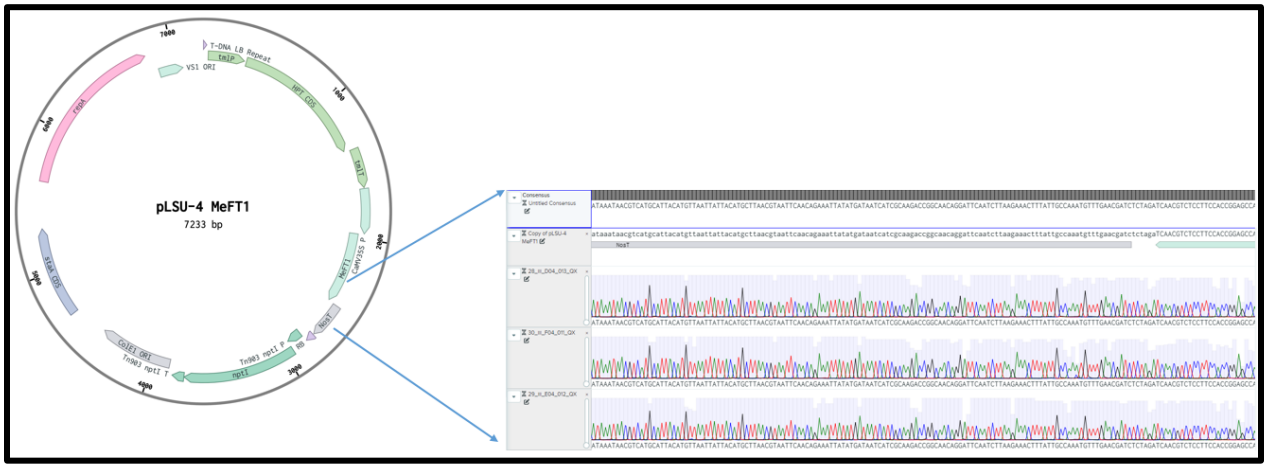

Figure 11: The pLSU-4 plasmid for constitutive expression. The plasmid contains *MeFT1* gene under the control of Cauliflower Mosaic Virus (CaMV) promoter (35S CaMV promoter) and nopaline synthase (NOS) terminator.; The hygromycin phosphotransferase coding sequence (HPT CDS) under the control of tumor large promoter (tmlP) and tumour large terminator (tmlT) served as the selectable marker for the stably transformed plants, while the kanamycin resistance marker gene (*nptI*) served as the selectable marker for the transformed *Agrobacterium* cells containing recombinant *E. coli* cells. LB: Left border; RB: Right border; Rep A: Replication A protein.

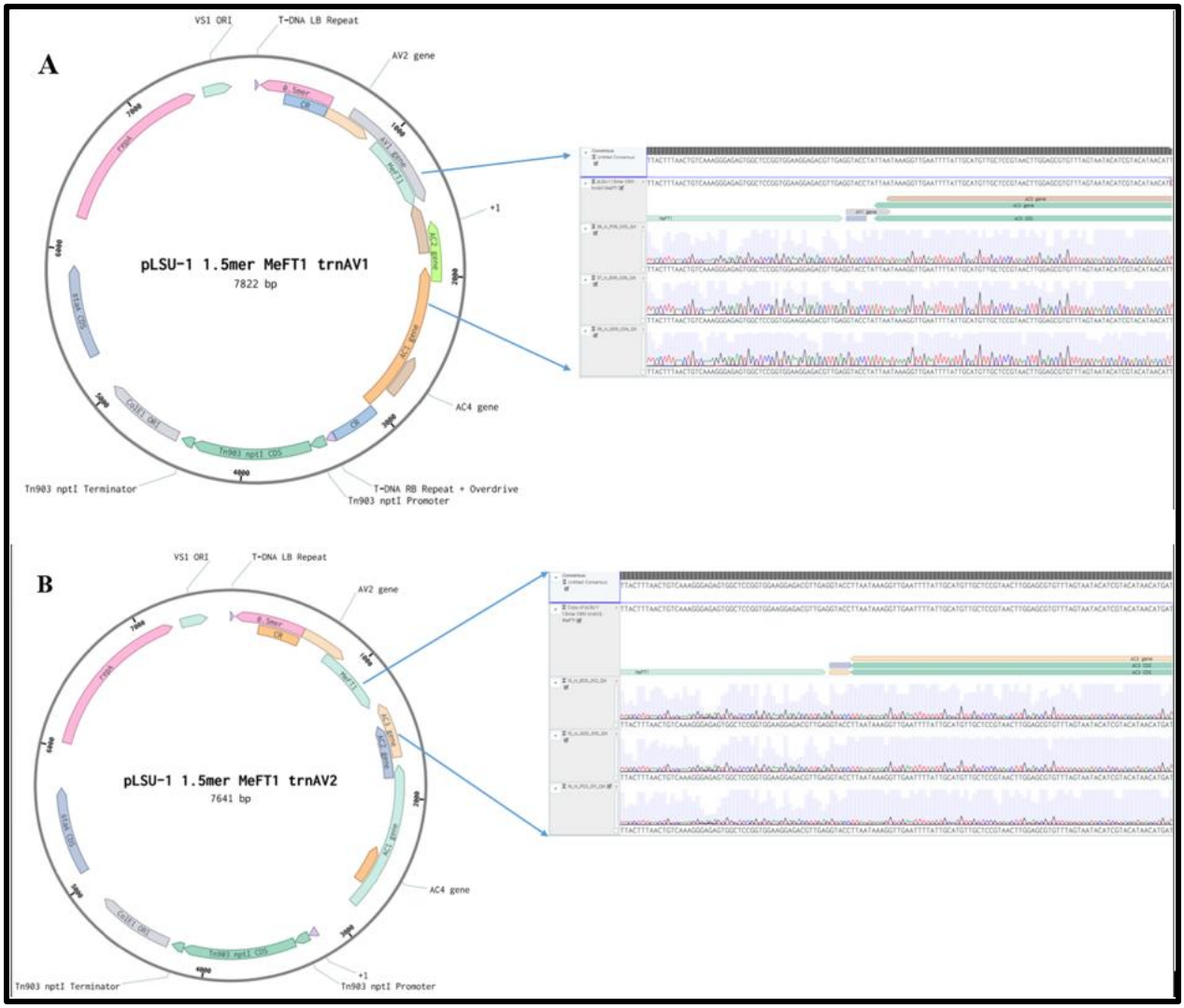

Figure 12: pLSU-1 plasmids for induced expression. The plasmids contain the *MeFT1* gene in the deconstructed virus backbone with truncated (A) pre-coat (AV1) and (B) coat (AV2) proteins. The kanamycin resistance marker gene (*nptI*) served as the selectable marker for the transformed *Agrobacterium* cells containing recombinant *E. coli* cells. CR: Common region; LB: Left border; RB: Right border; Rep A: Replication A protein AV1: Coat protein; AV2: Pre-coat protein; AC1: Replication-associated protein (Rep), AC2: Transcriptional activator protein (TrAP), AC3: Replication enhancer protein (REn), and AC4: RNA-silencing suppressor.

4.2 Cassava FEC production

The FECs were obtained for the four cassava cultivars 60444, TME 419, KBH 2016B/185, and KBH 2016B/521. After nodal explant initiation in the CAM medium, the enlarged axillary buds were carefully excised and moved into CIM medium, where they differentiated into organized embryogenic structures (OES) characterized with globular structures (Figure 12; upper panel). The OES were transferred to GD medium where they differentiated into friable embryogenic calluses (FECs) characterized by fine finger-like projections (Figure 12; lower panel).

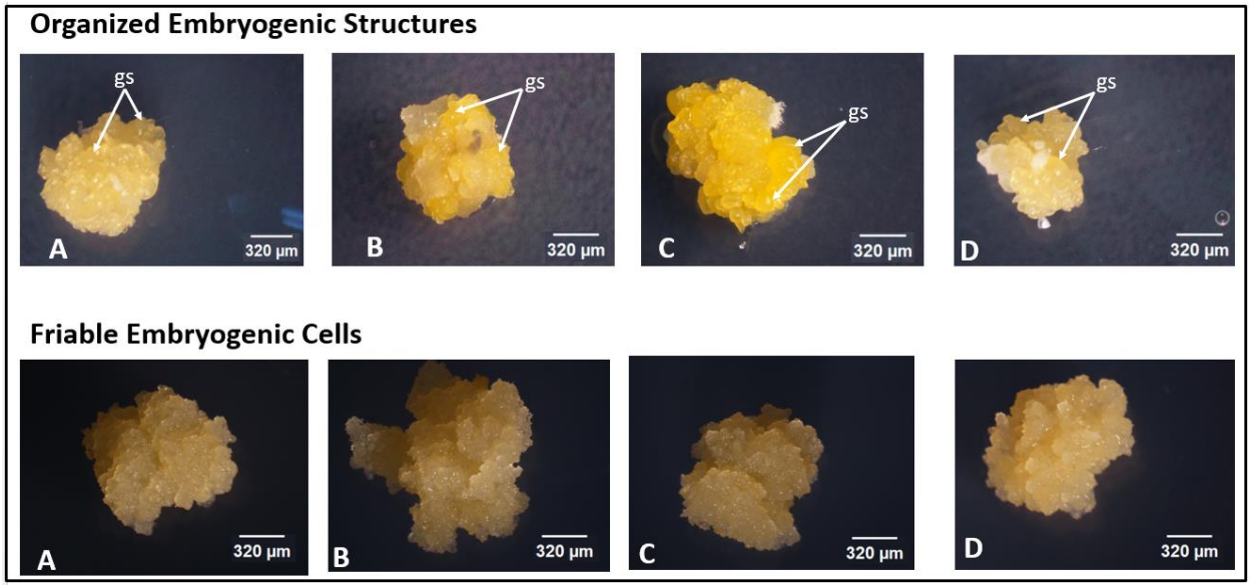

Figure 13: The organized embryogenic structures (OES) and friable embryogenic calluses (FECs) of the four cassava cultivars. A) TMS 60444, B) TME 419, C) KBH2016B/185, and D) KBH2016B/521. gs: globular structures. Scale: 4 pixels/µm.

4.3 Stable transformation and regeneration of cassava FECs

The FECs of the four cassava cultivars were placed in a suspension of *Agrobacterium* cells containing the *MeFT1* gene and were gently shaken at room temperature for transformation. The transformed FECs were moved in the selection medium for regeneration. The FECs of TMS 60444 regenerated forming cotyledons (Figure 13 A) but the FECs of the other three cultivars did not

form cotyledons. These cotyledons were transferred into CEM medium for shoot formation, followed by CMB media for rooting. Four plant lines were regenerated from the TMS 60444 cotyledons.

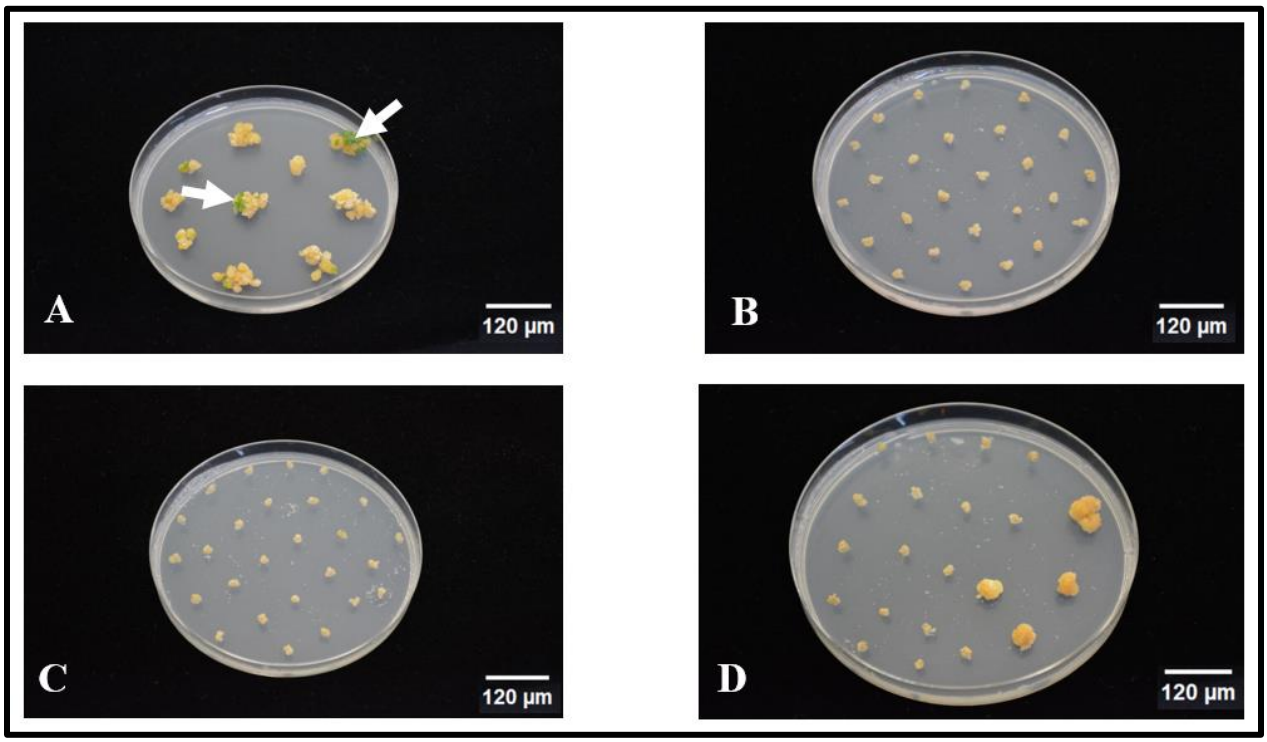

Figure 14: The transformed FECs of the four cassava cultivars. (A) TMS 60444, (B) KBH2016B/521, (C) KBH2016B/165, and (D) TME 419. The white arrows show the cotyledons of the TMS 60444. Scale: 50 pixels/µm.

4.4 Stable transformation and regeneration of *N. benthamiana*

Leaves of young *N. benthamiana* plants were cut into small pieces and placed in a suspension of *Agrobacterium* cells containing the *MeFT1* gene and were gently shaken at room temperature for transformation. The transformed leaves were then moved into selection medium for regeneration. The transformed leaves formed calli (Figure 8B) which regenerated into 15 plants lines.

4.5 Transient transformation of cassava and *N. benthamiana*

Cassava plants were transiently transformed by infiltrating the leaves and three sites close to the axillary meristems. The lower parts of the stem of the infiltrated cassava plants were also superficially cut with the same syringe made to increase the chance of vector uptake. *N. benthamiana* plants were transiently transformed by infiltrating the lower side of the third most open leaf. The infiltrated *N. benthamiana* plants had more flowers compared to the controls (Figure 14). The infiltrated *N. benthamiana* also flowered earlier and had more branches compared to the controls (Appendix 2). The infiltrated cassava plants did not flower (Figure 15) and thus no flowering data were collected on them.

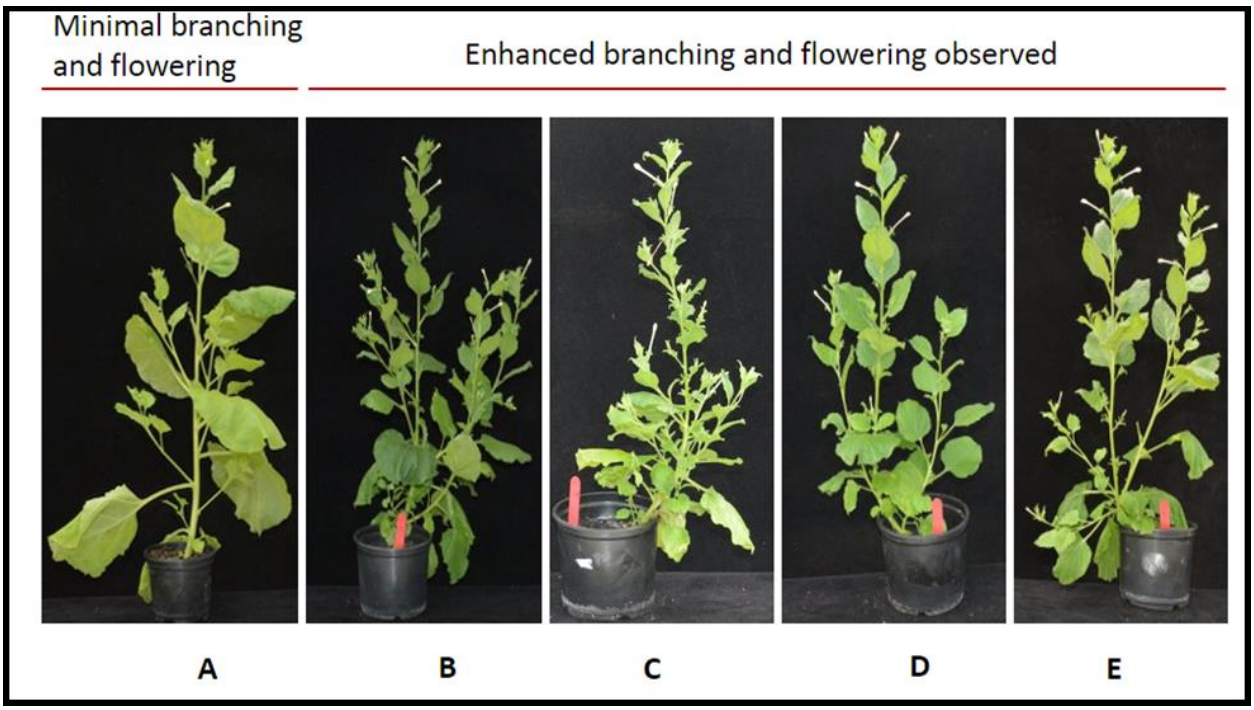

Figure 15: Agro-infiltrated *Nicotiana benthamiana* plants. (A) Control, (B) MeFT1 ΔV1, (C) MeFT1 ΔV1 + B genome, (D) MeFT1 ΔV2, and (E) MeFT1 ΔV2 + B genome.

Figure 16: The infiltrated cassava plants.

4.6 Molecular Analysis of the transformed cassava and *N. benthamiana* plants

4.6.1 PCR analysis of stably transformed cassava and *N. benthamiana* plants

Two lines of the regenerated *N. benthamiana* plants tested positive (Figure 16) for the transformed gene. The positive lines flowered earlier (2 weeks after transfer to the rooting media) (Figure 17) compared to the negative control lines. The four regenerated lines of cassava cultivar TMS 60444 were negative for the *MeFT1* gene.

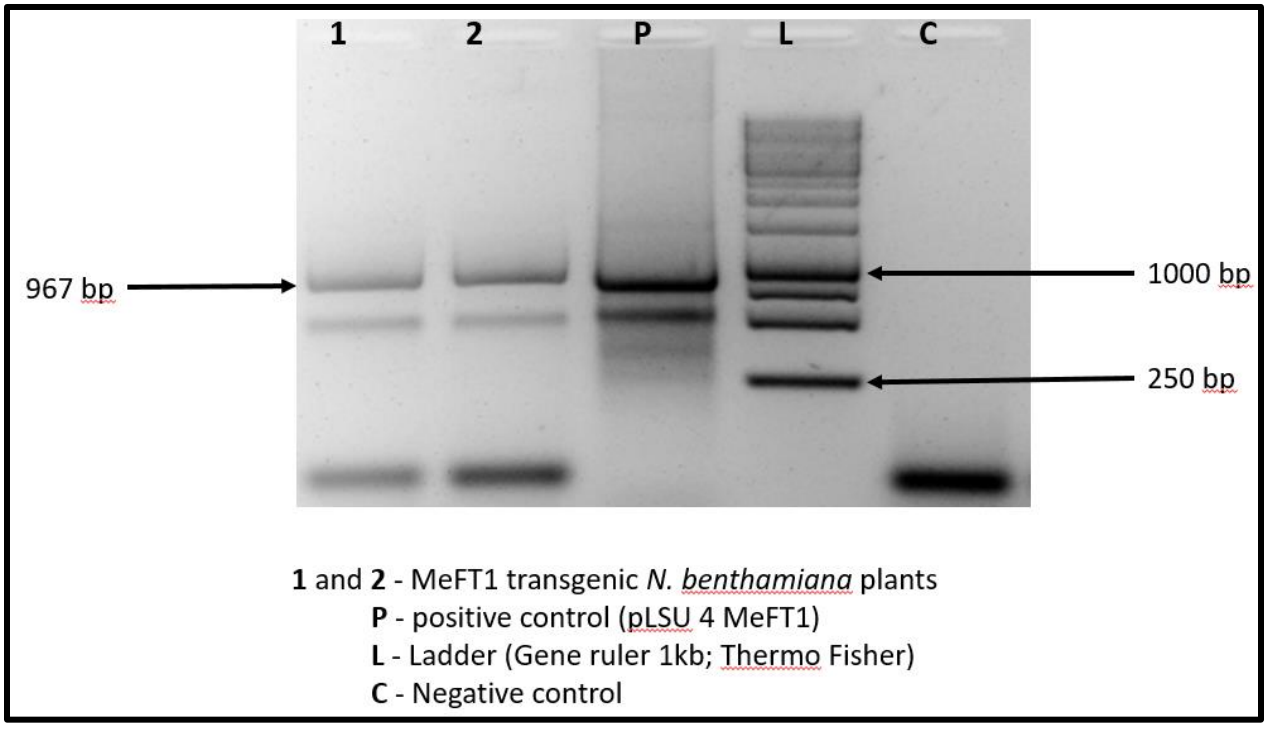

Figure 17: Gel image of the two positive lines of the MeFT1 transgenic *Nicotiana benthamiana* plants.

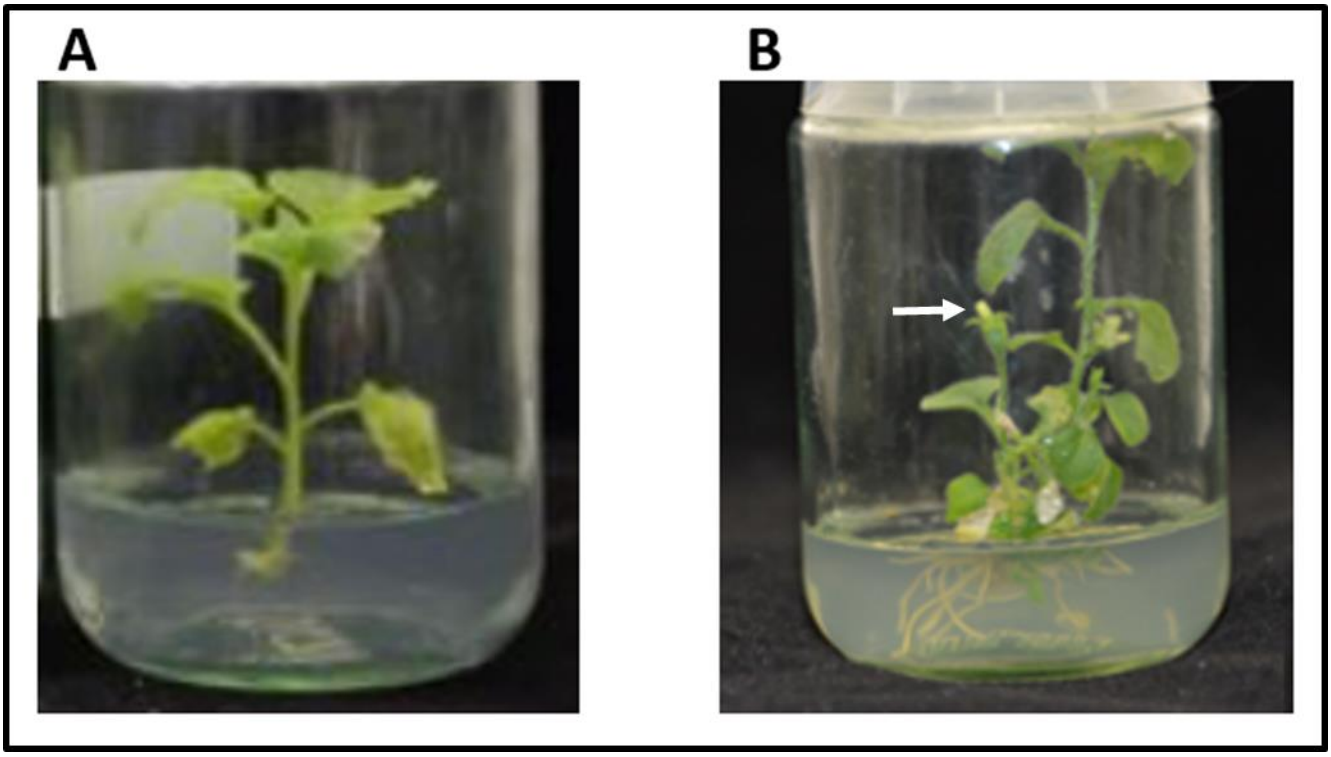

Figure 18: The non-transgenic (A) and transgenic (stably transformed) *Nicotiana benthamiana* (B). The transgenic plant flowered earlier than the control. The white arrow shows the flower.

4.6.2 PCR analysis of the transiently transformed cassava and *N. benthamiana* plants

The PCR analysis was conducted for the infiltrated plants to evaluate the systemic movement of the viral vector. The systemic *N. benthamiana* samples were positive for the viral vector (Figure 18), indicating that viral vector moved systematically in *N. benthamiana* plants only. The systemic cassava samples were PCR-negative. Furthermore, the transfer of the vector to the next generation was also analyzed for the infiltrated *N. benthamiana* plants. The results demonstrated that the F1 generation plants were negative for the viral vector (Figure 19). This indicated that the viral DNA was not integrated in the plant's genome.

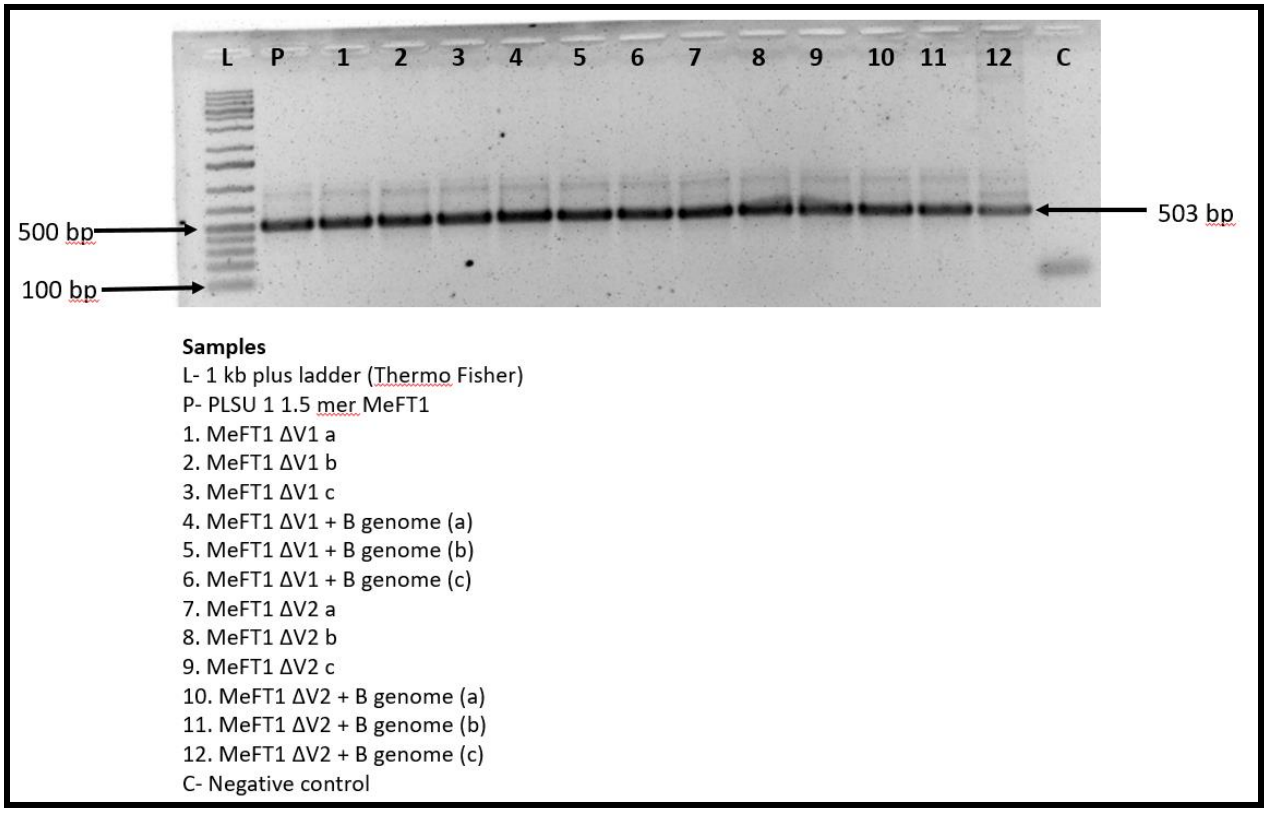

Figure 19: PCR analysis of the systemic samples from infiltrated *Nicotiana benthamiana* samples.

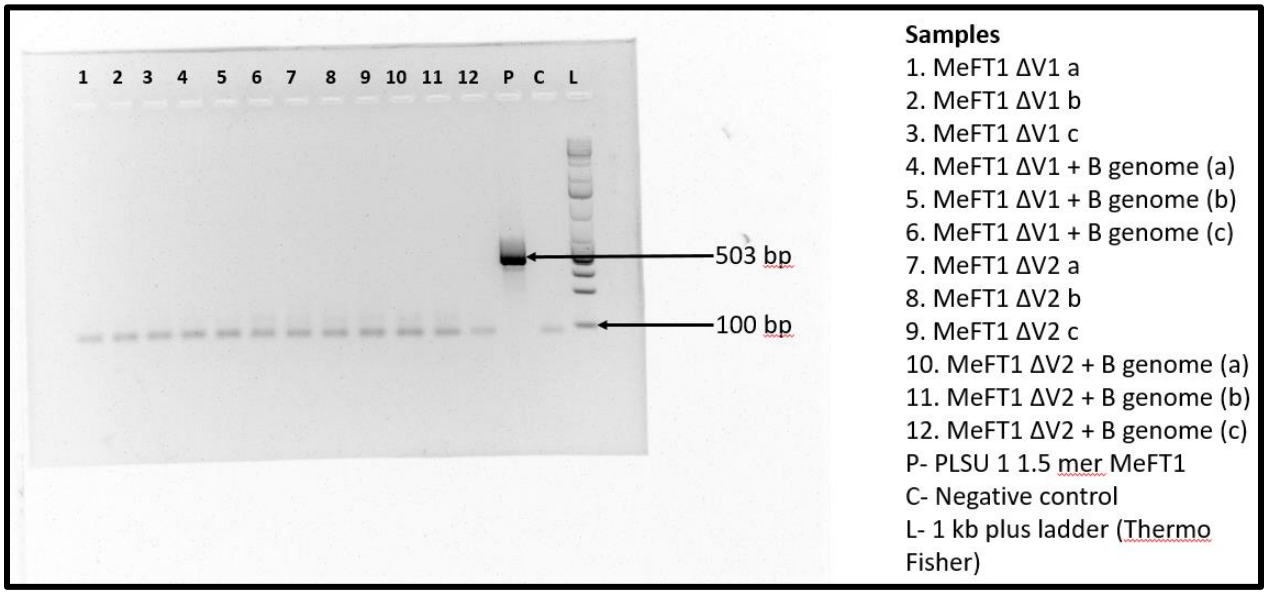

Figure 20: PCR analysis of the first genertaion (F1) of the infiltrated *Nicotiana benthamiana* plants.

4.6.3 Southern blot analysis of the transiently transformed cassava and *N. benthamiana*

The POI samples of the infiltrated *N. benthamiana* and cassava plants were subjected to southern blot analysis to check for the viral delivery and replication (Figure 20). The results showed that the viral vector was successfully delivered in the infiltrated cassava and *N. benthamiana* plants and replicated at the POI in *N. benthamiana* plants. The replication was shown by the presence of the different forms of the viral vector DNA, including supercoiled, open circular and linear, which were indicated by their different molecular weights (Figure 20). Since the systemic cassava samples tested negative for the PCR analysis, only systemic *N. benthamiana* samples were subjected to southern blot analysis. The southern blot analysis results showed that the systemic *N. benthamiana* samples were negative for the viral vector, possibly due the lower copy numbers which could not be detected via southern analysis.

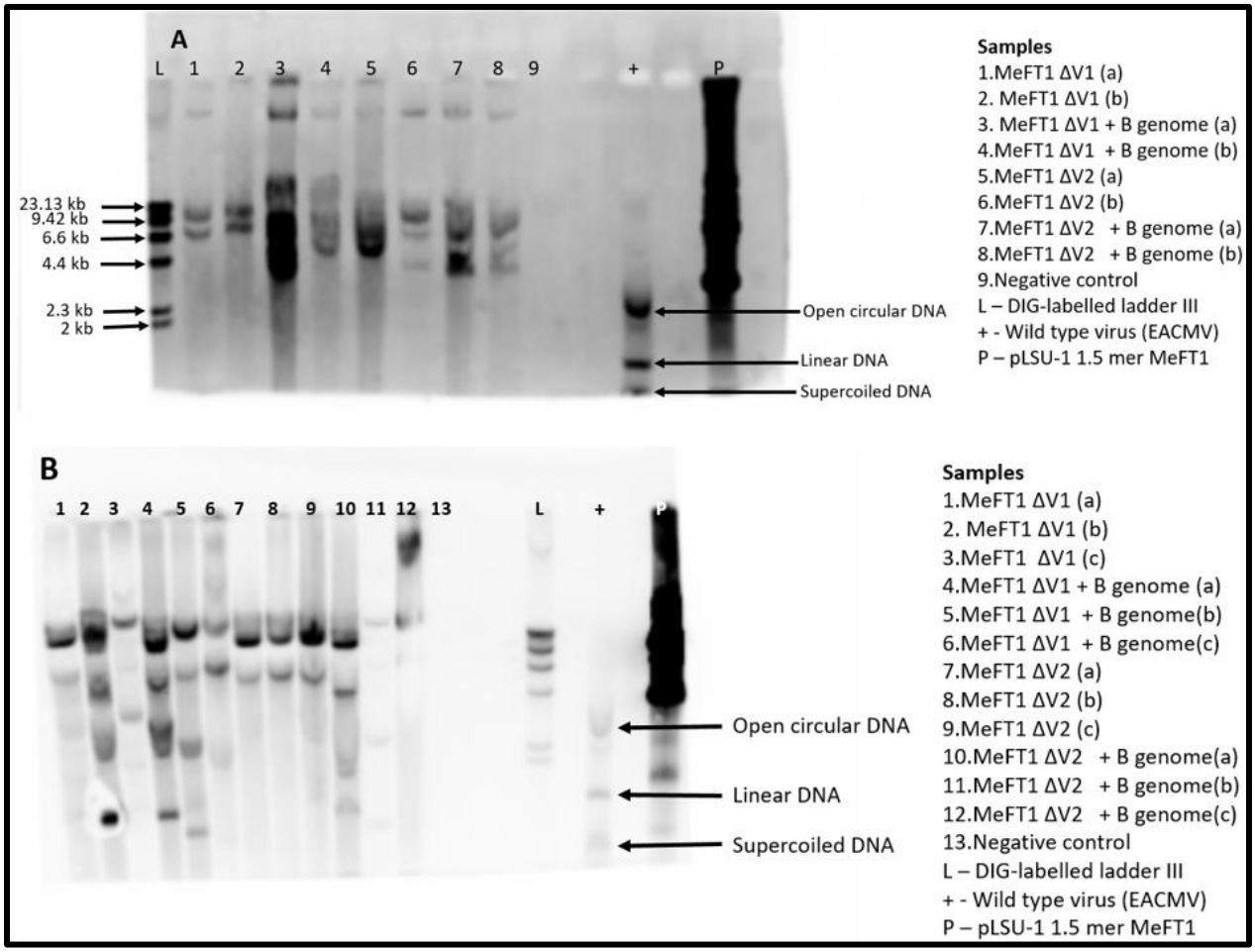

Figure 21: Southern blot analysis of the (A) cassava and (B) *Nicotiana benthamiana* point of infiltration (POI) samples.

4.7 Gene Expression Analysis

Since there was no systemic movement of the viral vector in the infiltrated cassava plants, the expression analysis was conducted using the infiltrated *N. benthamiana* plants. The wild-type flowering cassava plants served as the positive control for the assay. The infiltrated *N. benthamiana* samples and wild-type flowering cassava samples had undetermined Ct values (Appendix 1), indicating very low expression levels or possibility of non-amplification of the *MeFT1* gene.

CHAPTER FIVE

5.1 DISCUSSION

The main challenge in cassava breeding is the late and non-synchronous flowering of the farmer-preferred cultivars which most often need improvement from ever evolving pathogens. Flowering is induced by the *FT* gene, present in various plants as homologs. Two *FT* homologs have been reported in cassava, *MeFT1* and *MeFT2 (Adeyemo et al., 2019)*, among which *MeFT1* has been widely characterized and reported to induce flowering (Odipio *et al*., 2020). Therefore, this study over expressed *MeFT1* in cassava and *N. benthamiana* plants using the deconstructed EACMV vector to induce early flowering. Here, *N. benthamiana* served as the model species for the proof of concept, and stable transformation which allowed for the constitutive expression of the target gene, served as the positive control experiment for the study. The results showed that the *N. benthamiana* plants regenerated from the stable transformation were positive for the *MeFT1* gene and exhibited early flowering while still in tissue culture; however, the regenerated cassava plants tested negative for the gene. This might have been due to the cassava transformation and regeneration difficulties. Cassava genetic transformation requires suitable tissues with high totipotency, such as FECs, somatic embryo cotyledons and embryogenic suspension cultures for a successful regeneration of transgenic plants (Elegba *et al*., 2021; Hellen *et al*., 2021). Among these tissues, FECs are the most preferred for the *Agrobacterium*-mediated cassava transformation due to their ability to produce numerous independent transgenic events for different cassava cultivars (Ma *et al*., 2015; Nyaboga *et al*., 2015). FECs production is highly cultivar-dependent, necessitating individual cultivar optimization for successful FECs generation. This greatly limits its application in cassava transformation. Moreover, FEC-based cassava transformation is also limited by low regeneration rates of the transformed calluses and low conversion rates of their

somatic embryos into plantlets (Lentz, *et al*., 2018b). In the present study, FECs generated from four cassava cultivars (TMS 60444, TME 419, KBH 2016B/185, and KBH 2016B/521) were used for the *Agrobacterium*-mediated transformation. The transformed FECs of TMS 60444 produced cotyledons, which were later transferred into cassava shoot elongation media (CEM) for differentiation, but the FECs of the other three cultivars did not regenerate. However, the control (non-transformed) FECs of all the cultivars produced cotyledons. This indicated low regeneration rates of the transformed cassava FECs. Moreover, four lines were regenerated from the cotyledons of the transformed TMS 60444, demonstrating the low regenerated rates of their somatic embryos into plantlets. Similar cases of low regeneration rates of the cotyledonary embryos into plantlets have also been reported by previous studies. Chetty *et al*. (2013) reported only 33 transgenic plantlets from 514 cotyledonary embryos resistant to hygromycin for the cassava genotype T200. Similarly, Ihemere *et al*. (2006) obtained only 26 transgenic regerants from over 800 cotyledonary embryos with resistance to paromomycin for the cassava variety TMS71173. Another common phenomenon in cassava transformation is the regeneration of escapees. In this study, the four lines regenerated from the TMS 60444 cotyledonary embryos grew on hygromycin-treated media; however, they were all negative for the transformed *MeFT1* gene. Lentz *et al*. (2018a) transformed a Brazilian farmer-preferred variety Verdinha BRS 222 with a GUS reporter gene and obtained only one transgenic line out of the 35 regenerated lines. This indicated that 34 regenerated lines (97%) were escapees. Unlike cassava, *N. benthamiana* transformation is relatively simple with a short turnaround time; however, its regeneration is also associated with high-false positive rates. This is probably because *N. benthamiana* transformation involves transforming leaf tissues and not individual cells. In this study, two out of 15 lines regenerated from the transformed *N. benthamiana* leaves were transgenic. Yau *et al*. (2020) reported that only 38 out of the 58 tobacco

shoots regenerated from the kanamycin-resistant calli were positive for the kanamycin resistance gene, suggesting that the 20 shoots were escapees. Additionally, Nagl *et al*. (2005) transformed *N. tabacum*, *N. benthamiana* and *N. excelsior* with the beet necrotic yellow vein virus (BNYVV)-derived coat protein and found that 50-83% of the regenerants were non-transgenic despite the high transformation efficiency.

For transient expression, cassava and *N. benthamiana* plants were inoculated with constructs carrying the *MeFT1* gene.. The southern blot analysis showed the viral vector was successfully delivered in both cassava and *N. benthamiana* plants and replicated in *N. benthamiana* at POI. The replication was illustrated by the different forms of the replicating viral vector in the southern blot images. Replicating virus exist in three forms namely supercoiled, open circular and linear DNA. The supercoiled form, also known as covalently closed circle (ccc), is the native form of the virus *in vivo*, enabling it to fit inside the cell. Since the supercoiled conformation is highly compacted, it less restrained by the agarose matrix than the open circular and linear conformations. The open circular conformation tends to be bulky than the linear one, and thus, it more restrained by the agarose matrix than the linear DNA. Therefore, replicating virus produce three bands on agarose gel showing the movement of the supercoiled, open circle and linear conformations. In this study, the replication of the deconstructed viral vector was analyzed via southern blot analysis, using the wild type EACMV as the positive control. The wild type EACMV showed three distinct bands representing the supercoiled, open circle and linear conformations. During replication, viruses convert their circular DNA into linear forms, which are more stable and less prone to genetic loss, using proteins such as protelomorases (Knott *et al*., 2020). Therefore, the presence of the linear, open circular and supercoiled DNA of the viral vector in this study confirmed the viral replication in cassava and *N. benthamiana* plants at POI.

Viral vectors move through plants in two main ways; 1) cell-cell through the plasmodesmata and 2) systematically through the vascular system (Khakhar & Voytas, 2021). Plasmodesmata movement is a slow process which relies on the movement and shuttle proteins, while the systemic one occurs when the virus moves via the vascular system for rapid trafficking to the different parts of the plant. The systemic movement of certain viruses has been reported to be partly mediated by tRNA-like sequences present in their genomes (Zhang *et al*., 2016). In the present study, newly formed leaves (the third most open) were sampled from the infiltrated cassava and *N. benthamiana* plants at 10 dpi to analyze the viral vector systemic movement. *N. benthamiana* samples showed that the viral vector moved systematically; however, the movement was only detectable via PCR and not southern blot analysis. This suggests that the vector was present in low copy numbers, which could not be detected via southern blot analysis. A study by Kyallo *et al*. (2017) reported systemic infection of *N. benthamiana* plants with Deinbollia mosaic virus, demonstrating the ability of Begomoviruses to move systematically in *N. benthamiana*. The lack of systemic viral movement in infiltrated cassava plants might have been due to the poor uptake of the constructs associated with the difficulty in infiltrating cassava. The hydrophobic leaf surface and sunken stomata make the conventional agro-infiltration of cassava difficult (Duff-Farrier *et al*., 2019). Using surfactants and keeping the plants in the dark post-infiltration have been shown to improve the uptake of agro-infiltrated constructs in various plants (Donini & Marusic, 2019; Duff-Farrier *et al*., 2019). Additionally, Ellison *et al*. (2020) reported that the systemic movement of the viral vectors can be improved by including exogenous tRNA-like sequences into the vector. Gene expression quantification is important in understanding how much of the gene is transcribed in an organism. The most common method used to quantify the gene expression level is qPCR analysis, which determines the expression level based on the cycle threshold (Ct) values (Ma *et*

al., 2021). Lower Ct values indicate that the gene has high copy numbers, translating to higher expression levels. On the other hand, higher Ct values suggest that the gene has low copy numbers, indicating lower expression levels. In some cases, the Ct values may be undetermined, indicating that the gene of interest has a below-the-detection-limit expression level or the gene was not amplified (Ma *et al*., 2021). In this study, the infiltrated *N. benthamiana* and the wild-type flowering cassava plants had undetermined Ct values for the *MeFT1* gene. This indicated very low expression levels or non-amplification of the *MeFT1* gene. The flowering gene, *FT*, has been characterized in *N. tabacum* but not in *N. benthamiana*. Harig *et al*. (2012) isolated four *FT* genes from *N. tabacum* (*NtFT1*, *NtFT2*, *NtFT3*, and *NtFT4*), among which three (*NtFT1*, *NtFT2* and *NtFT3*) inhibit flowering and one (*NtFT4*) induces flowering. This suggested that *FT* genes can antagonistically regulate floral initiation. Thus, it could be possible that some endogenous *N. benthaniana FT* genes acted as antagonistic regulators and suppressed the expression of the cassava *FT* homolog, *MeFT1*, in the infiltrated *N. benthamiana* plants. Therefore, characterizing the *FT* gene in *N. benthamiana* would provide insight into their role in floral initiation. The *MeFT1* gene has not been characterized in different cassava cultivars, and the very low expression or no-amplification of the *MeFT1* in the wild-type flowering cassava used in this study might suggest possible differences in the *MeFT1* gene among different cassava cultivars. This is because the wild-type flowering cassava used in this study was TME 214, but the *MeFT1* gene was amplified from TMS60444. Thus, characterizing *MeFT1* gene across different cassava cultivars would aid in understanding the differences and functions of their flowering genes.

Transiently transformed genes do not get integrated into the host's genome. These genes are expressed for a short period of time in the somatic cells and are lost after several days via cell division, indicating that the genes are not passed to the next generations (Krenek *et al*. 2015). Upon

infection, CMV persists within the plant for a prolonged period and since the virus is not vertically transmitted, the F1 generations obtained by planting the seeds of the infected parental lines are usually free from the virus (McGarry and Ayre, 2012). In this study, F1 plants obtained from the agro-infiltrated *N. benthamiana* plants were analyzed to determine the presence of the EACMV-based vector. The F1 generation plants tested negative for the viral vector, indicating that the gene was not passed down from their transgenic parents. The infiltrated *N. benthamiana* plants flowered earlier and had more branches and flowers compared to the controls. Therefore, if optimized in the future, the EACMV-based could also induce early flowering in cassava.

5.2 Conclusion

Cassava transformation is limited by several factors, including difficulty in FEC production and transformation, low regeneration rates of the transformed FECs and high false positive rates of the regenerated plantlets. Four lines were regenerated from the transformed FECs of the cassava cultivar TMS 60444 but the plants tested negative for the transformed gene. The transient overexpression of the *MeFT1* gene induced early flowering and profuse branching in *N. benthamiana*. Southern blot analysis demonstrated the viral replication at the point of infiltration of the cassava and *N. benthamiana* plants. Therefore, the findings of this study provide a theoretical basis for developing viral-induced expression systems in plants, using *N. benthamiana* as the model plant. The study reports a successful delivery of the EACMV-based vector in cassava, which could be adopted in the future studies for cassava genetic improvement.

5.3 Recommendations

1. Optimizing the infiltration methods using surfactants and keeping plants in the dark post-infiltration could improve the uptake of agro-infiltrated constructs.
2. Improving the vector structure by incorporating exogenous tRNA-like sequences can enhance the systemic movement through the plants.
3. Increasing the amount of the infiltrated vectors could ensure efficient uptake of the viral vector by cassava plants, which could in turn promote viral systemic movement and induce flowering in cassava.
4. Characterizing the *MeFT1* gene across different cassava cultivars to determine their differences.
5. Characterizing *N. benthamiana FT* genes to determine their functions.

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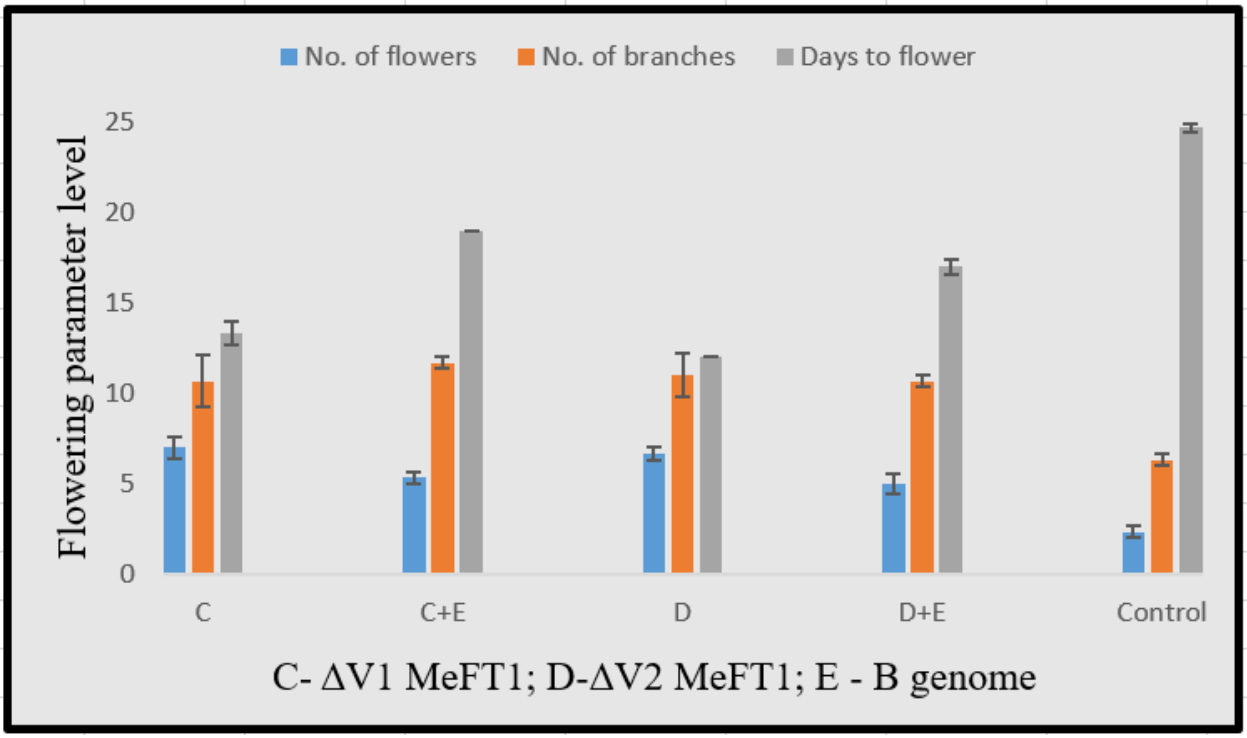
APPENDICES

Appendix 1: The qPCR results of the infiltrated *N. benthamaniana* plants and wild-type flowering cassava plants

Sample	Target gene	Ct value
pLSU 1 1.5 mer ΔV1 MeFT1 + B genome (a)	MeFT1	Undetermined
pLSU 1 1.5 mer ΔV1 MeFT1 + B genome (b)	MeFT1	Undetermined
pLSU 1 1.5 mer ΔV1 MeFT1 + B genome (c)	MeFT1	Undetermined
pLSU 1 1.5 mer ΔV2 MeFT1 + B genome (a)	MeFT1	Undetermined
pLSU 1 1.5 mer ΔV2 MeFT1 + B genome (b)	MeFT1	Undetermined
pLSU 1 1.5 mer ΔV2 MeFT1 + B genome (c)	MeFT1	Undetermined
Field-flowering cassava (a)	MeFT1	Undetermined
Field-flowering cassava (b)	MeFT1	Undetermined
Field-flowering cassava (c)	MeFT1	Undetermined
Plasmid (pLSU 1 1.5 mer ΔV1 MeFT1)	MeFT1	6.168
Plasmid (pLSU 1 1.5 mer ΔV1 MeFT1)	MeFT1	6.238
Plasmid (pLSU 1 1.5 mer ΔV1 MeFT1)	MeFT1	5.991
Ctrl a (non-infiltrated Nicotiana)	MeFT1	Undetermined
Ctrl b (non-infiltrated Nicotiana)	MeFT1	31.540
Ctrl c (non-infiltrated Nicotiana)	MeFT1	31.834
No template	MeFT1	Undetermined
No template	MeFT1	Undetermined
No template	MeFT1	Undetermined
pLSU 1 1.5 mer ΔV1 MeFT1 + B genome (a)	Me26S	Undetermined
pLSU 1 1.5 mer ΔV1 MeFT1 + B genome (b)	Me26S	Undetermined
pLSU 1 1.5 mer ΔV1 MeFT1 + B genome (c)	Me26S	Undetermined
pLSU 1 1.5 mer ΔV2 MeFT1 + B genome (a)	Me26S	Undetermined
pLSU 1 1.5 mer ΔV2 MeFT1 + B genome (b)	Me26S	Undetermined
pLSU 1 1.5 mer ΔV2 MeFT1 + B genome (c)	Me26S	Undetermined
Field-flowering cassava (a)	Me26S	Undetermined

Field-flowering cassava (b)	Me26S	24.309
Field-flowering cassava (c)	Me26S	23.602
Plasmid (pLSU 1 1.5 mer ΔV1 MeFT1)	Me26S	Undetermined
Plasmid (pLSU 1 1.5 mer ΔV1 MeFT1)	Me26S	Undetermined
Plasmid (pLSU 1 1.5 mer ΔV1 MeFT1)	Me26S	Undetermined
Ctrl a (non-infiltrated Nicotiana)	Me26S	Undetermined
Ctrl b (non-infiltrated Nicotiana)	Me26S	Undetermined
Ctrl c (non-infiltrated Nicotiana)	Me26S	Undetermined
No template	Me26S	Undetermined
No template	Me26S	Undetermined
No template	Me26S	Undetermined
pLSU 1 1.5 mer ΔV1 MeFT1 + B genome (a)	NPP2	21.951
pLSU 1 1.5 mer ΔV1 MeFT1 + B genome (b)	NPP2	22.846
pLSU 1 1.5 mer ΔV1 MeFT1 + B genome (c)	NPP2	22.944
pLSU 1 1.5 mer ΔV2 MeFT1 + B genome (a)	NPP2	22.937
pLSU 1 1.5 mer ΔV2 MeFT1 + B genome (b)	NPP2	23.267
pLSU 1 1.5 mer ΔV2 MeFT1 + B genome (c)	NPP2	21.454
Field-flowering cassava (a)	NPP2	Undetermined
Field-flowering cassava (b)	NPP2	Undetermined
Field-flowering cassava (c)	NPP2	Undetermined
Plasmid (pLSU 1 1.5 mer ΔV1 MeFT1)	NPP2	Undetermined
Plasmid (pLSU 1 1.5 mer ΔV1 MeFT1)	NPP2	Undetermined
Plasmid (pLSU 1 1.5 mer ΔV1 MeFT1)	NPP2	Undetermined
Ctrl a (non-infiltrated Nicotiana)	NPP2	21.985
Ctrl b (non-infiltrated Nicotiana)	NPP2	Undetermined
Ctrl c (non-infiltrated Nicotiana)	NPP2	22.303
No template	NPP2	Undetermined
No template	NPP2	Undetermined
No template	NPP2	Undetermined

Appendix 2: Flowering parameters of the infiltrated *N. benthamiana* plants.



The infiltrated plants flowered earlier and had more flowers and branches compared to the controls.

C: pLSU-1 ΔV1 MeFT1; C+E: pLSU-1 ΔV1 MeFT1 + B genome; D: C: pLSU-1 ΔV2 MeFT1; D+E: pLSU-1 ΔV2 MeFT1 + B genome.

Appendix 3: Southern Blot Buffers

20X Sodium citrate dihydrate (SSC)

SSC (88.23 g) and NaCl (175.32 g) were dissolved in 1L of distilled water and the pH was adjusted to 7. The solution was autoclaved and stored at room temperature.

20% Sodium dodecyl sulfate (SDS)

SDS powder (200 g) was dissolved in 1L of distilled water to make a 20% SDS solution.

Depurination solution

Concentrated HCl (20.6 ml) was added to 979.4 ml of distilled water to make the depurination solution.

Denaturation solution

The solution was prepared by dissolving 87.75 g of NaCl and 20 g of NaOH in 1L of distilled water, followed by autoclaving and storage at room temperature.

Neutralization solution

Tris (60.57 g), Nacl (87.75 g) and EDTA (0.3724 g) were dissolved in 1L of distilled water to prepare the neutralization solution. The pH was adjusted to 7.5 before autoclaving.

Maleic Acid Buffer

Maleic acid (11.6 g) and NaCl (8.75 g) was dissolved in 1L of distilled water and the solution was adjusted to a pH of 7.5 before autoclaving.

Detection buffer

Detection buffer was prepared by mixing 100 ml of 1M Tris-HCl solution with 20 ml of 5M Nacl solution and topping up the solution to 1 L using distilled water.

Low stringency buffer

A mixture of 20X SSC solution (10 ml) and 20% SDS solution (5 ml) was reconstituted to 100 ml with distilled water to prepare the low stringency buffer.

High stringency buffer

High stringency buffer was prepared by topping up 2.5 ml of 20X SSC solution and 5 ml of 20% to 100 ml with distilled water.

Washing buffer

Maleic Acid containing 0.3% Tween 20.

Blocking solution

The blocking solution was prepared by weighing 1 g of the blocking powder (Sigma) in 100 ml of maleic acid. The mixture was dissolved by heating it in the microwave. Thereafter, 60 ml was

allocated as the blocking solution and the remaining 40 ml was used as the antibody solution, in which 4 µl of the antibodies (AP conjugates) was added to make a 1:10,000 dilution.

Appendix 4: Synthesis of the Southern Blot Probe

Component	**DIG-Labeled Probe**	**Unlabeled DNA control**	**Labeled kit control**
Nuclease-free water	32.25 µl	32.25 µl	29.25 µl
10X buffer with $MgCl_2$	5 µl	5 µl	5 µl
DIG-labeled dNTP mix	5 µl	-	5 µl
dNTP stock	-	5 µl	-
Forward primer (AGGCGCAGTGATGAGTTCCCCT)	5 µl	5 µl	5 µl (Kit primers)
Reverse primer (AGGACGGGGAAGACGATGTGGG)	5 µl	5 µl	5 µl (Kit primers)
Enzyme mix	0.75 µl	0.75 µl	0.75 µl
DNA template	2 µl	2 µl	5 µl (Kit template)

The reaction reagents were thawed on ice, vortexed, and briefly centrifuged, followed by addition

into a sterile microtube to form a reaction mixture of 50 µl. The PCR reaction conditions were as follows: one cycle of 95 ℃ for 5 minutes; 35 cycles of 95 ℃ for 30 seconds, 60 ℃ for 30 seconds, 72 ℃ for 30 seconds; and a final cycle of 72 ℃ for 7 minutes. Thereafter, probe labeling was confirmed by gel electrophoresis using 2 ul of the PCR product.

Hybridization probe was prepared by adding 10µl of the PCR product into 40µl of distilled water and heating the mixture in boiling water for 5 min to denature the probe. The mixture was immediately chilled in ice for 2 min and vortexed briefly. The denatured probe was then added into a 10 ml of pre-warmed hybridization buffer.

Appendix 5: Preparation of the DIG-labeled marker

The DIG-labeled marker was prepared by mixing 5 µl of DIG-labeled marker (DNA Molecular Weight Marker VI, Sigma), 3 µl of the loading dye and 11 µl of distilled water.

Appendix 6: Preparation of plant selection antibiotics

Cefotaxime

Cefotaxime (300 mg/ml) was prepared by dissolving 15 g of Cefotaxime sodium in 50 ml of distilled water, followed by filter sterilization using a 0.2 µm filter. The solution was then aliquoted into sterile 1.5 ml tubes and stored at 4 °C.

Kanamycin

Kanamycin (50 mg/ml) was prepared by dissolving 500 mg of Kanamycin monosulfate in 10 ml of distilled water, followed by filter-sterilization using a microfilter (0.2 µm) and storage at 4 °C.

Hygromycin

Hygromycin (10 mg/ml) was prepared by weighing 100 mg of hygromycin into 10 ml of distilled water, filter-sterilized with a 0.2 µm filter and stored 4 °C.

Acetosyringone

Acetosyringone (100 mM) was prepared by dissolving 196 mg of acetosyringone in 1 ml of 70 % ethanol. The solution was then topped up to 10 ml using distilled water, followed by filter-sterilization using a 0.2 µm filter and storage at 4 °C.

Rifampicin

Rifampicin (25 mg/ml) was prepared by weighing 250 mg of rifampicin into 10 ml of dimethyl sulfoxide (DMSO), filter-sterilized with a 0.2 µm filter and stored at 4 °C.

Appendix 7: Media preparation

Cassava basal medium (CBM)

CBM was prepared with 4.4 g/L of MS salts with vitamins (Duchefa) (Murashige and Skoog, 1962) and 2 % (w/v) sucrose supplemented with 2mM CuSO4. The pH was adjusted to 5.8 before adding 0.2 % (w/v) gelrite and the media was autoclaved at 121 °C and 15 psi for 15 minutes.

Cassava axillary medium (CAM)

The media contained MS salts with vitamins (Duchefa) (4.4g/L), 2mM CuSO4, BAP (10 mg/L) and 2 % (w/v) sucrose. The pH was adjusted to 5.8 before adding 0.8 % (w/v) Noble Agar and the media was autoclaved at 121 °C and 15 psi for 15 minutes.

Cassava embryo induction medium (CIM)

The media contained MS salts with vitamins (Duchefa) (4.4g/L), 2mM CuSO4, picloram (6 mg/L), and 2 % (w/v) sucrose. The pH was adjusted to 5.8 before adding 0.8 % (w/v) Noble Agar and the media was autoclaved at 121 °C and 15 psi for 15 minutes.

Gresshoff and Doy (GD) medium

The GD medium contained GD salts with vitamins (Duchefa) (2.7g/L) (Greshoff and Doy, 1972),

picloram (6 mg/L), biotin (0.2 mg/L), and 2 % (w/v) sucrose. The pH was adjusted to 5.8 before adding 0.8 % (w/v) Noble Agar and the media was autoclaved at 121 °C and 15 psi for 15 minutes.

Cassava embryo regeneration medium (MSN)

MSN components were MS salts with vitamins (Duchefa) (4.4g/L), NAA (1 mg/L), and 2 % (w/v) sucrose. The pH was adjusted to 5.8 before adding 0.8 % (w/v) Noble Agar and the media was autoclaved at 121 °C and 15 psi for 15 minutes.

Cassava shoot elongation medium (CEM)

CEM was prepared using MS salts with vitamins (Duchefa) (4.4g/L), 2mM CuSO4, BAP (4 mg/L), and 2 % (w/v) sucrose. The pH was adjusted to 5.8 before adding 0.8 % (w/v) Noble Agar and the media was autoclaved at 121 °C and 15 psi for 15 minutes.

Benthamiana co-cultivation medium (MS3)

MS3 medium contained MS salts with vitamins (Duchefa) (4.4g/L) supplemented with 3 % (w/v) sucrose. The pH was adjusted to 5.8 before autoclaving at 121 °C and 15 psi for 15 minutes.

Cassava co-cultivation medium (MSNB)

The medium was prepared with MS salts with vitamins (Duchefa) (4.4 g/L), BAP (1 mg/L), NAA (0.1 mg/L), and 3 % (w/v) sucrose. The pH was adjusted to 5.8 before adding 0.8 % (w/v) Noble Agar and the media was autoclaved at 121 °C and 15 psi for 15 minutes.

Benthamiana rooting medium (MS3+FeSO4)

MS3 supplemented with FeSO4

Appendix 8: Preparation of chemo-competent *E. coli* cells

Glycerol stock of DH5α *E. coli* cells was streaked onto an LB plate with no antibiotics and incubated overnight at 37 °C. A single colony was selected from the plate and inoculated in

10 ml of LB broth, followed by an overnight incubation at 37 ℃ in a rotary shaker to prepare the starter culture. Thereafter, 5 ml of the overnight culture was added in 100 ml of LB media and incubated for 3 hrs at 37 ℃ until the OD600 reached 0.5. The culture was immediately chilled in ice for 25 min and the cells were harvested by centrifugation at 4 ℃ for 10 min at 4000 g. The supernatant was discarded and the pellets were gently resuspended in 10 ml of ice-cold 0.1M CaCl2, followed by ice-incubation for 45 minutes. The solution was centrifuged at 4 ℃ for 10 min at 4000 g and the pellet resuspended in 10 ml of ice-cold 0.1M CaCl2 + 10% glycerol after discarding the supernatant. Thereafter, 50 ul of the suspension were aliquoted into sterile ice-chilled microtubes and snap-frozen in liquid nitrogen, followed by storage at -80 ℃ until use.

Appendix 9: Transformation of chemo-competent *E. coli* cells by heat-shock method

Plasmid DNA (50 ug) was mixed with 50 µl of thawed competent E. coli cells and incubated on ice for 20 min. The cells heat-shocked at 42°C for a minute and immediately chilled on ice for 2 min. Thereafter, 1 ml of LB broth was added to the cells, followed by incubation at 37 ℃ for 2 hrs. The transformation mixture (100 ul) was then plated on LB containing 50 mg/l of kanamycin and incubated at 37 ℃ overnight, after which the colonies were screened for plasmid presence via colony PCR.

Appendix 10: Preparation of electro-competent *Agrobacterium* cells

The *Agrobacterium* culture was grown in 20 ml of LB medium containing rifampicin (25 mg/ml) and kanamycin (50 mg/ml) and incubated with shaking at 28 ℃ until an of 0.8 at OD600 was reached. The culture was placed on ice for 20 min and centrifuged at 4 ℃ for 20 min at a speed of 3000g. The supernatant was discarded and the pellets were washed twice by resuspending in 20 ml of 10% glycerol, followed by centrifugation at 4 ℃ for 20 min at a

speed of 3000g. Thereafter, the washed cells were resuspended in 400 ul of LB broth containing 10% glycerol. The cell suspension was then used to make 50 ul aliquots, which were snap-frozen in liquid nitrogen and stored at -80 ℃ until use.

Appendix 11: Transformation of electrocompetent *Agrobacterium* cells by electroporation method

The competent *Agrobacterium* cells, plasmid DNA and electroporation cuvettes (2 mm) were ice-chilled, and 100 ng of the plasmid DNA was added to the competent *Agrobacterium* cells. The cells-plasmid mixture was transferred into the electroporation cuvette and incubated on ice for 15 min. The Gene Pulser machine (Bio-Rad Gene Pulser™) was set to 2.5 kV (field strength), 200Ω (controller) and 25µFD (capacitance), and the cuvette containing the mixture was then in the Gene pulser for electroporation. After that, 800 ul of LB broth was immediately added to the mixture.

Appendix 12: Preparation of the DNA and RNA extraction buffer

The CTAB extraction buffer was prepared by mixing 10% CTAB, with 1M Tris-HCl (pH 8.0), 0.5M EDTA (pH 8.0), 5M NaCl, and an appropriate amount of distilled water (depending on the volume of the buffer). Mercaptoethanol (20 µl/ml) was added under the fume hood before use.

Appendix 13: Preparation of 1X Tris-acetate-EDTA (TAE) buffer

The working concentration (1X) of TAE buffer (2L) was prepared by mixing 50 ml of 50X of the same buffer (2 M Tris base, 1 M glacial acetic acid and 0.05 M EDTA (pH 8)) with 150 ml of distilled water.

Appendix 14: Preparation of Agarose gel

Agarose gel (1.2%) was prepared by weighing 1.2 g of agarose powder (Duchefa) into 100 ml of

1X TAE buffer and heating in the microwave until the gel dissolves. Gel red (Thermo Fisher Scientific GelRed Nucleic Acid Gel Stain) (1 µl) was then added to the dissolved gel, mixed well and the gel was poured on a casting tray to solidify.