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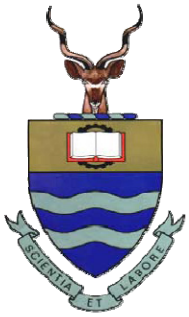
CASSAVA AXILLARY BUD TRANSFORMATION AND PRODUCTION OF SOMATIC EMBRYOS OF SELECTED CASSAVA CULTIVARS

Claudia Rossin



UNIVERSITY OF THE
WITWATERSRAND

A thesis submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in the School of Molecular and Cell Biology.

Johannesburg, 2008

DECLARATION

I declare that this is my own, unaided work. It is being submitted for the degree of Master of Science in the School of Molecular and Cell Biology, to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

_____

Claudia Rossin

This _____ day of _____ 2008.

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PREFACE

I sincerely thank Prof MEC Rey for her guidance and support throughout my MSc. I also wish to thank everyone that has helped me to achieve this goal in any manner; be it ordering goods, experimental inputs and assistance and for friendships developed that kept me sane.

ABSTRACT

Genetic transformation is essential for introducing new traits into cassava. However, the current protocols for cassava transformation are inefficient. In this study, the aims were to develop a protocol for *Agrobacterium tumefaciens*-mediated genetic transformation of cassava axillary buds and direct regeneration thereof, to screen selected cultivars for somatic embryogenesis (SE) potential and tobacco leaf discs were transformed with a 221bp Rep transgene derived from *South African cassava mosaic virus* (SACMV) to determine the efficiency of the antisense transgene to silence SACMV. Various explant pre-treatments were tested prior to transformation, followed by *Agrobacterium*-infiltration. Co-cultivation at different temperatures (22 and 25ºC), photoperiod (16h light 8h dark, and complete darkness) as well as co-cultivation time periods, were evaluated. GUS assays showed that putative transgenic plants had not been transformed. The most widely used explants for cassava transformation are friable embryogenic callus (FEC) and somatic cotyledons. In this study, 9 cassava cultivars were tested for SE and FEC induction. Media containing various plant growth regulators and various explants (40 explants per experiment) were used for the production of SE. The optimal media and explants for SE were shown to be axillary buds on MS2 containing 50µM picloram, except for cultivar AR9-18 which showed increased SE production using immature leaf lobes on MS2 containing 50µM picloram. The cultivars with the highest SE efficiency were cultivars TMS60444 (model cultivar), T200, AR 9-18, MTAI16, CR25-4 and CM523-7. Low SE efficiency was found in BRA 1183, MCOL2261 and SM707-17 cultivars. Cultivars with low SE efficiency produced mostly globular stage embryos and friable embryogenic callus. Tobacco leaf discs were transformed to test the viral-silencing efficiency of the 221bp Rep construct against SACMV. Results showed that regenerated transgenic tobacco lines infected with SACMV showed reduced symptom development compared with untransformed infected plants, and statistical analysis from RT-PCR results showed that there was a significant decrease in the amount of virus present in four of the five transgenic lines compared with non-transgenic controls.

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ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
AB	Axillary buds
AC1	Replicase gene
ACMV	*African cassava mosaic virus*
BAP	6-Benzylaminopurine
CaMV	Cauliflower mosaic virus
CIAT	International centre for tropical agriculture
CMD	Cassava mosaic disease
CTAB	Cetyltrimethylammonium bromide
CP	Coat protein
CR	Common region
DIG	Digoxigenin
Dpi	Days post inoculation
dsDNA	Double-stranded DNA
EACMCV	*East African cassava mosaic Cameroon virus*
EACMMV	*East African cassava mosaic Malawi virus*
EACMV	*East African cassava mosaic virus*
EACMZV	*East African cassava mosaic Zanzibar virus*
FAO	Food and agriculture organization
FEC	Friable embryogenic callus
GA_3	Gibberellic acid
GD	Gresshoff and Doy medium
GUS	β-glucuronidase
hpRNA	Hairpin RNA
IBA	Indole-3-butyric acid
ICMV	*Indian cassava mosaic virus*
IITA	International institute of tropical agriculture
ILL	Immature leaf lobes
LB	Luria broth
MP	Movement protein
MS	Murashige and Skoog medium

NAA	α-naphthaleneacetic acid
PCR	Polymerase chain reaction
PDR	Pathogen derived resistance
PTGS	Post-transcriptional gene silencing
Rep	Replication-associated protein
RNAi	RNA interference
SACMV	*South African cassava mosaic virus*
SE	Somatic embryos
SLCMV	*Sri-Lankan cassava mosaic virus*
siRNA	Short interfering RNA
ssDNA	Single-stranded DNA
TMV	Tobacco mosaic virus
TNA	Total nucleic acids
Ti	Tumour-inducing
vir	Virulence genes

RATIONALE FOR THE STUDY

In Southern Africa cassava is cultivated in the Northern Province, Mpumalanga, Northern KwaZulu Natal, Namibia, Mozambique, Angola, Swaziland, Botswana and Zimbabwe. In sub-Saharan Africa cassava serves as a food security crop while in South Africa it is used for starch production with special interests in biofuel production. Statistics from FAO showed that 52.5% of the world's cassava output was produced in Africa in 1999. Cassava has many uses including food, flour, animal feed, ethanol, starches for sizing paper and textiles, sweeteners, prepared foods and bio-degradable products, all of which represent potential market development opportunities for South Africa. Cassava provides food for an increasing number of people worldwide. This crop is robust but also has constraints. One of these constraints is the susceptibility of cassava to cassava mosaic disease which causes high yield losses. The other is that breeding for characteristic traits is slow due to the crops' heterozygous nature.

Genetic engineering of cassava is promising for the introduction of useful traits such as virus resistance and increased starch and yield. There have been few reports on successful production of transgenic cassava plants. This thesis aimed at developing an efficient transformation method for routine utilisation and to improve agronomic values of cassava by introducing a virus-resistance trait. We first aimed at improving the transformation system by transforming cassava axillary buds as this is a rapid means of regenerating plants directly. However, since axillary bud transformation was unsuccessful, the most widely used explants for transformation and regeneration, namely friable embryogenic callus and somatic cotyledons both of which are derived from somatic embryos, should continue to be used for such experiments. The failure of axillary buds to transform led to the development of an efficient somatic embryo-inducing protocol for selected cultivars which could then be incorporated into further transformation and regeneration studies.

CHAPTER 1. GENERAL INTRODUCTION

1.1. Cassava, the crop

Cassava (*Manihot esculenta* Crantz), also known as manioc, mandioc, tapioca, or yucca, is a shrubby, tropical perennial plant belonging to the family *Euphorbiacae* and is Africa's second most important crop in terms of calories consumed. Its starchy tuberous roots yield 25 – 35% starch which provide food for over 500 million people for small-scale and subsistence farming in developing countries (Li *et al*., 1998). This crop is grown for different uses which include: famine reserve, rural food staple, cash crop and urban food staple, industrial raw material and livestock feed (Nweke, 2004). Cassava roots and leaves are prepared as food by different methods in different parts of Africa. The roots are eaten raw, roasted or boiled, and cooking the roots slowly destroys the cyanogens (Nweke *et al*., 2002). However, cassava leaves are a more convenient food product than the roots and are also edible. The leaves have a similar nutritive value as other dark green leaves and are valuable sources of vitamins A, C, iron, calcium and protein as shown in Table 1.1 (Nweke *et al*., 2002).

Table 1.1 Nutritional values of fresh cassava roots and leaves per 100 grams (Nweke *et al*., 2002)

Nutrient	Unit	Cassava roots	Cassava leaves
Energy	Calories	146	62
Water	Grams	62.5	80.5
Carbohydrate	Grams	34.7	9.6
Protein	Grams	1.2	6.8
Fat	Grams	0.3	1.3
Calcium	Milligrams	33	206
Iron	Milligrams	0.7	2.0
Vitamin A	I.U.	Tr	10 000
Vitamin B1	Milligrams	0.06	0.16
Vitamin B2	Milligrams	0.03	0.30
Niacin	Milligrams	0.06	1.80
Vitamin C	Milligrams	36	265

Traditional breeding of cassava is hindered by the crop's irregular flowering and by the low seed set and germination rates of the highly heterozygous plants (Zhang *et al*., 2000). Genetic engineering can be used to overcome these limitations by allowing the introduction of desirable traits into cassava. Valuable traits would include pest and virus resistance and improved root quality.

Cassava has been growing for thousands of years in Peru and Mexico. Portuguese traders introduced cassava to Africa from Brazil in the 16th century (Nweke *et al*., 2002). Cassava is now cultivated in approximately 40 African countries. It initially spread through Africa as a famine-reserve crop because of its hardy characteristics.

In South Africa, cassava can be found growing in KwaZulu-Natal, Mpumalanga and the Limpopo Provinces and is grown by small-scale and subsistence farmers. The following characteristics of cassava gives small-scale farmers security against famine: it is drought tolerant; it has the ability to grow in poor soils; is relatively resistant to weeds and insect pests; it produces more carbohydrate per hectare than other food staple, and can be left in the ground for a long time before harvesting (Nweke *et al*., 2002). Under such harsh conditions as these, where other crops struggle to survive, cassava would be an important industrial crop and staple food. Cassava starch has many industrial uses. Most of the starch in South Africa is made from maize but sophisticated starch products made from waxy maize are imported. However, cassava starch qualities are similar to those of waxy maize and thus could be used to manufacture local products instead of importing starch. Cassava is grown for commercial starch by cassava starch manufacturing companies in the Mpumalanga region and has a huge economic potential for South Africa. Cassava starch is used to manufacture many chemical products such as citric acid and is also used in papermaking, food processing, lubricants, adhesives and textiles (Nweke *et al*., 2002).

1.2. Diseases of cassava

There are problems surrounding the cultivation of cassava such as diseases, pests, high cyanide contents of the roots, a low nutritional quality in terms of protein content and the short shelf-life of the harvested tubers (Munyikwa *et al*., 1998; Schreuder *et al*., 2001).

Cassava bacterial blight, the cassava mealybug and the cassava green mite are threatening the cassava industry in Africa. Rodents and termites are also problematic for cassava growers. Due to the limited access to chemicals by African farmers, other methods have been used to control cassava pests and diseases including fallow rotation, crop rotation, and selection of resistant varieties (Nweke *et al*., 2002).

The major disease in sub-Saharan Africa is cassava mosaic disease (CMD) which causes major economic losses due to decreases in cassava yield. One of the viruses causing CMD is *South African cassava mosaic virus* (SACMV) which belongs to the genus *Begomovirus*, family *Geminiviridae* and is transmitted by the whitefly *Bemisia tabaci* as well as planting cuttings from diseased plants.

SACMV causes severe systemic mosaic (Figure 1.1) in South African cassava plants. Breeding for resistant varieties was an earlier effort to control CMD. Local cassava cultivars are being investigated currently to try and genetically improve these plants with regard to resistance to CMD caused by SACMV and other cassava-infecting begomoviruses. Other begomoviruses that infect cassava include: *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV), *African cassava mosaic virus* (ACMV), *Indian cassava mosaic virus* (ICMV) and *Sri-Lankan cassava mosaic virus* (SLCMV) (Legg and Fauquet, 2004).

Cassava mosaic disease (CMD) causes losses of between 20 – 80% of total yields throughout Africa and can result in complete crop failure (Fregene and Puonti-Kaerlas, 2002). Due to the heterozygous nature of cassava, breeding of new CMD-resistance cultivars using traditional methods may lead to the loss of favoured local landraces and improved lines, thus genetic transformation technologies may be necessary to transfer the desired traits to preferred varieties (Fregene and Puonti-Kaerlas., 2002).



Figure 1.1 Healthy cassava and ACMV-infected cassava.

1.3. Structure of geminiviruses

SACMV genome consists of two covalently closed circular single-stranded DNA molecules known as DNA A and DNA B of similar size and both are required for infectivity (Figure 1.2). DNA A is involved in the replication of both DNA components and virus replication rates. Both components are responsible for vector transmission and virus spread and are also necessary for the systemic infection of susceptible host plants (Fregene and Puonti-Kaerlas, 2002). DNA B is involved in cell-to-cell and long-distance virus spread and production of disease symptoms. DNA B is dependent upon the presence of DNA A for replication while DNA A can replicate autonomously. Both molecules are nearly identical in size (2.7 – 2.8 kb) and are encapsidated separately.

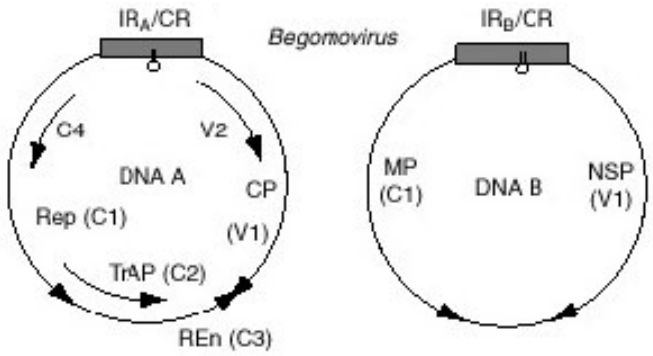


Figure 1.2 Genome organisation of bipartite geminiviruses (Palmer and Rybicki, 1997)

All geminiviruses have a conserved genome sequence in common (Hull, 2002). This sequence is termed the common region (CR) which has sequence similarities within both DNA components and contains modular *cis*-acting sequences involved in transcriptional regulation of certain viral genes and the sequence elements essential for virus replication (Idris and Brown, 1998). The CR is located in the 5' intergenic region on both DNA components and contains the viral origin of replication and the Rep protein binding domain. Rep is a multifunctional protein with the following characteristics: it is localised within the nucleus; has specific DNA recognition sites; has site-specific endonucleases and ligation activity for + / - strand viral DNA; has ATP/GTPase activity; activates the promoter for the coat protein gene mRNA; can repress its own promoter; can stimulate the expression of the proliferating cell nuclear antigen; and it interacts with retinoblastoma proteins (Hull, 2002). Geminivirus replication relies on DNA intermediates and takes place within the nucleus via two stages: by converting the genomic ssDNA into a dsDNA intermediate and amplification of viral ssDNA through the rolling-circle replication (Gutierrez *et al.*, 2004). The genomic ssDNA is then transported to neighbouring cells and is encapsidated to form mature viral particles.

1.4. Genetic engineering strategies for virus resistance

Introduction of a foreign sequence into a plant to obtain expression of that sequence is an approach for developing genetically engineered resistance. Genes derived from viral sequences are a source for protecting plants against viruses and are termed pathogen-derived resistance (PDR). PDR can interfere with the viral infection cycle through protein-based protection or through nucleic acid-based protection. However, none of the

approaches for PDR should interfere with essential host functions. Viruses encode specific genes for genome replication, spread from the original infection site through the host and the environment which have been proven to be excellent candidates for developing host resistance through PDR (Goldbach *et al*., 2003). Transgenic plants that are resistant to an infective virus have been developed by introducing a sequence of the viral genome into the target crop. Virus-resistant crops may be developed by introducing either the viral coat protein (CP) or sequences encoding the replicase gene (Dasgupta *et al*., 2003).

1.4.1. Protein-mediated resistance

PDR based on protein production confers resistance to a broad range of viruses (Beachy, 1997). Protein-mediated resistance requires high levels of transgenic protein expression but RNA silencing in plants may prevent this. Therefore, transgenic proteins should target early, essential processes which can be effectively disrupted by low levels of transgenic protein expression (Prins, 2003). The most widely used transgenes for protein-mediated resistance include the coat protein (CP), movement protein (MP) and replicase (Rep).

1.4.1.1. Coat protein-mediated resistance (CPMR)

CPMR was first reported in transgenic tobacco expressing the TMV CP gene (Hull, 2002). CPMR can provide broad or narrow protection. Narrow resistance was shown when the CP of TMV provided effective levels of resistance to closely related strains of TMV while levels of resistance tobamovirus decreased when CP sequences differ. Transgenic CP of soybean mosaic virus conferred resistance to two unrelated potyviruses, potato mosaic virus and tobacco etch virus (Dasgupta *et al*., 2003). Tobacco plants transformed with ACMV CP were found to contain low levels of mRNA and CP and the plants remained susceptible to ACMV. Broad resistance can be accomplished by combining genes from different viruses into one construct. CPMR blocks viral disassembly thus preventing infection.

1.4.1.2. Movement protein-mediated resistance (MPMR)

Movement proteins (MPs) facilitate viral spread between adjacent cells as well as systemically. Transgenic plants containing defective MPs from TMV showed resistance to several tobamoviruses, Alfalfa mosaic virus, cauliflower mosaic virus and others (Dasgupta *et al*., 2003). Knowledge of MP structure and function will allow the development of mutant proteins as dominant negative inhibitors to block local and systemic spread of viruses with high efficiency (Beachy, 1997). This is due to the competition for plasmodesmatal binding sites between the mutant and wild-type protein of inoculated virus. MPMR has a broad spectrum of efficiency which suggests that MPs of several viruses interact with the same plasmodesmatal components (Baulcombe, 1996).

1.4.1.3. Replicase- mediated resistance (RMR)

Resistance responses brought about by Rep do not require protein synthesis as it is mediated at the RNA level (Bendahmane and Gronenborn, 1997). Rep resistance remains confined to a narrow spectrum of viruses. To obtain a broader spectrum, genes from various dissimilar viruses can be pyramided. RMR is described in more detail under 'nucleic acid-mediated resistance'.

1.4.2. Nucleic acid-mediated resistance

One method to make cassava resistant to SACMV is to genetically engineer the crop using a fragment of the SACMV AC1 gene which encodes a replication-associated protein (Rep) since Rep is the only viral protein required for replication. Replicase-mediated resistance has been shown to be due to post-transcriptional gene silencing (PTGS) which is an inherent plant response. A PTGS-based strategy to control virus replication was shown when tobacco protoplasts, which were infected with ACMV and with a synthetic siRNA designed to target the AC1 gene of the virus, showed a decrease in AC1 mRNA levels by more than 90% and viral DNA by 70% (Vanitharani *et al*., 2005).

The essential functions of Rep make it an ideal target for obtaining virus resistance by suppressing its expression through transgenic RNA silencing (Bendahmane and Gronenborn, 1997). It has been shown that the resistance generated by using Rep sequences is tight thereby allowing transgenic plants to resist high dosages of input virus (Dasgupta *et al*., 2003).

PTGS is responsible for the degradation of nucleic acids in a sequence-specific manner including those of viruses and thus this strategy may be very effective in genetic engineering of virus resistance (Dasgupta *et al*., 2003). In PTGS, the elicitor dsRNA, which is produced during viral infection, is degraded to small interfering RNA (siRNA). A complex of cellular factors, such as RNA-dependant RNA polymerase, along with siRNA degrade RNA molecules bearing homology with the elicitor RNA and the degradation spreads within the entire organism (Dasgupta *et al*., 2003). This process has evolved as a plant defense mechanism against invading viruses with RNA or DNA genomes.

1.4.2.1. Post-transcriptional gene silencing (PTGS)

RNA silencing is a mechanism that suppresses gene expression by sequence-specific interactions with RNA at the post-transcriptional gene level. This silencing phenomenon is termed post-transcriptional gene silencing (PTGS) and is also known as RNA interference (RNAi). PTGS was discovered in petunia plants in 1990 by attempting to manipulate pigment synthesis genes. When the chalcone synthase and dihydroflavonol reductase genes were introduced into petunia using a strong viral promoter, mRNA levels decreased and the normally purple flowers became white as a result.

The natural functions of RNAi are to protect the plant genome against invasion by mobile genetic elements such as viruses and transposons, post-transcriptional and post-translational regulation of gene expression and epigenetic regulation of chromatin structure (Agrawal *et al.,* 2003; Matzke *et al.,* 2001). PTGS helps to maintain genome integrity by suppressing the activity of transposable elements.

RNAi uses double-stranded (ds) RNA as a trigger that targets homologous mRNAs for degradation. dsRNA that triggers RNAi are made in the nucleus or cytoplasm by

transcription through inverted DNA repeats, simultaneous synthesis of sense and antisense RNAs, viral replication and the activity of cellular or viral RNA-dependent RNA polymerases (RdRP) on ssRNA templates (Matzke *et al.,* 2001). Recessive gene disruption and dominant gene silencing are two approaches used to decrease the levels of undesirable gene products (Tang and Galili, 2004).

The dsRNA-triggered RNAi of dominant gene silencing is the most powerful in terms of its efficiency in the extent of gene silencing which is almost as complete as that achieved in a gene knockout approach (Tang and Galili, 2004). It must also be noted that PTGS of plant genes using the antisense strategy results in a restricted amount of silenced individuals while constructs encoding self-complementary hpRNA efficiently silence genes (Wesley *et al.,* 2001).

PTGS that starts locally in a plant can spread systemically throughout the plant and systemic silencing is triggered by dsRNA and siRNAs. A sequence-specific diffusible factor exists in PTGS which was established by grafting experiments between silent transgenic plants and non-silenced transgenic plants since the silencing signal was transmitted from the silenced scion to the non-silenced scion (Chicas and Macino, 2001). This diffusible factor could be made from dsRNA by the RdRP and may be responsible for the amplification of silencing. This explains why only a few molecules of dsRNA are sufficient to trigger RNAi and why an RdRP is required for dsRNA-induced PTGS (Chicas and Macino, 2001).

The most noticeable features of RNAi include:

- dsRNA induces silencing
- High degree of specific gene silencing with less effort
- Highly potent and effective (only requires few dsRNA for effective interference)
- Silencing introduced in different developmental stages
- Leads to systemic silencing
- No abnormality problems caused by knocked out gene in early stages
- Silencing effect passed through generations (Thakur, 2003).

RNAi technology is used to analyse the functions of many genes in a wide variety of organisms. Gene knockdown-related functional studies are carried out when transgenes are

present in the form of hairpin (or RNAi) constructs (Agrawal *et al.,* 2003). RNAi constructs are used to remove plant endotoxins if the toxin biosynthesis genes are targeted with these constructs (Agrawal *et al.,* 2003). This has been shown in the production of decaffeinated coffee plants in which the theobromine synthase was knocked down with the RNAi constructs (Ogita *et al.,* 2003).

RNAi is also used to improve nutritional values of crops, total crop yield, and virus-induced gene silencing to prevent crop losses. Traditional breeding for crop improvement has been successful although this process is time-consuming and crop genetic resource limitations has led to the reduction of traditional breeding as a means for improving crops (Tang and Galili, 2004).

1.4.2.1.1. PTGS mechanism

PTGS operates against transgenes, retroelements and RNA/DNA viruses. Sense RNA triggers PTGS which implies that this mechanism operates via an antisense RNA. Thus, gene silencing is induced by the simultaneous expression of sense and antisense RNA, and silencing is also induced by hairpin RNAs containing introns (Tang and Galili, 2004).

There are two models to explain how antisense RNA may be produced. The first model suggests that the insertion site of the transgene places it adjacent to an endogenous promoter that would transcribe antisense RNA (Hull, 2002). This is unlikely since there is no correlation between the direct transcription of antisense RNA and PTGS and suppression of the transgene leads to the loss of PTGS. The other model suggests that antisense RNA is produced indirectly from a sense transcript from the transgene. Transcription of integrated DNA gives rise to aberrant RNA and replication of RNA viruses involves the synthesis of complementary strands which are templates for the formation of dsRNA. RdRP is required for the transcription of antisense RNA from an RNA template. Other gene products are also involved inducing PTGS which include Dicer, AGOl, RNA helicase and DNA methylation.

There are at least three different pathways in the silencing mechanism:

1) Cytoplasmic short interfering (siRNAs) silencing

2) Silencing of endogenous mRNAs by micro RNAs (miRNAs)
3) DNA methylation and suppression of transcription (Vanitharani *et al.,* 2005).

Long dsRNA is cleaved into short-interfering (21 - 26 nucleotides) sense and antisense RNAs by a Ribonuclease Ill-like enzyme known as Dicer in an ATP-dependent processive manner. The antisense siRNAs produced by Dicer serve as guides for the RNA-induced silencing complex (RISC) which cleaves homologous ssmRNAs. An ATP-dependent unwinding of the siRNA duplex is required for the activation of RISC. RISC cuts the mRNA approximately in the middle of the region paired with antisense siRNA and thereafter the mRNA is further degraded in a sequence-specific manner as shown in Figure 1.3 (Matzke *et al.,* 2001).

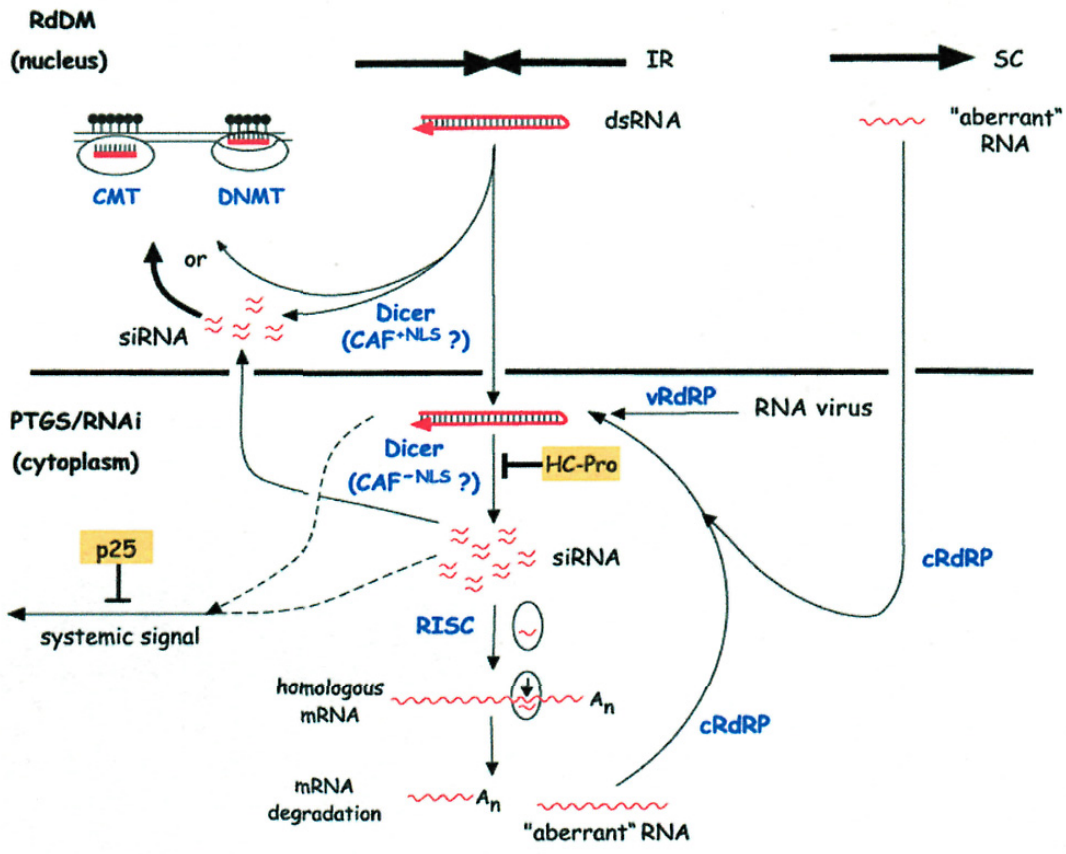


Figure 1.3 Model for RNA silencing.

RNA-directed DNA methylation (RdDM) and PTGS/RNAi are triggered by dsRNA that are cleaved by Dicer into siRNAs. The siRNAs are incorporated into RISC which becomes activated and degrades homologous mRNA (occurs in the cytoplasm) dsRNA triggers RdDM which interacts with the chromodomain of chromomethylase (CMT) and guide it to homologous DNA sequence, or unwound dsRNA might base pair with homologous ssDNA producing an RNA-DNA

duplex and ssDNA bulge. This unusual structure might attract a de novo DNA methyltransferase (DNMT). dsRNA can be made by transcribing through inverted repeats (IR) or by the activity of cellular RdRP acting on aberrant RNA templates synthesised from single-copy (SC) genes in the nucleus or generated in the cytoplasm by RISC cleavage of mRNA. RNA viruses produce dsRNA with the aid of a viral RdRP (vRdRP). Accumulation of siRNAs and systemic silencing is blocked by plant suppressors (Matzke *et al*., 2001).

1.4.2.1.2. RNA silencing by siRNAs, miRNAs and hpRNAs, and applications using these small RNAs

The siRNAs that are cleaved into RISC may be used as templates and converted into dsRNA thus increasing the level of siRNAs and enhancing RNAi. siRNAs confer viral resistance and prevent transposons hopping (Thakur, 2003). When dsRNA or siRNAs are introduced locally into the plants, they can trigger systemic silencing since they act as mobile trigger elements for systemic silencing.

21-25 nt-long endogenous RNAs have been detected in plants known as micro RNAs (miRNAs). They are an abundant family of non-protein-coding RNAs with a presumed post-transcriptional regulatory activity (Lai, 2003). These are single-stranded and are processed by Dicer-like enzymes from stem-loop precursor RNAs that are transcribed from intergenic regions (Voinnet, 2003). miRNAs resemble siRNAs in that they exhibit complete complementarity with the coding regions of their predicted targets (Voinnet, 2003; Lai, 2003). This suggests that plant miRNAs might act via an RNAi-like mechanism. miRNAs can program a plant-encoded RISC complex to retrieve and destroy endogenous transcripts.

miRNA is an endogenous siRNA-like RNA involved in developmental regulation of gene expression. Its precursor (pre-miRNA) is a small hpRNA with bulges in its stem region (Kusaba, 2004). The dsRNA, hpRNA and pre-miRNA are cleaved by Dicer into 21 nt RNA duplexes and the unwound ssRNA is incorporated into RISC (Figure1. 4). dsRNA and pre-miRNA are processed by Dicer-like proteins. miRNA cleaves mRNA and also inhibits translation. The majority of mRNAs that are predicted to be regulated by miRNAs are transcription factors involved in pattern formation. Thus, a major role of miRNAs may

be to clear cells of mRNAs encoding transcriptional regulators following the specification of cells (Lai, 2003).

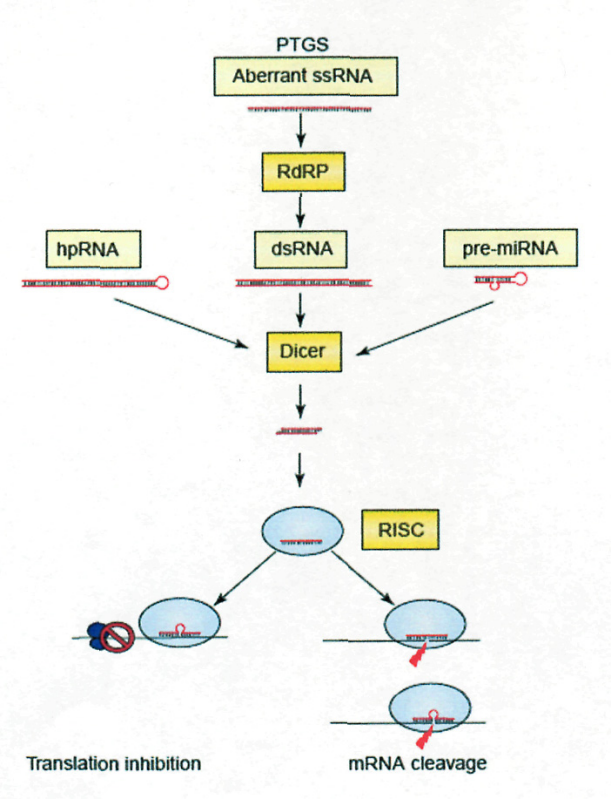


Figure 1.4 Mechanism of RNA silencing by hpRNAs and miRNAs (Kusaba, 2004)

Transgenes that encode hpRNA are effective inducers of PTGS of endogenous genes and transgenes and confer resistance to viruses (Wang and Waterhouse, 2001). Spliceable introns in the hpRNA transgene appear to enhance its silencing efficiency (Wang and Waterhouse, 2001). A target gene can be cloned as an inverted repeat spaced with an unrelated sequence in an hpRNA-producing vector driven by a strong promoter such as the 35S CaMV promoter (Kusaba, 2004).

If an intron is used as a spacer, the efficiency of silencing becomes high and almost all transgenic plants show gene silencing. The degree of silencing with intron-containing constructs showed 90 - 100% silencing in transgenic plants (Wesley *et al*., 2001). Wesley *et al.* (2001) used hpRNA constructs and obtained silenced *Arabidopsis,* cotton, rice and tobacco for every gene that was targeted (viral gene, transgene or endogenous gene) and silencing was uniform within tissues in which the hpRNA was expressed.

RNAi mediated by hpRNA has been used in cotton to remove two fatty acid desaturase genes to increase the production of nutritionally improved high-oleic and high stearic cotton-seed oils which are essential fatty acids for health of the human heart (Tang and Galili, 2004). Another example of hpRNA-induced RNAi is the rice mutant line LGC-1 (Low Glutelin Content-1) which is low in protein for patients with kidney diseases that require low protein diets (Kusaba, 2004). hpRNA is produced from an inverted repeat for *glutelin* which lowers the glutelin content in rice via RNAi. hpRNA-induced RNAi is inherited more stably than PTGS since hpRNA-induced RNAi does not require the generation of dsRNA mediated by RdRP for gene expression suppression (Kusaba, 2004). The efficiency of PTGS can be improved by using the hpRNA approach. The highest silencing obtained with an antisense construct was only as good as the least silenced plant with hpRNAi (Wesley *et al*., 2001).

1.4.2.1.3. Complications of PTGS

PTGS arose primarily to control native gene functions but their ability to recognise normal from deviant gene structure and function has led to major complications in plant biotechnology since transgenes are frequently recognised as being invasive and therefore subject to silencing.

Given these considerations, investigation of the mechanisms of gene silencing is likely to be valuable not only in overcoming difficulties in agricultural biotechnology but also in providing new insight to plant development and gene regulation. High copy numbers of the transgene could lead to the silencing of the transgene and possibly knock out plant genes required for plant growth and development. Genetic engineering of these plants can give rise to chimers and somaclonal variation which gives rise to transgenics that differ genotypically. The expression of transgenes may also give rise to unfavourable phenotypes. Another problem with PTGS is that plant viruses are able to suppress this silencing mechanism leading to the loss of crops since plants will become susceptible to these viruses.

1.5. Cassava transformation and regeneration

An efficient transformation system that is compatible with regeneration methods is a prerequisite for the development of transgenic cassava. Transformation and regeneration systems are often genotype-dependent and thus not all transformation methods are suited for certain cultivars. Genetic transformation techniques for cassava have been restricted by the lack of reproducible transformation and regeneration systems (Munyikwa *et al*., 1998). Current transformation protocols for cassava take long periods, plants are susceptible to somaclonal variation and transformation is insufficient because there is a low integration of genes of interest in the cassava genome (Msikita *et al*., 2006). Cassava transformation has remained between 3 and 5% compared with 10% in other agronomic crops.

Successful plant transformations require certain criteria that must be met. These standards include:

- The tissues to be transformed must be competent for propagation and regeneration
- DNA should be delivered efficiently into plants
- Selective agents are needed for transgenic plants
- A reasonable percentage of transgenic plants should be recovered
- The process must be simple, efficient, reproducible, genotype-independent and cost-effective
- Somaclonal variation should be avoided
- Stable integration of the transgene in the plant genome
- Safety and environmental considerations.

1.5.1. Transformation methods

Three methods of gene transfer have been used successfully in producing transgenic cassava. These techniques include: particle bombardment, electroporation and *Agrobacterium tumefaciens*-mediated transformation (Raemakers *et al*., 1997; Schreuder *et al*., 2001). There are other methods for plant transformation but these are often not used because of their impracticability at present.

1.5.1.1. Particle bombardment

Particle bombardment has a few advantages over other transformation methods which include: eliminating host range specificity problems that are sometimes found when transforming with *Agrobacterium*; it allows DNA transfer into intact tissues with relatively high efficiency; and DNA is transferred into cells without excessive damage to the tissues (Zhang *et al*., 2000). This method involves the acceleration of tungsten or gold particles through a partial vacuum under the pressure of helium gas. These particles are coated with purified plasmid DNA which are then accelerated through the walls of intact cells and the DNA integrates randomly into the plant's genome. The particles are innate and small thus preventing damage or affecting the cells and the pores in the cell walls, created by the particles, close by themselves.

Cassava friable embryogenic callus (FEC) and cotyledon-derived callus tissues have been successfully employed as target tissues for the integration of transgenes via microparticle bombardment although problems of somaclonal variation arose because plants were regenerated from embryogenic suspensions (Taylor *et al*., 2004). However, embryogenic suspension cultures contain a large amount of totipotent cells and thus FEC in liquid culture are ideal target tissues for use with direct gene transfer because insertion and integration of the genetic material is maximised (Schöpke *et al*., 1996; Taylor *et al*., 1996).

1.5.1.2. Electroporation

Electroporation may be used to transform protoplasts or suspension cultures. In this method, cells are subjected to the discharge of a capacitor creating transient openings in the plasmalemma through which the DNA can enter. Electroporation of protoplasts and somatic embryos has been performed by using the GUS reporter gene for selection which resulted in transient gene expression. Transgenic cassava plants were also produced by electroporation of protoplasts derived from FEC (Raemakers *et al*., 1997). However, the embryogenic capacity during long-time culture is lost and there is an increasing possibility of somaclonal variation (Msikita *et al*., 2006).

1.5.1.3. Agrobacterium-mediated transformation

Agrobacterium transformation is considered preferable to genetic transformations by artificial approaches such as electroporation because of the ease and low cost of the procedure and also due to the relatively low complexity of intact transgenes integrated into the plant genome (Gelvin, 2003). *Agrobacterium tumefaciens* is a Gram-negative soil bacterium that infects dicotyledonous plants and causes crown gall disease. The crown gall is the result of uncontrolled cell division and this bacterium also makes the plant produce sugars for its survival. Since this method is natural, it is mostly used in transferring genes. The plasmid with the gene of interest is inserted into *Agrobacterium* and then the plant is infected with the transformed bacterium.

Virulent *Agrobacterium* strains contain a tumour-inducing (Ti) plasmid which is responsible for the production of opines that the bacterium can utilise and this plasmid also encodes virulence (*vir*) genes (Hughes, 1996). There are two primary steps involved in *Agrobacterium* transformation: 1) binding of the *Agrobacterium* to the plant cell and 2) transfer of DNA to the plant cell. The region of the Ti plasmid that integrates into the plant genome is known as T-DNA (transfer DNA).

Chromosomal genes are necessary for binding of *Agrobacterium* to the plant cell and DNA transfer which include: *chv*A and *chv*B for the attachment of the bacterium to the plant cell; *chv*E for the induction of *vir* genes; and *chv*D, *ilv*, *mia*A and *att* which contribute to virulence.

Wounded plant cells release phenolic compounds that attract *Agrobacterium* to the wound site and stimulate the synthesis of Ti *vir* genes (Hughes, 1996). Acetosyringone can act as a chemical attractant *in vitro* and therefore may act as a chemotactic agent in nature. These phenolics activate the VirA protein which in turn transphosphorylates the VirG protein. This is a transcriptional activator for other *vir* genes.

T-strand production is initiated by VirD2 and VirD1 which serve as a strand and site-specific endonuclease (Tzfira and Citovsky, 2002). The T-strand is the bottom single-stranded DNA that has been cut at the left and right borders of the DNA. The virD2

protein covalently attaches to the 5' end of the T-strand following cleavage of the T-DNA borders with VirE2. The virD2 protein targets the DNA to the plant cell nucleus and integrates the DNA into the plant genome through the process of illegitimate recombination (Hughes, 1996). The *Agrobacterium*-mediated transformation process is summarized in Figure 1.5.

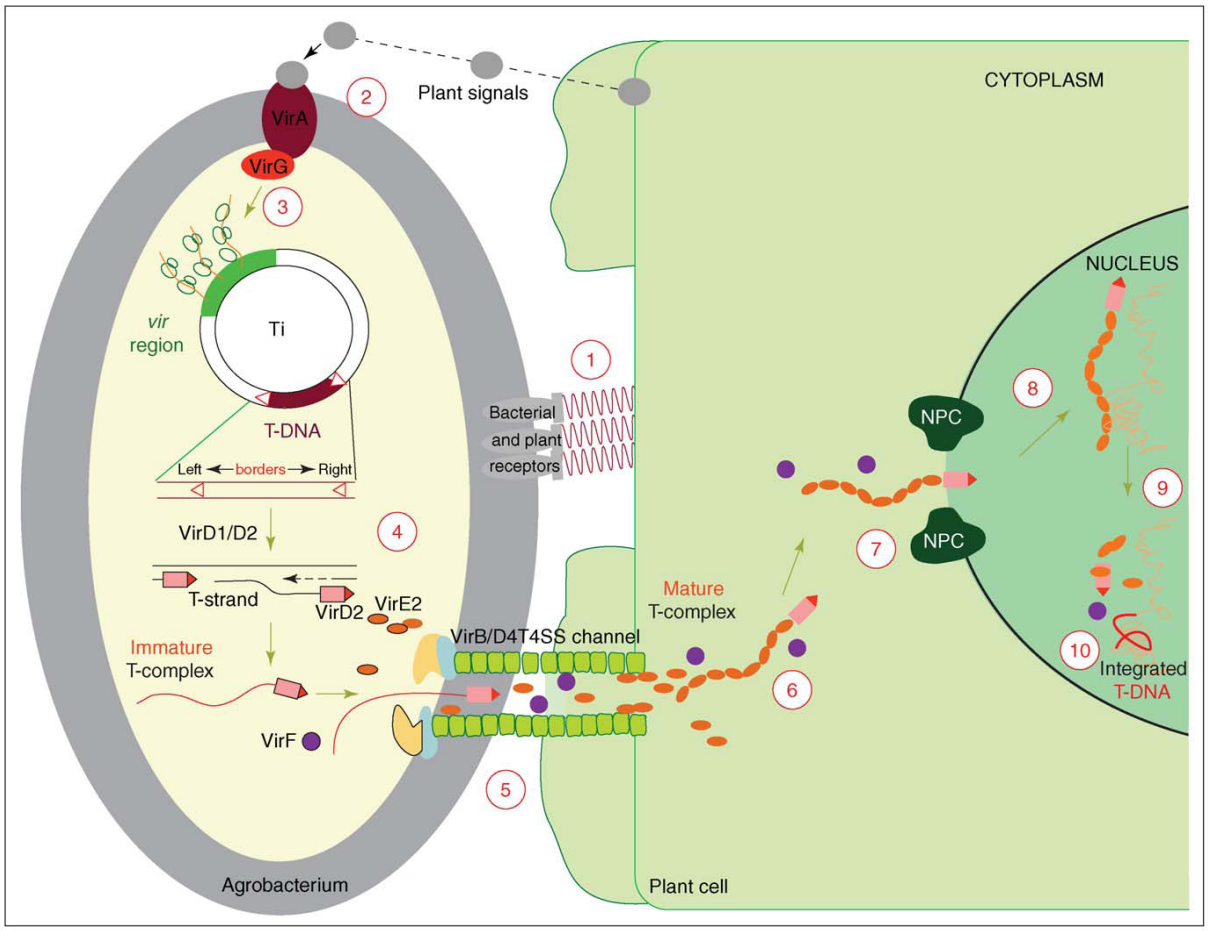


Figure 1.5 *Agrobacterium*-mediated transformation model.

Transformation starts with recognition and attachment of the *Agrobacterium* to the host cells (1) and the sensing of specific plant signals by the *Agrobacterium* VirA/VirG signal-transduction system (2). The *vir* gene region is activated (3) and a copy of the T-DNA is generated by VirD1/2 proteins (4) which is delivered as a VirD2-DNA complex with other Vir proteins into the host cytoplasm (5). VirE2 binds to the T-DNA and travels through the cytoplasm (6) into the nucleus (7). The T-DNA is brought to the integration point (8) where the Vir proteins are removed (9) ant the T-DNA integrates into the host genome (10) (Tzfira and Citovsky, 2002).

There are three general approaches for transformation with *Agrobacterium*:

1. Infection of wounded plants – the plant wound is inoculated with an *Agrobacterium* culture. A tumour is produced which is excised to produce an axenic culture.
2. Co-cultivation – isolated explants are incubated in an *Agrobacterium* suspension.
3. Leaf disc method – explants are incubated in an *Agrobacterium* suspension. Explants are then grown on a bacteriostatic medium to remove excess *Agrobacterium* and then moved to a selection medium to select for cells that have taken up the target gene.

In this procedure, the T-DNA-encoded oncogenes are deleted and replaced with genes of agronomic value that are subsequently transferred to and expressed within plant cells. The advantages of using *Agrobacterium* for transformation include: infection of intact cells, tissues and organs; a large number of DNA fragments can be transferred, the stability of a gene is excellent and there is a low incidence of transgene silencing. The disadvantage of using this method is that cells are difficult to transform and integration of concatamers of the entire binary vector may occur. It was shown that root transformation in *Arabidopsis* resulted in vector transfer in 33% of transformants while vacuum infiltration resulted in 62% vector transfer (Kohli *et al*., 2003).

1.5.1.4. Other methods of transformation

Silicon carbide whisker (SCW)-mediated transformation involves the use of silicon carbide crystals which are mixed in liquid medium in the presence of DNA and plant cells (Smith *et al*., 2001). The crystals pierce the plant cell wall which facilitates DNA entry into the plant cell. The first reported stable transformation of tobacco and maize cells was in 1992 by Kaeppler *et al*. However, the use of these carbon fibers requires caution because of their potential carcinogenic effects (Hansen and Wright, 1999). Aluminium borate whisker (ABW)-mediated transformation is similar to SCW transformation except that there have been no reports of mutagenicity of ABW to organisms and thus is a safer gene-transfer method than SCW (Mizuno *et al*., 2005). ABW has been used to transform tobacco calli, although only 3 out of 50 calli were obtained from hygromycin selection and of those 3, 1 expressed the GUS gene (Mizuno *et al*., 2005).

Polyethylene glycol (PEG) can be used as another direct gene transfer method for protoplasts that causes permeabilisation of protoplast plasma memebranes allowing macromolecules to enter the cell. However, electroporation has improved the simplicity of protoplast transformation and the reproducibility of high-frequency DNA delivery (Smith *et al*., 2001).

Agrolistic transformation combines microprojectile bombardment and *Agrobacterium*-mediated transformation to produce transgenic plants lacking superfluous vector sequences and contain a single copy of the transgene (Smith *et al*., 2001). Agrolistics involves co-transformation approach with plasmids carrying *Agrobacterium vir* genes v*irD1* and v*irD2* using bombardment. These *vir* genes are involved in T-DNA insertion events from plasmids that have been delivered to plant cells via bombardment. The introduction of *virE2* gene to the mixture protects the bound DNA from nuclease activity thereby decreasing the number of degraded transgene integrations (Smith *et al*., 2001). Tobacco calli that were agrolistically transformed exhibited 20% DNA integration after the action of *virD1* and *virD2* gene products and similar amounts of calli contained both agrolistic and biolistic events (Hansen and Chilton, 1996). Less transgene copies are inserted into the plant genome using agrolistics than with the normal biolostics procedure (Hansen and Chilton, 1996).

1.5.2. Improving Agrobacterium-mediated transformation efficiency

One of the challenges to plant transformation technologies is to improve transformation efficiencies especially for commercially important crops that are used for high-throughput applications. Plant and bacterial genes as well as environmental factors affecting DNA transfer from *Agrobacterium* to plants have been identified as important features which could improve transformation (Zambre *et al*., 2003). Environmental factors would include co-cultivation duration and temperature conditions as well as light conditions (continous light/dark or photoperiod), while acetosyringone has been shown to increase *vir* gene production in *Agrbacterium* which is necessary for T-DNA transfer from the bacteria to the plant (Veluthambi *et al*., 2003 and Zambre *et al*., 2003). Other factors include: *Agrobacterium* strain and plant genotype; explant type, quality, preculture, hormone treatment, wounding or infiltration; and use of antibacterial agents, antioxidants, ethylene

and methylation inhibitors to decrease damage and gene silencing respectively in treated plant cells (Birch, 1997). It has also been shown that low-dose X-ray irradiation causes double-strand breaks in plants which enhances transformation rates in plants (Zhu *et al*., 2006). In this regard diploid protoplasts treated with UV light showed a 4 to 10-fold increase in transformation rate (Smith *et al*., 2001).

1.5.2.1. Effect of light on Agrobacterium-mediated transformation

Zambre *et al.* (2003) co-cultivated *Arabidopsis* roots and bean calli with *Agrobacterium* under continuous darkness, under a 16h light/8h darkness photoperiod or under continuous light at a constant temperature of 22ºC. Explants were extensively wounded and bacteria were pre-cultured with acetosyringone before co-cultivation. GUS assays revealed that co-cultivation in darkness severely inhibited T-DNA transfer compared with the light regimes in both plant species (Zambre *et al*., 2003). Exposure to light during co-cultivation was essential for efficient T-DNA transfer, irrespective of explant type, plant species or genotype, *Agrobacterium vir* plasmids or co-cultivation periods. Thus, light promotes *Agrobacterium*-mediated T-DNA transfer to plant cells. It has also been reported that co-cultivation of *Brassica* with *A.rhizogenes* under continuous light increased transformation rates (Zambre *et al*., 2003). Wounding of green tissues prior to transformation was noted to be unnecessary when co-cultivation took place in continuous light as the stomata remained open and thus allowed for *Agrobacterium* penetration into plants without the need for wounding (Escudero and Hohn, 1997).

1.5.2.2. Effect of temperature on Agrobacterium-mediated transformation

Dillen *et al.* (1997) have shown that temperature affects the transfer of T-DNA into plants. Bean calli and tobacco leaf discs were transformed at 15, 19, 22, 25, 27 and 29°C. The highest level of GUS expression was observed at 22°C and decreased as the temperature increased. Very little expression was found at 27 and 29°C. A possible explanation for the temperature sensitivity of gene transfer is that regulation of the *vir* regulon is temperature dependent which was shown in previous studies (Dillen *et al*., 1997). VirD2 and VirG

production and *virB* induction were high between 20 and 25°C while low levels of transgene expression were found at 27°C.

The temperature at which the *Agrobacterium* is grown prior to transformation is also important for *vir* gene induction. *Vir* gene induction occurs at 28ºC while increasing temperatures during bacterial growth starts to decrease the induction of these genes. This was shown by Jin *et al*, (1993) when they monitored the expression of *vir* genes at 28, 32 and 37ºC by measuring the β-galactosidase activity in *Agrobacterium tumefaciens* A243MX which contains a *virB*::*lacZ* fusion (Jin *et al.*, 1993). *Vir* gene expression was dramatically decreased as the temperature increased. An explanation for the decline in *vir* gene expression is that VirA and VirG – which are required for *vir* gene induction – signal transduction is sensitive to temperature of 32°C and above. VirA autophosphorylation and phosphate transfer to VirG are inhibited at elevated temperatures and *vir* gene expression is inactivated.

1.5.2.3. Effect of phenolic compounds and explant pre-incubation on Agrobacterium transformation

Pre-incubation of explants and the addition of phenolic compounds such as acetosyringone increases transformation efficiency due to various mechanisms. Wounded plant tissues in their active metabolic state produce and secrete *vir*-inducing molecules into the surrounding medium. Actively dividing cells resulting from wound-induced cell division were considered to be more prone to transformation than non-dividing cells. Newly synthesized cell wall is essential for *Agrobacterium* attachment. Active DNA replication machinery of rapidly dividing cells was also proposed to be important for T-DNA integration (Sunilkumar *et al.*, 1999).

It was shown by Sunilkumar *et al.* (1999) that the *vir*-inducing activity of tobacco leaf segments increased with an increase in the pre-incubation period. Pre-incubation for 24, 48 and 72 hours resulted in a 2.3, 3.5 and 4.5-fold increase in the level of *virE* induction, respectively. An increase in pre-incubation period resulted in an increase in transformation efficiency. Stimulation of plant cell division and activation of DNA replication during pre-

incubation may play an important role in the integration of T-DNA leading to stable transformation.

1.5.3 Transformation systems using various types of explants

Explants are initiated from sterile pieces of a whole plant and may consist of pieces of organs such as leaves or may be specific cell types such as pollen. Many explant features are known to affect the efficiency of culture initiation and transformation. Younger, more rapidly growing tissue or tissue at an early stage of development is most effective. Several explant types have been used in regeneration and transformation studies of cassava including FEC, callus, somatic embryos, cotyledons, and protoplasts (as shown in Figure 1.6) as well as axillary buds and chloroplasts.

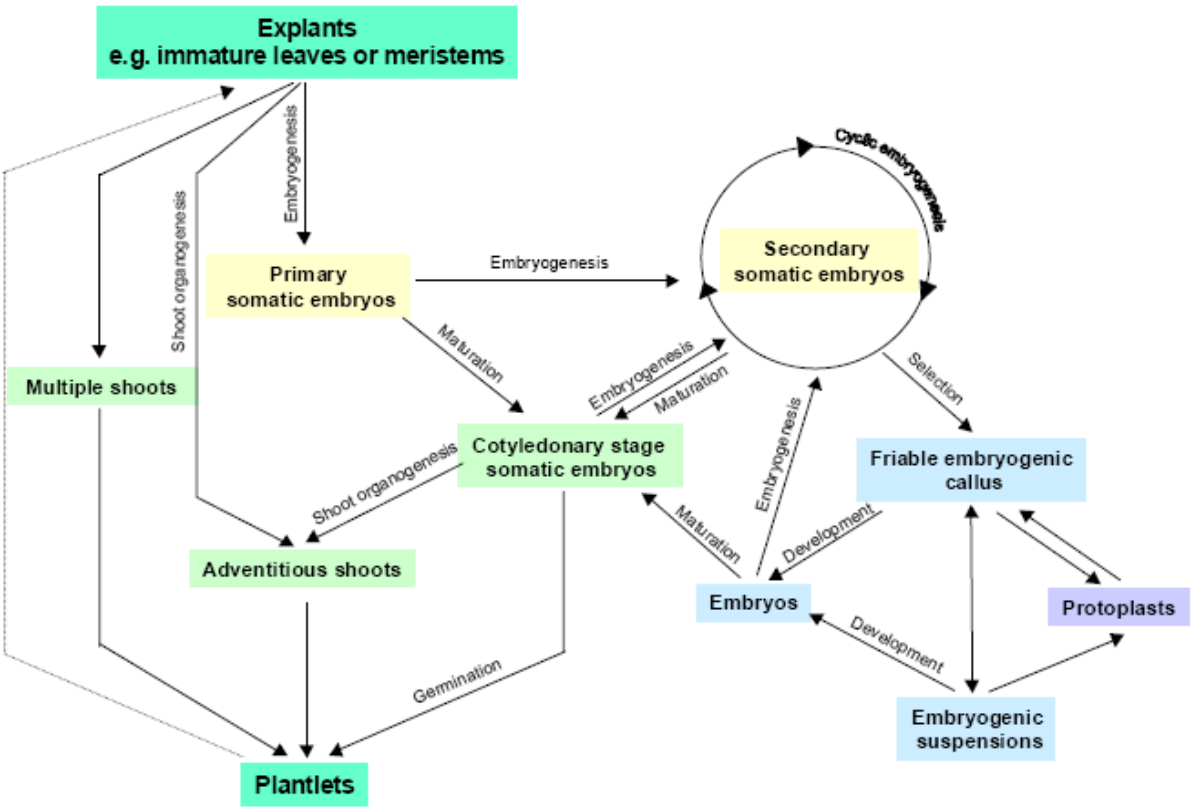


Figure 1.6 Schematic representation of various regeneration steps in cassava (Zhang, 2000)

1.5.3.1. Embryogenic tissue transformation

Somatic embryos are produced from somatic tissues such as immature leaf lobes and axillary buds by placing these explants on MS media containing 2,4-D/Picloram or NAA. Primary somatic embryos are induced by 2,4-D and picloram (Li *et al*., 1996; Taylor *et al*., 2001; Zhang and Puonti-Kaerlas, 2005) and secondary somatic embryos can be induced by using high concentrations of NAA (Ma and Xu, 2002). Cotyledons for transformation procedures are produced by transferring mature (secondary) somatic embryos to media containing a cytokinin such as BAP. FEC is induced from organised embryogenic structures on Gresshoff and Doy salts and vitamins supplemented with picloram. FEC is of a single cell origin and proliferates rapidly thus the tissue has a reduced risk of chimerism however; the long span of tissue culture *in vitro* may cause somaclonal variation (Schreuder *et al*., 2001; Zhang, 2000).

Four transformation systems have been employed using *Agrobacterium* for the transfer of genes to cassava (Figure 1.7.). These systems rely upon the production of embryogenic tissues from *in vitro* leaf-lobe and axillary bud explants but differ in the way in which these are manipulated to the gene transfer systems (Taylor *et al*., 2004). However, regeneration of plants from somatic embryos induced from cotyledons of zygotic embryos, immature leaves, primary somatic embryos and embryogenic suspensions are based on the conversion of somatic embryos to whole plants and require extended tissue culture periods or are poorly compatible with transformation protocols (Li *et al*., 1998).

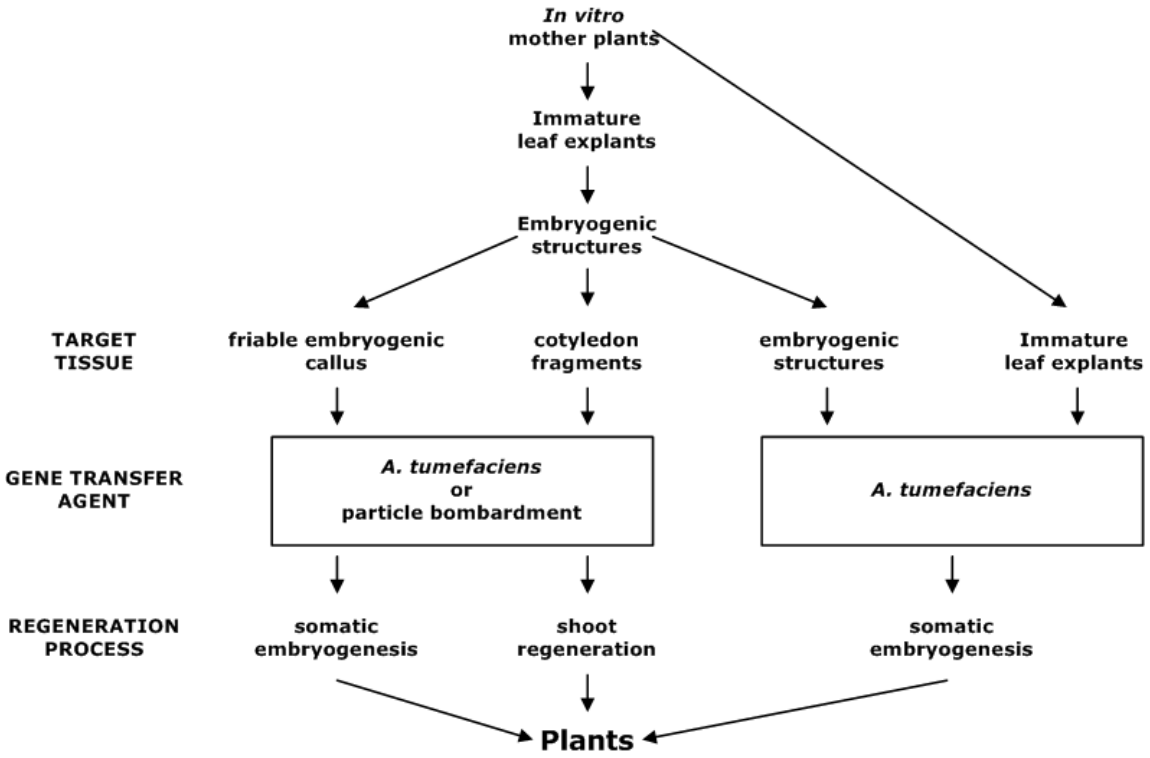


Figure 1.7 The four systems that are used to recover genetically transformed cassava plants (Taylor *et al*., 2004)

Transformation efficiencies using the tissues mentioned have been shown to be low. Secondary somatic embryos used for plant transformation contained GUS activity in only 1% of the newly formed embryos (Raemakers *et al*., 1997). Similar results were found during plant regeneration from transgenic callus and embryogenic suspension cultures: a low rate of transformed calli producing somatic embryos and a low conversion rate of embryos into plants (González *et al*., 1998).

The transformation frequency of cassava somatic embryos via particle bombardment is between 0.6% and 1.2% (Zhang *et al*., 2000). Only 11 out of 958 TMS60444 explants were shown to be independent transgenic lines after selection, and 60% of these shoots expressed the *uidA* gene in GUS assays (Zhang *et al*., 2000). Similarly, a low transformation efficiency of 1% was found when somatic-embryo-derived cotyledons were used as a source of explants for *Agrobacterium*-mediated transformation to generate Basta resistant plants based on Southern tests (Sarria *et al*., 2000).

The use of FEC as an explant source for transformation yielded few transgenic plants. Transformation efficiencies of FEC were ascertained as follows: 1037 callus lines were co-cultivated with *Agrobacterium*, 526 showed GUS expression, 219 FEC formed somatic embryos, 37 lines that were transferred to germination media produced plants (Shreuder *et al*., 2001). Therefore, transformation efficiency based on explant regeneration was determined to be 6%.

Another problem encountered in cassava transformation is that not all cultivars are amenable to both transformation and regeneration. *Agrobacterium*-mediated transformation of cassava cultivar MCol.22 cotyledon pieces were able to regenerate into plants which could be due to competence of a certain cell type to transform and regenerate shoots (Hankoua *et al*., 2006). However, in TME 8 and TMS 91/02327 cotyledons did not regenerate into plants (Hankoua *et al*., 2006). Thus, cells that are competent for transformation may not be competent for organogenesis and vice-versa. Many factors play a role in the transformation and regeneration process. A possible explanation for the lack of regeneration of TME 8 and TMS 91/02327 is that the *hpt* gene may have been methylated and thus silenced thereby preventing the growth of plants on hygromycin-containing media or that ethylene induced by wounding cotyledons may have interfered with the transformation process (Hankoua *et al*., 2006).

However, the use of organised tissues and organs such as somatic embryos that are capable of regenerating into full plants are used to avoid somaclonal variation. At present, the use of organised tissues – such as somatic cotyledons and FEC – is the only practical transformation and regeneration methods for cassava. Somatic embryos and FEC have been used to obtain transgenic plants using particle bombardment, electroporation, and *Agrobacterium tumefaciens*-mediated transformation as shown in Table 1.2. Protoplast culture is one of the methods that will prevent chimeras (Raemakers *et al*., 1997). It has to be shown which are the most optimal procedures regarding efficiency, experimental duration, transgene stability and the integrity of the original genotype (Raemakers *et al*., 1997).

The preferred method of gene transfer for cassava in all four systems was by using *Agrobacterium*. More transgenic plants containing single-copy insertions of the transgene were recovered by using *Agrobacterium*-mediated transformation than in microparticle

bombardment (Taylor *et al*., 2004). Li *et al*. (1996) transformed somatic embryo-derived cotyledons using *Agrobacterium* and were able to regenerate transgenic shoots from these explants via organogenesis.

Table 1.2 Summary of used transformation procedures in cassava (Raemakers *et al*., 1997).

Explant	Regene-ration	Gene transfer technique	Selection genes	Result
somatic embryo	SE	electroporation	NPTII/GUS	chimeric transformed embryo
somatic embryo	SE	particle bombardment	NPTII/LUC	chimeric transformed embryo
somatic embryo	SE	particle bombardment	NPTII/GUS	chimeric transformed embryo
somatic embryo	AS	*Agrobacterium*	NPTII/GUS/HPH	transgenic plants
somatic embryo	PR	electroporation	LUC	transgenic callus
FEC	SE	particle bombardment	NPTII/GUS	transgenic plants
FEC	SE	particle bombardment	PAT/LUC	transgenic plants
FEC	PR-SE	electroporation	LUC	transgenic FEC

AS = adventitious shoot regeneration, GUS = _-glucuronidase, HPH = hygromycin phosphotransferase, LUC = luciferase, NPTII = neomycin phosphotransferase, PAT = phosphinothricin acetyl transferase gene, PR(-SE) = protoplast regeneration (via somatic embryogenesis), SE = somatic embryogenesis.

1.5.3.2. Protoplast transformation

Protoplasts are plant cells whose cell walls have been enzymatically removed. Protoplasts are transformed by using PEG or electroporation, but have also been transformed using *Agrobacterium* (Twyman *et al*., 2002). Transformed protoplasts are placed on selective medium and allowed to regenerate new cell walls. The cells proliferate to form callus from which embryos or shoots can be regenerated using appropriate hormone treatments (Twyman *et al*., 2002). Protoplast transformation is limited by the ability to regenerate into whole plants and is disadvantageous because of long culture periods. Cassava is very recalcitrant to plant regeneration from protoplasts and shoot regeneration from protoplasts has only been reported once by Shahin and Shepard in 1980 (Sofiari *et al*., 1998).

1.5.3.3. Axillary bud transformation

Time constraints and regeneration rates could be overcome by using axillary buds since a short period of time is required for explant preparation for transformation and direct regeneration occurs because axillary buds give rise to shoots without the need for explant differentiation. Axillary bud transformation has been used for various plants including sugarcane (Manickavasagam *et al*., 2004), *Acacia mangium* (Xie and Hong, 2002), chicory (Frulleux *et al*., 1997), eucalyptus (Yao and Lin-Wang, 2005), and kenaf (Kojima and Ueda-Shi., 2004).

Manickavasagam *et al*. (2004) used axillary buds of sugarcane for transformations with *Agrobacterium* to make the plant resistant to the herbicide BASTA. Meristematic regions of the axillary buds were wounded 4 – 5 times with a needle before *Agrobacterium* infiltration. Transgenic shoots were obtained in approximately 5 months with repeated proliferation of shoots in selection media to eliminate chimeric transformants (Manickavasagam *et al*., 2004). Their findings showed that the transformation efficiency was 49.6% with axillary buds which is higher than that of shoot meristems and callus explant transformations (Manickavasagam *et al*., 2004).

Xie and Hong (2002) transformed axillary buds of *Acacia mangium* using *Agrobacterium* and their results showed that after 6 months of selection, 80% of the resistant multiple adventitious shoots stained an intense blue colour. Internode stem segments of eucalyptus were transformed using *Agrobacterium*. Results showed that 1 – 3 hybridisation bands were detected in all 6 independent transgenic plants that were tested (Kojima and Ueda-Shi, 2004).

Kenaf plants have been transformed by conventional methods such as callus or cells in tissue culture using *Agrobacterium*. *In planta* transformation of kenaf has been reported by Kojima *et al*. (2004). Wounded lateral meristems of 3 month-old plants were inoculated with an *Agrobacterium* suspension by using a cotton swab (Kojima *et al*., 2004). Plants were grown for a further 3 – 4 days until lateral buds were 3cm in length and the smaller buds were removed. Plants were grown to maturation to obtain seeds. Seven randomly-selected seeds were grown and young leaves were tested for the transgene. An

Agrobacterium-removal step was not included in their protocol. Thus to test for contaminating *Agrobacterium*, leaf homogenate was placed on LB plates containing appropriate antibiotics for a few days which revealed that no *Agrobacterium* was present in the leaves (Kojima *et al*., 2004). The transgene was detected in all 4 T_0 transformants and 6 of the 7 T_1 transformants contained the transgene (Kojima *et al*., 2004).

Advantages of using axillary buds for genetic transformations include direct shoot proliferation from the buds which avoids an intervening callus stage to avoid somaclonal variation and a decrease in the time between transformation and regeneration. This allows for transgenic plants to maintain their characteristics as well as being produced rapidly.

1.5.3.4. Organelle transformation

Nuclear transformation has been used in plant genetic engineering to produce transgenic crops. However, there are a number of disadvantages of using this method of transformation due to environmental concerns. This is because of development of resistance against the transgene due to low levels of expression, and the presence of cotransferred plasmid DNA, bacterial antibiotic genes and plant genome DNA may spread to wild relatives in the environment (Daniell and Dhingra, 2002; van Bel *et al*., 2001). Vector sequences associated with the transgene may integrate into the plant genome which could lead to unpredictable endogenous and transgenic expression of the transgene when using nuclear transformations (van Bel *et al*., 2001).

Chloroplast transformation is an alternative to nuclear transformation which has many advantages over nuclear transformation regarding environmental safety and protein expression. These advantages include:

- High levels of transgene expression and high accumulation of foreign proteins leading to low cost production of biopharmaceuticals
- Introduction of multiple genes in a transformation event due to the highly polyploidy plastid genome thus decreasing the chances of transgene resistance
- Transgene containment through maternal inheritance thus preventing out-crossing of transgenes to crops through pollen dispersal
- Lack of gene silencing leading to more stable transgene expression

- Engineering transgenes without antibiotic resistance genes thus allowing for the production of edible vaccines
- Elimination of position effects because of site specific transgene integration thus preventing transgene expression interference
- Elimination of vector sequences
- Chloroplasts form disulphide bonds and are able to fold human proteins which are leading to the production of biopharmaceuticals
- Minimizing toxic protein effects because of chloroplast compartmentalisation (Altpeter *et al*., 2005; Daniell *et al*., 2002; Kumar *et al*., 2004; van Bel *et al*., 2001).

Chloroplast transformation is used in order to prevent gene pollution via pollen dispersal. Plastid genes are inherited uniparentally in a maternal manner (Kwon *et al*., 2001). The plastid DNA is lost during the process of pollen maturation and is thus not transmitted to the next generation nor would it be transferred to relatives of the transformed crop. The plastid chromosome-encoded protein is not produced in the pollen and would therefore not affect insects that feed on the pollen (Kwon *et al*., 2001).

The first report of stable chloroplast transformation was for the alga *Chlamydomonas reinhardtii* and the first successful transformation in a higher plant was in tobacco to introduce spectinomycin resistance (Primrose *et al*., 2001). More than 40 transgenes have been stably integrated and expressed via the tobacco chloroplast genome to confer important agronomic traits and to express valuable industrial biomaterials and therapeutic agents as seen in Figure 1.8 (Grevich and Daniell, 2005). Efficient plastid transformation has been achived via somatic embryogenesis using species-specific chloroplast vectors in soybean, carrot and cotton (Grevich and Daniell, 2005).

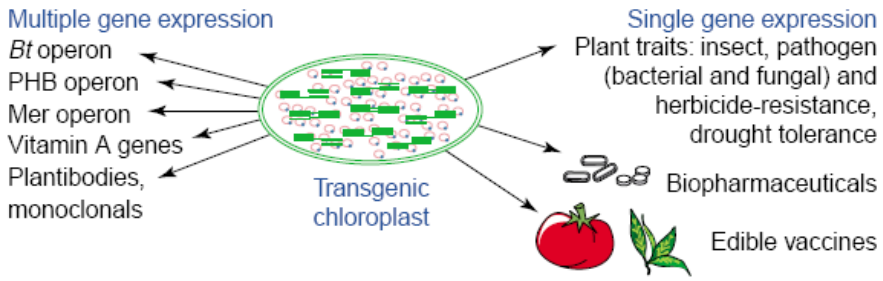


Figure 1.8 The numerous products obtained from chloroplast transformation (Daniell *et al*., 2002)

There are a few technical difficulties in transforming chloroplasts. The transgene must be present in each copy of the plastid genome within each cell for successful transformation to occur (Figure 1.9) otherwise there is rapid somatic segregation and genetic instability; and selection of transformants takes many months after which the undifferentiated cells have to be regenerated into plants (Bock and Khan, 2004; Gewolb, 2002). Plants, other than tobacco, tomato, potato and the night-shade plant family, remain difficult to regenerate as the techniques are species-specific (Gewolb, 2002).

Genetic manipulation of chloroplasts is important because it will provide plants that grow more efficiently through more proficient use of energy produced by the chloroplast. Plastid transformations have been used to investigate chloroplast gene functions by reverse genetics and for trait modification and molecular farming in crop plants (Altpeter *et al*., 2005). Transplastomic plants have been successfully produced to confer pest/ herbicide/ disease resistance, drought and salt tolerance, and are also used for phytoremediation and metabolic engineering (Altpeter *et al*., 2005). This method of transformation has also been used in the production of edible vaccines, monoclonal antibodies and biopharmaceuticals (Altpeter *et al*., 2005).

Direct DNA transfer methods are used for transforming plastids which include particle bombardment (Figure 1.9 A) and PEG (Heifetz, 2000). Particle bombardment is the preferred method because it is faster, easier and more versatile, and detailed methodologies for this transformation have been described. Selection of transformants is performed by using appropriate antibiotics or with betaine aldehyde if vectors do not contain an antibiotic selection gene (Bock and Khan, 2004).

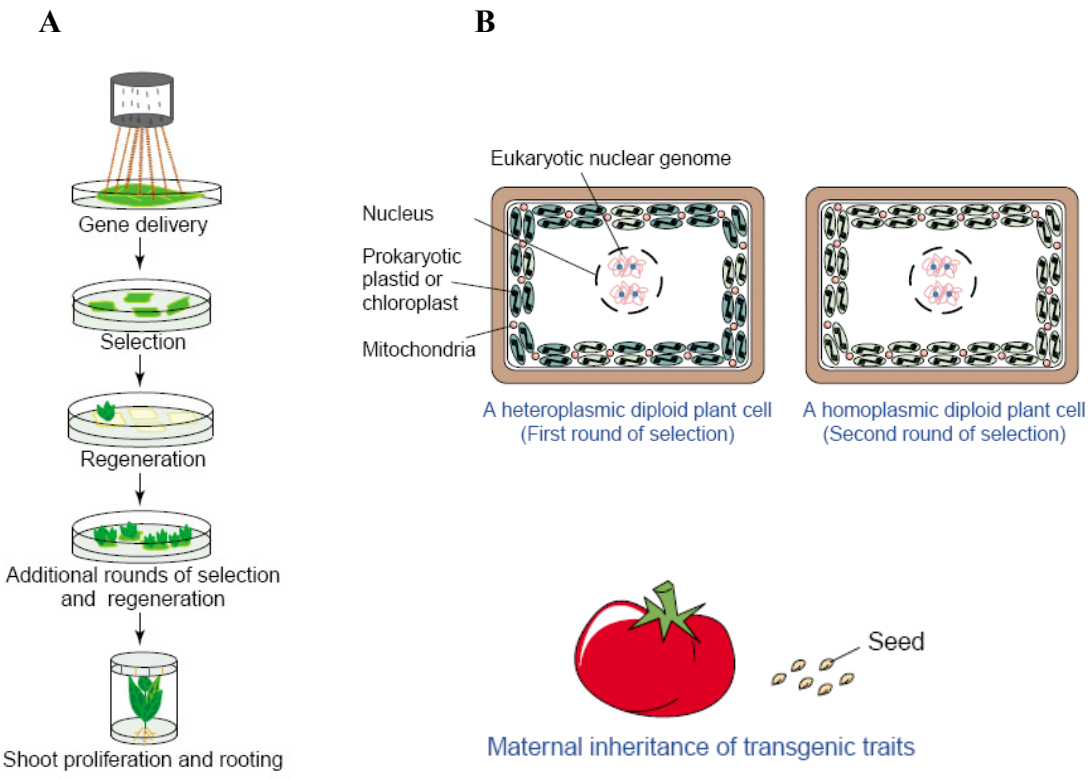


Figure 1.9 A and B Chloroplast transformation via particle bombardment.
The tungsten coated with the transformation vector is shot into the explants. Explants are regenerated on media containing appropriate selection antibiotics thus selecting only transformed plastids. The primary transformants are heteroplastic, and regeneration under antibiotic selection eliminates residual wild-type genomes. 100% of seeds germinate on selective media because traits are inherited maternally without segregation (Bock and Khan, 2004).

The first round of selection leads to the production of heteroplasmic cells which are a mixture of transformed and wild-type chloroplast genomes (Figure 1.9 B). Homoplasmic cells are obtained after long periods of selection (Daniell *et al*., 2002).

1.6. Objectives and thesis plan

The general objectives and aims were to improve cassava transformation and regeneration for SACMV resistance.

The specific aims of this thesis were:

1. Attempt to transform cassava axillary buds via *Agrobacterium*-mediated transformation and regenerate them directly
2. Screen elite selected cassava cultivars for genetic capability to induce somatic embryos
3. Transform tobacco to evaluate a transgene against SACMV.

A limited number of research groups have been successful at transforming and regenerating cassava. A suitable transformation programme requires reliable transformation methods and means of regenerating transformants. Chapter 2 attempted to transform and regenerate directly from axillary buds without an intervening callus stage. Because non-transgenic plants were obtained, the results of this study provided an essential guide for designing new experiments which will be conducted in our laboratory. Chapter 3 aimed at screening nine elite selected cassava cultivars (including the SA landrace, T200) for efficient production of embryogenic structures (somatic embryos/friable embryogenic callus) for subsequent transformation and regeneration experiments in our laboratory. Chapter 4 aimed to screen for the efficiency of an antisense transgene against SACMV in transgenic tobacco. Chapter 5 is the general discussion of the work undertaken in this thesis.

CHAPTER 2. CASSAVA AXILLARY BUD TRANSFORMATION

2.1 INTRODUCTION

Cassava is an important food staple and is also used for industrial applications such as ethanol production (Nweke *et al*., 2002). Cassava is grown by subsistence farmers in South Africa and is also produced commercially for starch production. However, the yield is severely affected by cassava mosaic diseases (CMD) which causes losses of up to 20 – 80% of total yields throughout Africa (Fregene and Puonti-Kaerlas., 2002). Transgenic plants resistant to viruses have been developed by introducing a viral sequence into plants which leads to the expression of the introduced viral sequence. This interferes with the virus in the plant through various mechanisms such as replication inhibition or prevention of systemic viral spread by RNA silencing through antisense and sense or short-hairpin constructs.

Developing regeneration and transformation procedures is a prerequisite for improving cassava genetic engineering. Constraints to transformation include amenability to regeneration and/or transformation, an efficient means of DNA transfer and stable integration, as well as methods to detect for and select transgenic plants. Various explants have been used for cassava transformation. Currently, the use of organised tissues – such as somatic cotyledons and friable embryogenic callus (FEC) – is the only practical transformation and regeneration methods for cassava (Taylor *et al*., 2004). Somatic cotyledons and FEC have been used to obtain transgenic plants via particle bombardment, electroporation, and *Agrobacterium tumefaciens*-mediated transformation as shown in Figure 1.7. However, somatic cotyledon and FEC transformation and regeneration are cultivar-dependent, and may result in somaclonal variation due to continuous cell differentiation *in vitro* and explants take long periods to regenerate. Cassava axillary buds have been used for transformation experiments where somatic embryos were induced followed by shoot regeneration (Msikita *et al*., 2006). However, the transformation efficiency using this system ranges between 1 and 5% (Msikita *et al*., 2006). Successful axillary bud transformation has been achieved in sugarcane (Manickavasagam *et al*.,

2004), *Acacia mangium* (Xie and Hong, 2002), chicory (Frulleux *et al*., 1997), eucalyptus (Yao and Lin-Wang, 2005) and kenaf (Kojima *et al*., 2004) as shown in Table 2.1.

Table 2.1 Axillary bud transformation in various crops.

Plant	Selection	Transformation efficiency	GUS expression
Eucalyptus	4 weeks – 30mg/l kanamycin 10 – 11 weeks – 50mg/l kanamycin	8 – 12%	Chimeric
Acacia mangium	30 days – 30mg/l timentin (T) and 12mg/l geneticin (G) 60 days – 200mg/l T and 20mg/l G 30 days – 100mg/l T and 30mg/l G 30 days – 50mg/l T and 12mg/l G 60 days – 15mg/l G	30%	70% Chimeric, after 6 months selection 80% resistant multiple shoots stained blue
Sugarcane	Repeated proliferation of shoots on selection medium (phosphinothricin 5mg/l) eliminated chimeric transformants (15 weeks)	50%	70% Chimeric, after 15 weeks secondary shoots fully transgenic
Chicory	4 weeks – 100mg/l kanamycin. Developing green shoots were transferred to 200mg/l kanamycin for 4 months followed by 1 month on rooting media with 100mg/l kanamycin	10%	60% Chimeric: 64% GUS at wound sites, 39% in vascular tissues, 13% in meristematic zone of buds

Therefore, utilising cassava axillary buds as explants for transformation could lead to shorter regeneration and transformation periods as well as decreasing the chances of somaclonal variation.

2.2. GENERAL OBJECTIVES

There is a great need to control the spread and damage caused by cassava-infecting geminiviruses because of the severity of CMD and the growing economic importance of cassava as a food, starch and biofuels crop in South Africa. *South African cassava mosaic virus* (SACMV) has been identified as one of the begomoviruses causing CMD in South Africa and would thus adversely affect the production of cassava in this country. Other cassava viruses that cause major losses include *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV). While genetic engineering is a valuable tool for the improvement of cassava, low transformation efficiencies and lengthy periods required to generate tissues for transformation, and regeneration times, remain problematic.

The objective of this study was to increase the efficiency and decrease the time period of transformation of cassava by attempting *Agrobacterium*-mediated transformation of axillary buds. This is the first report of experimental trials for cassava axillary bud transformation via direct regeneration.

Specific aims

The specific aims of this research were to:

- Design a SACMV AC1 gene fragment, clone into pART7 in order to obtain the CaMV 35S promoter and octopine synthase terminator and subclone (promoter:insert:terminator) into pCAMBIA1305.1.
- Transform *Agrobacterium* strain Agl1.
- Transform cassava cultivars T200 and TMS60444 axillary buds with *Agrobacterium*.
- Vary experimental parameters: explant pre-treatments, vacuum infiltration, co-cultivation temperature, photoperiod (dark vs. continuous light) and duration to increase transgene transfer from Agl1 into plants.
- Test for transformation success: explant selection on hygromycin-containing media, GUS assays and PCR.

2.3. MATERIALS AND METHODS

2.3.1. Method for construct design

The SACMV AC1 (1070bp) gene sequence (accession number NC 003803) was placed into Primer 3 Output programme to select primers that amplify a region of this gene. Primers that amplify a 221bp fragment were chosen in an area of the SACMV AC1 gene that has high (63% and 65%) nucleotide similarity with ACMV, ACCMV, ACUMV, EACTMV, EACMV, EACMZV, SLCMV and ACMCV. ICVM has only 10% similarity compared with the other cassava-infecting viruses and thus was not used to design the

antisense construct. The cassava mosaic virus sequences were aligned using T-COFFEE Version 1.41 programme (Notredame *et al*., 2000).

2.3.2. Blunt-end cloning into pART7

Vector preparation

The pART7 plasmid DNA (4900 bp) was isolated from an overnight *E.coli* culture using the High Pure Plasmid Isolation kit (Roche). The samples were pooled and concentrated using a Speedvac. The plasmid was digested with *Sma*I (to generate blunt ends) for 3 hours at 30ºC followed by *Sma*I inactivation at 65ºC for 15 minutes. This plasmid was purified on a low-melting temperature agarose gel by excising the desired band followed by purification using the High Pure Plasmid Isolation kit (Roche). The linearised purified plasmid was concentrated in a Speedvac and the pellet was resuspended in 10µl water. The vector was blunt-end polished with T4 DNA polymerase (Fermentas) for 20 minutes at 11ºC followed by inactivation at 70ºC for 10 minutes. Samples were purified using DNace quickclean (Bioline) and concentrated in a Speedvac. The vector was dephosphorylated with Antarctic phosphatase (NEB) for 30 minutes at 37ºC and the enzyme was inactivated at 65ºC for 5 minutes. Samples were purified using the High Pure Plasmid Isolation kit (Roche). Vector DNA was concentrated and the pellet was resuspended in 5µl of 1X dilution buffer (Rapid DNA ligation kit – Roche).

Insert preparation

The SACMV AC1 gene was amplified using the Expand High Fidelity PCR System (Roche) using primers SACMV DNA-A AC1 F (forward) 5' – GAC AAG GAC GGA GAC ACC AT – 3' and SACMV DNA-A AC1 R (reverse) 5' – ATC GGA GGC TCC TGA AAA AT – 3'. These primers yield a fragment of 221 bp which starts at 535 bp and ends at 755 bp in the SACMV AC1 gene. A standard PCR reaction volume of 50µl was made up consisting of 4µl of DNA-A, 1µl of 10mM dNTPs mix, 1.5µl of each primer (10µM), 5µl of 10X AccuBuffer with MgCl (Bioline) and 2.5 units of ACCUZYME DNA polymerase (Bioline) which creates blunt ends. The appropriate cycling took place in

MyCycler PCR machine (BioRad) in which the *Rep* gene fragment was amplified by incubation at 94ºC for 2 minutes, followed by 30 cycles of 30 seconds at 94ºC, 30 seconds at 56ºC and 1 minute at 72ºC, and a final extension at 72ºC for 7 minutes. Insert DNA was purified using the High Pure Plasmid Isolation kit (Roche). The DNA was concentrated using a Speedvac. The insert was phosphorylated as follows: 1µg DNA was added to 2µl T4 Polynucleotide kinase (Bioline), 10µl 10X PNK buffer (bioline), 2µl 10mM dNTPs, 5µl ATP solution (provided with PNK) and made up to a final volume of 100µl with water. Samples were incubated at 37ºC for 30 minutes followed by inactivation at 70ºC for 5 minutes. Insert DNA was purified using the High Pure Plasmid Isolation kit (Roche). The DNA was concentrated and the pellet was resuspended in 5µl of 1X dilution buffer (Rapid DNA ligation kit – Roche).

Ligation reactions

The purified vector concentration was 70ng/µl and insert concentration was 40ng/µl. The following molar ratios for blunt-end ligations were used: 1:2, 1:5, 1:7 and 1:10. The molar ratios were calculated using this formula:

$$\frac{\text{vector concentration (ng)} \times \text{insert length (kb)}}{\text{vector length (kb)}} \times \text{molar ratio} = \text{ng of insert DNA}$$

Vector and insert were ligated using the Rapid DNA Ligation Kit (Roche) as follows:

	Ligation control	**Transformation control**	**Vector and insert ratios**			
			1:2	**1:5**	**1:7**	**1:10**
Vector	1µl	1µl	1µl	1µl	1µl	1µl
Insert			0.2µl	0.5µl	0.6µl	0.9µl
1x dilution buffer	9µl	9µl	8.8µl	8.5µl	8.4µl	8.1µl
Total volume	10µl	10µl	10µl	10µl	10µl	10µl
T4 DNA ligation buffer	10µl	10µl	10µl	10µl	10µl	10µl
T4 DNA ligase	1µl	1µl	1µl	1µl	1µl	1µl

The samples were ligated at 16ºC for 10 minutes.

E.coli DH5α transformation

The ligation mixture (2 - 5µl) was added to competent *E.coli* cells and transformed using the Transformation and Storage Buffer (TSB) method (Chung *et al*., 1989). *E.coli* cells were grown overnight at 37ºC and 200 rpm. One ml of the overnight culture was inoculated into 30ml LB and grown to an OD_{600} of 0.3 – 0.6. One ml aliquots of culture were centrifuged at maximum speed (Eppendorf MiniSpin centrifuge) for 1 minute at room temperature. The supernatant was discarded and the cells were resuspended in 100µl chilled TSB by pipetting followed by incubation on ice for 10 minutes. The ligation mixture was added, mixed thoroughly and incubated on ice for 30 minutes. Nine hundred µl TSB and 18µl 1M glucose was added and incubated for at 37ºC (200 rpm) for 1 hour to activate antibiotic resistance genes. The cultures were spread (100µl and 450µl of each molar ratio) onto NA plates containing 100mg/l ampicillin (to select for transformants) at 37ºC for no longer than 17 hours. Restriction digests of extracted transformed plasmids with various enzymes were performed to determine the presence of the insert although this method was unsuccessful. Thus, PCR with sequencing primers for pART7 multiple cloning site (MCS) was used to determine insert integration as well as the number of inserts in the vector. PCR with pART7 MCS primers yield a fragment size of 106 bp, thus a vector that contains one copy of the insert (221 bp) will yield a fragment size of 327 bp. Only one out of six clones contained a single insert. The clone containing the insert was gel-purified and was sequenced to determine the direction of the insert i.e. sense or antisense integration into pART7.

2.3.3. Blunt-end cloning into pCAMBIA 1305.1

Vector preparation

The pCAMBIA 1305.1 vector (11846 bp) (provided by CAMBIA, Canberra, Australia) was prepared for cloning by digesting the vector with *Xba*I for 3 hours at 37ºC and *Xba*I was inactivated by adding 0.5M EDTA. The plasmid was blunt-end polished and dephosphorylated as in 2.3.2.

Insert preparation

The CaMV 35S promoter and octopine synthase terminator containing the 221 bp SACMV AC1 gene fragment in the antisense direction in the multiple cloning site was excised from pART7 by digesting with *Not*I for 3 hours at 37ºC followed by enzyme inactivation at 80ºC for 20 minutes. The *Not*I fragment of interest was purified on a low-melting temperature agarose gel followed by purification using the High Pure Plasmid Isolation kit (Roche). The DNA was concentrated using a Speedvac. The insert was blunt-end polished (as described in 2.3.2) and phosphorylated by including 2µl Klenow (Bioline) in the reaction. Insert DNA was purified using the High Pure Plasmid Isolation kit (Roche). The DNA was concentrated and the pellet was resuspended in 5µl of 1X dilution buffer (Rapid DNA ligation kit – Roche).

Vector and insert ligation

The purified vector concentration was 20ng/µl and insert concentration was 20ng/µl. The following molar ratios for blunt-end ligations were used: 1:5 and 1:10. The molar ratios were calculated using this formula:

$$\frac{\text{vector concentration (ng)} \times \text{insert length (kb)}}{\text{vector length (kb)}} \times \text{molar ratio} = \text{ng of insert DNA}$$

Vector and insert were ligated using the Rapid DNA Ligation Kit (Roche) as follows:

	Ligation control	**Transformation control**	**Vector and insert ratios**	
			1:5	**1:10**
Vector	1µl	0.4µl	1µl	1µl
Insert			0.9µl	1.8µl
1X dilution buffer	9µl	9.6µl	8.1µl	7.2µl
Total volume	10µl	10µl	10µl	10µl
T4 DNA ligation buffer	10µl	10µl	10µl	10µl
T4 DNA ligase	1µl	1µl	1µl	1µl

The samples were ligated at 16ºC for 10 minutes.

E.coli DH5α transformation

E.coli cells were transformed as described in section 2.3.2. PCR with sequencing primers for pART7 MCS was used to determine insert integration and number of inserts in the vector. The clone containing the insert was gel-purified and sequenced to determine the direction of the insert i.e. sense or antisense integration into vector.

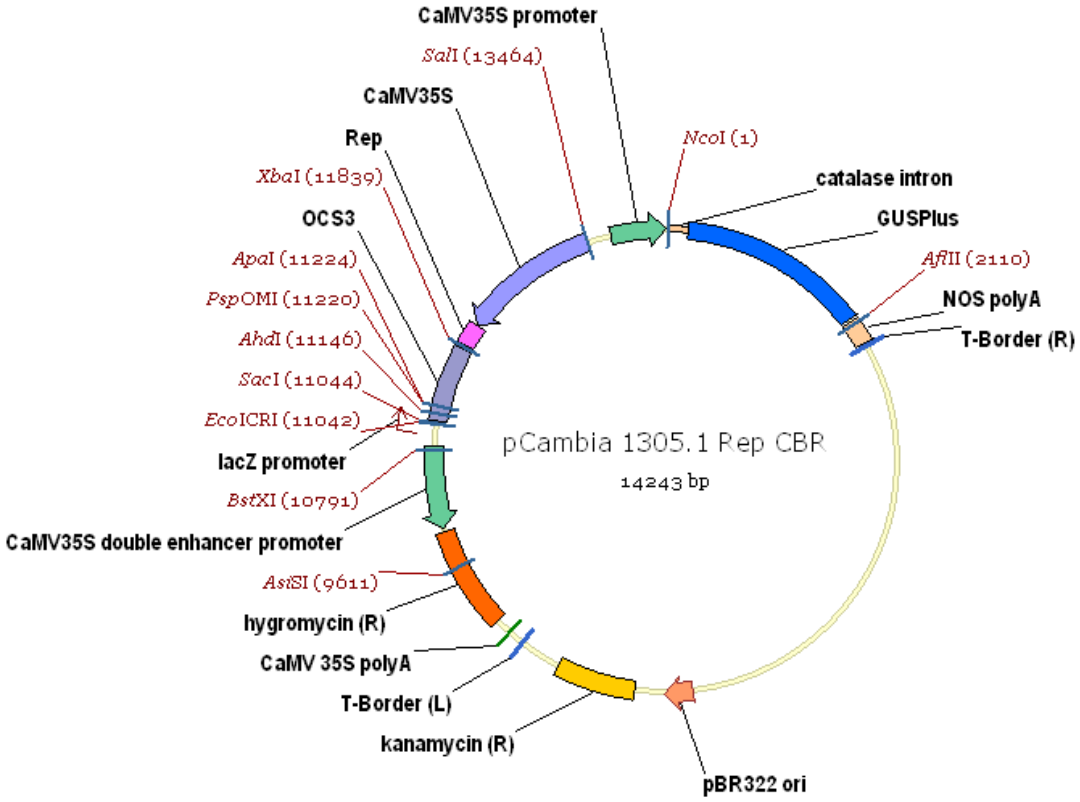


Figure 2.1 pCAMBIA 1305.1 containing *Rep* transgene.

2.3.4 Transformation of antisense clone into *Agrobacterium* Agl1 strain

Agrobacterium tumefaciens strain Agl1 (supplied by P.Chavarriaga, International Centre for Tropical Agriculture, Colombia) was transformed using the freeze-thaw method according to An *et al*. (1988). Agl1 was grown in 5ml LB (Luria Broth) medium supplemented with 100mg/l carbenicillin overnight at 140 rpm on a rotary shaker at 30ºC. Two ml of the overnight culture was transferred to 50ml LB containing 100mg/l carbenicillin and incubated at 140 rpm on a rotary shaker at 30ºC until the cultures reached

an OD_{600} of 0.5 – 1.0. These cell suspensions were chilled on ice followed by centrifugation at 3000g for 5 minutes at 4ºC. The supernatant was removed and the pellet was resuspended in 1ml of 20mM $CaCl_2$ solution (ice-cold). The resuspended cells (0.1ml) were aliquoted into prechilled Eppendorf test tubes. One µg of the pCAMBIA 1305.1 plasmid containing the 221 bp *Rep* gene fragment was added to the cells and frozen in liquid nitrogen. The GFP-containing pCAMBIA 1303 vector was also transformed into Agl1. The cells were thawed by incubating the tubes at 37ºC for 5 minutes. One ml of LB medium was added to the tubes and incubated at 30ºC for 2 – 4 hours with gentle shaking to allow bacteria to express the antibiotic resistance genes. The tubes were centrifuged for 30s at 13 400 rpm at 4°C. The supernatant was discarded and the pellet resuspended in 0.1ml LB medium which was spread onto LB agar plates containing 100mg/l kanamycin for selection. The plates were incubated at 30ºC for approximately 3 days. The plasmid DNA was extracted and transformants were screened by PCR with the 221 bp SACMV AC1 insert primers and pART7 MCS primers. PCR with either set of primers showed that all four colonies contained the pCAMBIA 1305.1 clone (Figure 2.1).

2.3.5. Plasmid extractions

Agrobacterium plasmids were extracted using the alkaline lysis minipreparation method according to Sambrook *et al*. (1989). One bacterial colony was grown in 5ml LB supplemented with 100mg/l carbenicillin and 100mg/l kanamycin overnight at 30ºC at 140rpm. The overnight culture (1.5 ml) was aliquoted into Eppendorf tubes and centrifuged at 13 400 rpm for 3 minutes at 4ºC. The supernatant was discarded and the dried pellet was resuspended in 100µl of ice-cold solution I (50mM glucose, 25mM Tris.Cl pH 8.0, 10mM EDTA [ethylenediaminetetraacetic acid], made up to final volume with sterile distilled water) by vortexing vigorously and incubating at room temperature for 5 minutes. Two hundred µl of freshly prepared solution II (0.2M NaOH, 1% SDS, made up to final volume with sterile distilled water) was added, mixed by inverting rapidly 5 times and incubated on ice for 5 – 10 minutes. A 150µl aliquot of ice-cold solution III (3M potassium acetate, 5M glacial acetic acid, made up to final volume with sterile distilled water) was added, mixed vigorously and incubated on ice for 5 – 10 minutes. The samples were then centrifuged at 13 400 rpm for 5 minutes at 4ºC followed by transferring the supernatant to a new tube. A 350µl aliquot of isopropanol was mixed with the

supernatant and incubated at room temperature for 5 minutes. The DNA was precipitated by centrifuging at 13 400 rpm for 10 minutes at 4ºC. The supernatant was discarded and the pellet was washed in 1ml 70% ethanol by centrifuging at 13 400 rpm for 10 minutes at 4ºC. The ethanol was discarded and the dried pellet was resuspended in 14µl TE buffer (10mM Tris.Cl, 0.1mM EDTA pH 8.0) and 1µl RNAse (20mg/ml stock) for several hours. Samples were stored at -20ºC. Extracted plasmids were used to screen for transformants.

2.3.6. Plant material

In vitro cassava cultivars T200 (local cultivar), and TMS60444 (model cultivar) were maintained as shoot cultures on Murashige and Skoog (MS) medium supplemented with 20g/l sucrose (MS2), solidified with 7.8g/l agar, pH 5.8, at 25ºC under a 16 h photoperiod (3000 Lux). Explants were subcultured every 4 – 6 weeks.

2.3.7. Explant pretreatment, transformation by *Agrobacterium* infiltration and co-cultivation, selection of putative transformed explants and regeneration of transformed plants

Preparation of *Agrobacterium* suspension for transformation:
Transformed *Agrobacterium* strain Agl1 was grown in 30ml LB containing 100mg/l kanamycin overnight at 140 rpm on a rotary shaker at 30ºC. Two ml of the overnight culture was transferred to 30ml LB with 100mg/l kanamycin and 200µM acetosyringone, and incubated at 140 rpm on a rotary shaker at 30ºC until the cultures reached an OD_{600} of 0.8 – 1.0. The cultures were centrifuged at 3000g for 15 minutes at 4ºC. The pellet was resuspended in an equal volume of liquid MS2 supplemented with 200µM acetosyringone.

The pre-treatment of explants, conditions for transformation and type of transformant screening is summarised in Table 2.2.

Table 2.2 shows the parameters used for explants pre-treatments and transformation of cassava axillary buds.

Experiment	Cultivar	Pretreatment	Explant treatment	Transformation conditions	Transformation vector	Transformation screening
A	T200 and TMS60444	2 days on 4E* media	Internodes injured with needle or treated with 0.1% Tween 20	Agro-infiltrated for 15 min, co-cultivated for 3 days at 25°C in the dark	pCAMBIA1305.1 with *Rep* insert	Selection on MS2 + 30mg/l hygromycin, surviving explants regenerated. TNA extracted for PCR with GUS Plus primers. **Negative results.**
B	T200 and TMS60444	3 days on 4E* media	Internodes injured in axillary bud region	Agro-infiltrated for 15 min, co-cultivated for 3 and 4 days at 25°C in continuous light	pCAMBIA1305.1 with *Rep* insert	Selection on MS2 + 30mg/l hygromycin, surviving explants regenerated. TNA extracted for PCR with GUS Plus primers. **Negative results.**
C	T200 and TMS60444	3 days on 4E* media	Axillary buds with stem removed below and above bud	Agro-infiltrated for 5 min, co-cultivated for 3 days at 22°C in continuous light	pCAMBIA1305.1 with *Rep* insert	Selection on MS2 + 30mg/l hygromycin, surviving explants regenerated. TNA extracted for PCR with GUS Plus primers. **Negative results.**
D	T200 and TMS60444	3 days on 4E* media	Axillary buds removed from stem	Agro-infiltrated for 15 min, co-cultivated for 3 days at 22°C in continuous light	pCAMBIA1305.1 with *Rep* insert	All explants were assayed for GUS expression. **Negative results.**
E	T200 and TMS60444	3 days on 4E* media	Agl1 injected through internode ends towards axillary buds	Agro-infiltrated for 15 min, co-cultivated for 30 min in Agl1 suspension, excess Agl1 removed, co-cultivated for 3 days at 22°C in continuous light	pCAMBIA1305.1 with *Rep* insert	All explants were assayed for GUS expression. **Negative results.**
F	T200 and TMS60444	2 days on 4E* media	Internodes injected with Agl1 in axillary bud region	Agro-infiltrated for 15 min, co-cultivated for 3days at 22°C in continuous light	pCAMBIA1303	All explants were assayed for GUS expression. **Chimeric expression of GUS gene was achieved.**

* 4E media – MS2 supplemented with 0.04 mg/l BAP, 0.05 mg/l GA_3 and 0.02 mg/l NAA, and 200µM acetosyringone was added to the 4E media.

2.3.8. DNA isolation from putative transgenic plants using the (cetyltrimethylammonium bromide) CTAB method

Total nucleic acids (TNA) were isolated using the CTAB extraction method developed by Doyle and Doyle (1987). Twenty to fifty mg of explant samples were ground to a fine powder using liquid nitrogen. 0.5ml pre-heated (65ºC) extraction buffer (2% CTAB, 20mM EDTA pH8.0, 1.4M NaCl, 100mM Tris.Cl pH 8.0) and 1µl β-mercaptoethanol (final concentration of 0.1% v/v) were added to the ground plant material and incubated at 65ºC for 30 – 60 minutes. The aqueous layer containing the total nucleic acids was extracted by adding 0.5ml chloroform: isoamyl alcohol (24:1), inverting, centrifuging at 13 400 rpm for 10 minutes at 4ºC. The aqueous layer was transferred to a new microfuge tube. This chloroform: isoamyl alcohol step was repeated. Nucleic acids were precipitated with an equal volume of isopropanol (0.5ml), followed by centrifugation at 13 400 rpm for 10 minutes at 4ºC. The supernatant was discarded and the pellet was washed with ice-cold 70% ethanol (0.5ml) and spun at maximum speed for 10 minutes at 4ºC. The dried pellet was resuspended in 20 - 50µl of TE buffer and 1µl RNAse (20mg/ml stock) and incubated at room temperature for several hours. TNA samples were quantified using a spectrophotometer to determine the purity and DNA concentrations before and after restriction digests. Samples were stored at -20ºC.

2.3.9. GUS Assays

Explants were assayed for expression of the *gus* gene carried in the pCAMBIA1305.1 plant transformation vector following the histochemical procedure supplied by Fermentas. Explants were incubated in a staining buffer with final concentrations of 0.1mM sodium phosphate pH 7.0, 10mM Na-EDTA, 1mM potassium ferricyanide, 2mM X-Gluc (5-bromo-4chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt) and 0.1% (v/v) Triton X-100 at 37ºC for 12 – 24 hours. Explants were rinsed several times with 70% ethanol to remove chlorophyll in order to bleach the explants and enhance the blue stain.

2.3.10. Polymerase chain reaction (PCR) analysis

DNA samples from putative transgenics isolated using the CTAB method were used for PCR analysis. A region of the GUS Plus gene amplified using primers GUS Plus F (forward) 5' - GGT CAC AAC CGA GAT GTC CT - 3' and GUS Plus R (reverse) 5'- CAA CAT CCT CGA CGA TAG CA -3' to yield a 181bp fragment.

A standard PCR reaction volume of 50µl was made up consisting of 1µg DNA sample, 10mM dNTPs mix, 10µM of each primer, 10x Taq buffer (Invitrogen) and 2.5 units of Taq DNA polymerase (Invitrogen). The appropriate cycling took place in MyCycler PCR machine (BioRad) in which a fragment of the β-glucuronidase gene was amplified by incubation at 94ºC for 2 minutes, followed by 30 cycles of 30s at 94ºC, 30s at 55ºC and 30s at 72ºC, and a final elongation at 72ºC for 10 minutes.

2.4. RESULTS

2.4.1 Cloning

The pART7 vector (4900 bp) was successfully prepared for cloning (Figure 2.2).

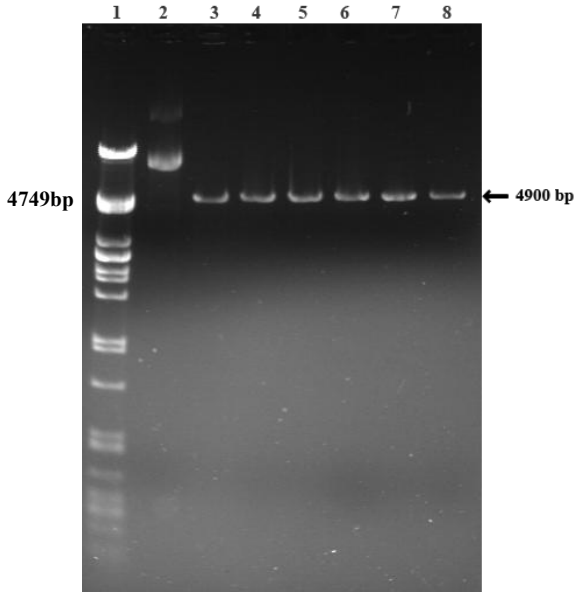


Figure 2.2 pART7 plasmid digested with *Sma*I for cloning.
Lane 1: MWM, 2: undigested pART7 control, 3 – 8: *Sma*I-digested pART7.

The SACMV AC1 gene was successfully amplified (Figure 2.3).

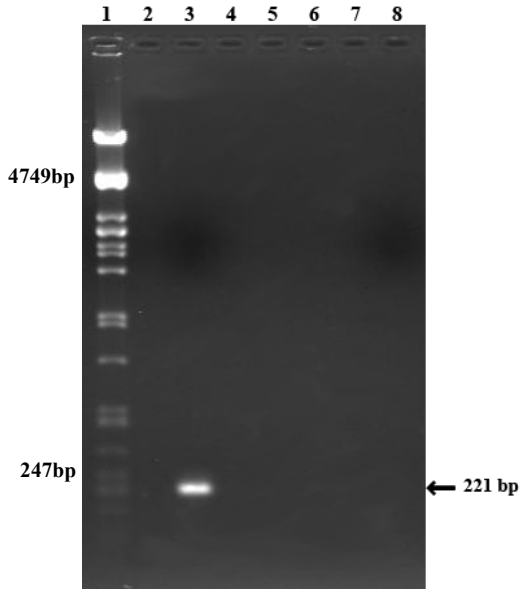


Figure 2.3 PCR amplification of a 221 bp gene fragment from SACMV AC1 gene.
Lane 1: MWM, 3: DNA-A from SACMV, 8: no template control.

The SACMV AC1 21bp fragment was successfully cloned into pART7 (Figure 2.4). Sequencing showed that the 221bp Rep fragment was integrated in the antisense orientation.

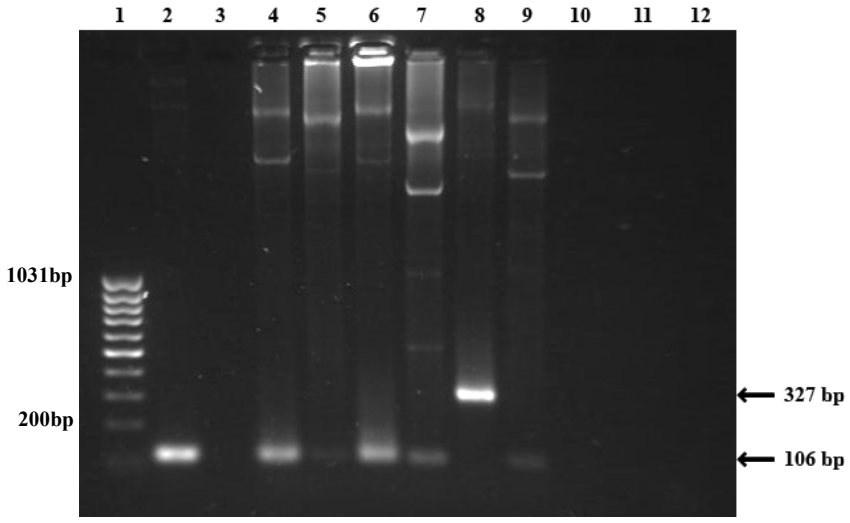


Figure 2.4 PCR amplification of pART7 MCS.
Lane 1: MWM, 2: pART7 control, 4 – 9: clones 1 – 6, 12: no template control. Only the clone in lane 8 contains a single insert.

The pCAMBIA 1305.1 vector successfully prepared for sub-cloning (Figure 2.5).

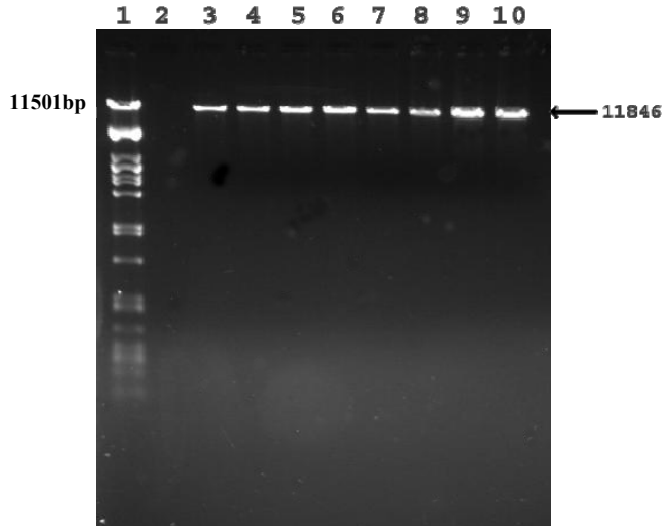


Figure 2.5 pCAMBIA 1305.1 plasmid digested with *Xba*I for cloning.
Lane 1: MWM, 3 – 10: *Xba*I-digested pCAMBIA 1305.1.

The CaMV 35S promoter and octopine synthase terminator containing the 221bp SACMV AC1 gene fragment in the antisense direction in the multiple cloning site was successfully prepared for sub-cloning (Figure 2.6).

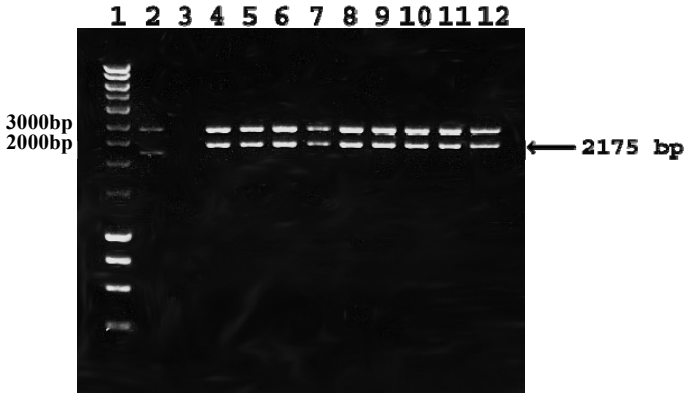


Figure 2.6 pART7 *Not*I fragment containing 221 bp antisense insert.
Lane 1: MWM, 2: pART7 control, 4 – 12: *Not*I fragment with antisense insert.

PCR with sequencing primers for pCAMBIA1305.1 CaMV 35S promoter and PlacZ promoter determined that insert integration into this plasmid had been achieved (Figure 2.7).

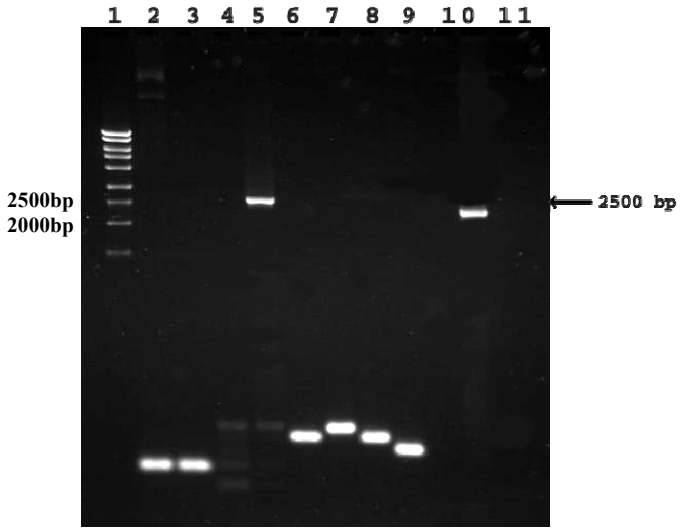


Figure 2.7 PCR with pCAMBIA 1305.1 primers.
Lane 1: MWM, 2: pCAMBIA 1305.1, 3 – 9: clones A – G, 10: pART7, 11: no template control.

The clone containing the insert was successfully transformed into *Agrobacterium* Agl1 (Figures 2.8 – 2.10).

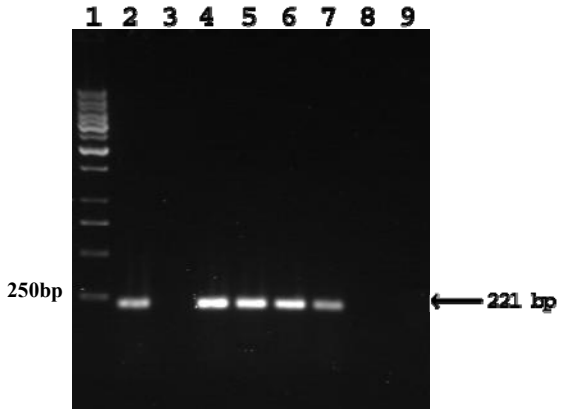


Figure 2.8 PCR with SACMV AC1 primers.
Lane 1: 1Kb MWM, 2: DNA-A, 4 – 7: transformed Agl1, 10: no template control.

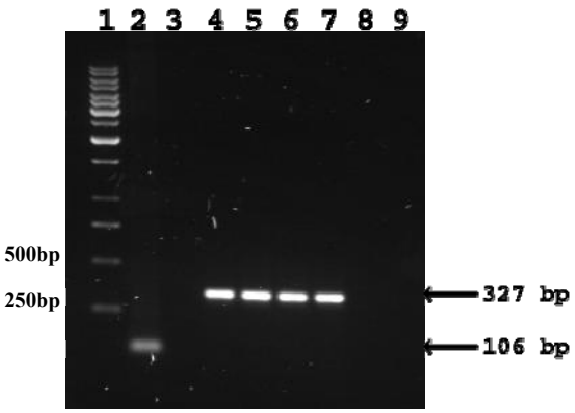


Figure 2.9 PCR with pART7 MCS primers.
Lane 1: MWM, 2: pART7 control, 4 – 7: transformed Agl1, 9: no template control.

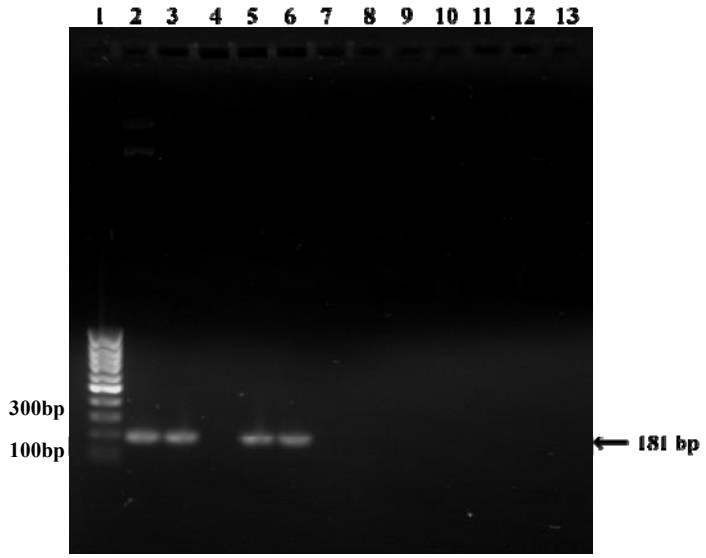
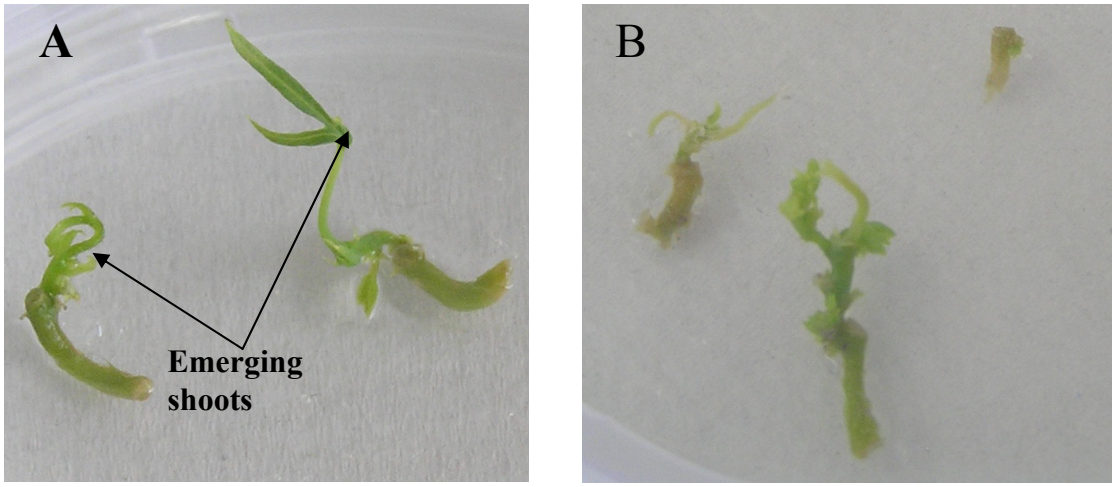


Figure 2.10 PCR with GUS Plus primers.
Lane 1: MWM, 2: pCAMBIA 1305.1, 3 – 6: transformed Agl1, 8: untransformed Agl1, 9: DNA-A, 10: untransformed T200, 13: no template control.

2.4.2. Plant transformation

Experiments A – C

Forty six % of the wounded T200 explants and 49% of Tween 20-treated T200 survived 2 months of selection on medium containing the antibiotic hygromycin (Figure 2.11). These explants were transferred to MS2 without antibiotics to allow regeneration.



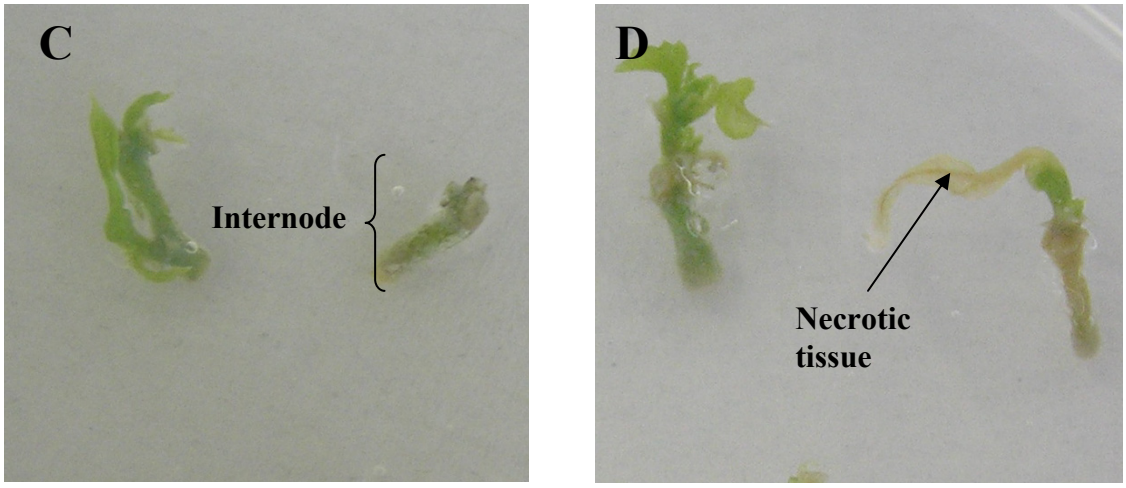


Figure 2.11 A – D: T200 axillary bud transformation from experiment A.
A: Tween-treated T200 internodes after the 1st week of selection on hygromycin-containing medium; B: Tween-treated T200 internodes after the 2nd week of selection on hygromycin-containing medium; C: T200 internodes with injured axillary buds after the 1st week of selection on hygromycin-containing medium; D: T200 internodes with injured axillary buds after the 2nd week of selection on hygromycin-containing medium.

Regenerated explants were tested for GUS expression as well as PCR using GUS Plus primers (Figure 2.12). Both tests showed negative results in all explants from experiments A and B, however, some explants in experiment C showed positive PCR results. Although regenerated plants that were assessed for GUS expression showed negative results thereby suggesting that the PCR products were false positives (Figure 2.12 lanes 17 and 18).

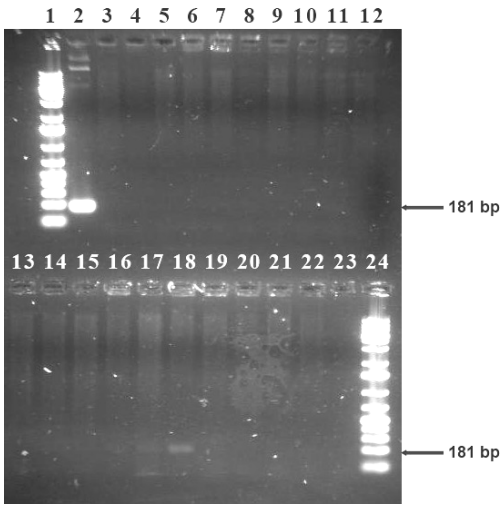


Figure 2.12 PCR with GUS Plus primers.
Lane 1: 1 Kb MWM; 2: pCAMBIA 1305.1 (positive control); 3 – 23: samples; 24: 1Kb MWM.

Experiments D and E

Axillary buds that had been carefully removed from the stem (Figure 2.13) were tested for transformation.

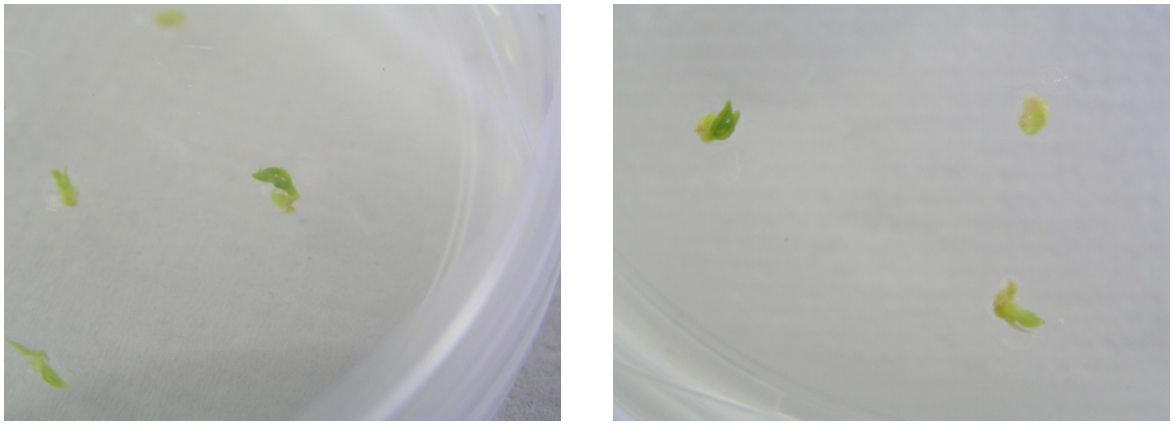


Figure 2.13 TMS60444 axillary buds that have been removed from the stem 7 days after transformation (1st day on selection plate).

All explants were bleached after the second week on selection medium and therefore the axillary bud alone may not be used for transformation.

Experiment F

The plant transformation vector pCAMBIA 1303 containing GFP and GUS (provided by CAMBIA, Canberra, Australia) was used for transformations. GUS assays were performed on all explants. Not all explants tested showed blue staining and staining was only observed at the cut ends of the stem and wounded sites (Figure 2.14). Thus, erratic staining could be due to the expression of the reporter proteins by the remaining *Agrobacterium* in the explants as the pCAMBIA 1303 vector does not contain an intron for gusA.



Figure 2.14 GUS assays of cassava axillary buds transformed with Agl1 containing pCAMBIA 1305.1.

Various explants screened after 1 week of Agl1 elimination and 1 week selection. Not all explants tested showed GUS activity and emerging leaves did not contain any blue spots. Only chimeric GUS expression was observed at wounded sites.

2.5. DISCUSSION

In this study, an attempt to transform cassava axillary buds/internodes and direct organogenesis was made in order to improve the efficiency and timeframe for the transformation of cassava with viral (SACMV) genes. Transformation efficiencies using cassava FEC or somatic embryos have been shown to be low (3 – 5%) and explants require extended tissue culture periods (Raemeakers *et al*., 1997). Time constraints and regeneration rates may be overcome by using axillary buds as the explant source for transformation since a short period is required for explant preparation for transformation and direct regeneration occurs because the axillary buds give rise to shoots without the need for explant differentiation. Axillary bud transformation has successfully been used for various plants including sugarcane (Manickavasagam *et al*., 2004), *Acacia mangium* (Xie and Hong, 2002), chicory (Frulleux *et al*., 1997), and eucalyptus (Yao and Lin-Wang, 2005) (Table 2.1).

Transformation parameters in this study (as shown in Table 2.2) were chosen based on results from previous research for optimal transformation efficiencies (T-DNA transfer from *Agrobacterium* into the plant genome) (Zambre *et al*., 2003). These parameters included using the hypervirulent *Agrobacterium* strain Agl1; *Agrobacterium* infiltration; co-cultivation temperatures (22ºC or 25ºC) and duration of 3 – 4 days (Dillen *et al*., 1997; Jin *et al*., 1993); photoperiod (light or dark) (Zambre *et al*., 2003); explant preculture on medium containing acetosyringone (*vir* gene inducer) (Sunilkumar *et al*., 1999) and explant wounding prior to transformation (Escudero and Hohn, 1997). The use of acetosyringone is employed routinely in the transformation of recalcitrant crops such as rice, maize, barley and wheat (Veluthambi *et al*., 2003).

The first step of this experiment was to determine the regeneration potential of the following explants on MS2 media: axillary buds removed from the stem; axillary buds attached to a piece of stem segment (1 cm in length) (internodes); and axillary buds with stem segments removed just above and below the axillary bud. Results showed that axillary buds alone and those attached to a piece of stem segment regenerated after a long culture period (approximately 2 – 3 months) while explants with intermodal tissue

regenerated within 2 – 3 weeks. All explants were used for subsequent transformation procedures.

Various explant pre-treatments and co-cultivation conditions were tested for potential transformation efficiency such as swelling of axillary buds on 4E media, injuring axillary buds prior to transformation, Agro-infiltration, co-cultivation at different temperatures, photoperiods and duration. Some explants survived after 3 months of antibiotic selection and were regenerated to test for transgene integration. SB and PCR were negative indicating that transformation was unsuccessful. However, a few explants in experiment C showed positive PCR results, yet negative for GUS expression. The positive PCR amplification is most likely an artefact since GUS assays were negative. An alternative explanation for these findings could be explained by low levels of transgene expression or chimaeric gene expression. Internodes, injected with *Agrobacterium* at the cut-end of the stem segments as well as along the stem and the axillary bud, revealed GUS expression only at the wounded sites and only one blue spot were visible at the axillary buds on few of the explants (Figure 16). In contrast, studies conducted by Manickavasagam *et al*., (2004); Xie and Hong, (2002); Frulleux *et al*., (1997); and Yao and Lin-Wang, (2005) indicated that internodes/axillary buds were markedly superior for transformation in terms of regeneration potential.

Manickavasagam *et al*., (2004) used 6-month-old axillary buds (1cm in size) of sugarcane for transformations with *Agrobacterium* for resistance against the herbicide BASTA. Meristematic regions of the axillary buds were wounded 4 – 5 times with a needle before being subjected to *Agrobacterium* infiltration for 10 minutes. Two *Agrobacterium* strains (EHA105 (hypervirulent) and LBA4404 (less virulent)) were chosen to evaluate transformation efficiency. It was found that the average number of explants producing GUS-positive shoots was increased by using EHA105 (Manickavasagam *et al*., 2004). Transgenic shoots were obtained in approximately 5 months with repeated proliferation of shoots in selection media to eliminate chimeric transformants (Manickavasagam *et al*., 2004). Their findings showed that the transformation efficiency was 49.6% with axillary buds which was higher than that of shoot meristems and callus explant transformations in sugarcane and regeneration through axillary buds causes minimal genetic changes (Manickavasagam *et al*., 2004).

Xie and Hong (2002) transformed stem segments (0.4 – 0.8cm in length) containing axillary buds of *Acacia mangium* by immersing explants in the *Agrobacterium* suspension for 15 minutes followed by a 3 day co-cultivation period. Results showed that after 6 months of repeated antibiotic selection of emerging shoots, 80% of the resistant multiple adventitious shoots stained an intense blue colour uniformly in elongated shoots. A similar transformation procedure was used for eucalyptus transformation where internode stem segments were transformed using *Agrobacterium*. Results showed an average transgene copy number of 1 – 3 in all 6 independent transgenic plants that were tested (Yao and Lin-Wang, 2005). Most eucalyptus transformation protocols use explants derived from embryos, young seedlings, cotyledons and hypocotyls however, these are not satisfactory for commercially important species as regeneration involves a callus induction phase (Yao and Lin-Wang, 2005). In this study, 2 – 3 months of selection on antibiotic medium was performed, where emerging shoots were transferred every 2 – 3 weeks to medium containing 30mg/l hygromycin and necrotic tissue was removed, but no GUS expression was observed in the regenerated plants. The hygromycin concentration should be in the range that inhibits cell division and regeneration from untransformed cells without killing the cells rapidly.

An explanation for the lack of ability of cassava axillary bud transformation could be that the meristematic region of the bud was not targeted efficiently during transformation because cassava axillary buds are small (1mm in diameter). For example, sugarcane axillary buds are 1cm in diameter and thus are easy to target (Manickavasagam *et al*., 2004). Furthermore, cassava axillary buds are covered by leaf primordia therefore rendering access to *Agrobacterium* difficult. *Agrobacterium* transformation of axillary buds alone and axillary buds attached to a piece of stem resulted in tissue browning and necrosis. This has also been shown in many monocotyledonous and dicotyledonous plants such as poplar, wheat, tomato, grape, sorghum, pepper and lettuce (Khanna *et al*., 2007). It has also been reported that maize callus infected with *Agrobacterium* undergoes a swift hypersensitive type of cell death (Khanna *et al*., 2007). Similar results were found with sugarcane meristems and attempts were made to by-pass necrosis by using antioxidant compounds to allow cell survival and gene transfer competence (de la Riva *et al*., 1998). Several antinecrotic treatments on the *Agrobacterium*-mediated DNA transfer and callus formation were tested and findings showed that genetically transformed calli could be

obtained only when a mix of antinecrotic compounds with remarkable antioxidative activity was added (de la Riva *et al*., 1998).

Despite the use in this study of the hypervirulent Agl1 strain of *Agrobacterium*, wounding of cassava axillary buds and the addition of acetosyringone, direct transformation was disappointingly unsuccessful. The virulence of 3 *Agrobacterium* strains (octopine LBA4404, nopaline C58C1 and hypervirulent EHA105) were tested in pea transformation. Results showed that LBA4404 produced 1 transgenic plant per 100 explants, 2.2 for C58C1 and 8.2 for EHA105 and thus indicates that the choice of correct strain may increase transformation frequency (Orczyk and Orczyk, 2000). The EHA105 strain also showed good results in transformation of other recalcitrant grain legumes and monocotyledonous plants (Orczyk and Orczyk, 2000).

It may be possible to explore further approaches to access transformable meristematic tissues within the axillary buds of cassava. In conclusion, transformation of internodes/axillary buds was unsuccessful thus FEC and somatic cotyledons should remain the explant source of cassava for transformation procedures.

CHAPTER 3. SOMATIC EMBRYOGENESIS OF SELECTED CASSAVA CULTIVARS

3.1. INTRODUCTION

Cassava is an important food crop for over 500 million people in developing countries. It is a reliable crop because of its tolerance to adverse environmental conditions and grows in marginal soils where other crops fail to grow. Genetic engineering of cassava requires efficient regeneration and transformation methods. For cassava, several explant types have been used in regeneration and transformation studies using various types of starting materials which include friable embryogenic callus (FEC), callus, somatic embryos, cotyledons, and protoplasts. FEC and cotyledons remain the most widely used explants for transformation and regeneration due to their success in these procedures (Raemakers *et al*., 1997). FEC is induced from organised embryogenic structures on Gresshoff and Doy salts and vitamins supplemented with picloram (Taylor *et al*., 2001). FEC is of a single cell origin and proliferates rapidly thus the tissue has a reduced risk of chimerism however; the long span of tissue culture *in vitro* may cause somaclonal variation.

Somatic cotyledons and FEC are induced from somatic embryos and it is these explants that are used for transformation. Somatic embryogenesis was first described by Stamp and Henshaw (1982) and numerous somatic embryogenesis and regeneration systems have been reported to date. Explants are initiated from decontaminated pieces of a whole plant and may consist of pieces of organs such as leaves or may be specific cell types such as pollen. Many explant features are known to affect the efficiency of culture initiation and transformation. Younger, more rapidly growing tissue or tissue at an early stage of development is most effective. Somatic embryos have been induced from various explants including: immature leaf lobes (Hankoua *et al*., 2005; Taylor *et al*., 2001; Sofiari *et al*., 1997; Li *et al*., 1998; Raemakers *et al*., 1997; Guohua, 1998; Atehnkeng *et al*., 2006; Hankoua *et al*., 2006; Guohua and Qiusheng, 2002; Danso and Ford-Lloyd, 2002; Zhang and Puonti-Kaerlas, 2005); shoot apical meristems (Hankoua *et al*., 2005; Raemakers *et al*., 1997; Atehnkeng *et al*., 2006; Hankoua *et al*., 2006); zygotic embryos or floral tissue

(Raemakers *et al*., 1997), and on various media containing different plant growth regulators (Table 3.1).

Somatic embryogenesis begins with the induction of primary embryos from the mentioned explants by culturing on MS2 supplemented with either 2,4-D or picloram followed by the production of secondary embryos on MS2 media containing lower concentrations of 2,4-D whereas media supplemented with NAA had a higher capacity to induce secondary embryos and at a faster rate (Raemakers *et al*., 1997). It has been shown that in certain cassava genotypes, Dicamba and Picloram were superior to 2,4-D for primary somatic embryo induction while some genotypes such as MCOL22 have the same results as 2,4-D (Raemakers *et al*., 1997). Mature somatic embryos are regenerated into cotyledons by placing explants on media containing BAP (Hankoua *et al*., 2006). The generation of embryogenic structures should be optimised for various cassava cultivars as not all cultivars are amenable to somatic embryogenesis and regeneration as well as transformation.

This chapter reports on the capabilities of selected cassava cultivars to produce somatic embryos. These cultivars were chosen because of their importance in the production of planting materials for the cassava industry in South Africa and genetic transformation.

Table 3.1 Cassava cultivars tested for their embryogenic competence.

Cultivars	SE production	Reference
TME13, 127, 8, 1, TMS I 91/02327 and 60444	AM and ILL on 50 Pi for primary SE and OES.	Hankoua *et al*., 2006
MCOL22, 1505, TMS90853, Gading, Adira1, Adira4 and Line 11.	ILL on 4.5 – 36.2 µM 2,4-D or 0.5 – 215.1 µM NAA for primary SE for 20 days then transferred to 0.4 µM BA for secondary SE.	Sofiari *et al*., 1997
MCOL22	ILL on 4mg/l 2,4-D for 14 days for primary somatic embryos.	Groll *et al*., 2001
TMS60444, 6042, 83350, 90853,Bonoua Rouge, Kataoli, Okouta, Line 2, Line 11, M.Aus7, MCOL22, 1505, 2215, MTAI5, 25	ILL on 50µM Picloram.	Taylor *et al*., 2001
TME8, 594, 596, SL80/40, I60142, I 90/0528, 96/0016, 96/0035, 96/0860, 96/1439 and Z 95/0826	Axillary meristems and ILL on 8 and 12mg/l Picloram for 30 days.	Atehnkeng *et al*., 2006
MCOL22 and TMS60444	Axillary buds on stem onto 10mg/l BAP for 6 days, enlarged buds removed and placed onto 50 Pi, or ILL on 50 Pi.	Zhang and Puonti-Kaerlas, 2005
Nanzhi 188 (Chinese major cultivar)	ILL onto 18.1µM 2,4-D (darkness for 20 days then 14hr photoperiod for 10 days). Primary SE to same media for secondary SE.	Guohua, 1998
TME1, 2, 5, 8, 12, 13, 127, 203, TMS282, 70775, 30572, 92/0057, 30001, 4(2)1425, 92/0666(3x), 91/02327 and 92/02324	AM and ILL cultured on 50 Pi for primary SE in the dark for 1 week then transferred to light for 3 weeks.	Hankoua *et al*., 2005
MCOL22, 1505, MPer183 and TMS60444	ILL on 2 – 12mg/l 2,4-D, 8mg/l optimum.	Li *et al*., 1998
Nanzhi 188	ILL on: 4mg/l 2,4-D, 40mg/l NAA, 2mg/l BA, 2mg/l BA and 40mg/l NAA or 2mg/l BA and 4mg/l 2,4-D.	Guohua and Qiusheng, 2002
Água Morna, Amansa Burro, Aparecida, Mata Fome, Milagrosa, Rosa, Rosinha and Sacai	Shoot apices and shoots onto 1, 3, 6, 9, 12, or 15mg/l Pi for 3 weeks in the dark.	Feitosa *et al*., 2007
KU 50 and Hanatee (Asian cultivars)	Apical buds, lateral buds and ILL onto 6 and 12mg/l picloram, dicamba and 2,4-D for 3 weeks in dim light (2000 lux).	Saelim *et al*., 2006
MCOL22, Thai cultivars: T5, KU50 and Hanatee	AM and ILL on 50 Pi for 2 weeks in the dark. Primary SE transferred to same media for cycling secondary embryos.	Zhang *et al*., 2001
CMC40, MCOL22, MPer183 and TMS60444	AM and ILL on 50 Pi or 12mg/l 2,4-D for 2 weeks in the dark. Transferred to same media after 2 weeks x3.	Zhang *et al*., 2000

AM: shoot apical meristem; SE; somatic embryos; ILL: immature leaf lobes; OES: organised embryogenic structures; 50 Pi: MS2 supplemented with 50µM Picloram.

3.2. GENERAL OBJECTIVES

Cassava transformation systems rely upon amenable regeneration from explants. FEC and cotyledons are the most widely used explants for transformation and are both induced from SE. Thus, the objective of this study was to determine the efficiency of somatic embryogenesis from the selected elite cassava cultivars.

Specific aims

- Test immature leaf lobes and axillary bud explant sources for the optimum production of SE
- Test picloram and 2,4-D for the optimum production of SE
- Evaluate which cultivar is best for inducing SE.

3.3. MATERIALS AND METHODS

3.3.1. Plant material

In vitro cassava cultivars T200 (local commercially grown cultivar), TMS60444 (model cultivar), BRA 1183, MTAI 16, CM 523-7, AR 9-18, CR 25-4, SM 707-17 and MCOL2261 were maintained as shoot cultures on MS (Murashige and Skoog) medium supplemented with 20g/l sucrose (MS2), solidified with 7.8g/l agar, pH 5.8, at 25ºC under a 16/8 h photoperiod (3000 Lux). Explants were subcultured every 4 – 6 weeks.

3.3.2. Embryogenic tissue induction

Three treatments were used to produce somatic embryos.

Treatment 1: Four – six weeks old i*n vitro* immature leaf lobes (ILL) were excised from mother plants using fine forceps and a scalpel and placed with the abaxial surface in contact with the medium on MS2 supplemented with 12mg/l picloram and cultivated in

the dark at 25ºC. Ten Petri dishes per cultivar, per treatment were used with each Petri dish containing ten leaf explants (100 explants per cultivar). Explants were transferred to fresh medium after 2 weeks in culture and production of embryogenic tissues from leaf lobe explants was scored after 6 weeks in culture.

Treatment 2: Four – six weeks old i*n vitro* immature leaf lobes (ILL) were excised from mother plants using fine forceps and a scalpel and placed with the abaxial surface in contact with the media on MS2 supplemented with 8mg/l 2,4-D and cultivated in the dark at 25ºC. Ten Petri dishes per cultivar, per treatment were used with each Petri dish containing ten leaf explants (100 explants per cultivar). Explants were transferred to fresh medium after 2 weeks in culture and production of embryogenic tissues from leaf lobe explants was scored after 6 weeks in culture.

Treatment 3: Four – six weeks old internodes were placed on MS2 supplemented with 10mg/l BAP to induce bud swelling for 6 days. Axillary buds (AB) were removed with a sterile needle and placed on MS2 supplemented with 12mg/l picloram and cultivated in the dark at 25ºC. Ten Petri dishes per cultivar, per treatment were used with each Petri dish containing ten leaf explants (100 explants per cultivar). Explants were transferred to fresh medium after 2 weeks in culture and production of embryogenic tissues from leaf lobe explants was scored after 6 weeks in culture.

A 0 – 5 range scale system developed by Taylor *et al.* (1996) was used for scoring SE production efficiency where 0 indicates the absence of embryogenic structures and 5 indicates that the entire leaf margin contained embryogenic structures.

3.3.3. Statistical analysis

Analysis of variance (ANOVA) and other statistical analysis were done with GraphPad InStat 3.01 software (GraphPad Softwre, Inc., San Diego, CA).

3.4. RESULTS

The formation of somatic embryos (SE) was observed in several cassava cultivars (Figure 3.1). High efficiency organised embryogenic structures (OES) were successfully achieved from axillary bud and immature leaf explants on MS2 media supplemented with 50µM picloram from cultivars TMS60444, T200, AR9-18, MTAI16, CR25-4 and CM523-7 while BRA1183, MCOL2261 and SM707-17 produced mostly friable callus with some mature friable embryogenic callus (FEC) and very few to no SE. Induction of SE from immature leaf lobes on medium supplemented with 8mg/l 2,4-D was low compared with the other treatments in all cultivars tested. The high efficiency SE-producing cultivars mainly showed mature somatic embryos after 6 weeks co-cultivation on SE-inducing medium.

Cultivars within the same treatment and cultivars in different treatments (1 – 3) were compared to find the most efficient explant and medium for somatic embryogenesis. From Figure 3.2 it can be seen that the explant source as well as the auxin type had varying effects on the production of SE. Cultivars T200, TMS60444, AR9-18, MTAI16, CR25-4 and CM523-7 produced the highest amount of SE (2.5 – 4.0). BRA1183 and SM707-17 produced SE at low efficiencies (< 1.5) while MCOL2261 showed the lowest frequency of SE (< 0.5) with mostly friable embryogenic tissue. No significant differences were found between T200 ILL and AB on MS2 supplemented with 50µM picloram as well as TMS60444 ILL and AB on MS2 supplemented with 50µM picloram. However, there were significant differences ($p < 0.01 – 0.001$) when compared with SE produced from ILL on MS2 supplemented with 8mg/l 2,4-D. In general, SE formation was better with AB except in AR9-18 (Figure 3.2) which showed best production using ILL on MS2 supplemented with 50µM picloram. Table 3.2 shows the explant source and type of media for the most efficient production of SE for the various cultivars.

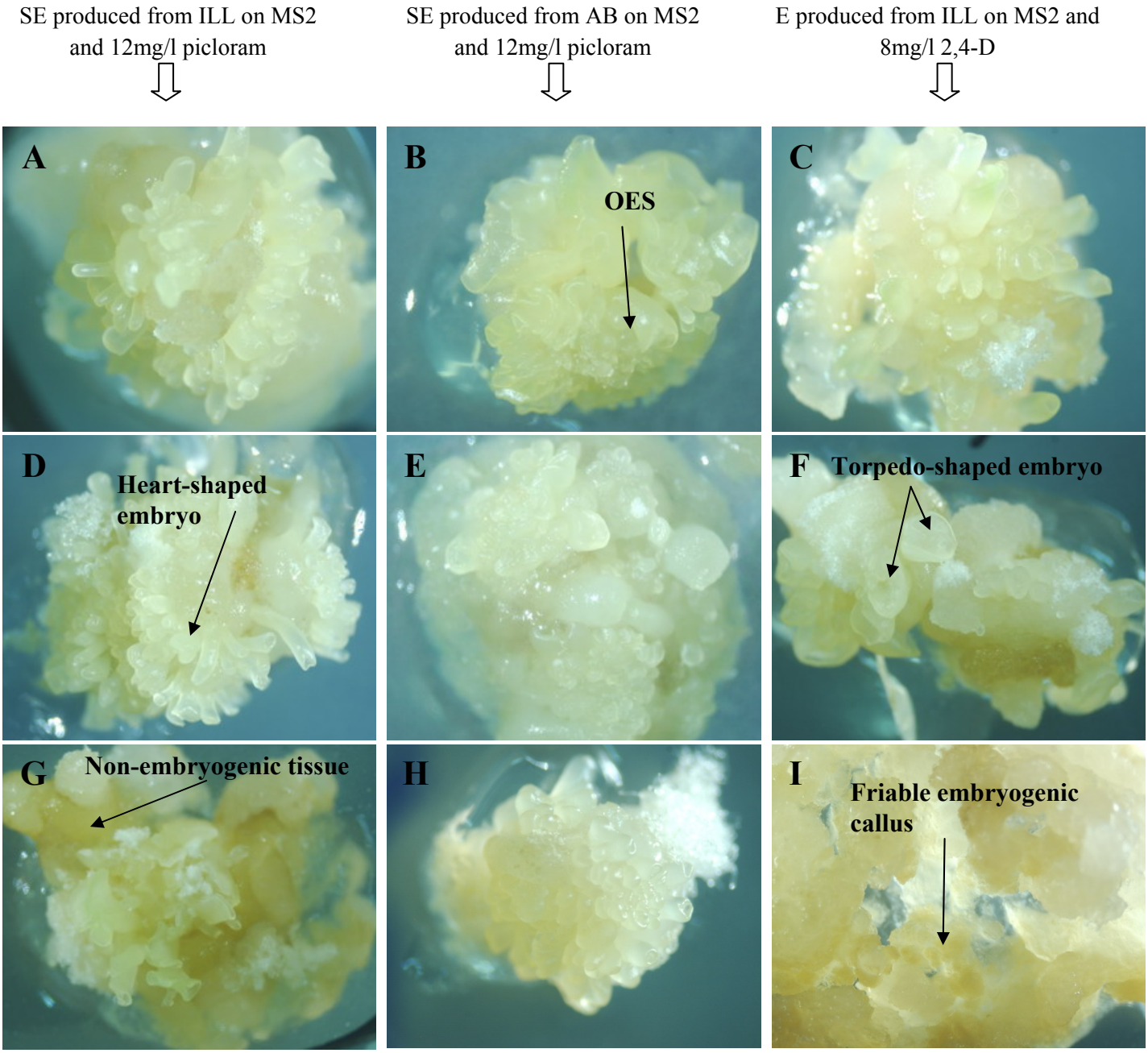


Figure 3.1 Organised embryogenic structures produced by ILL/AB on medium supplemented with 12mg/l picloram and 8mg/l 2,4-D. A – C: T200; D – F: AR9-18; G – I: MCOL2261.

TMS60444, T200, AR9-18, MTAI16, CR25-4 and CM523-7 produced efficient SE thus T200 and AR9-18 were used to illustrate the different stages of somatic embryogenesis. The remaining cultivars produced few SE thus MCOL2261 was used to illustrate this.

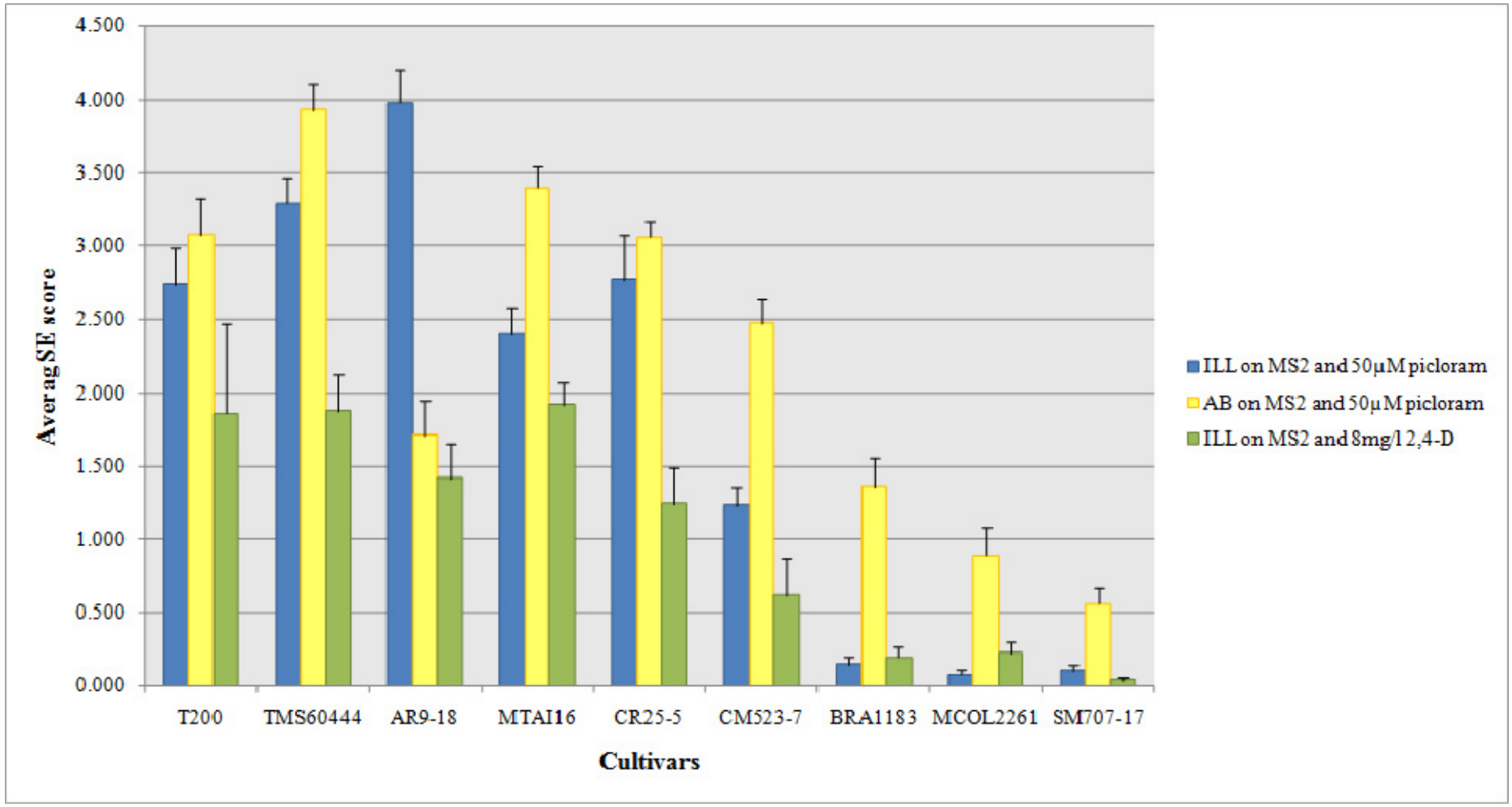


Figure 3.2 Influence of various media and explants on the production of SE in selected cassava cultivars.
Error bars represent the standard error of the mean. A scoring system developed by Taylor *et al.* (1996) was used as described in section 3.3.2.
n = 100.

Table 3.2 Summary of superior explants and media for the production of SE

Cultivar	Source and Traits	Explant	Medium
T200	South Africa. South African landrace and high starch	ILL/AB	MS2 with 50µM picloram
TMS60444	FAO/IITA. Model cultivar	ILL/AB	MS2 with 50µM picloram
AR9-18	CIAT-breeding line. CMD and CGM resistance	ILL	MS2 with 50µM picloram
MTAI16	CIAT/FAO. High starch and wide adaptation	AB	MS2 with 50µM picloram
CR25-4	CIAT-elite variety. CMD resistance	ILL/AB	MS2 with 50µM picloram
CM523-7	CIAT-elite variety. High starch	AB	MS2 with 50µM picloram
BRA1183	Brazil/FAO. High altitude and good adaptation to low temperature	AB	MS2 with 50µM picloram
MCOL2261	CIAT-elite variety. Good adaptation to low temperature	AB	MS2 with 50µM picloram
SM707-17	CIAT-elite variety. Good adaptation to low temperature and high altitude	AB	MS2 with 50µM picloram

3.5. DISCUSSION

In this current study, cassava cultivars with selected useful traits (Table 3.2) were selected from germplasm obtained from CIAT. Somatic embryos were successfully achieved from immature leaf lobes and axillary buds on media supplemented with 50µM picloram. The formation of SE was observed in several cassava cultivars. Some cultivars were evaluated after 3 months and it was shown that MTAI16 and CM523-7 produced more SE with longer cultivations and at later developmental stages (torpedo-shaped embryos). The differences in the amount of SE produced and the type of developmental stages occurring (such as globular or torpedo embryos) may be explained by the genotype-explant

combination. The ability of cassava genotypes to produce somatic embryos is influenced by the explant type (such as ILL or AB) as well as the type of auxin (for example picloram and 2,4-D) and concentration. The results of this study, on the genotypic variation in SE, concur with previous reports of other cassava cultivars from various countries (Atehnkeng *et al*., 2006; Hankoua *et al*., 2005). The most commonly used explants type to induce SE is ILL (Atehnkeng *et al*., 2006; Danso and Ford-Lloyd, 2002; Guohua, 1998; Guohua and Qiusheng, 2002;Hankoua *et al*., 2006; Hankoua *et al*., 2005; Li *et al*., 1998; Raemakers *et al*., 1997; Sofiari *et al*., 1997; Taylor *et al*., 2001; Zhang and Puonti-Kaerlas, 2005), followed by shoot apical meristems (Atehnkeng *et al*., 2006; Hankoua *et al*., 2006; Hankoua *et al*., 2005; Raemakers *et al*., 1997). In this study, various explants and PGRs were tested for the induction of SE. It was shown that AB placed on MS2 supplemented with 50µM picloram induced the highest efficiency (4/5) of SE for most cultivars. For cultivars T200, TMS60444, MTAI16, CR25-4 and CM523-7, somatic embryos could be induced from both explants in medium with picloram. It should be noted that AB had a high trend of SE induction frequency compared with ILL which gave slightly lower frequencies. In contrast, AR9-18 gave the highest SE frequencies from ILL on medium with picloram. All cultivars showed relatively low SE frequencies on medium with 2,4-D compared with picloram-containing medium. SE of TMS60444, T200 and AR-19 SE that had been transferred to MS2 supplemented with 0.1mg/l BAP induced cotyledons from all SE clumps transferred.

Table 3.1 shows the various cultivars and induction media that have been used to test for SE competence in order to produce somatic cotyledons or FEC for transformation experiments. Hankoua *et al*., (2005) observed the ability of African cassava genotypes to form primary somatic embryos using AM and ILL on MS2 supplemented with 50µM picloram and 2µM $CuSO_4$ (Table 3.2). Significant differences were found between cultivars with respect to the type of explants used to initiate SE. SE induced from AM from TME 1, 5, 203, 12 and TMS 91/02324 produced more SE than using AM from TME 127, TMS 91/02327, 30001, 70775 and 93/0665(x3) (Hankoua *et al*., 2005). However, higher SE efficiencies were found in TME 8, 282, 203, 127, 5, 12, TMS 93/0665(x3) and 91/02327 using ILL as the starting material (Hankoua *et al*., 2005). Maturation frequencies of the various cultivars also varied. Genotypes with the highest maturation frequencies include TMS 30001, 70775, 91/02327, 30572, 93/0665(x3) and TME 1 while lowest frequencies were found in TME 203 (Hankoua *et al*., 2005). The ability for these SE to

regenerate shoots was also tested and it was shown that TME 8 and TMS 91/02327 had the highest regeneration potential (Hankoua *et al*., 2005). Similar results (variation of SE efficiency and regeneration in various cultivars) have been reported for cultivars from other countries (Atehnkeng *et al*., 2006; Feitosa *et al*., 2007; Hankoua *et al*., 2005; Saelim *et al*., 2006).

Cultivars MCOL2261 (Figure 3.2) and SM707-17 produced insignificant amounts of SE, however, loose callus and FEC were the predominant tissue type found. FEC was also found in BRA1183, CM523-7 and CR25-4. This is an interesting observation since FEC are normally produced from SE transferred to GD2 supplemented with 50µM picloram and not directly from the initial explant. No previous reports to our knowledge have been found on direct FEC production without an intervening SE phase. This can be explained by the embryogenic propagules being unable to develop beyong the preglobular stage and instead break up into new embryogenic propagules (Raemakers *et al*., 1999)

Somatic embryogenesis is the most commonly used regeneration method for cassava (Fregene and Puonti-Kaerlas, 2002). However, it is restricted to meristematic and embryogenic tissues and is induced by a limited number of explants such as immature leaf lobes and cotyledons. Genotypes that are able to form SE might not be able to regenerate through direct shoot organogenesis and thus it appears that these processes are controlled by different and independently inherited traits. Therefore it is important to evaluate somatic embryogenesis in various cassava cultivars as this is the basis for the production of cotyledons and friable embryogenic callus for transformations and their regeneration abilities. In this study, we demonstrated, for the first time, high SE potential in several cassava cultivars exhibiting important traits that could be exploited for commercial cultivation of cassava in South Africa.

CHAPTER 4. TOBACCO LEAF DISC TRANSFORMATION WITH SACMV 221BP REP TRANSGENE

4.1. INTRODUCTION

Cassava is an important food staple and an industrial crop. However, cassava mosaic disease (CMD) causes considerable crop loss and thus genetic engineering is necessary to confer resistance against cassava viruses (Fregene and Puonti-Kaerlas, 2002). The main driving force for producing transgenic crops that are resistant to viruses is the impact the viral diseases have on crop productivity worldwide. Plant viruses have small genomes which make them suitable targets for engineered resistance. Transgenic plants resistant to viruses have been developed by introducing a viral sequence into the plant genome. This leads to the expression of the introduced viral sequence which interferes with the virus in the plant. This method of genetic engineering is known as pathogen-derived resistance (PDR) (Goldbach *et al*., 2003).

Viruses encode specific genes essential for replication and encapsidation of their genome and these have been proven excellent candidates for developing host resistance based on PDR. Post-transcriptional gene silencing (PTGS) has been achieved by using an antisense strategy although hairpin construct with introns have been shown to have a greater effect on silencing (90 – 100%) in infected transgenic plants (Wesley *et al*., 2001). This technology is based on blocking gene expression via PTGS. Antisense technology has been applied to many crop plants to modify various traits such as flower colour, fruit ripening, producing longer last flowers and generating male sterility for hybrid seed production (Bhalla and Singh, 2004).

One approach to engineer cassava resistance to SACMV is by using the SACMV *Rep* gene since Rep is the only viral protein required for replication. Virus-resistant crops may be developed by introducing either the viral coat protein (CP) or sequences encoding the *replicase* gene (Dasgupta *et al*., 2003). Replicase-mediated resistance has been shown to be due to PTGS which is an inherent plant response. PTGS is responsible for the degradation of nucleic acids in a sequence-specific manner including those of viruses and thus this strategy may be very effective in genetic engineering of virus resistance

(Dasgupta *et al*., 2003). Geminiviruses (ssDNA) replicate in the nucleus through a rolling-circle mechanism that generates dsDNA intermediates which are templates for replication and transcription; transcription being bidirectional and the presence of complementary RNA strands provides a source of dsRNA (Voinnet, 2005). In PTGS the elicitor dsRNA, which is produced during viral infection, is degraded to small interfering RNA (siRNA). A complex of cellular factors, such as RNA-dependant RNA polymerase, along with siRNA degrade RNA molecules bearing homology with the elicitor RNA and the degradation spreads within the entire organism (Dasgupta *et al*., 2003). This process has evolved as a plant defense mechanism against invading viruses with RNA or DNA genomes. ACMV-resistant transgenic cassava plants have been successfully produced by expressing ACMV genes of *Rep*, *TrAP* and *REn* in an antisense orientation (Vanderschuren *et al*., 2007). A PTGS-based strategy to control virus replication was shown when plant cells that were infected with ACMV and with a synthetic siRNA designed to target the AC1 gene of the virus showed a decrease in AC1 mRNA levels by more than 90% and viral DNA by 70% (Vanitharani *et al*., 2005).

The essential functions of *Rep* make it an ideal target for obtaining virus resistance by suppressing its expression through transgenic RNA silencing (Bendahmane and Gronenborn., 1997). It has been shown that the resistance generated by using *Rep* sequences is tight thereby allowing transgenic plants to resist high dosages of input virus (Dasgupta *et al*., 2003). However, the level of resistance is dependent upon stable and high level of expression of the transgene while the level of gene expression relies on the type of construct, site of insertion into the plant genome and the number of genes inserted (Fauquet *et al*., 1992).

Cassava remains a difficult crop to transform and regenerate, thus *Nicotiana benthamiana* was used in this study as a model crop to test an antisense SACMV *Rep* construct, due to its ease of transformation and regeneration and because it is a host for cassava viruses (Fregene and Puonti-Kaerlas, 2002; Goodwin *et al*., 2008).

In this study, efficiency of a 221bp SACMV AC1 *Rep* transgene was evaluated for induction of resistance potential against SACMV infection in tobacco plants.

4.2 GENERAL OBJECTIVES

The objective of this study was to produce transgenic tobacco (*Nicotiana benthamiana*) plants with resistance against SACMV.

Specific aims

The specific aims of this research were to:

- Transform tobacco leaf discs with a virulent strain of *Agrobacterium* (Agl1) containing the SACMV AC1 *Rep* transgene
- Direct regeneration of tobacco leaf discs
- Test for transformation success: explant selection on hygromycin-containing media, GUS expression assays and PCR.
- Challenge acclimatised transgenic and non-transgenic plants with SACMV and screen for resistance against the virus

4.3. MATERIALS AND METHODS

4.3.1. Plant material

In vitro tobacco (*Nicotiana benthamiana*) plants were maintained as shoot cultures on MS (Murashige and Skoog) medium supplemented with 20g/l sucrose (MS2), solidified with 7.8g/l agar, pH 5.8, at 25ºC under a 16 h photoperiod (3000 Lux). Explants were subcultured every 4 – 6 weeks.

4.3.2. Transformation of tobacco leaf discs, regeneration and acclimatisation

In vitro N.benthamiana plants were used for transformation.

Preparation of *Agrobacterium* suspension for transformation: Transformed *Agrobacterium* strain Agl1 (Chapter 2) was grown in 30ml LB containing 100mg/l kanamycin overnight

at 200 rpm on a rotary shaker at 30ºC. Two ml of the overnight culture was transferred to 30ml LB with 100mg/l kanamycin and 200µM acetosyringone, and incubated at 200 rpm on a rotary shaker at 30ºC until the cultures reached an OD_{600} of 0.8 – 1.0. The cultures were centrifuged at 3000g for 15 minutes at 4ºC. The pellet was resuspended in an equal volume of liquid MS2 supplemented with 200µM acetosyringone.

Leaves from young *in vitro* plants were cut aseptically into 1 cm^2 strips and placed into the bacterial cell suspension followed by vacuum infiltration at 85 kPa for 10 – 15 minutes. Excess Agl1 was removed and explants were co-cultivated for 3 days at 25ºC under a 16h photoperiod. After the bacterial-explant tissue co-cultivation, explants were washed with MS2 supplemented with 500mg/l cefotaxime for 4 – 5 hours at ~ 100 rpm under continuous light. The medium was removed and explants were rinsed with MS2 supplemented with 500mg/l cefotaxime, blotted dry onto sterile filter paper and transferred to MS2 supplemented with 500mg/l cefotaxime, 30mg/l hygromycin, 1.0mg/l benzylaminopurine (BAP) and 0.1mg/l α-naphthaleneacetic acid (NAA) for selection. Emerging shoots were transferred into bottles containing MS2 supplemented with 500mg/l cefotaxime. Two ml eppendorf tubes containing sterilised potassium permanganate (7.43 mg/ml) mixed with perlite were added to the bottles to remove ethylene produced. Shoots with well developed roots were transferred into soil for hardening off. Plantlets were placed in containers covered with cling wrap which was uncovered gradually until plants were well acclimatised into the growth chamber conditions (25 - 28ºC, 16h/8h photoperiod).

4.3.3. DNA isolation from putative transgenic plants using the CTAB (cetyltrimethylammonium bromide) method

As per method described in 2.3.8.

4.3.4 GUS assays

As per method described in 2.3.9.

4.3.5. Polymerase chain reaction (PCR) analysis

DNA samples from putative transgenic plants isolated using the CTAB method were used for PCR analysis. A region of the GUS Plus gene was amplified using GUS Plus primers as described in section 2.3.10 and Rep F 5' ATC GGA GGC TCC TGA AAA AT 3' and Rep R 5' GAC AAG GAC GGA GAC ACC AT 3' to yield a 221bp fragment.

A standard PCR reaction as described in section 2.3.10 was used. The appropriate cycling took place in MyCycler PCR machine (BioRad) in which a fragment of the β-glucuronidase gene was amplified by incubation at 94ºC for 2 minutes, followed by 30 cycles of 30s at 94ºC, 30s at 59ºC for Rep and 55ºC for GUS, and 30s at 72ºC, and a final elongation at 72ºC for 10 minutes.

4.3.6. Challenge of transgenic plants with SACMV, quantification of viral load using Real-Time quantitative PCR and scoring disease symptoms

Transgenic (T_0) and healthy (non-transgenic control) tobacco plants were agro-inoculated with full-length head-to-tail dimers of DNA-A and DNA-B components (pBINSA A and pBINSA B constructed and supplied by L. Berrie) of SACMV using an adapted method of Hayes *et al* (1988). One millilitre aliquots of log-phase (OD_{600} 0.6) recombinant *A.tumefaciens* strain C58C1 containing each of the genomic clones was pelleted at 8000 rpm, washed in sterile distilled water and resuspended in 200µl of LB. Equal volumes of each culture was mixed and used to inoculate plants at the 6 – 8 leaf stage.

A total of 60µl of recombinant C58C1 per plant was injected at 3 – 4 positions along the stem using a 1ml syringe with 0.1mm needle. Untransformed control seedlings were inoculated with DNA-A and B and the vector pBIN only. Transgenic plants were inoculated with DNA-A and B. Plants were incubated in a greenhouse at 25 – 28ºC with a 16h/8h photoperiod and monitored for symptom development.

A standard curve was generated using known 1:10 serial dilution concentrations of SACMV DNA-A cloned into plasmid pBluescript. The concentrations ranged from 100ng

to 0.1pg. The standard curve constructed represents a linear relationship between the cycle threshold (the time at which fluorescent intensity is greater than background fluorescence.) and the initial concentration of DNA added. The copy number (molecules/ng) present for each concentration was calculated using Avagadro's constant. The equation incorporating Avogadro's constant [Xg/µl DNA/(plasmid length +insert length in basepairs x 660) x Avagadro's constant x 10^{-12}] was used for each 10-fold dilution which was calculated and found to range from $1.54x10^8$ molecules/ng to $1.54x10^4$ molecules/ng.

Viral titer was quantified using the TNA extracted from 21dpi and 35dpi emerging leaves and source leaves (leaves below emerging leaves) as well as 100pg standard of DNA-A in pBluescript on the LightCycler (Roche Applied Science). Quantitative PCR was carried out using the LightCycler ® FastStart DNA Master SYBR Green I kit (Roche Applied Science). 20µl LightCycler PCR master mix was prepared according to manufacturer's instructions to contain an optimal concentration of $MgCl_2$, forward and reverse primers, 2µl of LightCycler FastStart DNA Master SYBR Green I and 2ul (200ng TNA for unknown and 200pg for standard DNA-A) of sample DNA. Primers designed to amplify a 153bp fragment of the SACMV core coat protein (CCP) were used in Real-Time PCR reactions. Primer sequences were as follows: CCP F (forward primer) -5' GCACAAACAAGCGTCGAT 3' and CCP R (Reverse primer) -5' CTGCCAGTATGCTTAACGTCA 3'. Cycling conditions of an initial activation cycle of 95ºC for 10min, followed by 30 cycles of denaturation at 95ºC for 5 sec, annealing at 60ºC for 10 sec and elongation at 72ºC for 10 sec. The intensity of fluorescent emission of the SYBR Green dye present in each LightCycler capillary was measured at 520nm at the end of each elongation phase.

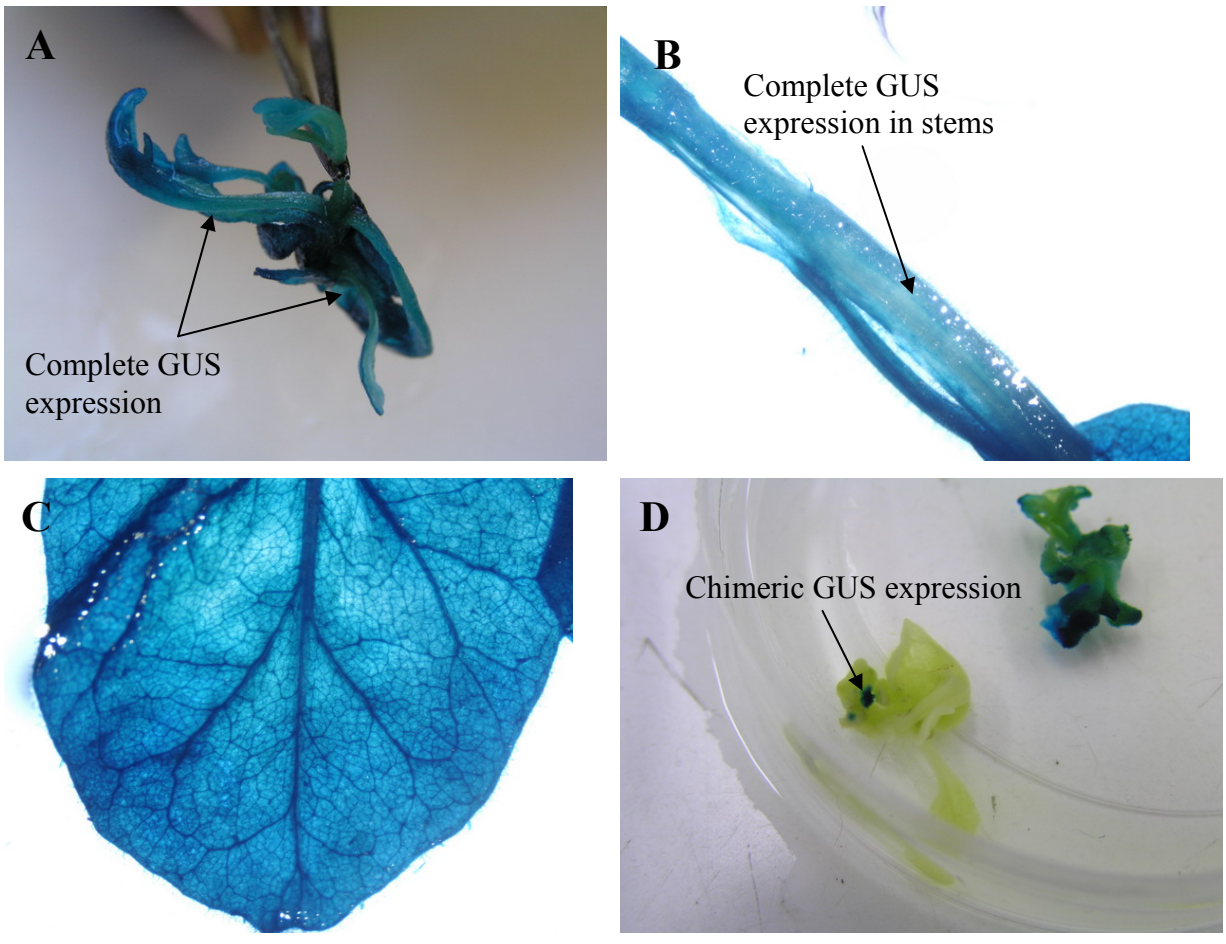
Symptoms caused by SACMV were recorded at 21 days post inoculation and plants were scored as follow: 0 = no symptoms; 1 = local chlorotic spots and/or mild leaf curling of young systemic leaves; 2 = severe leaf curling, only on young systemic leaves; 3 = curling of various systemic leaves (different level of severity could be observed); 4 = large chlorotic spots on infected and systemic leaves, curling is severe in all symptomatic leaves; 5 = typical mosaic symptom (Sangaré *et al*., 1999).

4.3.7. Statistical analysis

Analysis of variance (ANOVA) and other statistical analysis were done with GraphPad InStat 3.01 software (GraphPad Softwre, Inc., San Diego, CA).

4.4. RESULTS

N.benthamiana was successfully transformed as shown by GUS expression of emerging shoots and regenerating plant leaves. Different levels of expression were observed *in vitro*: some explants showed complete transgene integration (Figure 4.1 A, B and C), and only a few showed chimeric expression (Figure 4.1 D and E) or no expression (Figure 4.1 F). Chimeric expression in all 5 lines was observed *ex vitro* 21 days post inoculation (Figure 4.1 G and H). PCR amplification with GUS Plus primers and 221bp Rep primers indicated that plants showing GUS expression did indeed contain the transgene (Figures 4.2 and 4.3).



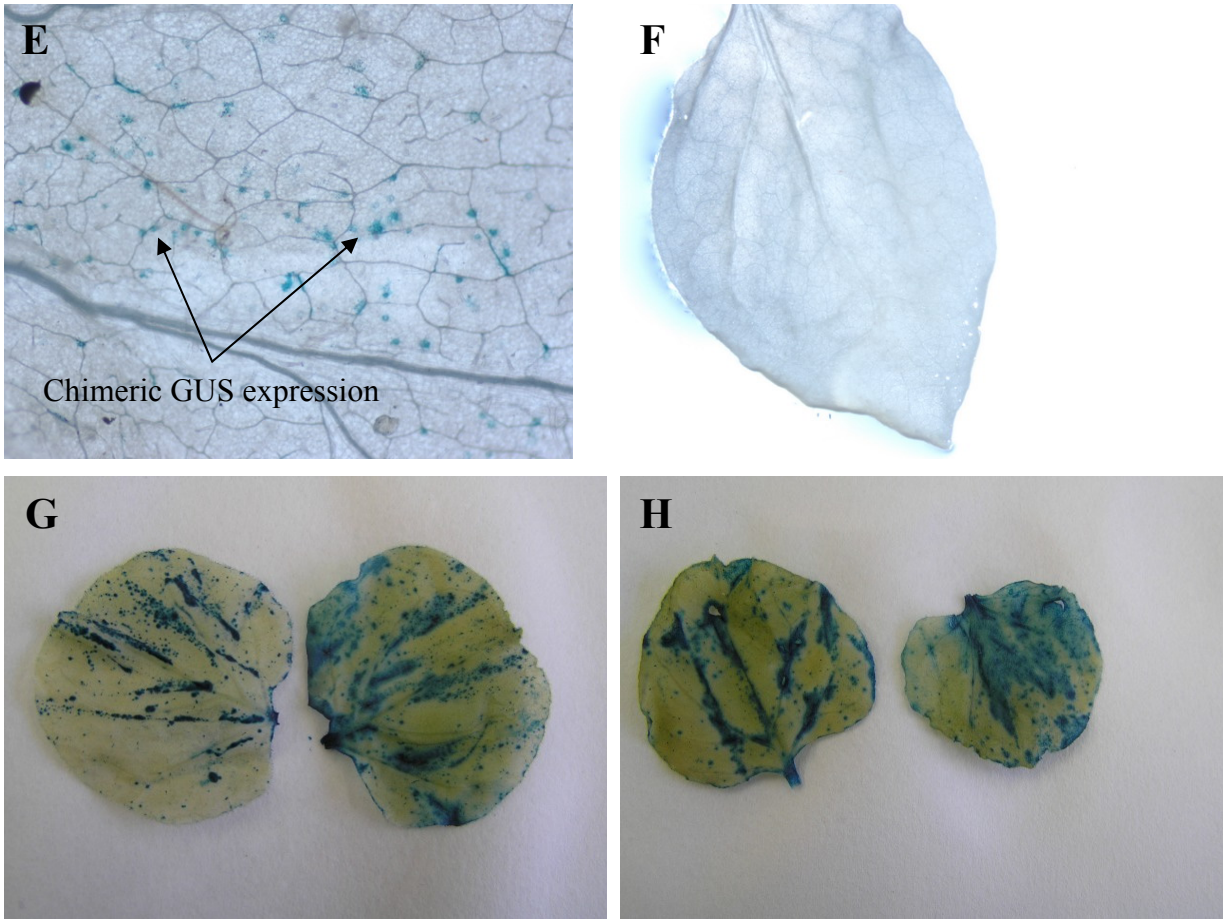


Figure 4.1 GUS assays of *in vitro* emerging shoots and regenerated plant leaves from tobacco leaf disc transformation (A – F) and from acclimatised greenhouse (*ex vitro*) transgenic lines infected with SACMV 21 days post inoculation (G and H).

A, B and C: transgenic plants showing complete GUS expression; D, E and F: chimeric GUS expression; G and H: acclimatised infected transgenic lines showing chimeric GUS expression.

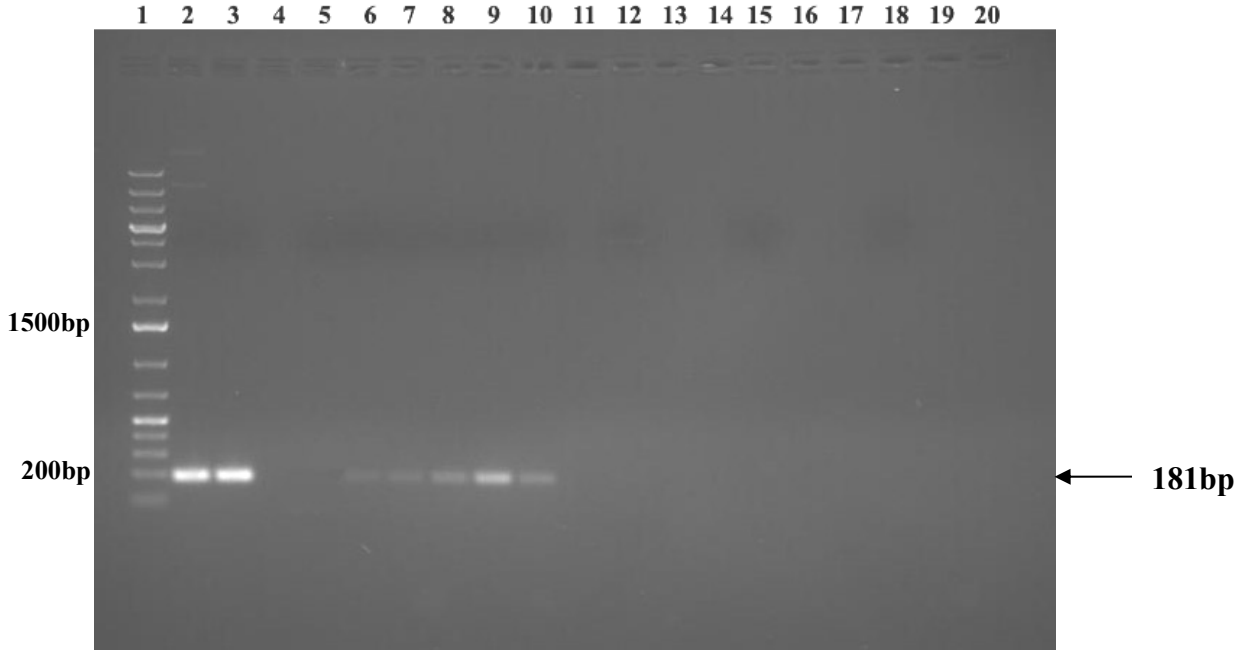


Figure 4.2 PCR with 181bp GUS Plus primers.
Lane 1: MWM, 2: pCAMBIA 1305.1, 3: pCAMBIA 1305.1 containing Rep transgene; 6 – 10: 5 transgenic tobacco lines; 12: DNA – A; 15: untransformed tobacco control; 18: no template control.

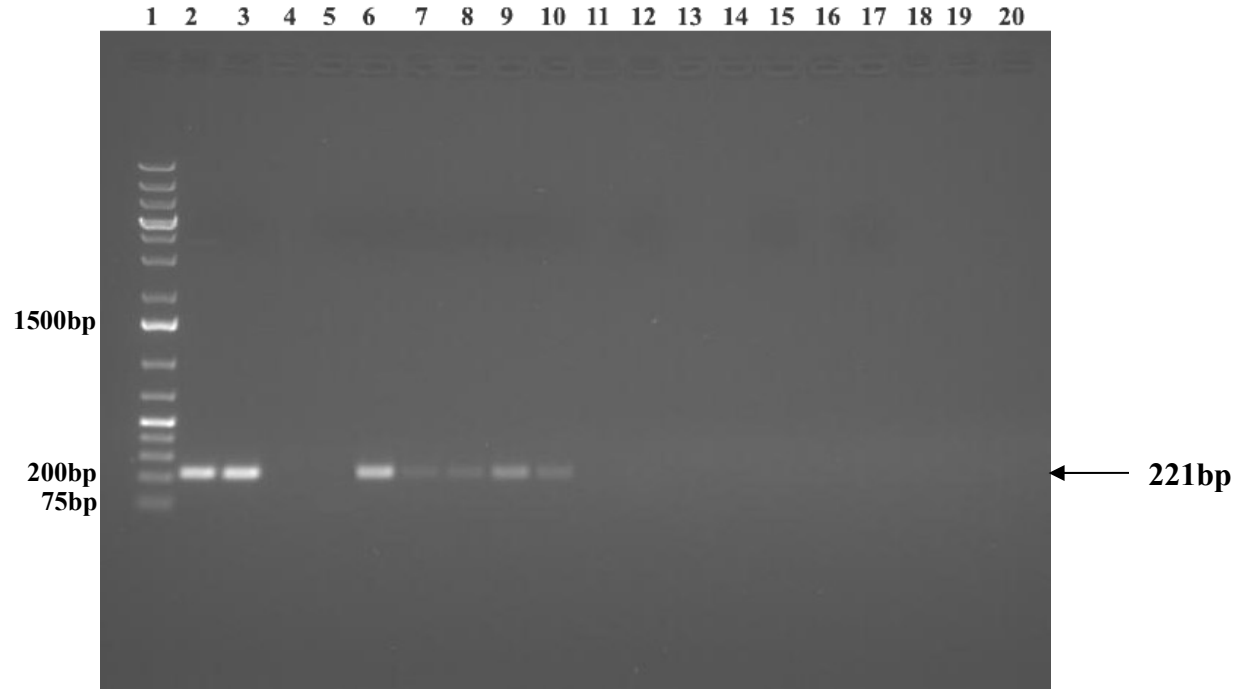


Figure 4.3 PCR with SACMV AC1 primers.
Lane 1: MWM, 2: DNA-A, 3: pCAMBIA 1305.1 containing Rep transgene; 6 – 10: 5 transgenic tobacco lines; 12: pCAMBIA 1305.1; 15: untransformed tobacco control; 17: no template control.

Symptom scoring and viral quantification was carried out at 21 and 35 dpi. At 21dpi only 2 of the 6 untransformed SACMV-inoculated tobacco control plants were showing severe symptoms and at 35dpi, the 2 infected plants were severely stunted (Table 4.1). Four of the transgenic lines showed mild symptom development at 21dpi, however, symptoms were observed in few of the plants in each line (Table 4.1). Transgenic plants showed increased symptom development at 35dpi, with most plants in each of the 5 lines showing infection, but not as severe as the untransformed plants.

Real-time PCR of SACMV DNA from total nucleic acid (TNA) from untransformed infected tobacco versus transgenic infected lines 21 days post inoculation (21 dpi) showed that 4 of the 5 lines were able to reduce viral concentration significantly ($p < 0.001$) (Figure 4.4). Viral titer increased significantly in 4 of the 5 transgenic lines at 35dpi (Figure 4.4). Fold decrease in viral titer was up to 7.18 at 21dpi followed by an increase at 35dpi although fold decrease was 2.12 compared with control plants (Table 4.2).

Table 4.1 Symptom scoring of transgenic and untransformed control tobacco plants at 21 and 35 days post-inoculation

Tobacco line	21 dpi		35 dpi	
	# plants with symptoms		# plants with symptoms	
Untransformed, uninfected	0		0	

Untransformed , infected with SACMV DNA-A and B	2		2	
Transgenic line 1 infected with SACMV DNA-A and B	0		2	

Transgenic line 2 infected with SACMV DNA-A and B	2		3	
Transgenic line 3 infected with SACMV DNA-A and B	0		4	

Transgenic line 4 infected with SACMV DNA-A and B	1		3	
Transgenic line 5 infected with SACMV DNA-A and B	1		3	

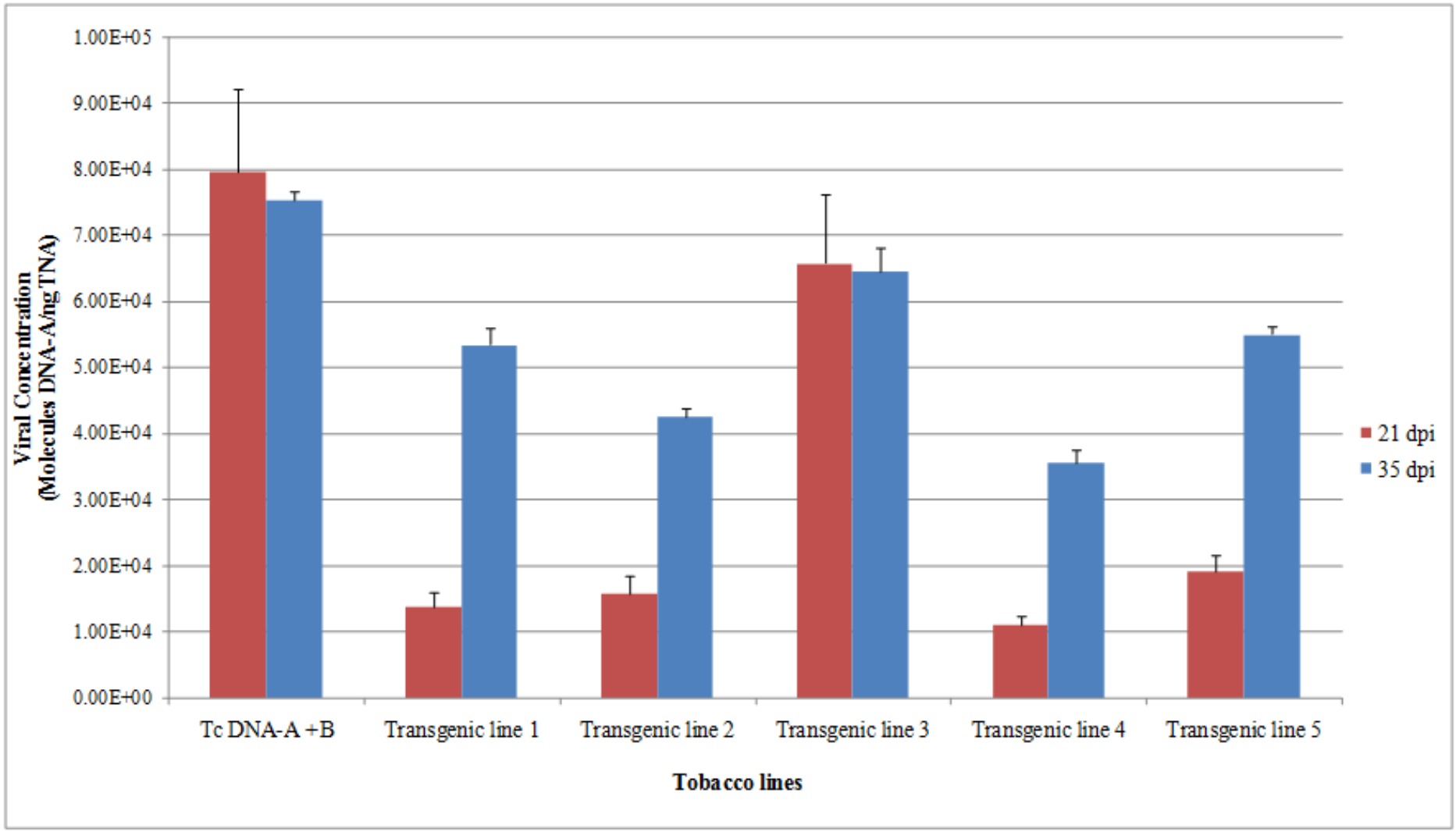


Figure 4.4 Viral concentration from 21 and 35 dpi infected plants calculated from Real-time PCR.
Error bars represent the standard error on the mean. n = 6.

Table 4.2 Characteristics of plants 21 and 35 dpi with respect to transgene presence, GUS expression, viral concentration, symptom severity and fold decrease in virus of transgenic versus non-transgenic plants

Line	Transgene (Fig. 4.2 and 4.3)	GUS expression *in vitro*	GUS expression 21 dpi *ex vivo*	Average symptom severity (scale 0 – 5)	Mean viral DNA 21 dpi (molecules DNA-A/ng TNA)	Fold decrease at 21 dpi	Mean viral DNA 35 dpi (molecules DNA-A/ng TNA)	Fold decrease at 35 dpi
Untransformed, uninfected	-	-	-	-	0	0	0	0
Untransformed, infected with SACMV DNA-A and B	-	-	-	1.3	7.97E+04	0	7.55E+04	0
Transgenic line 1	+	Chimeric	Chimeric	0.75	1.37E+04	5.82	5.35E+04	1.41
Transgenic line 2	+	+	Chimeric	1.17	1.58E+04	5.04	4.26E+04	1.77
Transgenic line 3	+	+	Chimeric	1.5	6.59E+04	1.21	6.47E+04	1.17
Transgenic line 4	+	+	Chimeric	1.17	1.11E+04	7.18	3.56E+04	2.12
Transgenic line 5	+	+	Chimeric	1.6	1.91E+04	4.17	5.50E+04	1.37

4.5. DISCUSSION

Cassava is devastatingly affected by CMD. Genetic engineering with various viral constructs has been demonstrated to control plant viral diseases efficiently.

In this study, a *Rep* antisense region of SACMV has shown to decrease viral concentration by up to 7.18 fold 21dpi (Table 4.2) in transgenic tobacco plants. The type and extent of protection by a transgene is variable depending on several factors including the type of virus and integration events in different transgenic lines. Variation can occur among different plants derived from the same line. Results from this study demonstrated that transgenic plants 21dpi with SACMV showed fewer viral symptoms than control plants, spread of infection was delayed and reduced, and reduced virus titer was seen at 21dpi but increased at 35dpi (Figure 4.4 and Table 4.2) although less than in control plants.

Only mild symptoms were found in two of the six transgenic lines 21 days post inoculation with SACMV (Table 4.1). This variation could be due to the number of transgenes present .If multiple copies of the transgene had integrated into the plant genome there could have been ectopic pairing of homologous DNA leading to transcriptional or post-transcriptional gene silencing. It could also be due to the position of the transgene in the plant genome – the transgene may have integrated in a domain lacking features of the DNA or chromatin that are essential for high expression levels (Angell and Baulcombe, 1997). Symptoms were observed in most plants in each line at 35dpi thus infection efficiency of transgenic plants was higher than that of control plants. However, since samples of each line and the control were pooled, an average of viral load was determined and was found to be higher in untransformed plants (Figure 4.4). Therefore, had viral infection efficiency increased in the control plants, a much lower viral titer would have been found compared with control plants. Generally, there is a correlation between symptom appearance and viral replication, as shown by comparing symptom development at 21 and 35dpi and viral titer at these 2 time points.

The low level of resistance in one transgenic line (Table 4.2) may indicate that transgene expression in plants is problematic in terms of transgene copy number, and there might be a selection for cells that do not express the transgene or do so at low levels (Noris *et al*.,

1996). GUS expression also varied from *in vitro* plants to *ex vitro* (acclimatised) plants. Variation depends on the developmental and physiological state of the tissue, as well as the growth environment (Serres *et al*., 1997). Similar findings have been observed in transgenic cranberries. Differences in GUS expression in transgenic cranberry were found to occur between different plant tissues, within a plant tissue, over time and may also be influenced by interfering secondary plant compounds (Serres *et al*., 1997). Acclimatised transgenic cranberry plants containing *gusA* showed more GUS expression in younger leaves than older ones from the same plant. When environmental conditions were varied (*in vitro*, greenhouse or field), different GUS activity was observed and expression decreased as conditions changed to those of outdoors (Serres *et al*., 1997). Serres *et al*., (1997) found that inhibition of endogenous GUS activity by phenolic compounds was counteracted by the addition of polyvinylpolypyrolidone (PVPP) since PVPP absorbed the phenolic compounds.

Antisense strategies for viral resistance interfere with viral infection by either inhibiting gene expression or interfering with viral replication (Grumet, 1994). This antisense strategy has been tested in several systems with varying results. Antisense versus sense sequences of the CP genes of *Cucumber mosaic virus* (CMV), *Potato virus* X (PVX), *Tobacco mosaic virus* (TMV) and *Zucchini yellow mosaic virus* (ZYMV) were compared in transgenic plants (Grumet, 1994). It was found that plants expressing the sense CP genes yielded higher resistance levels than antisense genes. Others found that antisense or sense genes were equally effective at conferring resistance against *Potato leaf roll virus* (PLRV) in transgenic potato plants (Grumet, 1994). An antisense sequence of the replicase gene of *Tomato golden mosaic virus* (TGMV) (AL1 gene necessary for replication) was shown to significantly decrease expression of TGMV.

Rep-mediated resistance against TMV was first reported in tobacco plants containing a 54kDa putative *Rep* gene and this type of resistance has also been demonstrated against other viruses (Dasgupta *et al*., 2003). This type of resistance demonstrated higher levels of protection against TMV than coat protein-mediated resistance (Scholthof *et al*., 1993). Full-length, truncated and mutated *Rep* gene constructs have been used for resistance. *Nicotiana benthamiana* plants transformed with a truncated C1 region of the monopartite

Tomato yellow leaf curl Sardinia virus (C1 also known as AC1 in bipartite geminiviruses) conferred strong and specific inhibition of viral replication (Brunetti *et al*., 2001).

Transgenic tomato plants expressing a 1086bp region of the *Rep* gene of *Tomato leaf curl virus* in an antisense orientation was shown to confer high levels of resistance and inheritability up to second generation plants when challenged with the virus (Praveen *et al*., 2005). Of the 6 lines tested, 2 contained a single transgene insert, 2 contained double inserts and 2 contained triple inserts. Northern analysis revealed nearly equal transcript level in all 6 lines. All first generation lines showed 50 – 77% resistance when challenged with the virus; plants containing a single insert showed 70 – 77% resistance while those with multiple inserts showed 50 – 57% resistance (Praveen *et al*., 2005). This variation in copy number reported in tomato could explain the variable resistance levels between lines in this study. However, the DIG SB method was unsuccessful and thus copy number could not be determined. Detection of copy number in transgenics can be problematic, and the use of radioactive-labeled probes may be more sensitive than DIG. Variability in transgene expression and virus resistance levels requires that many lines need to be evaluated to select for stable integration and virus control.

Furthermore, a PTGS-based strategy to control viral replication was shown when cassava cells were simultaneously infected with *African cassava mosaic viru*s (ACMV) and a synthetic siRNA designed to target the AC1 gene of the virus showed a decrease in the AC1 mRNA accumulation levels by more than 90% and viral DNA by 70% (Vanitharani *et al*., 2005). This strategy is highly promising for developing viral resistance and can be used as an alternative to antisense or sense RNA which can also induce silencing.

To obtain an ideal transformant, the transgenic plant would contain a single copy gene that would segregate as a mendelian trait, with uniform expression from one generation to the next. This has been reported by Oğraş and Gözükirmizi (1999) where the marker gene integration into the first and second generation seedlings derived from transgenic tobacco plants were analysed. It was shown that progenies inherited the transgene in a 3:1 Mendelian ratio and exhibited normal Mendelian segregation although, Mendelian and non-Mendelian transgene segregation have been reported (Oğraş and Gözükirmizi, 1999).

Currently T1 from self-pollinated seeds of the lines in this study are being tested for stability and inheritability of the transgene and conference of resistance against SACMV.

CHAPTER 5. GENERAL CONCLUSIONS

Cassava is an important food staple and economic crop. However, losses are high due to susceptibility of the crop to cassava mosaic disease therefore genetic engineering is a necessity to improve crop yield and quality. In this study, it has been shown that axillary bud transformation and direct regeneration thereof is not feasible. Thus somatic embryo-derived cotyledons and friable embryogenic callus remain the explant of choice for transformation. It is therefore imperative to determine the ability of individual cultivar genotypes to produce somatic embryos, cotyledons and friable embryogenic callus and their ability to be regenerated and transformed. It has been shown that various genotypes respond differently in the induction of somatic embryos from different explant sources and to the type of auxin used (Hankoua *et al*., 2005; Atehnkeng *et al*., 2006). In this study, cultivars that were efficient at producing somatic embryos included TMS60444, T200, AR9-18, MTAI16, CR25-4 and CM523-7 when using either axillary buds or immature leaf lobes on MS2 media supplemented with 50µM picloram. Cultivars that responded poorly to forming somatic embryos included BRA1183, SM707-17 and MCOL2261 and consequently these cultivars might not be considered suitable for genetic modification. Further studies are continuing in our laboratory to optimise cassava transformation by improving transformation and regeneration of cotyledons and friable embryogenic callus of selected elite cultivars, which have been shown in this study, which produced efficient SE. Given the importance of SE in the production of planting materials and transformation, it would be beneficial to evaluate as many African genotypes for their competency to produce SE and to apply current successful transformation systems in our lab to produce transgenic plants with valuable traits.

It has also been shown in this study that transgenic tobacco expressing an antisense 221bp *Rep* gene from SACMV AC1 confers resistance to this virus at varying levels in different lines. Transgenic lines showed up to a 7 fold decrease in viral concentration at 21 dpi. Antisense strategies have been successfully used to obtain transgenic cassava plants (Fregene and Puonti-Kaerlas, 2002). The antisense strategy was not efficient enough to inhibit viral replication perhaps due to the chosen gene region and thus various SACMV constructs are currently being developed in our laboratory to increase silencing efficiency

against SACMV by using hairpin constructs and multiple gene regions as theses are more promising at increasing silencing efficiency.

CHAPTER 6. REFERENCES

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