# THE DEVELOPMENT OF SACMV - RESISTANT CASSAVA USING PATHOGEN - DERIVED RESISTANCE STRATEGIES, TARGETING THE DNA - A COMPONENT

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## Abstract

*South African cassava mosaic virus* (SACVM) is a cassava mosaic disease- causing virus, belonging to the genus *Begomovirus* of the *Geminiviridae* family. It is a bipartite geminivirus, having two circular single-stranded DNA components, designated DNA-A and DNA-B. The AC1 open reading frame on the DNA-A component encodes for the Rep protein, which is absolutely required for virus replication. Plant virus silencing strategies exploit the use of the viral-derived sequences to confer resistance towards that particular virus, a concept termed pathogen derived resistance (PDR). PDR resistance strategies used to engineer resistance towards geminiviruses include the expression of viral proteins, the use of naturally occurring defective interfering molecules which delay and attenuate infection symptoms, the sequence specific degradation of viral transcripts in the host cytoplasm by inducing RNA silencing with sense, antisense or invert-repeat transgenes and the use of double-stranded RNA molecules to direct methylation of virus promoter sequences. In this study SACMV AC1-derived sequences in sense and invert-repeat transgene constructs were used to induce RNA silencing against SACMV infection in *Nicotiana benthamiana*. The replication of SACMV DNA-A was reduced by almost 100% in two *N. benthamiana* lines expressing untranslatable SACMV AC1 sense transcripts compared to antisense and control lines. Two size classes of transgene derived siRNAs were isolated from unchallenged leaf tissue; one size class being unique, amongst the sense lines, to the replication knockdown lines. Similarly, a sense arm sequence-modified invert-repeat construct resulted in almost 100% SACMV replication reduction in six transgenic lines. This construct was produced using a technique for the production of stable RNA silencing constructs. Unmodified, spliceable intron invert-repeat transgene constructs

transcribed to produce hairpin RNA molecules of 572-nt and 193-nt arm length, resulted in SACMV replication knockdown of 60 and 80% respectively. Towards the production of SACMV resistant cassava cultivars, TMS60444 FEC and MTAI 16 cotyledons were transformed with an invert-repeat RNA silencing construct, however regeneration of transformed plants failed. Cassava transformation and regeneration is inherently difficult and it has been proposed here that a minimal cassette technology-based method can be applied to achieve transformation of cassava with the RNA silencing constructs that showed significant SAMCV silencing in this study.

## Declaration

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

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Sarah Helen Taylor

_____ day of _____ 2009

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| | |
|---|---|
| **AC1** | ORF that encodes the replication associated protein (Rep) |
| **ACMV** | *African cassava mosaic virus* |
| **AS** | antisense |
| **BAP** | 6-Benzylaminopurine |
| **bp** | base pair |
| **CIAP** | Calf Intestinal Alkaline Phosphatase |
| **CTAB** | Cetyltrimethylammonium bromide |
| **DNA** | deoxyribonucleic acid |
| **dpi** | days post inoculation |
| **dsRNA** | double-stranded RNA |
| **EACMCV** | *East African cassava mosaic Cameroon virus* |
| **EDTA** | ethylene diamine tetra-acetate |
| **FEC** | friable embryogenic callus |
| **GD** | Gresshoff and Doy |
| **GUS** | β-glucuronidase |
| **hp** | hairpin |
| **hpRNA** | hairpin RNA |
| **IBA** | Indole-3-butyric acid |
| **IR** | invert repeat |
| **LB** | Luria broth |
| **l-hp** | long-hairpin |
| **MCS** | multiple cloning site |
| **MCT** | minimal cassette technology |
| **MS medium** | Murashige and Skoog medium |
| **MWM** | molecular weight marker |
| **NAA** | α-naphthaleneacetic acid |
| **nt** | nucleotide |
| **OES** | organised embryonic structures |
| **ORF** | open reading frame |
| **pBS** | pBluescript |
| **PCR** | polymerase chain reaction |
| **PDR** | pathogen derived resistance |
| **PTGS** | post-transcriptional gene silencing |

| | |
|---|---|
| **RDR/RdRP** | RNA-dependent RNA polymerase |
| **Rep** | replication associated protein |
| **RNA** | ribonucleic acid |
| **ssDNA** | single-stranded DNA |
| **ssRNA** | single-stranded RNA |
| **S** | sense |
| **siRNA** | small interfering RNA |
| **SACMV** | *South African cassava mosaic virus* |
| **U** | unit |
| **X-gluc** | 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid cyclohexylammonium salt |

# CHAPTER 1 - Literature review

## 1.1 THE CROP

### 1.1.1 Cassava

Cassava (*Manihot esculenta* Crantz; family Euphorbiaceae) is a perennial woody shrub, native to South America. It is grown in more than eighty countries between the latitudes 30°N and 30°S where the annual rainfall is greater than 750 millimetres and the mean annual temperature is greater than 18° to 20°C (Anonymous, 2008a; Puonti-Kaerlas, 1998). Cassava is tolerant to drought and low-nutrient soils and is grown in areas not normally conducive to crop production (IITA, 2008).

Cassava provides food for over 500 million people, especially in areas prone to drought and famine and in times of civil unrest. It is grown for its enlarged starchy tuberous roots, which contain a starch content of up to 85% dry weight. This amount of starch is reported to be close to the maximum theoretical concentration of starch on a dry weight basis among food crops (Anonymous, 2008a). The leaves, utilised as a vegetable, are rich in proteins, vitamins and minerals (Babaleye, 2008). Other uses of the crop include animal feed, flour and high-quality starch for industrial purposes (Anonymous, 2008a).

Two downfalls of cassava are the presence of cyanogenic glucosides and the short shelf-life of harvested roots. Extensive processing is required to reduce the cyanide content before consumption. Harvesting of roots can be delayed until required for up to thirty-six months, however, once harvested they have a shelf-life of two days (IITA, 2008; Hillocks, 2002). Cassava plantations are prone to

pests and disease including cassava green mite, cassava mealybug, cassava mosaic disease and cassava bacterial blight. Together with poor culture practices, pests and diseases can be responsible for yield losses of at least 50% in Africa (IITA, 2008) and total crop failure in extreme circumstances (Puonti-Kaerlas, 1988).

Global cassava production was 172 million tonnes in the year 2000, with Africa accounting for 54% of this production (IITA, 2008). Cassava is grown in all African countries south of the Sahara and north of the Limpopo river. Countries in southern Africa where cassava is cultivated include Namibia, Mozambique, Angola, Swaziland, Malawi, Botswana and Zimbabwe (Anonymous, 2008a). Nigeria is Africa's largest producer of cassava and in 1999, was the world's largest producer with 33 million tonnes (IITA, 2008).

Improving cassava using traditional breeding programmes is difficult. This is in part due to the high heterozygosity and allopolyploid nature and the low natural fertility of cassava plants (Li *et al.,* 1996). In addition, cassava takes a long time to breed and does not reproduce true to type via seeds (Puonti-Kaerlas, 1998). Biotechnology provides a means to improve the starch quality of cassava, resistance to pests and disease as well as to reduce the amount of cyanide-producing substances (Anonymous, 2008b).

### 1.1.2 Cassava in South Africa

Cassava spread to select regions of South Africa, mainly as a result of tribal movements in the 1830's and 1860's. This food crop is now grown in the Northern Province, Mpumalanga, Northern Kwa-Zulu Natal and neighbouring Mozambique and Swaziland (Anonymous, 2008a). These areas of the country are typically characterised by sandy and infertile soils, erratic rainfall and extreme heat, making cassava the ideal food crop for these regions. Unfortunately a taste preference for maize has ensured that cassava has not become a large-scale crop in South Africa, even in areas where it is ideally suited to the growth conditions (Daphne, 1980). Despite this, cassava is grown by small-scale farmers as a secondary staple food for food security and for sale locally or to traders from Swaziland and Mozambique (Mathews, 2000).

Where cassava is grown in South Africa, both the roots and the leaves are consumed. Individual plants from a cassava crop are harvested and eaten as required. The roots are peeled and eaten fresh, or chipped, dried and ground into a meal. The leaves are eaten fresh or crushed and dried and eaten with groundnuts (Daphne, 1980). Besides its use as a food crop, cassava can be grown as a cash crop, sold for industrial use. In South Africa over 80% of the cassava produced is used for starch and alcohol production. Cassava Starch Manufacturers (CSM) processes starch from cassava at their factory in Dendron in Limpopo Province. They have their own source of cassava as well as contracting farmers to produce cassava for the factory. The food, textile, paper, corrugated cardboard and mining industries are the main users of the starch produced by CSM (Mathews, 2000). The full potential of cassava in South Africa had yet to be discovered (Anonymous, 2008a).

## 1.2 THE PROBLEM

### 1.2.1 Cassava mosaic disease

Some of the main diseases of cassava are cassava bacterial blight, superelongation disease, frog disease and cassava mosaic disease. Of these, cassava mosaic disease (CMD) is the most important disease of cassava in Africa, leading to losses of up to 40-50% of total yields (Puonti-Kaerlas, 1998). In 2004 an incidence of more than 50% was reported in eleven of the eighteen top cassava producing countries in Africa (Sseruwagi *et al*., 2004). The causative agents of cassava mosaic disease are members of the *Geminiviridae* family of plant viruses and belong to one of four genera of the family; *Begomovirus* (Fauquet *et al*., 2005). In addition to cassava, diseases caused by geminiviruses are a limitation to other important tropical and sub-tropical crops including maize, bean, squash, cucurbits and tomato (Bisaro, 1996).

### 1.2.2 Cassava mosaic geminiviruses

In the latest taxonomy, six species of cassava mosaic geminiviruses have been classified each with a number of strains; *African cassava mosaic virus*, *East African cassava mosaic virus*, *East African cassava mosaic Cameroon virus*, *East African cassava mosaic Malawi virus*, *East African cassava mosaic Zanzibar virus*, *Indian cassava mosaic virus* and *South African cassava mosaic virus*. Sri Lankan cassava mosaic virus is classified as a strain of *Squash mild leaf curl virus* (Fauquet *et al*., 2005).

All cassava mosaic geminiviruses consists of a genome of two circular molecules of covalently closed, circular, single stranded DNA contained in

separate geminate particles. The two genome components are designated DNA-A and DNA-B, with both DNA components being required for infectivity. The sequence of the two components differs with the exception of the 'common region' (CR) of 193 nucleotides located within the intergenic region. A conserved inverted repeat that forms a stem-loop structure lies within the intergenic region. The sequence 5'TAATATTAC is located in the loop of the stem-loop and has been found in all geminiviruses characterised to date. This sequence is critical for the replication of geminiviruses (Bisaro, 1996; Castellano *et al.*, 1999).

Replication of the single-stranded DNA occurs in the nucleus of the host cell by a rolling-circle mechanism with the replicative cycle consisting of two stages. Firstly, genomic ssDNA is converted to dsDNA intermediates. This is followed by the formation of new dsDNA intermediates and mature ssDNA genomes by the rolling-circle mechanism, involving the viral replication initiator protein (Rep) (Castellano *et al.*, 1999; Gutierrez, 2000). Rep protein is encoded by the AC1 ORF on the DNA-A component and is required for the initiation and termination of rolling-circle viral replication and regulates viral gene expression. Rep is a ~40-kDa sequence-specific DNA binding protein with site-specific endonucleolytic activity (Gutierrez, 1999). Rep protein has been shown to introduce a specific nick at the initiation site (↓) within the invariant TAATATT↓AC sequence to initiate rolling-circle replication (Bisaro, 1996).

DNA-A encodes the proteins necessary for replication and encapsidation. Besides Rep, other proteins encoded by the DNA-A component are the replication enhancer protein (REn), the transcriptional activator protein (TrAP) and the coat protein (CP; Bisaro, 1996; Palmer *et al.*, 1998; Gutierrez, 2000). Figure 1.1 shows the genome organisation of SACMV, an example of a bipartite

geminivirus. The ORFs on the DNA-B component of geminiviruses encode for proteins that are required for cell-to-cell and systemic spread of the virus from the inoculation site (Bisaro, 1996).

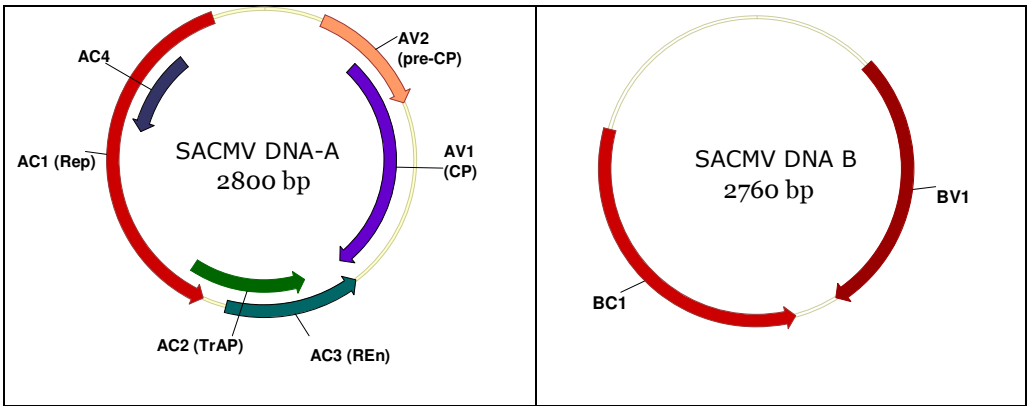


**Figure 1.1:** Genome organisation of a bipartite geminivirus, SACMV, consisting of two single-stranded circular DNA molecules, DNA-A and DNA-B (adapted from Gutierrez, 1999).

### 1.2.3 South African cassava mosaic virus

*South African cassava mosaic virus* (SACMV) is a distinct cassava-infecting geminivirus belonging to the genus *Begomovirus.* SACMV is a distinct bipartite Old World *Begomovirus* of cassava. It has been found to be most closely related to the monopartite tomato yellow leaf curl virus and to EACMV (Berrie *et al*., 1998). SACMV is transmitted by the whitefly *Bemisia tabaci* and to date contributes to cassava mosaic disease (CMD) in South Africa (SA), Zimbabwe, Swaziland and Madagascar (Berrie *et al*., 1998). Three variants exist, namely *South African cassava mosaic virus-[Madagascar:12}* (SACMV-[MG:12]), *South*

*African cassava mosaic virus-[South Africa]* (SACMV-[ZA]) and *South African cassava mosaic virus [Zimbabwe:Muzarabani]* (SACMV-[ZW:Muz]) (Fauquet *et al*., 2005).

## 1.3 TOWARDS SOLVING THE PROBLEM

### 1.3.1 The discovery of RNA silencing in plants

RNA silencing is a natural defence system found in plants, protecting against viruses and transposable DNA elements (Buchon *et al*., 2006). The phenomenon was first discovered in plants but the mechanisms are common across a diverse collection of eukaryotes. As the phenomenon was discovered in different organisms it was referred to by different terminology; posttranscriptional gene silencing (PTGS) in plants, quelling in fungi and RNA interference (RNAi) animals. In 2002 Hamilton *et al*. used the phrase 'RNA silencing' as a generic term to describe all the silencing mechanisms that were known (eg RNAi, PTGS) since it is clear now that they shared mechanistic similarities.

In the past decade a revolutionisation in the approaches that are being employed to induce silencing of plant pathogens has occurred (Waterhouse *et al*., 2001). This has come about with the discovery of RNA silencing and the elucidation of its mechanisms. We are now aware that foreign nucleic acids that enter the plant cell initiates the production of double-stranded RNA molecules, which are cleaved by Dicer into various size classes of single-stranded small-interfering RNAs (siRNAs). To date four DICER-LIKE (DCL) genes have been characterized in *Arabidopisis*. siRNA species are incorporated into a multi-subunit enzyme complex with RNA-directed nuclease activity, named RISC (RNA-

induced silencing complex) and direct sequence specific degradation of target mRNA. Presented here, is a review of the feature studies.

The idea of using a pathogens' own genetic material to generate resistance towards the pathogen was first considered in 1985, a concept referred to as pathogen derived resistance (PDR; Stanford *et al.*, 1985). PDR was applied in 1986 with the production of transgenic tobacco plants expressing the *coat protein* (*CP*) gene of *Tobacco mosaic virus* (TMV). This specific approach, whereby the expression of viral *CP* of a virus confers resistance to infection by that virus was named coat-protein mediated resistance (CPMR). Interestingly, a higher level of CP expression correlated with stronger virus resistance (Abel *et al.*, 1986). Besides coat-protein mediated resistance, virus replication and virus movement were identified as suitable targets for PDR against plant viruses. However, frequently the results obtained deviated from expected by way of no correlation between transgenic protein present and observed levels of resistance. With some reports of no protein present, it became apparent that the expression of the protein was not necessary (Prins and Goldbach, 1996).

A remarkable observation led to the realisation that a transgene and its homologous endogenous gene can be simultaneously suppressed, termed co-suppression. In 1990, attempts were made to over-express *chalcone synthase* (*chs*) in P*etunia*, which encodes a flower intermediate pigment biosynthesis enzyme. Purple flowers had identical phenotypes to non-transgenics and there was no increase in flower pigment in white flowers or in flowers with white and purple sectors. Unexpectedly, co-suppression of the endogenous *chs* and transgene had occurred. *chs* mRNA levels in white tissue was dramatically

reduced and the transcription rate of the transgene and endogenous gene was equal to that in purple tissues (Napoli *et al*., 1990).

The link between PDR and co-suppression became apparent following attempts to determine if different levels of expression of *Tobacco etch virus* (TEV) CP would result in resistance. Expecting resistance in the lines expressing TEV CP (CP+), the TEV challenged RNA control (RC) lines expressing untranslatable coat-protein mRNA were symptomless, indicating that the CP RNA sequence and not CP itself was responsible for the observed resistance. Further studies revealed recovery TEV CP+ and RC lines, which were susceptible to infection by other plant viruses (Lindbo *et al*., 1993). It was suggested that sequence-specific driven RNA degradation in the cytoplasm was activated by RNA, not protein. Thus the term RNA-mediated virus resistance was coined. In their paper, Lindbo *et al.* (1993) proposed the activity of a plant-encoded RNA-dependent RNA polymerase and double-stranded RNase. In 1996, it was shown that a nonviral transgene (GUS) could be silenced by the same sequence-specific RNA-degradation process (English *et al*., 1996).

Based on the idea that PTGS involved double-stranded RNA resulting from the hybridization of transgene RNA and target RNA, Waterhouse *et al.* (1998) concluded that gene constructs, which upon transcription produced duplex transcripts, increased the efficacy of PTGS compared to constructs that produced single stranded transcripts. This was a significant turning point for research aimed at initiating gene silencing in plants. Until this point several models had been proposed for the induction and operation of PTGS, all sharing the basic idea; degradation of RNA molecules sharing sequence homology with the transgene. Based on their results, Waterhouse *et. al.* (1998) proposed a model in

which PTGS is induced by dsRNA, which directs sequence-specific degradation of RNAs in the cytoplasm with the assistance of an RNA-dependent RNA polymerase. Concurrently was the discovery that dsRNA was the inducer of RNA silencing in nematodes (Fire *et al.*, 1998), protozoa (Ngo *et al.*, 1998) and insects (Kennerdell *et al.*, 1998). Co-suppression, antisense suppression and RNA-mediated resistance in plants were now collectively being referred to as PTGS.

A few years later Tenllado *et al.* (2001), who were working with *Pepper mild mottle virus* (PMMoV), *Tobacco etch virus* (TEV) and *Alfalfa mosaic virus* (AMV), delivered dsRNA directly into leaf cells by mechanical inoculation or *Agrobacterium*-mediated transient expression. The dsRNA derived from viral sequences was able to interfere with virus infection. No reduction in viral load was detected in inoculated plants when sense ssRNA, antisense ssRNA, DNA or nonhomologous dsRNA was delivered to leaf cells, once again confirming the requirement of sequence homology for PTGS.

The next significant discovery was the association of small RNA (sRNA) molecules with PTGS as shown by Hamilton *et al.* (1999). sRNA molecules were approximately 25 nucleotides in length, of sequence homology to the sequence that was being silencing and accumulated in a way that correlated with the silencing efficiency. Later these sRNAs became known as small-interfering RNAs (siRNAs) and the guides to target mRNAs. A multi-subunit enzyme complex was purified from *Drosophila* cell extract fractions showing PTGS, and always co-fractionated with sRNA species. RISC (RNA-induced silencing complex) has RNA-directed nuclease activity and was proposed as a mediator of PTGS (Hammond *et al.*, 2000).

Dicer enzyme was identified in 2001, and is a member of the RNase III family of nucleases, which show specificity for dsRNA. After determining that *Drosophila* CG4792 results in the processing of dsRNA into ~22-nt fragments, independent of the sequence of the RNA substrate, it was proposed that CG4792 was responsible for the initiation step of RNAi, producing guide sequences. The enzyme was named Dicer (*Dcr*) due to its ability to digest dsRNA (Bernstein *et al*., 2001). Dicer is evolutionarily conserved in worms, flies, fungi and mammals. Dicer structure includes a helicase domain, dual RNase III motifs and a PAZ domain. The PAZ sequence can be found in the *Drosophila* ARGONAUTE protein as well as in related proteins in *Arabidopsis, C. elegans* and *Neurospora*. It is the association of the PAZ domain with an AGRONAUTE relative, which in turn is associated with an RNase that allows for the guide RNA to direct the RNase to single-stranded mRNA targets that share sequence homology with the guide RNA (Balucombe, 2001). The work by Bernstein *et al*. (2001) complements that of Yang *et al*. (2000) who provided evidence that guide RNAs are directly derived from dsRNA in *Drosophila* embryos and Fagard *et al.* (2000) who showed the involvement of *Arabidopsis* AGRONAUTE1 in PTGS. Evidence proving that plant siRNAs are direct products of endonucleolytic cleavage of long dsRNA came from a study using wheat germ extracts to mimic aspects of RNA silencing in plant using an *in vitro* system. Two distinct classes of siRNAs (as discovered by Hamilton *et al*., 2002) were generated from exogenous dsRNA in wheat germ extracts. A 5' adenosine in the longer class indicated that different enzymes were responsible for the generation of the two classes, they functioned in different effector complexes, or both (Tang *et al*., 2003).

RNA-dependent RNA polymerase (RdRP) plays an essential role in RNA silencing in plants. Schiebel *et al.* (1998) isolated an RdRP encoding gene

activated upon virus infection in tomato. In 2000 a model, which involved the action of RdRP, was proposed to explain the ability of sense transgenes to trigger PTGS (Dalmay *et al*., 2000). In this model, RdRP generates dsRNA from transgenes. A similar prediction was made by Serio *et al*. (2001) for the generation of dsRNA from antisense transgenes and they alternatively proposed that hybridisation of a sense endogene transcript hybridises with the antisense transgene transcript. Using mutagenesis, Dalmay and colleagues (2000) identified four genetic loci in *Arabidopsis* that control PTGS. *SDE1* was of particular interest since SDE1 is the plant homolog of *N. crassa QDE*-1, which encodes an RNA-dependent RNA polymerase and shares extensive similarity with RdRP of tomato. This led to the proposal that the SDE1 RdRP was responsible for dsRNA synthesis, rather than using it as a template, as previously thought. This means that SDE1 is involved in the initiation of transgene-induced PTGS but not RNA virus-induced PTGS, wherein viral RdRP would synthesise dsRNA, the replication intermediate.

Concurrently, Mourrain *et al.* (2000) reported the successful cloning of the *Arabidopsis SGS2* and *SGS3* genes from PTGS-impaired mutants. SGS2 protein was shown to be similar to the tomato RdRP, as well as to QDE-1 and *C. elegans* EGO-1 thus confirming a mechanistic relationship between PTSG, RNAi and quelling. The authors could not elucidate the role of SGS3 since no similarities could be found between it and any other protein, even though *sgs2* and *sgs3* mutants shared the same molecular and phenotypic characteristics. In addition, *sgs2* or *sgs3* mutants showed enhanced susceptibility to CMV infection with CMV tolerance being restored when the SGS2 or SGS3 gene was introduced into the respective mutant. This provided confirmation that PTGS is a virus resistance mechanism in plants.

The silencing signal is able to move from the point of initiation. Systemic acquired silencing was shown when transgene-specific post-transcriptional silencing was transmitted by grafting from silenced stocks to non-silenced scions (Palauqui *et al.*, 1997)**.** A diffusible sequence-specific signal that allowed for the systemic transmission of the silencing signal was proposed. In 1998 Voinnet *et al.* identified that the systemic spread of sequence-specific transgene RNA degradation in plants could be initiated by localized introduction of ectopic promoterless DNA. PTGS was triggered in transgenic plants following the localized introduction, using biolistics, of exogenous DNA, homologous to the transgene. Also since the exogenous DNA was promoterless this confirmed that the expression of the incoming DNA is not a requirement for gene silencing. Twenty-five nucleotide siRNAs were implicated in systemic silencing when two size classes of siRNAs (21-22nt and 25nt) were associated with local and systemic silencing of GFP in *N. benthamiana* respectively (Hamilton *et al.*, 2002).

PTGS can be suppressed by virus-encoded proteins. The effect of viral suppressors on local silencing was tested (Hamilton *et al.*, 2002). Using P1 protein of rice yellow mottle virus, P19 protein of tomato bushy stunt virus, Hc-Pro of potato virus Y, 2b protein of cucumber mosaic virus and the AC2 protein of African cassava mosaic virus, a mixture of a T-DNA construct carrying the 35S-GFP transgene to initiate silencing and a T-DNA construct carrying the viral suppressor sequence was agro-infiltrated into the leaves of GFP transgenic *N. benthamiana*. The level of two size classes of GFP siRNA (21-22nt and 25nt) was reduced to various extents in the presence of each of the viral suppressors. However, there was in inverse relationship between the short GFP siRNA accumulation and GFP mRNA levels. In the presence of a viral suppressor, the accumulation of short siRNAs was associated with the reduction of GFP mRNA.

The level of GFP siRNA in the upper leaves of infiltrated plants indicated a strong correlation between the production of the long siRNA species and systemic silencing. The authors proposed that the 21-22nt (small) class of siRNAs are responsible for guiding the RISC to the target RNA, and it is unlikely that the long class of siRNAs acts as a guide. Furthermore, synthetic siRNAs were found to be inactive in an RISC assay if they were longer than 23nt (Elbashir *et al*., 2001).

A number of endogenous RNA silencing pathways act in parallel in plants, To date the microRNA, trans-acting siRNA, natural-antisense siRNA and repeat-associated siRNA pathways have been elucidated (Eamens *et al*., 2008). Micro RNAs (miRNAs) are processed from single-standed RNA transcripts, transcribed by MIR genes and repress the expression of complementary mRNAs by transcript cleavage. The transacting-siRNA pathway has been implicated in the control of gene expression for normal development in plants. Like miRNAs, tasiRNAs guide cleavage and degradation of homologous, cellular transcripts. Each TAS gene encodes several siRNAs that regulate the expression of unlinked genes. NatsiRNA molecules target cis-acting gene pair transcripts for cleavage, whilst the rasiRNA pathway regulates gene expression at the transcriptional level through the inactivation of promoter sequences by DNA methylation. In *Arabidopsis* different Dicer like enzymes (DCL) have been identified in the production of these small RNAs. DCL1 is responsible for the production of miRNAs from the pre-miRNA precursor transcript. Repeat-associated siRNAs and ta-siRNAs are produced by DCL3 and DCL4 respectively (Brodersen *et al*., 2006 and Eamens *et al*., 2008).

### 1.3.2 Geminiviruses and RNA silencing

Geminiviruses are targets of RNA silencing even though DNA viruses do not produce dsRNA in their replication cycle. The replicative forms of DNA viruses were identified to be a possible target of RNA silencing by Pooggin *et al*., (2003). *Tomato yellow leaf curl Sardinia virus* (a monopartite begomovirus), African cassava mosaic virus-[Cameroon],, *East African cassava mosaic Cameroon virus*, *East Afican cassava mosaic virus,*, Sri-Lankan cassava mosaic virus and *Indian cassava mosaic virus* have been shown to produce virus-specific siRNAs (Lucioli *et al*., 2003; Chellappan *et al*., 2004). One model predicts that dsRNA can be produced as a result of the bi-directional transcription that occurs in these geminiviruses, producing transcripts that overlap at their 3' ends (Chellappan *et al.,* 2004). Alternatively, the AC1 transcript produced abundantly and early in the infection cycle, can provide a template for RdRP to make dsRNA or the secondary structure of the geminivirus transcripts that contains a certain amount of folding would present areas of dsRNA (Vanitherani *et al*., 2005).

Additionally geminiviruses have been found to express suppressors of RNA silencing. Specifically, the AC4 and AC2 ORFs of cassava mosaic geminiviruses have been identified as expressing silencing suppressors (Vanitherani *et al*., 2004; Butaye *et al*., 2005). Cassava mosaic geminiviruses can be classified as recovery or non-recovery phenotype. The recovery-type viruses such as Sri Lankan cassava mosaic virus and African cassava mosaic virus [Cameroon] portray this phenotype possibly as a result of the production of virus derived siRNAs which result in a reduction in viral DNA and mRNA. Work by Chellappan *et al.* (2004b) identified that ACMV[CM] siRNAs are predominantly derived from the C-terminus of AC1 that overlaps the N-terminus of the AC2

ORF. The non-recovery type cassava mosaic geminiviruses such as: *East African cassava mosaic Cameroon virus*,, *East African cassava mosaic virus*, and *Indian cassava mosaic virus* were shown to trigger the host RNA silencing mechanism however, siRNA production is low. In Sri Lankan cassava mosaic virus and ACMV[CM] AC4 acts as a silencing suppressor, whilst AC2 is a silencing suppressor in *East African cassava mosaic Cameroon virus* and ICMV.

### 1.3.3 Biotechnology

A prerequisite for the successful improvement of cassava using biotechnology is the development of a reproducible transformation system to facilitate gene transfer techniques. Originally, the regeneration of cassava plants through somatic embryogenesis was the only available method. Unfortunately, using the generated embryogenic structures as target tissues for genetic transformation yielded only chimaeric embryos. Several improved regeneration systems have been developed including the regeneration of transgenic cassava plants based on organogenesis (Li *et al.*, 1996), a friable embryogenic system (Taylor *et al.*, 1996) and the regeneration of transgenic cassava plants from embryogenic suspension cultures (Schöpke *et al.*, 1996).

### 1.3.4 Introducing dsRNA to initiate an RNA silencing response

Wesley and colleagues (2001) have reported a mechanism that induces PTGS with almost 100% efficiency. Plants are transformed with constructs encoding self-complementary 'hairpin' RNA (hpRNA) to provide resistance to

viruses. The generic intron-spliced hpRNA (ihpRNA) vectors pHANNIBAL (with bacterial ampicillin resistance genes) and pKANNIBAL (with bacterial kanamycin resistance genes) were subsequently designed. A PCR fragment of choice can be inserted in the sense and anti-sense orientation producing a hpRNA of sequence homology to the targeted viral sequence (Wesley *et al.*, 2001).

### 1.3.5 Cassava mosaic disease control strategies

Various pathogen derived resistance-based strategies, developed for the control of begomovirus proliferation in plants, include the expression of viral proteins, the use of naturally occurring defective interfering molecules which delay and attenuate infection symptoms and the use of double-stranded RNA molecules to direct methylation of virus promoter sequences. Coat protein-mediated resistance has successfully induced resistance to some begomoviruses [e.g. *Tomato yellow leaf curl virus* (Kunik *et al.*, 1994), *Tomato mottle virus* (Sinisterra *et al.,* 1999)], whilst several attempts have failed for example transgenic beans expressing the CP of *Bean golden mosaic virus* (Azzam *et al.*, 1996) and transgenic *Nicotiana benthamiana* expressing the CP of *African cassava mosaic virus* (Frischmuth *et al.*, 1998). Resistance against a wider range of different viruses have successfully been induced using the movement protein-mediated resistance strategy compared with the coat protein-mediated resistance strategy (Lapidot and Friedmann, 2002).

Defective-interfering (DI) viral DNAs present another strategy for controlling cassava-infecting geminiviruses. Subgenomic DNAs occur naturally in

geminivirus-infected plants together with the DNA-A and DNA-B genomic components (Frischmuth and Stanley, 1993). These subgenomic DNAs have the ability to delay symptom development and ameliorate symptoms, thus behaving as defective-interfering (DI) DNA, as demonstrated by Stanley and colleagues in 1990. Transgenic *Nicotiana benthamiana* plants expressing a DNA-B DI of ACMV showed a reduction in symptom severity and viral replication following inoculation with ACMV (Stanley *et al*., 1990). There are no recent reports in the literature of DI-mediated resistance strategies.

Transcriptional gene silencing (TGS) results from the methylation of target promoter sequences and can be exploited as a geminivirus silencing strategy. DNA constructs expressing dsRNAs homologous to the bi-directional promoter and the common region of *Mungbean yellow mosaic virus* (MYMV), when bombarded into MYMV-infected *Vigna mungo* plants, resulted in their recovery (Pooggin *et al*., 2003). *African cassava mosaic virus* resistance has been achieved in cassava by expressing dsRNA homologous to the ACMV DNA-A promoter (Vanderschuren *et al*., 2007).

Since mixed infections are common, expecially in cassava mosaic geminivirus infections, broad spectrum resistance is important. This has been achieved using an invert-repeat construct targeting non-coding conserved regions of a number of viruses belonging to the Tomato yellow leaf curl virus complex (Abhary *et al*., 2006). A modified version of the ACMV–[KE] AC1 ORF containing a point mutation at position 238 was used to produce transgenic cassava plants. Transgenic lines exibited resistant to EACMCV-[CM] and SLCMV, two non-homologous cassava mosaic geminiviruses. High level resistance was also achieved when transgenic lines were exposed to dual

inoculation with ACMV-[CM] and EACMCV-[CM]. Moreover, the modification was reported to increase the frequency of transgenic plant recovery (Chellappan *et al.*, 2004)

A further strategy employed to abolish viral replication of geminiviruses involves the expression of Rep protein mutants. NPT-binding and DNA-nicking domains are essential for geminiviral DNA replication. The expression of Rep protein mutated in these domains, acts as a *trans*-dominant-negative mutant to suppress viral replication as shown in numerous studies. Mutant *Rep* genes expressed in *trans* in a tobacco suspension culture system resulted in inhibiton of Bean *golden mosaic virus* replication (Hanson, *et al.*, 1999). A similar strategy was emolyed to confer ACMV resistance (Sangare et al., 1999) and *Tomato yellow leaf curl Sardinia virus* resistance (Bruneti *et al.*, 2001) in transgenic *N. benthamiana.*

The sequence specific degradation of viral transcripts in the host cytoplasm by inducing RNA silencing with sense, antisense or invert-repeat transgenes have been used extensively for the control of geminiviruses, and is the strategy that was used in this study to prevent the replication of *South African cassava mosaic virus.* Successes and failures resulting from the use of sense, antisense and invert-repeat transgene towards the control of geminiviruses will be highlighted throughout this report.

## 1.4 OBJECTIVE AND AIMS

### 1.4.1 Objectives

Sense, antisense and invert-repeat RNA silencing constructs,derived from the SACMV-AC1 ORF sequence are hypothesied to induce the production of siRNAs when expressed *in planta* and significantly reduce *South African cassava mosaic virus* DNA-A replication. To this end, *Nicotiana benthamiana*, a known host of SACMV, was transformed with either sense, antisense and invert-repeat RNA silencing constructs.

### 1.4.2 Specific aims

The specific aims of this study were to:

1) investigate the replication efficiency of SACMV DNA-A in challenged transgenic *N. benthamiana* lines, expressing a truncated SACMV AC1 transcript in the antisense or the sense orientation,

2) produce invert-repeat constructs that would express intron-hpRNA *in vivo* using the pHannibal vector system and to test the efficacy of the constructs to reduce SACMV replication in *N. benthamiana* and

3) develop an alternative method of producing invert-repeat constructs to alleviate difficulties encountered when cloning perfectly matched invert-repeat sequences.

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# CHAPTER 2 - Sense transgene mediated RNA silencing towards South African cassava mosaic virus resistance

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**Presented:**

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Taylor, S.H. and Rey, M.E.C. (2008) Sense transgene expression towards *South African cassava mosaic virus* resistance in *Nicotiana benthamiana*. BIO08 Conference, Grahamstown, South Africa. (poster)

## 2.1 ABSTRACT

*South African cassava mosaic virus* (SACMV) has a single-stranded circular bipartite genome (DNA-A and DNA-B). The replication efficiency of SACMV DNA-A in challenged transgenic *Nicotiana benthamiana* plants, expressing untranslatable SACMV AC1 transcripts in the antisense (p811AS) or sense (p811S) orientation was investigated. Stable transgene integration was confirmed in ten sense and eleven antisense lines. Transgene expression was confirmed in five sense and three antisense lines, and these lines were challenged with SACMV DNA-A and DNA-B. Two sense lines (S2 and S20) displayed a reduction in SACMV DNA-A replication of almost 100% and showed no virus symptoms, compared to the non-transformed control and other SACMV-challenged transgenic lines. This is the first reported case of significant knockdown of SACMV replication in transgenic *N. benthamiana.* Two size classes of presumptive siRNAs (between 22- and 30-bp) were detected in unchallenged S2 and S20 plants, suggesting that an RNA silencing mechanism is responsible for the efficient disruption of SACMV DNA-A replication in S2 and S20 transgenic lines. This study clearly demonstrates that sense AC1 constructs of SACMV are able to induce RNA silencing and result in effective reduction of virus replication in transgenic *N. benthamiana.*

## 2.2 INTRODUCTION

### 2.2.1 Background

RNA silencing in plants includes several distinct pathways involved in host defence against invading nucleic acids and endogenous gene regulation, all initiated by the production of small RNAs (Brodersen *et al.*, 2006; Horiguchi, 2004; Vazquez, 2006). There are numerous examples in the literature describing geminivirus silencing strategies involving antisense RNA-directed silencing (Day *et al.*, 1991; Bejarano *et al.*, 1994; Bendahmane *et al.*, 1997; Praveen *et al.*, 2005; Zhang *et al.*, 2005; Mubin *et al.*, 2007) and sense RNA-directed silencing (Noris *et al.*, 1996). However with the constant flow of new information in the field of RNA silencing in plants, it has become clear that these strategies are in fact very closely linked to hairpin RNA mediated silencing, having double-stranded RNA (dsRNA) in common. It is these dsRNA molecules that induce the RNA silencing pathway (Béclin *et al.*, 2002). The mechanism of sense transgene-initiated post-transcriptional gene silencing is clearer now, as studies have shown that it is an RNA-dependent RNA polymerase (RDR6 in *Arabidopsis*) that allows for the conversion of ssRNAs, transcribed from a sense transgene, to dsRNA molecules (Dalmay *et al.*, 2000).

The cassava mosaic geminivirus replication-associated protein (Rep), encoded by the AC1 ORF on the DNA-A component is involved in both viral replication (Hanley-Bowdoin *et al.*, 1990; Lazarowitz *et al.*, 1992; Fontes *et al.*, 1992; Fontes *et al.*, 1994) and transcriptional regulation (Sunter *et al.*, 1993; Eagle *et al.*, 1994). It is the only viral protein absolutely required for replication of viral DNA (Elmer *et al.*, 1988; Etessami *et al.*, 1991), making it an ideal target for virus silencing mechanisms.

### 2.2.2 A previous study

In a study conducted by Berrie (unpublished), cassettes were made for the expression of untranslatable SACMV full-length AC1 (1330-bp; but lacking the AC1 promoter) or 3'-truncated (811-bp; Figure 2.1) transcripts in the sense and antisense orientations. In each construct, the AC1 sequence was driven by the CaMV 35S promoter. The resulting four expression cassettes (p1330S and p1330AS, not shown; p811S and p811AS, Figure 2.2) were used in a pilot study wherein a *Nicotiana benthamiana* transient callus assay system was used to test the efficacy of each construct to reduce SACMV DNA-A replication. *N. benthamiana* callus co-bombarded with SACMV DNA-A and DNA-B dimers in pBIN19 and the p811AS construct showed a significant reduction in viral DNA-A replication four days after bombardment, compared to vector only bombarded callus. Based on the results it was decided not to pursue the full-length constructs, and to test the truncated constructs in a stable expression system. Forty *N. benthamiana* lines transformed with either the p811S or p811AS constructs were independently produced from *N. benthamiana* transformed leaf discs. $T_1$ plants were grown from seed collected from selfed $T_0$ plants. Thirteen lines either did not grow, or did not produce sufficient quantities of seed to allow for these lines to be grown to the second generation, leaving 27 lines to test (Figure 2.3).

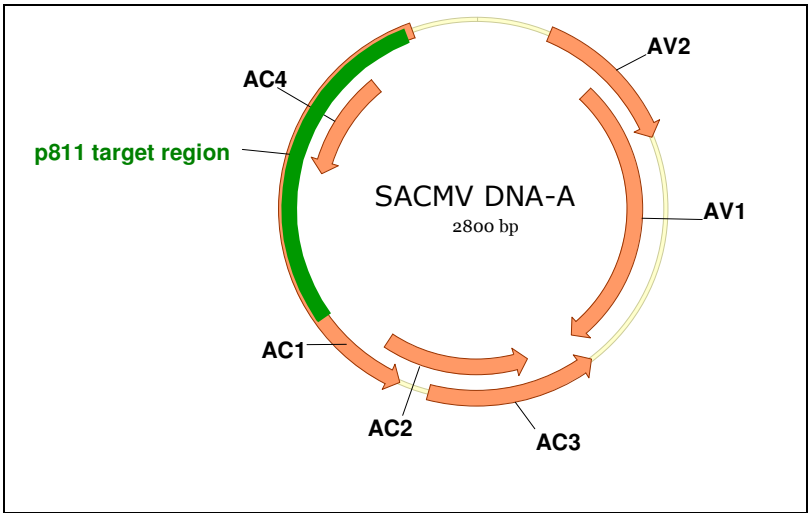


**Figure 2.1:** SACMV DNA-A showing the target region for the p811 construct

### 2.2.3 Objective and aims

The objective of this study was to test for SAMCV replication knockdown in a number of previously generated *N. benthamiana* lines transformed with the 3'-truncated SACMV AC1 sense or antisense construct (p811S or p811AS). The specific aims (Figure 2.3) were to:

1. establish transgene stability in $T_2$ plants,
2. detect GUS expression in p811S lines,
3. confirm and quantify transgene expression,
4. detect siRNAs,
5. challenge transgenic lines with infectious SACMV DNA-A and DNA-B dimers and monitor symptoms and
6. quantify viral DNA-A in challenged plants twenty-one days post infection.

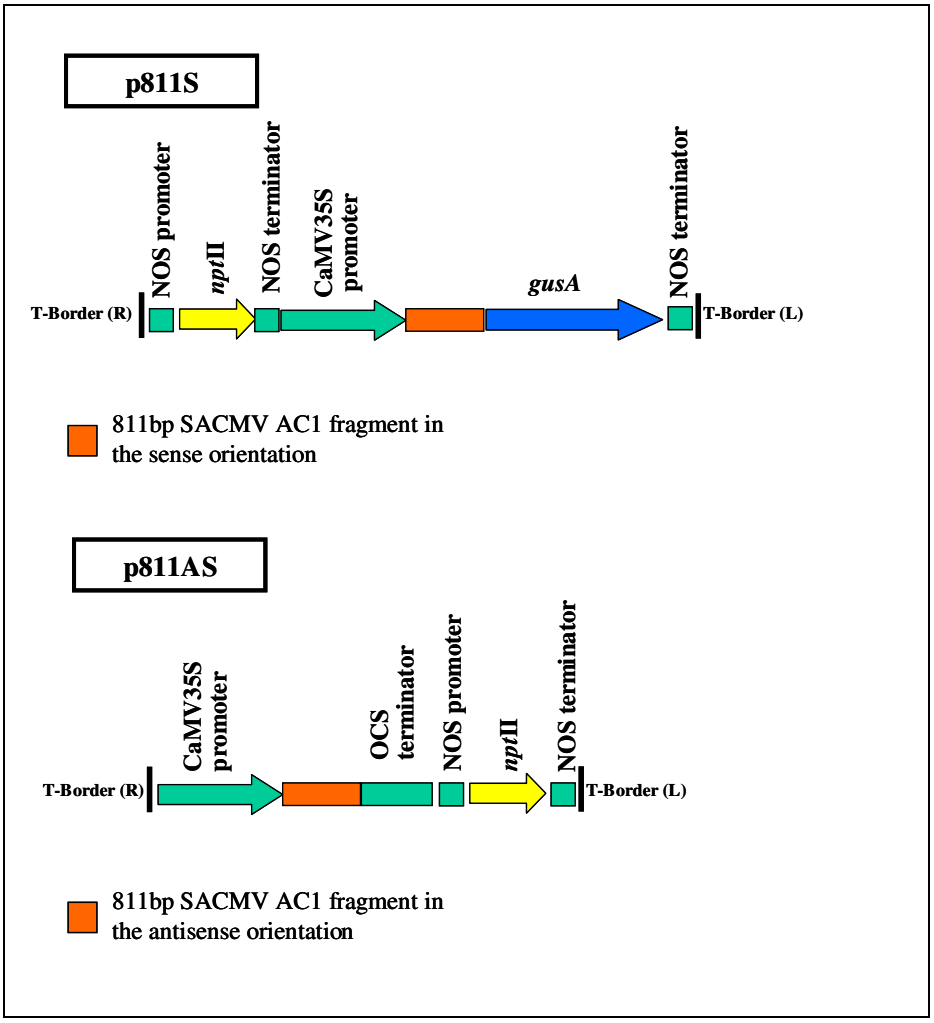


**Figure 2.2:** A schematic representation of truncated AC1 constructs in either the sense p811S) or antisense (p811AS) orientation. In each construct, transcription of the AC1 sequence is driven by the *Cauliflower mosaic virus* 35S promoter (CaMV 35S). Arrows indicate the direction of transcription. *npt*II - kanamycin resistance gene; *gusA* -glucuronidase gene. The 811bp fragment was cloned in to pART27 in the antisense orientation, and in to pBI121 in the sense orientation.

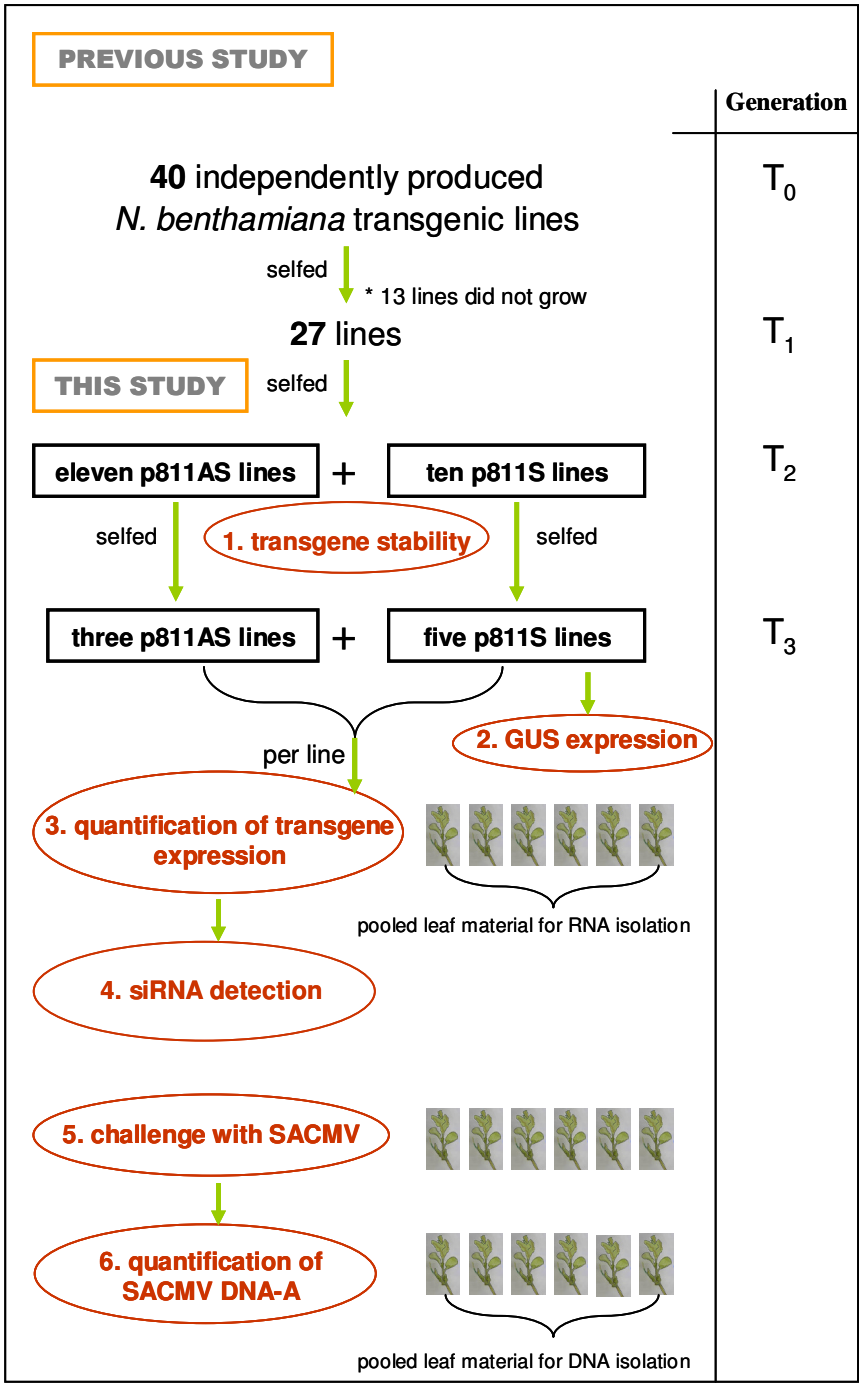


**Figure 2.3:** An outline of the methods used in this study to analyse p811S and p811AS transgenic *N. benthamiana* lines. The $T_0$ and $T_1$ lines were produced in a previous study and the $T_2$ and $T_3$ generation plants were produced and analysed in this study.

### 2.2.4 Transgene integration and stability

Second generation ($T_2$) p811AS and p811S transgenic plants, germinated from seed of selfed $T_1$ plants, were grown in soil in a controlled temperature growth-room at 25℃ under a sixteen hour photoperiod. DNA was isolated from 50mg leaf tissue per plant, using the CTAB method (Doyle and Doyle, 1987). The degenerate primers PCRC2 and PAV1978 (Rojas *et al*, 1993) were used to amplify a 698-bp region of the SACMV sequence of the transgene using a step-wise PCR profile (94℃ for 2 minutes; 2 cycles of 94℃ for 45 seconds, 60℃ for 45 seconds and 72℃ for 90 seconds; 2 cycles of 94℃ for 45 seconds, 58℃ for 45 seconds and 72℃ for 90 seconds; 30 cycles of 94℃ for 45 seconds, 56℃ for 45 seconds and 72℃ for 90 seconds; final extension at 72℃ for 7 minutes; MyCycler, BioRad). The PCR amplicon was resolved and visualised on 1% agarose gels stained with ethidium bromide.

### 2.2.5 $T_3$ generation plants

$T_3$ plants were grown from seed collected from selfed $T_2$ plants. Seeds were germinated in peat pellets (Jiffy) and incubated under a 16 hour photoperiod regime at 25℃ in a controlled growth room.

### 2.2.6 Quantification of transgene expression

The expression of the transgene in unchallenged lines was confirmed and quantified by absolute real-time PCR. An equal amount of leaf tissue (new leaves at the apex of each six week old seedling) was collected from six replicate plants per line in duplicate. RNA was isolated from 100mg pooled leaf tissue per line using Trizol® (Sigma-Aldrich), following the manufacturer's recommendations. Similarly, RNA was isolated from a non-transgenic control plant. The RNA was treated with Deoxyribonuclease I (Fermentas) to remove contaminating DNA. First strand cDNA was synthesised using the oligo-p$(dT)_{15}$ primer (First Strand cDNA synthesis kit, Roche Biochemicals) following the manufacturer's recommendations. cDNA was quantified using The NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc.) and amplified in duplicate by absolute real-time PCR using SYBRGreen fluorescent dye (LightCycler FastStart DNA Master$^{PLUS}$ SYBR Green I kit, Roche Biochemicals) with the LightCycler 1.5 Instrument (Roche). Reactions were set up according to manufacturer's instructions to which 2µl cDNA and 2µM of each PCR primer (p811F: 5' ggctagttcccggattacat; p811R: 5'gacaaggacggagacacc; primers designed using LightCycler Probe Design Software 2.0) was added. The following PCR conditions were used to amplify the 150-bp amplicon: 5 min at 95ºC; 40 cycles of 5 sec at 95ºC, 10 sec at 60ºC and 10 sec at 72ºC.

For quantification purposes, a standard curve was generated by amplification of SACMV DNA-A in pBluescript (kindly donated by L. Berrie). Serial concentrations of DNA-A (1000pg, 100pg, 10pg, 1pg, 0.1pg) were amplified (p811R and p811F primers) in duplicate by real-time PCR using SYBRGreen fluorescent dye (LightCycler FastStart DNA Master$^{PLUS}$ SYBR Green I kit, Roche

Biochemicals) with the LightCycler 1.5 Instrument (Roche). The same cycling conditions and primer concentrations were used as described above. The amount of transgene RNA in each sample was calculated from the standard curve, and the following equation was used to express the amount as molecules/ng DNA added to the PCR reaction:

$$\text{molecules/ng DNA} = \frac{[\text{calculated concentration (pg)} \times 1.56\text{x}10^{5} \text{ molecules}]}{\text{cDNA added to PCR reaction (ng)}}$$

This equation assumes that one plasmid (DNA-A in pBluescript) has one copy or molecule of DNA-A therefore if 100ng plasmid DNA = 0.026pmol plasmid then, using Avagadro's number, 1pg plasmid DNA = $1.56\text{x}10^{5}$ molecules.

The data was analysed using Microsoft Excel and GraphPad InStat 3.01 software (GraphPad Software, Inc., San Diego, CA)

### 2.2.7 Histochemical detection of β-glucoronidase

GUS assays were performed on unchallenged leaf material from sense transgenic plants. Leaf and stem tissue from six week old $T_3$ plants was placed in staining buffer (1M sodium phosphate (pH 7.0); 0.5M EDTA; 10% Triton X-100; 50mM $K_3Fe(CN)_6$; 0.1M X-Gluc (Fermantas)) and incubated at 37ºC for 24 hours followed by several washes in 50% ethanol. The tissue was viewed under a dissecting microscope (Olympus SZ-STS) and photographed with a Nikon DXM 1200 digital camera.

### 2.2.8 siRNA detection in unchallenged p811AS and p811S lines

A highly sensitive solution hybridisation and RNA-protection-based assay was used to detect siRNAs in p811AS and p811S $T_3$ lines. The *mir*Vana™ miRNA probe construction kit (Ambion®) was used to generate two $_{32}$P-labelled antisense RNA probes from oligonucleotide DNA templates (Figure 2.4). An 8-bp sequence complementary to the 3' end of the T7 promoter primer was added to the 3' end of each DNA oligonucleotide sequence (Table 2.1). The target-specific oligonucleotide was annealed to the T7 promoter primer and a dsDNA transcription template was generated using Exo-Klenow enzyme (Ambion®). A complementary 5' to 3' RNA transcript was transcribed *in vitro* by T7 phage RNA polymerase, using the 3' to 5' strand of the dsDNA as a template. Radiolabeled UTP [α-$_{32}$P] was incorporated into the transcript during the transcription reaction. All reactions were performed according to manufacturer's instructions. Full-length labelled transcripts were resolved on a 15% denaturing polyacrylamide gel, visualised by autoradiography and gel-purified to separate full-length transcripts from prematurely terminated transcription products and unincorporated nucleotides. The specific activity of each probe was determined (TriCarb Liquid Scintillation counter, Packard).

**Table 2.1:** DNA oligonucleotide sequences used as templates for the *in vitro* transcription of antisense RNA probes. The T7 promoter sequence is identified by bold letters at the 3' end of the sequence.

| Probe | DNA oligonucleotide sequence |
|---|---|
| AC1 probe | 5' ttgaggtaatgggggtcgacgt**cctgtctc** |
| AC4 probe | 5' attgttgtccgccgcgagcaga**cctgtctc** |

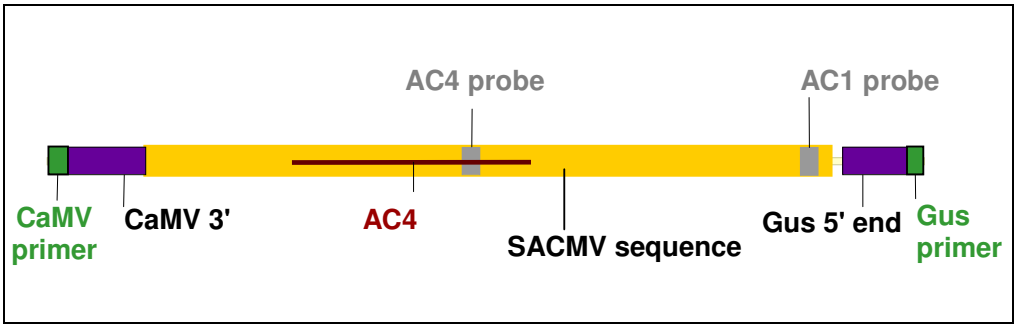


**Figure 2.4:** A region of the p811S transgene showing PCR primers (CaMV primer and Gus primer) and siRNA detection probe (AC4 probe and AC1 probe) binding sites.

Total RNA was isolated from 100mg leaf tissue from p811S and p811AS plants individually using Trizol® (Sigma-Aldrich) according to manufacturer's instructions and resolved on 1% TAE agarose gels (80V for 1 hour) and visualised by staining with ethidium bromide to check RNA integrity. RNA was quantified with a spectrophotometer (The NanoDrop® ND-1000, NanoDrop Technologies, Inc.). Hybridisation reactions were carried out according to manufacturer's recommendations (*mir*Vana™ miRNA detection kit, Ambion®). Sample RNA (5µg per reaction) was mixed with 5.0 x $10^4$ cpm of each radiolabeled antisense RNA probe in separate reactions. The RNA was heat denatured and incubated at 42℃ for 16 hours for hybridisation. After hybridisation, single stranded RNA, including the unhybridised sample RNA and excess RNA probe was digested with ribonuclease and the remaining dsRNA was precipitated. Radiolabeled protected RNA probe fragments were resolved on 12% denaturing polyacrylamide gels (20mA) and visualised by autoradiography.

### 2.2.9 Inoculation with SACMV DNA-A and DNA-B

Six week old $T_3$ seedlings were challenged with SACMV DNA-A and DNA–B dimers by agroinoculation, using pBINSA-A and pBINSA-B transformed *Agrobacterium tumefaciens* (kindly donated by L. Berrie). For control purposes, six non-transgenic *N. benthamiana* seedlings were challenged in the same way. A Hamliton syringe was used to deliver a mixture of pBINSA-A and pBINSA-B transformed *A. tumefaciens* (O.D.= 0.6 each; equal volumes mixed) into the stem of the seedlings. Twenty microlitres of the mixture was injected at three different positions along the stem. Inoculated plants and unnoculated controls were maintained at 25ºC with a 16 hour photoperiod, and viral symptoms were scored every seven days until 28 days post inoculation (dpi) using the six point symptom severity score (Fauquet and Fargette, 1988; 0 = no symptoms, 1 = mild chlorosis without deformation, 2 = clear mosaic with or without slight leaf deformation, 3 = strong mosaic all over the leaflets with leaf deformation, 4 = same as score 3 except with severe leaf deformation, 5 = severe mosaic and severe reduction of leaf size).

### 2.2.10 Quantification of DNA-A in SAMCV challenged plants

The amount of viral DNA-A present in SACMV inoculated plants at twenty one days post inoculation (dpi) was quantified using absolute real-time PCR. Leaf tissue was collected, in duplicate, from six replicate plants per line and pooled to total 50mg, selecting only the new leaves at the apex of each plant. DNA was isolated from leaf tissue using the CTAB method (Doyle and Doyle, 1987) and quantified using The NanoDrop® ND-1000 spectrophotometer (NanoDrop

Technologies, Inc.). A region of the AV1 ORF of SACMV DNA-A was amplified, in duplicate, by real-time PCR using SYBRGreen fluorescent dye (LightCycler FastStart DNA Master$^{PLUS}$ SYBR Green I kit, Roche Biochemicals) with the LightCycler 1.5 Instrument (Roche). Reactions were set up according to manufacturer's instructions to which 2µl of 1:10 diluted DNA and 2µM of each PCR primer (AV1F: 5' gcacaagcgtcgat; AV1R: 5' ctgccagtatgcttaacgtca) was added. The following PCR conditions were used to amplify the 154-bp fragment: 5 min at 95ºC; 40 cycles of 5 sec at 95ºC, 10 sec at 60ºC and 10 sec at 72ºC.

For quantification purposes, a standard curve was generated by amplification of SACMV DNA-A in pBluescript as described in section 2.3.3, using the above-mentioned PCR primers (AV1F and AV1R). The amount of SACMV DNA-A per sample was calculated from the standard curve as described in section 2.3.2 and the data was analysed using Microsoft Excel and GraphPad InStat 3.01 software (GraphPad Software, Inc., San Diego, CA).

### 2.2.11 Sequence analysis of the AC1 region of p811S transgene

DNA was isolated (CTAB method; Doyle and Doyle, 1987) from representative p811S transgenic plants. The AC1 region of the transgene was amplified by PCR using EconoTaq (Invitrogen) and primers that anneal upstream and downstream of the AC1 sequence of the transgene (CaMV primer: 5' ggattgatgtgatatctccactg; GUS primer: 5' ctgtggaattgatcagcgtt; Figure 2.4). The PCR amplicon was TA cloned into the pTZ57R cloning vector (Fermantas) according to manufacturer's instructions and sequenced with the M13 reverse

and forward primers. The reverse and forward sequences were aligned using DNAMAN (Version 4.0; Lynnon BioSoft).

## 2.3 RESULTS

### 2.3.1 Transgene integration in $T_2$ lines

Seeds from selfed $T_1$ p811S and p811AS transgenic lines (six per line) were grown to produce $T_2$ generation plants for each line. DNA was amplified by PCR using transgene specific primers (Figure 2.5; line AS6 shown). A PCR amplicon was confirmation of the presence of the transgene in the $T_2$ generation and indicated transgene stability. Lines AS1, AS3, AS4, AS6, S2, S7 and S10 had a transgene integration frequency of 1.00 (Figure 2.6; blue bars), however these lines, with the exception of AS6, S7 and S10 had a replicate number of less than six, since some plants did not grow (Figure 2.6; yellow bars). In lines AS14, AS16, AS23, AS26 and S9, four of six replicates had retained the transgene, whilst in lines S8 and S20 the transgene was present in five of six replicates (Figure 2.6). Lines were selfed to produce seed and $T_3$ plants were grown. Based on the growth success of these plants, three antisense (AS3, AS4 and AS8) and five sense (S2, S3, S7, S10 and S20) lines were used for the remainder of the study.

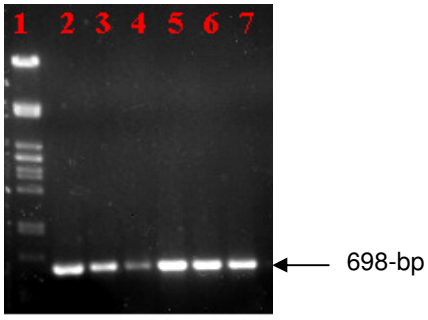


**Figure 2.5:** PCR amplification of a 698-bp region of the p811AS transgene in line AS6. Lane 1: Lambda DNA *Pst*I MWM. Lanes 2-7: Six replicate plants.

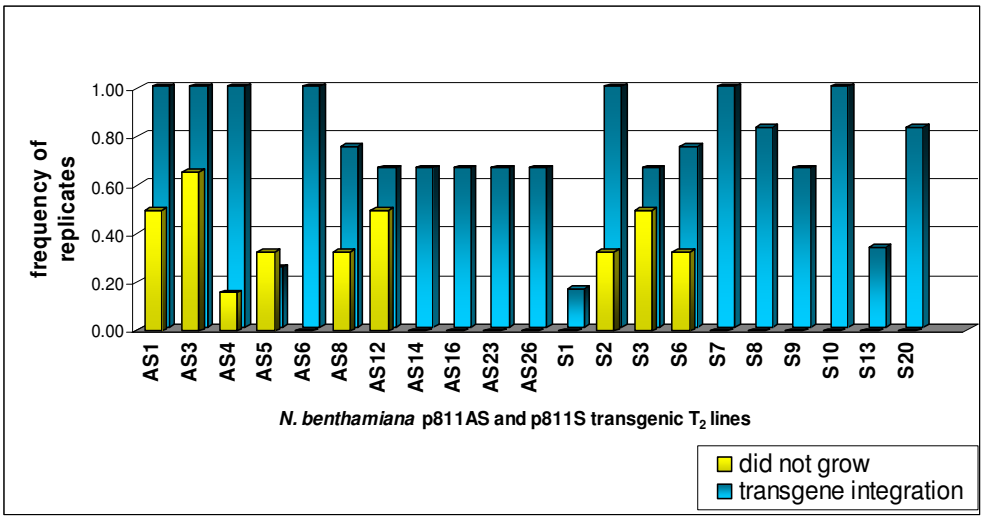


**Figure 2.6:** Transgene stability analysis of p811AS and p811S $T_2$ lines. Transgene integration was based on successful PCR amplification of a 698-bp region of the transgene. The blue bars show the frequency of plants with the transgene. N = 6 unless in the case of lines where some replicates did not grow, in which case the yellow bars represent the frequency of plants that did not grow per line.

### 2.3.2 Quantification of transgene RNA in $T_3$ lines

Transgene expression was confirmed and transgene RNA was quantified in newly emerged leaves of unchallenged $T_3$ plants (n=6) from lines AS3, AS4, AS8, S2, S3, S7, S10 and S20 using absolute real-time PCR. For RNA quantification purposes a standard curve was prepared (Figure 2.7), which was acceptable, according to manufacturer's recommendations, with respect to slope (-3.880) and error value (0.176) and the amplification efficiency of the standards was confirmed to equal that of the unknown samples (1.81).

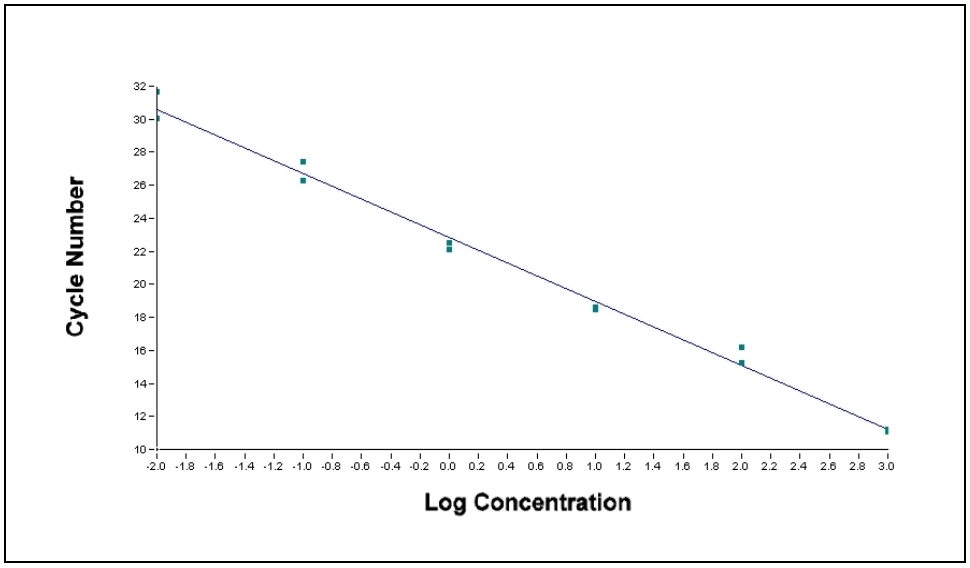


**Figure 2.7:** The standard curve generated by PCR amplification of DNA-A (p811F and p811R primers), used to quantify transgene RNA in p811S and p811AS lines.

Transgene expression was confirmed in all lines, with the amount of transgene RNA varying between lines, ranging from $10^{1}$ molecules/ng cDNA (line S20) to $10^{-4}$ molecules/ng cDNA (line AS8) (Figure 2.8). The amount of transgene RNA detected in S3 was significantly more than that detected in lines AS3, AS4, AS8, S2 and S7 ($p< 0.05$).

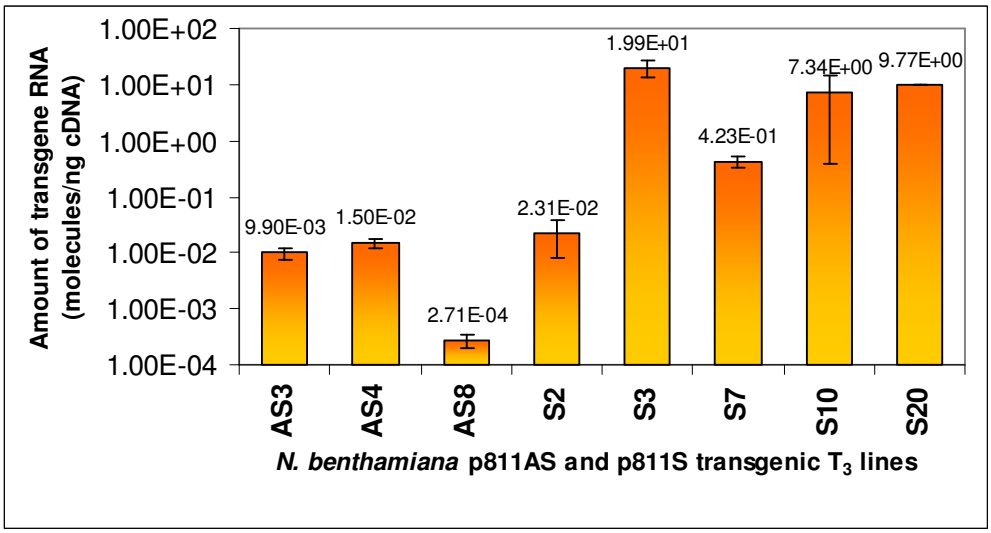


**Figure 2.8:** Quantification of transgene RNA in newly emerged leaves of *N. benthamiana* p811AS and p811S lines. cDNA was amplified by absolute real-time PCR. The amount of transgene RNA is expressed as molecules/ng cDNA and is the mean of six replicates per line. Non-transgenic control had a value of 0 molecules/ng cDNA. Error bars represent standard error of two quantification reactions. Y-axis is logarithmic scale.

### 2.3.3 Histochemical detection of β-glucoronidase in p811S lines

In the sense construct used in this study, the AC1 and *gusA* sequences are driven by the same CaMV 35S promoter. GUS assays were performed on leaves and stems from unchallenged p811S transgenic $T_3$ plants. The newly emerged leaves and stems of lines S3, S10 and S20 were completely blue, indicating a high level of GUS expression in these tissues. In addition, the fully expanded leaves of S10 had isolated blue spots around the leaf blade. GUS expression was minimal in line S7, confined to isolated spots around the edges of fully expanded leaves. Line S2 showed only slight traces of GUS expression, with occasional faint blue staining on the stem (Figure 2.9).

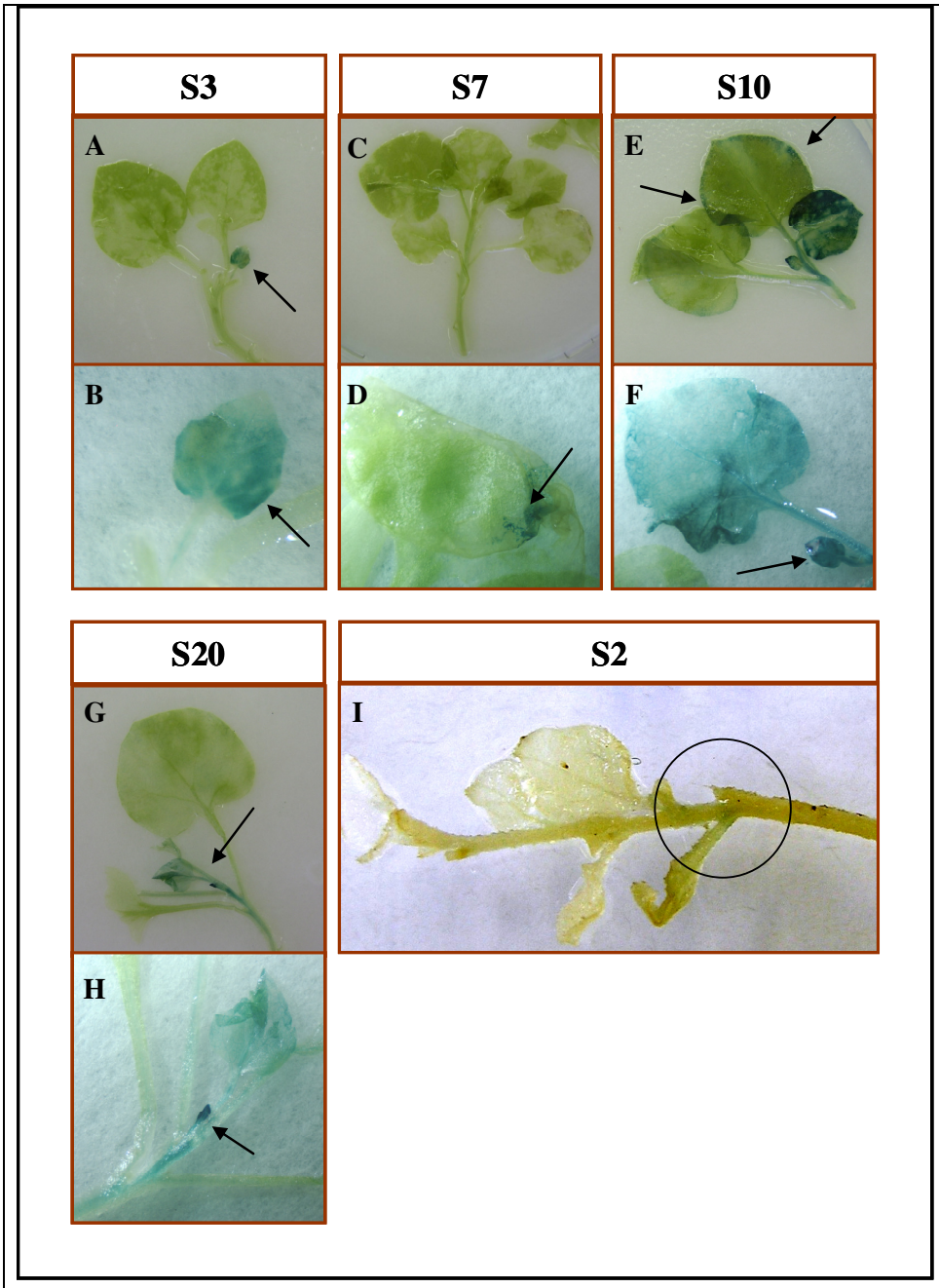


**Figure 2.9:** GUS assays of p811S lines. Leaves and stems from five transgenic lines were assayed for GUS activity. Panels A, B, F, G and H: completely blue newly emerged leaves from lines S3, S10 and S20. Panel C: no GUS expression in S7 except for isolated blue spots around the edges of fully expanded leaves (panel D). Panel E: Isolated blue spots around edges of S10 fully expanded leaves. Panel I: Very faint blue staining on stem of S2.

### 2.3.4 Sequence analysis of the AC1 region of the p811S transgene

Total nucleic acid was isolated from S2 and S20 transgenic plants, and the AC1 region of the p811S transgene was successfully amplified by PCR (data not shown). The 1089-bp PCR amplicon was TA-cloned in the pTZ57R cloning vector (Fermentas) and sequenced with M13F and M13R primers. The sequences were aligned to obtain the full sequence for the AC1 region. The sense orientation was confirmed for both the S2 and S20 transgenic lines and the sequence was used to design probes for siRNA detection.

### 2.3.5 Detection of transgene derived siRNAs in unchallenged $T_3$ lines

A RNase protection assay based method was used to detect siRNAs in p811AS (AS3, AS4 and AS8) and p811S (S2, S3, S7, S10 and S20) lines. Two 32-nt $_{32}$P-labelled antisense RNA probes were made and hybridised to RNA isolated from unchallenged plants from each line. Two size classes of siRNAs of between 22-nt (minimum protected sequence) and 30-nt were detected with the AC1 probe in lines AS3, AS4, S2 and S20 (Figure 2.10). The exact size of the detected siRNAs was unable to be determined. The molecular weight marker used (Decade™ Marker System, Ambion) has bands of 10nt increments, the lowest being 10nt. This marker therefore does not allow for sizing within the increments. The minimal sequence protected from digestion by ribonuclease was 22-nt, which is the probe length excluding the T7 promoter sequence. Therefore the size classes could be estimated to between 22-nt and 30-nt (from the RNA marker). The large size class varied in intensity between the lines with AS4 and

S2 having more of this class of siRNA than AS3 and S20. One size class of siRNAs, corresponding to the smallest of the two size classes were detected in lines AS8, S3, S7 and S10 (Figure 2.10; AS8 shown). The AC4 probe was hybridised to lines S2 and S20 only, and one size class of siRNAs was detected in these lines (results not shown).

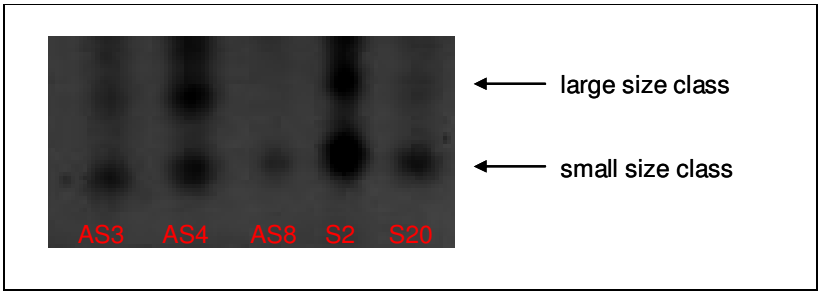


**Figure 2.10:** siRNA detection with $^{32}$P-labelled antisense AC1 probe. Two size classes of siRNAs were detected in lines AS3, AS4, S2 and S20, whilst only the small size class was detected in AS8. Molecular weight marker, nuclease-treated probe and untreated probe controls not included in figure,

### 2.3.6 Symptom severity in SACMV challenged in $T_3$ lines

Six replicate plants from eight $T_3$ transgenic lines (AS3, AS4 AS8, S2, S3, S7, S10 and S20) were challenged with SACMV DNA and monitored for symptom development until twenty eight days post infection (dpi). Symptom severity was scored using the six point symptom severity score (Fauquet and Fargette, 1988) at 7, 14 (data not shown) 21 (Table 2.2) and 28 dpi (data not shown). Typical symptoms for SACMV infection in *N. benthamiana* include reduction in leaf size, leaf curling, leaf yellowing and mosaic patterns on the leaves. Symptoms were absent in all replicates of S2 and S20 transgenic lines

(score = 0) upto 28dpi. At 21dpi, AS4, S7 and S10 scored 3, showing strong mosaic on leaflets and leaf deformation. Mild symptoms were recorded on S3 and AS3 at 21dpi, scoring 2. AS8 had severe leaf deformation (score 4) and the most severe symptoms (score = 5) were recorded on the non-transgenic control (Table 2.2). The unchallenged replicate from each transgenic line did not display symptoms upto 28dpi. Symptoms continued to develop on challenged plants and at 28 dpi, each line had increased by one symptom score, with the exception of S2 and S20, which remained symptomless.

### 2.3.7 Quantification of SACMV DNA-A in challenged $T_3$ lines

Leaf material was collected from SACMV-challenged transgenic plants at 21dpi and SACMV DNA-A replication was quantified by absolute real-time PCR. A standard curve was prepared (Figure 2.11), which was acceptable according to manufacturer's standards with respect to slope (-3.673) and error value (0.0952) and the amplification efficiency of the standards was confirmed to equal that of the unknown samples (1.87). Two sense lines (S2 and S20) showed a significant reduction ($p<0.05$) in viral DNA replication 21dpi ($10^0$ molecules/ng DNA) compared to the challenged non-transgenic control ($10^5$ molecules/ng DNA). There was no significant difference in the amount of viral DNA in the remaining lines compared to the control (Figure 2.12).

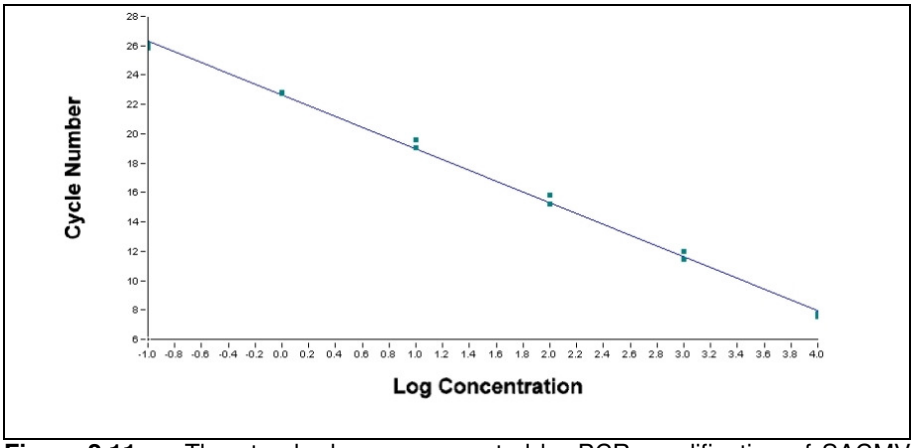


**Figure 2.11:** The standard curve generated by PCR amplification of SACMV DNA-A (AV1F, AV1R primers), used to quantify SACMV DNA-A

**Table 2.2:** **Symptom severity data for SACMV- challenged p811S and p811AS transgenic *N. benthamiana* lines. Symptoms score at 21 days post inoculation is shown.**

| Line | Symptoms (21dpi) | Symptom severity score |
|---|---|---|
| AS3 |  • reduction in leaf size and slight leaf curling | 2 |
| AS4 | strong mosaic; leaf deformation | 3 |
| AS8 |  •severe leaf deformation<br>• strong mosaic | 4 |
| S2 | no symptoms | 0 |
| S3 | Slight leaf deformation; mild chlorosis | 2 |
| S10 and S7 | strong mosaic; leaf deformation | 3 |
| S20 | no symptoms  | 0 |
| Non-transgenic control |  • severe leaf deformation<br>• severe mosaic | 5 |

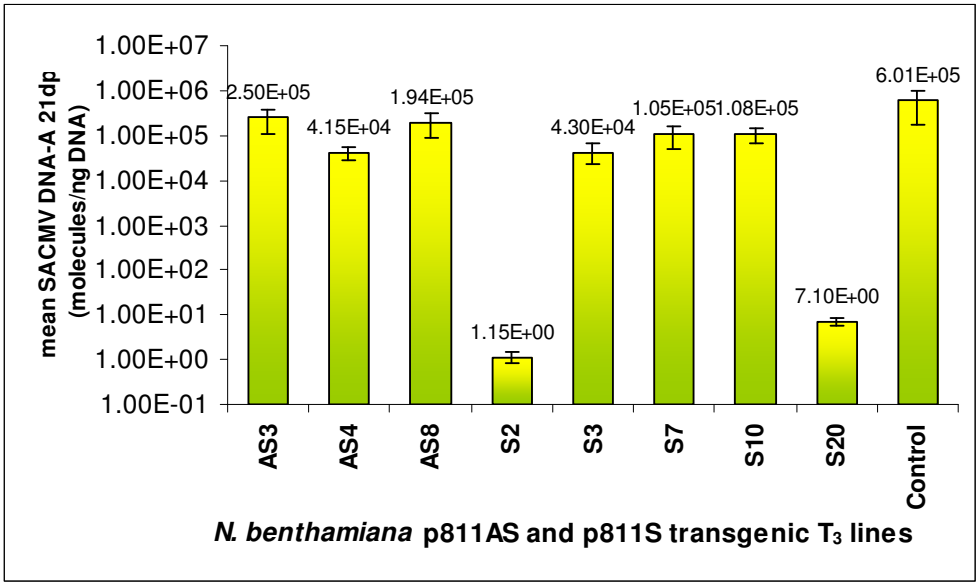


**Figure 2.12:** SACMV DNA-A quantified at 21dpi in SACMV-challenged *N. benthamiana* p811AS and p811S lines (n=6). Control - non-transgenic SACMV-challenged *N. benthamiana*. The amount of SACMV DNA-A is expressed as molecules/ng DNA and is the mean of six replicates per line. Error bars represent standard error of two quantification reactions. Y-axis is logarithmic scale.

### 2.3.8 Results summary

Two size classes of AC1 sequence origin siRNAs were detected in transgenic lines. The two SAMCV knockdown lines were unique amongst the sense lines in that they also had a large size class of siRNA. There was no correlation between the transgene transcript level and knockdown phenotype in the sense or antisense lines. When the transgene transcript and GUS levels were compared in newly emerged leaves of the sense lines, there was a positive correlation between transcript concentrations and GUS activity (Table 2.3).

**Table 2.3:** Summary of results obtained for *N. benthamiana* p811S and p811AS lines before and after challenging with SACMV DNA

| Line | GUS activity in newly emerged leaves[1] | GUS activity in fully expanded leaves and in stems[1] | Transgene transcript levels in newly emerged leaves[2] | siRNA detection[3] | | | SACMV replication knockdown[3] |
|---|---|---|---|---|---|---|---|
| | | | | AC1 probe | | AC4 probe | |
| | | | | Small size class | Large size class | | |
| S2 | _ | _ | + + | + | + | + | + |
| S3 | + + + + | _ | + + + + | + | _ | Not tested | _ |
| S7 | _ | + | + + + | + | _ | | _ |
| S10 | + + + + | + | + + + + | + | _ | | _ |
| S20 | + + + + | _ | + + + + | + | + | + | + |
| AS3 | Not applicable | | + + | + | + | Not applicable | _ |
| AS4 | | | + + | + | + | | _ |
| AS8 | | | + | + | _ | | _ |

1: + isolated blue spots around the leaf blade; ++++ completely blue leaf; - no activity detected

2: + lowest transgene transcript level; ++++ highest transgene transcript level

3: + present; - absent

## 2.4 DISCUSSION

In this study, the replication efficiency of SACMV DNA-A in challenged transgenic *Nicotiana benthamiana* plants, expressing truncated SACMV AC1 transcripts in the antisense (p811AS) or sense (p811S) orientation was investigated. Stable transgene integration was confirmed in ten sense and eleven antisense lines. Lines were selected and transgene expression was confirmed in five sense (S2, S3, S7, S10 and S20) and three antisense lines (AS3, AS4 and AS8), and these lines were subjected to further investigation.

Using an absolute real-time PCR method, the level of transcript RNA in newly emerged leaf tissue from unchallenged plants was quantified using AC1 specific primers. In sense transgenic tobacco lines S3, S10 and S20, the level of transgene transcripts in newly emerged leaves was higher (319-fold to 864-fold) than S2. There was only eighteen times more AC1 RNA in S7 than S2. Significantly more AC1 transgene RNA was present in S3 than AS3, AS4, AS8, S2 and S7. Statistical analysis of S10 was inconclusive due to high standard error between PCR reactions (n=2). RNA was isolated from pooled leaf tissue from six replicates per line. Each PCR result was therefore the average amount of transgene RNA per line. A direct comparison cannot be made between the levels of RNA expression in the sense and antisense lines since the construct design was different. The AC1 fragment was cloned in the antisense orientation in to pART27, whilst it was cloned in to pBI121 in the sense orientation. pBI121 has the glucuronidase gene sequence, whilst pART27 does not. It is however interesting to note that the level of AC1 RNA was consistently highly in the sense lines that in the antisense lines.

There was a clear correlation between the amount of transgene RNA and GUS activity in newly emerged leaves from the five sense lines. In the sense construct, *gusA* is positioned downstream of the AC1 fragment, with the two gene sequences being driven by one CaMV 35S promoter. Staining the newly emerged leaves of lines S3, S10 and S20 with X-Gluc resulted in completely blue tissue indicating a high level of *gusA* expression as expected since *gusA* and the AC1 sequence are on the same transcript. In these three sense lines GUS activity was absent to almost absent in older leaves on the same shoots, and is consistent with observations of cranberry leaves as GUS activity was found to be dependent on developmental and physiological state of the leaf tissue in cranberry (Serres *et al*., 1997). S2 had the lowest level of transgene RNA and the least visible GUS activity compared to the other sense lines. This is in line with the findings of Beltrán *et. al.* (2008) wherein lines with highest mRNA levels presented the highest intensities of GUS and visa versa.

Transgenic plants ($T_3$) from five sense (S2, S3, S7, S10 and S20) and three antisense (AS3, AS4 and AS8) lines were challenged by agro-inoculation with SACMV DNA-A and DNA-B. Symptom development was monitored and the same symptoms were recorded for all replicates in a particular line. Lines AS3, AS4, AS8, S3, S7 and S10 portrayed typical symptoms of SACMV infection at 21dpi, varying only with respect to the extent of leaf deformation. At 21dpi there was no significant difference in the amount of replicating SACMV DNA-A in these three antisense and three sense lines compared to the non-transgenic control. Two sense lines (S2 and S20) displayed no virus symptoms and showed a significant reduction in SACMV DNA-A replication ($p<0.05$) compared to the non-transformed control and other SACMV-challenged transgenic lines. This is the

first reported case of significant knockdown of SACMV replication in transgenic *N. benthamiana.*

Varying degrees of success have been achieved when using antisense-induced RNA silencing against geminiviruses. In an attempt to reduce replication of Tomato leaf curl New Delhi virus, Praveen *et al.* (2005) demonstrated that antisense technology could specifically suppress the expression of the virus. It is interesting that in studies where sense and antisense transgenes are compared, sense seemed to outperform antisense. Noris *et al.* (1996), when comparing *N. benthamiana* lines expressing sense or antisense 3' truncated *Tomato yellow leaf curl virus* C1 found that seven sense and three antisense transgenic lines showed no symptoms or detectable amounts of viral DNA. In four sense lines, 6-26% of plants were resistant whereas in the best performing antisense line, only 5% of plants were resistant. Similarly, sense lines performed better than antisense in studies conducted by Waterhouse *et al*. (1998). Our study shows some sense lines with resistance to SACMV, whilst other sense lines and all antisense lines did not show resistance. This study did not include the evaluation of transgene copy number, which could be an attributing factor to this phenomenon, as could the positioning of the transgene in the genome.

An RNA silencing mechanism is most likely responsible for the efficient disruption of SACMV DNA-A replication in S2 and S20 transgenic lines. Two size classes of transgene transcript derived siRNAs of between 22-nt and 30-nt were detected in unchallenged transgenic lines. The small size class was detected in all lines but the large size class was unique to the two knockdown lines (S2 and S20) and AS3 and AS4. The small size class of siRNAs was detected in S2 and S20 using an AC4 probe. Hamilton *et al*. (2002) first reported two size classes of

siRNAs, associated with local and systemic silencing of GFP in *N. benthamiana.* The 24-26nt siRNAs were as abundant as the 21-22nt size class in local silencing tissue, whereas in the systemic silencing tissue the longer siRNAs are much more abundant. Chellappan *et al.* (2004) detected siRNAs corresponding to these two size classes in uninfected leaf material transformed with a modified full-length *African cassava mosaic virus AC1* transgene when probing with a full-length AC1 sequence. Similarly, in this study, we have detected two size classes of siRNAs in unchallenged transgenics expressing an untranslatable SACMV AC1 transcript. Contrary to these findings, Zhang *et al.* (2005) did not detect any siRNAs in uninfected cassava plants expressing ACMV antisense RNA.

The low amount of transgene RNA and the lack of GUS expression in S2 could be a result of a small interfering RNA inducing the degradation of all homologous transcripts, including the transgene transcript itself. It is noteworthy that the two knockdown lines had vastly different amounts of transgene RNA, possibly correlated to the amount of siRNAs being produced, yet both lines effectively reduced SACMV DNA-A replication. The target region of the p811S and p811AS constructs includes the complete AC4 ORF. The presence of AC4 protein was not confirmed, however siRNAs of AC4 sequence identity were detected in transgenic lines S2 and S20 (other lines were not tested). This implies degradation of the AC4 transgene RNA, preventing translation.

The size class of siRNA produced from the cleavage of long perfect dsRNAs depends on the Dicer-like protein to produce siRNAs of 21-22-nt and 24-nt. The smaller of the two size classes is associated with mRNA degradation, whilst the 24-nt size class is responsible for RNA-dependent DNA methylation and histone modification (Blevins *et al.*, 2006). The production of two size classes

of siRNAs indicates that at least two RNA silencing pathways are involved in the two sense knockdown lines, but not in the sense lines that did not show reduction in SACMV replication. Interestingly, only the small siRNAs were generated in the knockdown lines from the region of the transcript overlapping the AC4 ORF. Although none of the antisense lines tested here showed knockdown capability, siRNAs were detected in these lines. Two size classes were detected in AS3 and AS4 whilst the smaller size class was detected in AS8, presenting a correlation in that AS8 portrayed more severe symptoms at 21 dpi than either AS3 or AS4.

Plant hosts have evolved antiviral RNA-silencing mechanisms and plant viruses are now known to express proteins that act as suppressors of RNA-silencing (Roth *et al*., 2004). The first geminivirus silencing suppressor to be identified was the AC2 protein (Butaye *et al*., 2005). A study of the synergistic interaction between two cassava mosaic viruses identified a second geminivirus silencing suppressor protein. A mixed infection of *East African cassava mosaic Cameroon virus* and African cassava mosaic virus [Cameroon]) results in severe cassava mosaic disease and was shown to involve the DNA-A components of both viruses. It was revealed that the *East African cassava mosaic Cameroon virus* AC4 protein acts as a silencing suppressor (Vanitharani *et al*., 2004).

The 811-bp AC1 sense construct used in this study to effectively reduce SACMV infection in *N. benthamiana* induced presumptive siRNAs complementary to the AC4 ORF of SACMV in lines S2 and S20. The AC4 protein is a strong silencing suppressor in recovery-type viruses (Vanitharani, *et al*., 2004). As yet no in-depth studies have been done on SACMV to determine whether it behaves as a recovery- or non-recovery-type virus in either *N. benthamiana* or cassava. However, based on observations in our lab, initial

indications are that SACMV is a recovery-type virus, and if this is indeed true, degradation of the SACMV AC4 transcript via the production AC4 transgene-derived siRNAs may explain the highly effective knockdown of SACMV replication in these two lines when challenged with SACMV resulting in a symptomless phenotype.

The mechanism by which single-copy transgene inserts producing sense transcripts can trigger PTGS (S-PTGS) is becoming clearer. Transgene transcripts with abnormal features, such as the lack of 5' capping or a poly(A) tail, can be recognised by an RNA-dependent RNA polymerase (RDR6 in *Arabidopsis*) and converted into dsRNA. Although uncapped mRNAs are usually degraded by 5'-3' exonucleases, it has been shown that they can accumulate in the absence of exonuclease activity (Brodersen and Voinnet, 2006). Therefore the reaction to an invading virus is immediate in transgenics with a sense transcript, since transgene derived siRNAs can be present in the cell prior to infection as shown in this study. The currently accepted model requires an antisense transgene transcript to wait for a complementary mRNA from the invading virus to generate dsRNA and induce the RNA silencing pathway (Serio *et al*., 2001), therefore eliciting a slower response. However, our study shows antisense transgene derived siRNAs are present prior to infection.

This study demonstrates the potential of sense transgene mediated RNA silencing to protect against SACMV infection in cassava. Eight transgenic *N. benthamiana* lines were challenged with SACMV DNA-A and DNA-B. Viral replication was significantly reduced in two lines expressing untranslatable SACMV AC1 sense transcripts, to levels where no disease symptoms were visible on infected plants upto 28dpi. Sense transgene mediated RNA silencing

relies on an endogenous RdRP to make a dsRNA molecule from the ssRNA transgene transcript, thereby triggering RNA silencing pathways (Dalmay, 2000). In *Arabidopsis*, gene silencing using inverted repeat–PTGS (IR-PTGS) has been shown to be a more efficient inducer of RNA silencing than S-PTGS (Vaucheret, 2006). However, the design and cloning of invert-repeat constructs can be problematical due to inherent genetic instability resulting from the formation of four-way helical junctions, known as a cruciform structure (Duckett *et al*., 1988). A successful sense-induced RNA silencing strategy, such as the one adopted in this study can be applied in cassava.

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# CHAPTER 3 - Minimal cassette technology to test the efficacy of invert-repeat constructs to reduce SACMV DNA-A replication

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Rey, M.E.C., Salman, Z., Makwarela, M., Berrie L.C., van Rooyen, S.L. and Taylor, S.H. (2003) Development of pathogen-derived resistance strategies for begomoviruses in cassava. *8th International Congress of Plant Pathology*, Christchurch, New Zealand, p. 243. (paper)

Taylor, S.H. and Rey, M.E.C. (2004) A *South African cassava mosaic virus* resistance strategy using a dsRNA approach. *4th International Geminivirus/ 2nd International ssDNA comparative Virology Symposium*, Cape Town, South Africa, p. P1-2. (poster)

## 3.1 ABSTRACT

Two *South African cassava mosaic virus* (SACMV) truncated AC1 invert-repeat (IR) constructs were produced using the pHannibal gene-silencing vector system but due to the perfectly-matched nature of the invert-repeat sequence, attempts to sub-clone the IR constructs into a binary vector for plant transformation failed. This allowed for the exploration of minimal cassette technology, and a technique was developed to test the efficacy of the IR constructs in a *Nicotiana benthamiana* transient system, using minimal cassette technology and particle bombardment. Two minimal gene expression cassettes containing SACMV AC1 invert-repeat sequences with a spliceable intron were produced and particle bombardment cartridges were prepared for each minimal gene expression cassette. *N. benthamiana* callus was (i) co- bombarded with the minimal gene expression cassettes and SACMV DNA-A and DNA-B dimers or (ii) bombarded with the cassettes and then after 48 hours bombarded with SACMV DNA-A and DNA-B dimers. Replicating SACMV DNA-A was quantified using absolute real-time PCR four days after challenging. There was a 60-80% reduction in replicating SACMV DNA-A in callus tissue that was co-bombarded with either of the AC1 minimal gene expression cassettes compared to the control. There was no significant reduction in the amount of replicating SACMV DNA-A when the minimal gene expression cassette and SACMV DNA were bombarded separately. This study can be furthered by testing the constructs in a stable expression system in *N. benthamiana* or cassava regenerated from bombarded callus.

## 3.2 INTRODUCTION

### 3.2.1 Background

Until recently, the extent of vector backbone sequence integration in transgenic plants was underestimated. *Agrobacterium*-mediated transformation relies on the left and right border sequences for T-DNA transfer. T-DNA transfer was assumed to be initiated at the right border and terminated at the left border until it was documented that sequences beyond the T-DNA left and right borders were being integrated into the host's genome. In 1997, Kononov *et. al.* reported 75% of transgenic tobacco plants, produced by *Agro*-mediated transformation contained vector backbone sequences. These backbone sequences were transferred into the plant genome either independently of or linked to the T-DNA left or right borders, thus dispelling the idea that only the sequence between the T-DNA borders is integrated. Other accounts show that the extent of integration varies widely and the crops that are affected are numerous. Lange *et al.* (2006) obtained 48% of transgenic barley lines that contained vector backbone sequences whilst Abdal-Aziz *et al.* (2006), Zhang *et al.* (2008) and Sahab *et al.*, 2008 found that 65.7% of transgenic strawberry lines, 31% of transgenic cotton and 60-80% of cassava transformants, respectively, contained vector backbone integrated sequence. In each case, the crop was transformed by *Agro*-mediated transformation. De Buck *et. al.* (2000) proposed that read-through at both borders was due to inefficient recognitions of the borders as initiation and termination sites. This was confirmed by the finding that two or three copies of the left border are efficient at preventing read-through and integration of vector-derived sequences (Kuraya *et. al.*, 2004). With the public demand being for superfluous,

foreign DNA free transgenic plants, minimal cassette technology has become recognized as an important transformation tool.

Minimal cassette technology (MCT) employs the use of a minimal gene expression cassette comprised only of promoter, open reading frame and terminator sequences for transformation. Transformation is achieved by particle bombardment and results in stable integration of the transgene into the host genome (Kumar *et al.*, 2006). Since the technology makes use of a direct gene transfer technique, vector backbone sequences are not required. Selection of transgenics is achieved by the co-bombardment of two gene cassettes, one carrying the gene of interest and the second carrying the selectable marker gene of choice. Since the two genes are integrated at difference loci in the host genome, this allows for the selectable marker gene to be removed by segregation in some plant species (Kumar *et al.*, 2006).

The use of minimal cassettes in plant transformation was first reported by Fu *et al*. (2000). In co-transformation experiments of selectable (*hpt*) and non-selected (*gusA*) cassette, co-integration and co-expression of co-bombarded cassettes and stable transgene expression was confirmed and no transgene silencing was evident for four generations. MCT has successfully been used to transform rice (Fu *et al.*, 2000; Loc *et al.*, 2002), potato (Romano *et al.*, 2003), wheat (Yao *et al.*, 2006) and grapevine (Vadal *et al*, 2006). Furthermore, bombarding regenerable tissue with minimal cassettes can lead to transformed (stable expression) whole plants (Fu *et al.*, 2000; Vidal *et al*, 2006; Yao *et al.*, 2006)

The pHannibal vector allows for the construction of RNA silencing constructs. A PCR product from a gene of interest is directionally cloned into polylinkers in the sense orientation (*Xho*I.*EcoR*I.*Kpn*I polylinker) and in the antisense orientation (*Cla*I.*Hind*III.*BamH*I.*Xba*I polylinker) either side of a spliceable intron (Figure 3.1). Gene fragment length of 300-bp to 600-bp was recommended for maximum silencing efficiency (Helliwell *et al*., 2003). The process of intron-splicing is thought to align the arms of the hpRNA, thereby increasing the efficiency of hpRNA production. Double-stranded RNA is produced with a loop of 30-50-bp depending on the restriction sites used during cloning. The invert-repeat sequence is driven by a constitutive 35S promoter (Wesley *et al*., 2001).

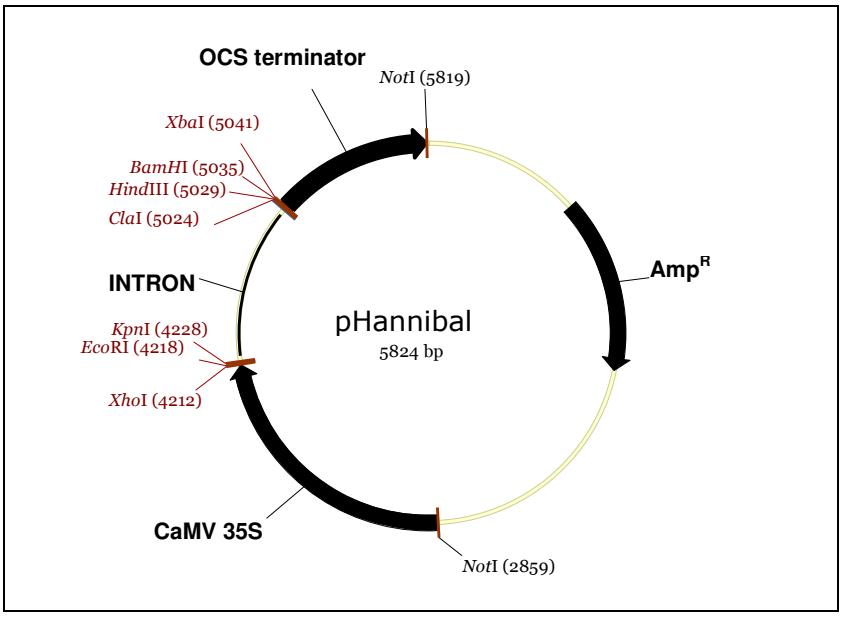


**Figure 3.1:** A schematic representation of the pHannibal gene-silencing vector (accession number AJ311872). The vector includes a ~700-bp spliceable intron, a CaMV 35S promoter, the ocs terminator sequence and ampicillin resistance gene ($Amp^R$).

### 3.2.2 Target region

The SACMV AC1 transcript was targeted for RNA silencing in this study. A 572-bp and 193-bp region at the N-terminus of the AC1 sequence was used to generate two invert-repeat constructs. Both regions overlap the 5' end of the AC4 ORF (Figure 3.2). AC4 is a silencing suppressor (Vanitharani, *et al.*, 2004) and it is predicted that inclusion of a silencing suppressor sequence in a RNA silencing construct can reduce transgene variation between transgenic lines caused by RNA silencing (Butaye *et al*, 2005). The fragment lengths were selected based on available restriction sites. Two invert-repeat constructs with different arm length were used to determine the effect of arm length on silencing efficacy. An Invert-repeat of arm length 20-bp can induce RNA silencing, however the chance of off-target silencing is significantly increased with shorter IR constructs.

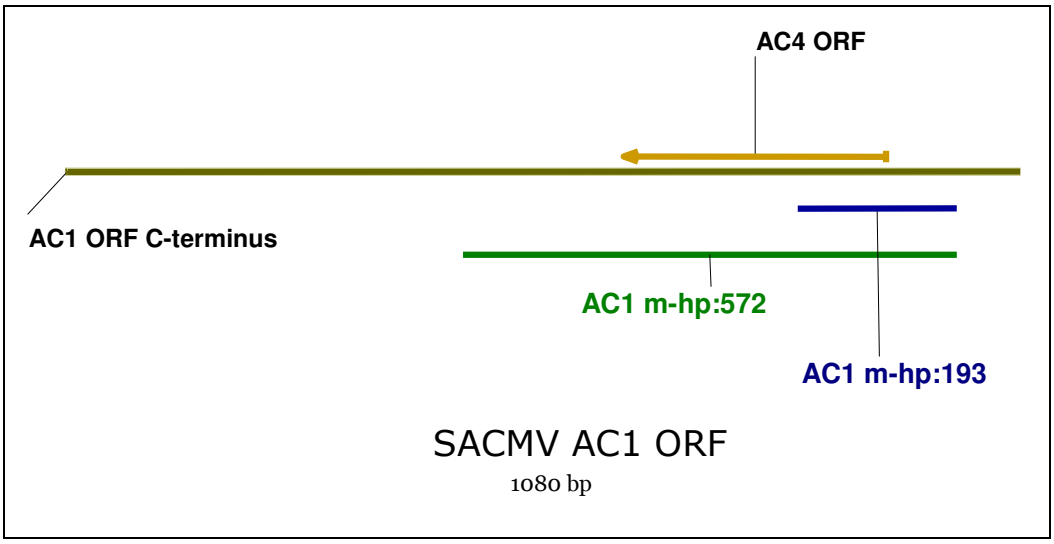


**Figure 3.2:** A schematic representation of a portion of the SACMV AC1 ORF (AF155806.1 nts 1583-2647) overlapping showing the target region for the invert-repeat constructs generated in this study (AC1 m-hp:572 and AC1 m-hp:193).

### 3.2.3 Objective and aims

The objective of this study was to generate two invert-repeat constructs, using the pHannibal gene-silencing vector, to induce RNA silencing of SACMV in *Nicotiana benthamiana*. The constructs would produce a dsRNA molecule with arm length of 572-bp or 193-bp on expression. As the invert-repeat constructs could not be sub-cloned into a plant transformation vector, it was decided to use the constructs as minimal gene expression cassettes and introduced them into *N. benthamiana* callus by particle bombardment. The specific aims (Figure 3.3) were to:

1. produce two invert-repeat constructs that differed in arm length for comparative purposes,
2. introduce each construct into *N. benthamiana* callus tissue by particle bombardment and
3. challenge callus tissue with SAMCV DNA-A and DNA-B and quantify replicating SACMV DNA-A .

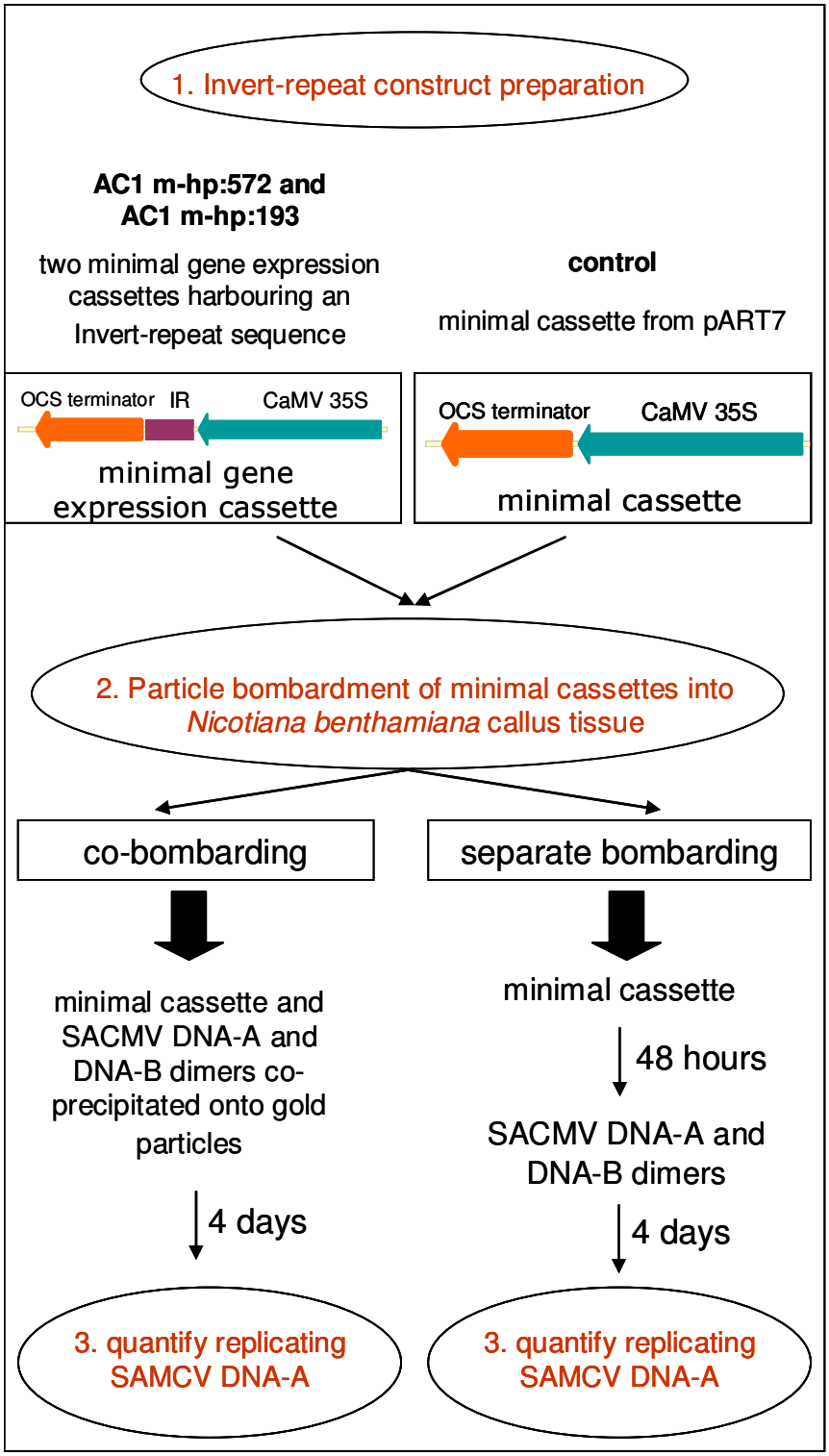


**Figure 3.3:** An outline of the methods used to fulfill the aims of this study.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Invert-repeat construct preparation

The pHannibal gene-silencing vector was used to generate invert-repeat constructs with a 742-bp intron. A 583-bp region of SACMV AC1 ORF was PCR amplified (MyCycler, BioRad). PCR reactions were set up according to manufacturer's recommendations (Expand High Fidelity PCR System; Roche Biochemicals). Inclusion of restriction enzyme sites at the 5' end of the PCR primers (Table 3.1) was to allow for the downstream directional cloning of amplified viral DNA into the pHannibal MCS in the sense and antisense orientations, either side of the intron. A step-wise PCR profile was used to amplify the region of interest from SACMV DNA-A in pBlueScript (kindly donated by L.C. Berrie); 94°C for 2 minutes; 2 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 2 minutes; 2 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 2 minutes; 26 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 2 minutes; final extension at 72°C for 7 minutes.

**Table 3.1:** PCR primer pairs used to amplify a 583-bp region of the SACMV AC1 ORF. [1]

| Primer set | Primer | Sequence |
|---|---|---|
| **Set A** | pHanRep***Xho*I**<br>pHanRep***EcoR*I** | 5' cctcgaggtactcggtctccatggcc<br>5' ggaattccactctctcgaaagaagcgg |
| **Set B** | pHanRep***BamH*I**<br>pHanRep***Cla*I** | 5' cggatccgtactcggtctccatggcc<br>5' catcgatgactctctcgaaagaagcgg |

.

[1] The inclusion of restriction sits allowed for directional cloning into the pHannibal vector. Underlining identifies nucleotides that anneal to the template DNA.

Directional cloning of the PCR amplified viral DNA fragments into the pHannibal vector failed and a blunt-end sub-cloning strategy was employed. The DNA fragment obtained using primer set A (Table 3.1) was prepared for blunt-end cloning by gel purifying the fragment from a 1% agarose gel. The purified fragment was blunt-end polished with Klenow enzyme (Roche Biochemicals) and phosphorylated with T4 Polynucleotide Kinase (Roche Biochemicals). pHannibal vector DNA was linearised with *EcoR*I, blunt-ended with Klenow enzyme (Roche Biochemicals) and dephosphorylated with CIAP (Roche Biochemicals). All reactions were set up according to manufacturer's recommendations. The prepared DNA fragment and vector DNA were ligated with T4 DNA ligase (Roche Biochemicals) at 25°C for 18 hours. *E. coli* DH5α was transformed with the resulting circularised DNA and transformants were selected for on Luria Agar supplemented with ampicillin (100µg/ml). Putative clones were screened for by double digestion with *EcoR*I and *Xho*I (Promega). Positive clones were subsequently screened for the correct orientation of the insert by digestion with *Pst*I (Promega) to yield 1630-bp and 4785-bp fragments (Figure 3.4). Selected clones (pHannibal:AC1 sense) harbouring the insert in the correct orientation were sequenced (Inqaba Biotech, South Africa) with the ST2 sequencing primer (5' cta tcc ttc gca aga ccc; Figure 3.4) and aligned with the SACMV sequence (AF155806) using DNAMAN ((Version 4.0; Lynnon BioSoft).

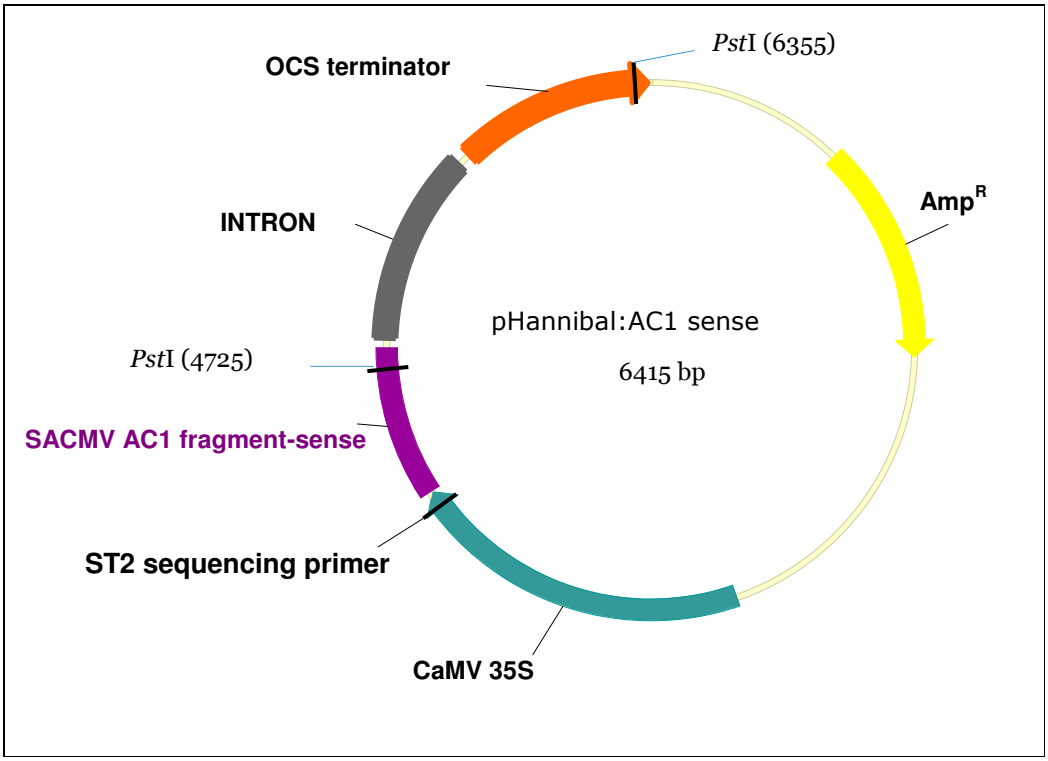


**Figure 3.4:** The pHannibal gene-silencing vector harbouring the SACMV derived AC1 fragment in the sense orientation.

To sub-clone the SACMV AC1 fragment in the antisense orientation, the DNA fragment obtained from PCR with primer set B (Table 3.1) was prepared for blunt-end cloning as previously described. The pHannibal clone containing AC1 fragment in the antisense orientation was linearised with *BamH*I, rendered blunt-end and dephosphorylated as previously described. The DNA fragment and vector DNA were ligated with T4 DNA ligase (Roche Biochemicals) at 25℃ for 18 hours. *E. coli* DH5α was transformed with the resulting circularised DNA and transformants were selected for on Luria Agar supplemented with ampicillin (100µg/ml). Putative clones were screened for presence of the insert and correct orientation by *Pst*I digestion to yield 4785bp, 1226bp and 984bp fragments (Figure 3.5). Selected clones (pHannibal:AC1 m-hp) were sequenced (Inqaba

Biotech, South Africa) with the ST1 sequencing primer (5'gat aga tca tgt cat tgt g; Figure 3.5) and aligned with the SACMV sequence (AF155806) using DNAMAN (Version 4.0; Lynnon BioSoft).

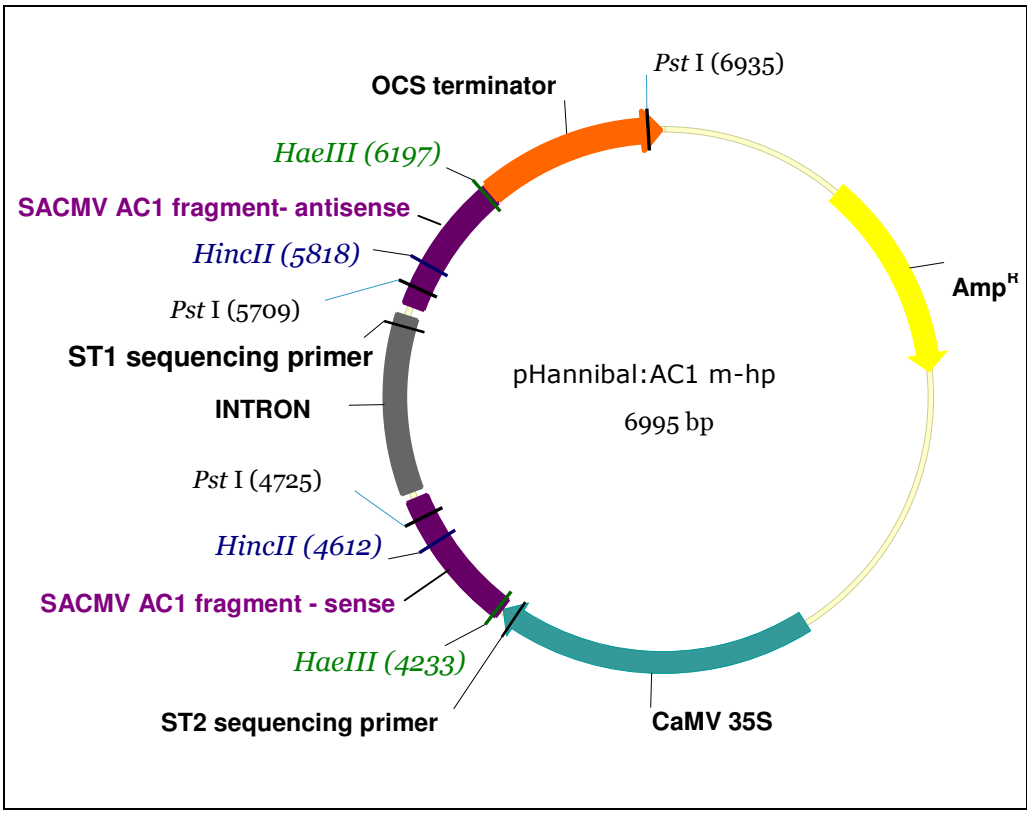


**Figure 3.5:** The pHannibal gene-silencing vector harbouring the SACMV derived AC1 fragment in the sense and antisense orientations either side of the intron to create an invert-repeat construct.

### 3.3.2 Invert-repeat constructs with different arm lengths

pHannibal:AC1 m-hp plasmid was digested with *Hae*III (Promega) to generate a invert-repeat construct with a 572-bp arm length (Figure 3.5). A

second invert-repeat construct with a 193-bp arm length was generated from the pHannibal:AC1 m-hp clone by restriction with *Hinc*II (Figure 3.5). The respective fragments (1964bp or 1206bp) were agarose gel purified, rendered blunt-end and dephosphorylated as described in section 3.3.1. The invert-repeat fragments were ligated (T4 DNA ligase; Roche Biochemicals; 25°C for 18 hours) with *Sma*I linearised pART7 vector DNA to generate expression cassettes (promoter: IR sequence: terminator). *E. coli* DH5α was transformed with the resulting circularised DNA and transformants were selected for on Luria Agar supplemented with ampicillin (100µg/ml). Putative clones were screened for the presence of the insert with *Pst*I digestion and sequenced (Inqaba Biotech, South Africa) with a sequencing primer designed to anneal to the MCS of pART7 (5' ccc act atc ctt cgc aag). Attempts to sub-clone the expression cassettes into pCambia plant transformation vector (CAMBIA, Australia) failed. The constructs were used to generate minimal gene expression cassettes.

### 3.3.3 Preparation of minimal gene expression cassettes for particle bombardment

DNA was isolated from clones pART7:AC1 m-hp:572 and pART7:AC1 m-hp:193 (High Pure Plasmid Isolation Kit; Roche Biochemicals) and digested with *Msc*I and *Pvu*I and the respective DNA fragments, as indicated in Table 3.2, were gel-purified from 1% agarose gels (Gel purification kit, Qiagen). The resulting minimal gene expression cassettes are illustrated in Figure 3.6. The DNA was used to coat gold particles for delivery into plant tissue by particle bombardment.

**Table 3.2:** Vectors or clones and respective restriction enzymes used to generate DNA fragments for particle bombardment.

| Vector | Restriction enzyme/s | Gel-purified fragment |
|---|---|---|
| pART7 only (control) | *Msc*I and *Pvu*I | 2363-bp |
| pART7:AC1 m-hp:572 | *Msc*I and *Pvu*I | 4327-bp |
| pART7:AC1 m-hp:193 | *Msc*I and *Pvu*I | 3569-bp |

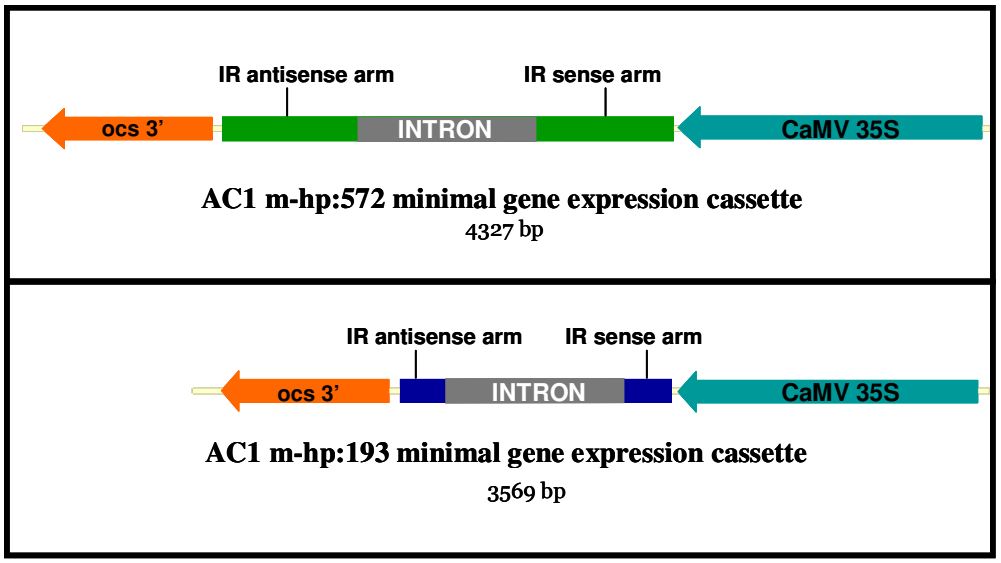


**Figure 3.6:** Schematic representation of minimal gene expression cassettes used for particle bombardment

### 3.3.4 *N. benthamiana* callus induction

*Nicotiana benthamiana* seeds were germinated and seedlings were grown in tissue culture bottles on Murashige and Skoog (MS) medium plus 3% sucrose and 0.6% agar (pH 5.8). One $cm^2$ leaf discs were cut from newly expanded tissue culture leaves and placed on MS medium plus 1% sucrose and 0.5% agar supplemented with 50µM picloram (Sigma-Aldrich) and incubated at 28℃ for a

twelve hour photoperiod in a growth chamber (Binder) to initiate callus. Callus tissue was sub-cultured every two weeks.

### 3.3.5 Pre-bombarding preparation of callus tissue

Six week old *N. benthamiana* callus tissue was sub-cultured four days prior to bombarding. Pieces of callus of approximately 5mm diameter were placed on MS medium plus 1% sucrose and 0.5% agar, supplemented with 50µM picloram (Sigma-Aldrich) and incubated at 28℃ for a twelve hour photoperiod in a growth chamber (Binder). Four hours prior to bombarding, callus tissue was transferred to osmotic medium (MS plus 1% sucrose, 200µM D-mannitol, 50mg/L myo-insitol, 50 µM picloram and 0.5% agar) in petri plates and incubated in the dark at room temperature. The medium was covered with a piece of sterile filter paper and eight pieces of callus were placed in a circle (<60mm diameter) in the center of each plate on the filter paper.

### 3.3.6 Particle bombardment cartridge preparation

The Helios™ Gene Gun System (BioRad) was used to deliver the minimal gene expression cassettes and SACMV DNA into the callus tissue. SACMV DNA-A and DNA-B dimers in pBIN19 (kindly donated by L. Berrie) were co-precipitated (0.15µg DNA-A/shot; 0.15 µg DNA-B/shot) together with one of the minimal gene expression cassettes (AC1 m-hp:572 or AC1 m-hp:193; 0.25µg/shot), or the pART7 minimal cassette for the control (0.25µg/shot) For the

separate delivery of a minimal gene expression cassette and SACMV DNA, separate cartridges were prepared with either minimal gene expression cassette DNA (0.25µg/shot), pART7 minimal cassette (0.25µg/shot) or SACMV DNA dimers in pBIN19 (0.15µg DNA-A/shot; 0.15 µg DNA-B/shot). Gold particles were 0.6µm in size and 0.25mg gold was used per shot. All cartridges were prepared according to Helios™ Gene Gun System Instruction Manual.

### 3.3.7 Co-bombardment of minimal gene expression cassettes and SAMCV DNA

Plastic covers, 80mm in height and 60mm in diameter with a 35mm hole in the center to accommodate the spacer of the gene gun were sterilized in 0.35% sodium followed by 100% ethanol. For each bombarding event, a plastic cover was placed over the pre-prepared calluses to prevent the calluses from being lost by the force of the shot (Carsono *et al.*, 2008). Each minimal gene expression cassette was co-bombarded with SACMV DNA-A and DNA-B dimers in pBIN19, co-precipitated onto gold particles. The pART7 minimal cassette co-precipitated with the SACMV dimers served as the control. Four sets of eight calluses were bombarded once for each minimal gene expression cassette using 200psi helium pressure under sterile conditions. After bombarding, calluses were incubated for 16 hours at 25℃ in the dark, following which they were transferred to callus medium, pH 5.8 (MS medium plus 1% sucrose and 0.5% agar, supplemented with 50µM picloram (Sigma-Aldrich)) and incubated at 28℃ for a twelve hour photoperiod in a growth chamber (Binder) for a further four days.

### 3.3.8 Separate bombarding of minimal gene expression cassettes and SACMV DNA

Four sets of eight calluses were bombarded as described in section 3.3.7 with each minimal gene expression cassette only. The pART7 expression cassette served as the control. After bombarding, calluses were incubated on osmotic medium (section 3.3.5) for forty-eight hours and then bombarded with SACMV DNA–A and DNA-B dimers in pBIN19 co-precipitated onto gold particles. Calluses were then incubated for 16 hours at 25℃ in the dark, following which they were transferred to callus medium, pH 5.8 (MS medium plus 1% sucrose and 0.5% agar, supplemented with 50µM picloram (Sigma-Aldrich)) and incubated at 28℃ for a twelve hour photoperiod in a growth chamber (Binder) for a further four days.

### 3.3.9 Viral load quantification

**DNA isolation from bombarded callus tissue**

DNA was isolated from bombarded callus tissue using a method adapted from Doyle and Doyle (1987). Eight pieces of callus (one bombarding event) were pooled and pulverised in a 2.0ml microfuge tube using a pestle. For DNA isolation 0.5ml 65℃ extraction buffer (2% CTAB, 20mM EDTA, 1.4M sodium chloride, 100mM Tris pH 8.0) and 1µl β-mercaptoethanol was added to the pulverised tissue. The mixture was heated at 65℃ for 30 minutes, after which total nucleic acid was extracted twice using 0.5ml chloroform:isoamyl alcohol (24:1), precipitated with isopropanol and pelleted by centrifugation (14000rpm, 4℃). Finally, the nucleic acid pellet was washed in 70% ethanol, dried in a vacuum drier and resuspended in 100 µl TE buffer (10mM Tris pH 8.0, 1mM

EDTA) supplemented with 0.1mg/ml RNase A (Fermentas). DNA was quantified using The NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). One microgram of each DNA sample was digested with *Dpn*I (Fermentas), an enzyme that requires the presence of N6-methyl-adanine within the recognition sequence to cleave DNA. Since non-replicating viral DNA is methylated, only replicating viral DNA and not input viral DNA will be present in the sample after *Dpn*I restriction. After digestion, each sample was purified using the PCR Purification Kit (Roche Biochemicals)

**Quantification of replicating SACMV DNA-A in bombarded callus**

The amount of viral DNA present in bombarded callus tissue 4 days post bombardment with SACMV DNA-A and DNA-B was quantified in duplicate by absolute real-time PCR. A region of the AV1 ORF of SACMV DNA-A was amplified using SYBRGreen fluorescent dye (LightCycler FastStart DNA Master$^{PLUS}$ SYBR Green I kit, Roche Biochemicals) with the LightCycler 1.5 Instrument (Roche). Reactions were set up according to manufacturer's instructions to which 6µl of purified DNA and 2µM of each PCR primer (AV1F: 5' gcacaagcgtcgat; AV1R: 5' ctgccagtatgcttaacgtca) was added. The following PCR conditions were used to amplify the 154-bp fragment: 5 min at 95ºC; 40 cycles of 5 sec at 95ºC, 10 sec at 60ºC and 10 sec at 72ºC.

For quantification purposes, a standard curve was generated by amplification of SACMV DNA-A in pBluescript (kindly donated by L.Berrie). Serial concentrations of DNA-A (1000pg, 100pg, 10pg, 1pg, 0.1pg) were amplified in duplicate by real-time PCR using SYBRGreen fluorescent dye (LightCycler FastStart DNA Master$^{PLUS}$ SYBR Green I kit, Roche Biochemicals) with the

LightCycler 1.5 Instrument (Roche). The same cycling conditions and primer concentrations were used as for the samples. The amount of transgene RNA per sample (pg) was calculated from the standard curve, and expressed as molecules/ng DNA using the following equation, based on the assumption that one plasmid (DNA-A in pBS) has one copy or molecule of DNA-A therefore if 100ng plasmid DNA = 0.026pmol plasmid then using Avagadro's number, 1pg plasmid DNA = $1.56 \times 10^5$ molecules:

$$\text{molecules/ng DNA} = \frac{[\text{calculated concentration (pg)} \times 1.56 \times 10^5 \text{ molecules}]}{\text{DNA added to PCR reaction (ng)}}$$

Data was analysed using Microsoft Excel and GraphPad InStat 3.01 software (GraphPad Software, Inc., San Diego, CA). Standard errors of the means were calculated for four bombardment events.

## 3.4 RESULTS

### 3.4.1 Invert-repeat construct

Attempts to directionally clone the sense and antisense fragments into the pHannibal vector failed with the double digestion steps being the most problematic. Cloning then shifted to a blunt-end strategy with the first attempt to blunt-end clone the SACMV AC1 fragment in the sense orientation in the pHannibal vector being successful. Blunt-end cloning is not ideal as it adds an extra screening step, but in this case eight clones were obtained with insert (twenty screened), two with the insert in the correct orientation. Attempts to sub-clone the SACMV AC1 fragment in the antisense orientation were more challenging. Some time was spent optimising the dephosphorylation step since the early results indicated preferential re-ligation of vector DNA. Ultimately seven of eleven clones screened had insert in the correct orientation. A pHannibal clone containing the sense and antisense inserts (pHannibal:AC1 m-hp) was used to generate invert-repeat constructs with shorter arm lengths therefore losing the promoter and terminator sequences. It was then necessary to include an additional sub-cloning step into pART7. Attempts to sub-clone the expression cassette into pCambia failed. Screening the putative pCambia clones by restriction digestion was not successful as unexpected sized bands were obtained, most likely due to the secondary structure within the DNA sequence causing the fragments not to run as linear fragments on the agarose gels. Heat denaturing the fragments and running them on formaldehyde gels did not resolve the problem. Southern blots of the clones confirmed that sequence rearrangement had occurred despite the fact that *E.coli* SURE® cells (Stratagene) were used (Figure 3.7). SURE® cells lack components of the pathways that

catalyse the rearrangement and deletion of non-standard secondary and tertiary structures. At this point it was decided to abandon attempts to generate pCambia vectors harbouring the invert repeat constructs. Instead, minimal gene expression cassettes were successfully generated from pART7 clones harbouring the invert-repeat sequences (pART7:AC1 m-hp:572 and pART7:AC1 m-hp:193). The minimal gene expression cassettes were subsequently bombarded into tobacco callus.

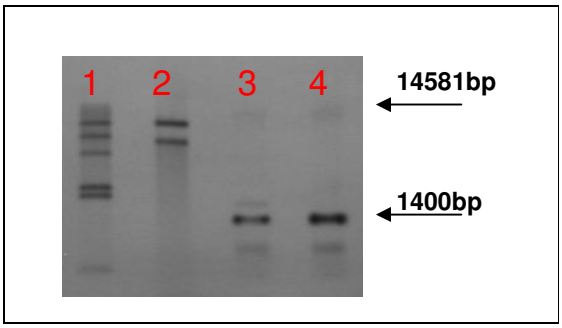


**Figure 3.7:** Southern blot analysis of a putative pCambia clone. Lane 1: Molecular weight marker (HindIII digested λ DNA). Lane 2: Postitve control (pHannibal plasmid DNA).The probe should hybridise with the 14581-bp fragment of the putative clones (lanes 3 and 4). The hybridisation of the probe to the 1400-bp fragment indicates rearrangement of the DNA.

### 3.4.2 Transient expression of minimal gene expression cassettes in *N. benthamiana* callus: A pilot study

A pilot study was conducted to confirm that viral DNA was detectable in bombarded tissue after seven days. *N. benthamiana* callus tissue was bombarded with SACMV DNAs. DNA was isolated from bombarded callus four and seven days post bombardment and digested with *Dpn*I. Four replicate plates containing eight pieces of callus each were used per time point. Replicating

SACMV DNA-A was detected by real-time PCR for each time point, therefore demonstrating that replicating SACMV DNA-A was detectable four and seven days post bombardment.

### 3.4.3 Replicating viral DNA-A quantification

Minimal gene expression cassettes containing invert-repeat SACMV sequences of 572-nts (AC1 m-hp:572) or 193-nts (AC1 m-hp:193) were bombared into *N. benthamiana* callus. In the first experiment, an expression cassette was introduced in to callus cells by particle bombardment followed by SACMV-DNAs forty-eight hours later. There was no significant difference in the amount of replicating SACMV DNA-A when either of minimal gene expression cassettes and SACMV DNAs were bombarded separately compared to the control (pART7 minimal cassette; Figure 3.8, blue bars). Although the AC1 m-hp:572 construct resulted in a higher amount of replicating viral DNA when bombarded separately to the SACMV DNAs, there was no significant difference between this amount and the control (Figure 3.8, blue bars).

In the second experiment, an expression cassette was co-bombarded in to callus cells together with SACMV DNAs. This resulted in a 60% and 80% reduction in replicating SACMV DNA-A in callus tissue that was co-bombarded with the AC1 m-hp:572 or AC1 m-hp:193 minimal gene expression cassettes respectively, compared to the control ($p<0.05$ and $p<0.01$ respectively; Figure 3.8 green bars).

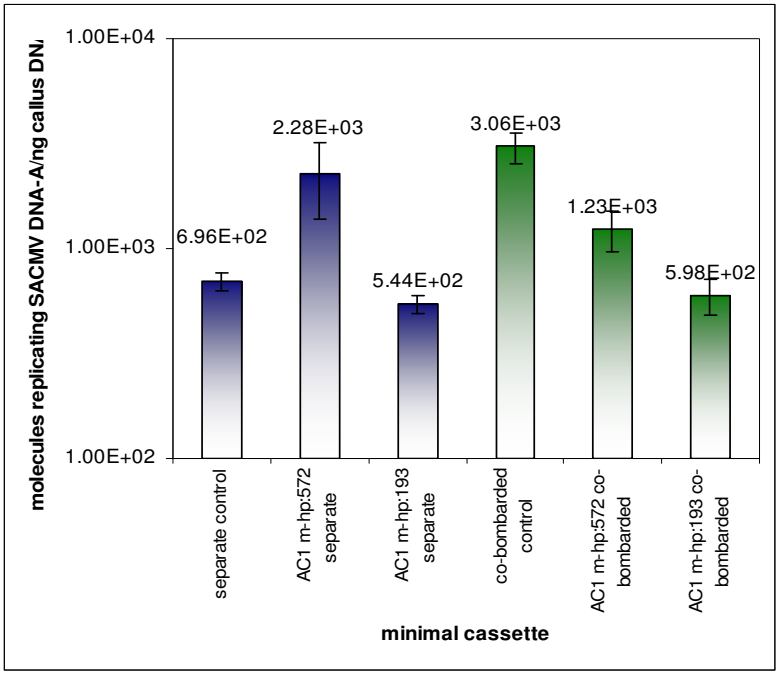


**Figure 3.8:** Quantification of replicating SACMV DNA-A in *N. benthamiana* callus by real-time PCR. Invert-repeat minimal gene expression cassettes (AC1 m-hp:572 and AC1 m-hp: 193) were bombarded separately (blue bars), or co-bombarded together with SACMV DNA (green bars). The control was pART7 minimal cassette. Data is the mean of four replicates and error bars represent the standard error. Y-axis is logarithmic scale.

## 3.5 DISCUSSION

Minimal cassette technology was used to test matched invert-repeat constructs for efficiency of SACMV DNA-A replication reduction. A minimal cassette, consisting of the promoter-invert-repeat sequence-terminator sequence was used to transform *Nicotiana benthamiana* callus tissue by particle bombardment, thus alleviating the need for a plant binary vector system and *Agrobacterium*-mediated transformation

Numerous challenges were encountered in the cloning steps to generate the invert-repeat constructs. Some challenges were able to be overcome and the end result was invert-repeat expression cassettes in pART7. Holliday junctions describe the four-way helical junction that is created between the two cruciform arms that result when interstrand base pairing occurs in palindromic DNA sequences (Duckett *et. al.*, 1988). Cruciform arms become problematic when trying to clone invert repeat DNA sequences since they alter the electrophoretic mobility of the DNA and they can render a sequence insensitive to a digestion by restriction endonucleases (Gellert *et. al.*, 1983). Problems can be encountered when transforming *E. coli* with DNA harbouring cruciform arms (Gellert *et al.*, 1983; Warren *et al.,* 1985; Lacomme *et al.,* 2003). Warren *et al.* (1985) and Lilley *et al.* (1981) independently report being unable to clone palindromic sequences in *E. coli*, citing instability and inviability as the likely causes. Interestingly these two authors were trying to clone sequences that differed in length by more than 500-bp (800-bp and 260-bp respectively). Lacomme *et. al.*, (2003) also reported being unsuccessful when trying to produce constructs with 100- and 200-bp direct inverted-repeats in a TMV-based vector in *E. coli*. However, cloning was

achieved when a 188-bp spacer fragment was positioned between the repeat sequences. In our study, although the matched sense and antisense AC1 sequences were cloned either side of a 742-bp intron in the pHannibal vector, this did not adequately stabilise the invert-repeat structure.

In this study, two minimal gene expression cassettes (promoter – invert-repeat sequence – terminator) containing matched AC1 invert-repeat constructs, with spliceable introns were prepared from pART7 clones. The cassettes differed with respect to the arm length of the invert-repeat sequence (572-nt or 193-nt). A pilot study was performed in which it was confirmed that quantifiable levels of replicating SACMV DNA-A could be detected in bombarded *N. benthaniama* callus tissue up to seven days after bombarding. A GUSPlus™ minimal gene expression cassette (CaMV 35S: GUSPLUS reporter gene sequence: NOS poly-A) released from pCambia vector 1305.1, was bombarded into callus tissue with the intention of quantifying transformation efficiency. Transformation efficiency was not established since GUS expression was not visible in the bombarded callus tissue 48 hours after bombarding (results not shown). The absence of transient *uidA* gene expression cannot be assumed as this was not confirmed with real-time PCR. It is more likely that the expression levels were low and therefore undetectable. Similar studies report GUS expression in *Pinus* embryonic tissue and mothbean hypocotyl explants three days and one week respectively following bombardment of the *uidA* reporter gene (Kamble *et al*., 2003; Salaj *et al*., 2005). In both cases however, *uidA* was under the control of a double CaMV 35S promoter, whereas in our study, a single CaMV 35S promoter was driving the expression of the GUS reporter gene and therefore expression may not have been high enough to detect histochemically. Another plausible reason for the undetectable levels of GUS expression may be tissue age and

helium pressure with which the DNA was delivered to the tissue. These two factors were reported by Ramesh *et al.* (2005) as affecting GUS expression in bombarded tissue. Expression levels of GUS were increased between five and ten fold in bombarded leaves by optimizing physical parameters including pressure with which the gold particles are delivered and amount of gold delivered. Similarly leaf age was reported to be a significant variable in GUS detection (Helenius *et al.*, 2000).

*N. benthamiana* callus was either co- bombarded with a minimal gene expression cassette and SACMV DNA-A and DNA-B or bombarded with a minimal gene expression cassette and after 48 hours bombarded with SACMV DNAs. In both cases DNA was isolated from bombarded callus tissue four days after bombarding with SACMV DNA and replicating SACMV DNA-A was quantified using absolute real-time PCR. There was significantly less replicating SACMV DNA-A in callus tissue that was co-bombarded with the AC1 m-hp:572 and AC1 m-hp:193 minimal cassettes compared to the control (pART7 minimal cassette). It is clear that there is a defence mechanism being initiated against SAMCV replication in the presence of the AC1 invert-repeat sequence, most likely RNA silencing. In a similar study, silencing of ACMV was achieved by agro-infiltrating *N. benthaniama* leaves with an ACMV AC1 hairpin gene construct and AC1 siRNAs were detected (Yadav *et al.*, 2008)

The invert-repeat minimal gene expression cassettes did not reduce the amount of replicating SACMV DNA-A when introduced into the tissue 48 hours before the introduction of SACMV DNAs. As there is no movement of the RNA silencing signal between cells in a transient callus expression system, only the cells that receive the invert-repeat construct have the potential to reduce SACMV

replication. However the challenging viral DNA may not necessarily enter cells that received the invert-repeat expression cassette in the first round of bombarding. There will be no siRNAs produced in these cells and SACMV replication will occur. Co-bombarding may increase the chance of the invert repeat construct and SACMV entering the same cells together. Local and not systemic resistance to ACMV was observed in transient leaf assay performed on *N. benthaniana* (Yadav *et. al*., 2008). Similarly, the mechanical inoculation of dsRNA into leaf cells does not trigger a systemic antiviral response and thus cells that receive virus particles but not dsRNA molecules are not protected (Tenllando *et al*., 2004).

The draw back of particle bombardment is that it requires large amounts of DNA. In this study, minimal cassettes were generated by restriction enzyme digests and fragment recovery by gel purification. An alternate way, as tested by Kumar *et. al*. (2006) is to amplify the expression cassette by PCR using a high fidelity DNA polymerase. This strategy however, could not be employed here, possibly due to the formation of a cruciform structure within the invert-repeat sequence that was not recognized by DNA polymerase. Rolling circle amplification is an option that was explored. A concatemer of expression cassettes is produced which can then be cleaved with a restriction enzyme. In this study, cleavage of the concatemer was unsuccessful. With the restriction digest method that was used, suitable restriction sites were available, allowing for the retention of a protection sequence on either side of the cassette (49-bp and 143-bp). It is believed that short protection sequences may be required to protect the expression cassette from end degradation by cellular nucleases after bombardment (Fu *et al*., 2000).

The introduction of minimal gene expression cassettes into regenerable plant tissue by particle bombardment can be used to generate transformed whole plants. The co-bombardment of a selectable marker gene minimal cassette with the invert-repeat minimal cassette is used to select for cells that have stable expression of the invert-repeat construct, which are subsequently regenerated into whole plants. Transformation by particle bombardment is not favourable due to the risk of transgene silencing resulting from multicopy transgene insertion (Matzke *et al.*, 1996; Kohli *et al.*, 2003). The particle bombardment of minimal gene expression cassettes has been shown to result in a low-copy number (one to two copies) compared to bombarding with supercoiled or linearised plasmid containing the transgene (Fu *et. al.,* 2000). Our study shows that matched invert-repeat constructs, introduced into plant tissue as minimal gene expression cassettes can reduce SACMV DNA-A replication when transiently expressed. The study can be furthered by regenerating plants from callus tissue and quantifying the knockdown of SACMV replication in a stable expression system. Towards the development of SACMV resistant cassava, the introduction of the matched invert-repeat constructs as minimal cassettes by particle bombardment is favourable with respect to vector backbone sequence incorporation, simple integration events and transformation method.

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# CHAPTER 4 - The induction of RNA silencing by a SACMV AC1-derived invert-repeat construct with sequence modifications

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## 4.1 ABSTRACT

A strategy to incorporate sequence modifications into the sense strand of an invert-repeat construct was used to alleviate the problems encountered when cloning with perfectly matched invert-repeat sequences. A 183bp region of *South African cassava mosaic virus* (SACMV) AC1 at the N-terminus was amplified by PCR from full-length SACMV DNA-A and treated with sodium bisulphite, resulting in the deamination of unmethylated cytosine residues. The modified fragment was ligated head-to-head with an unmodified fragment to generate an invert-repeat construct. The cytosine residues in the sense arm were converted to thymine resides resulting in mismatched basepairs along the length of the invert-repeat construct. It was predicted that a stable hpRNA structure would be formed upon transcription of the transgene. *N. benthamiana* leaf discs and cassava TMS 60444 FEC and MTAI 16 cotyledons were transformed with the invert-repeat expression cassette by *Agrobacterium*-mediated transformation. Ten *N. benthamiana* transgenic lines were challenged with SACMV DNA-A and DNA-B with five lines displaying no viral symptoms at 28dpi. Cassava transformation was confirmed however transformed tissues could not be regenerated. In this study, a technique for the rapid production of RNA silencing constructs has been developed and the results obtained with the construct tested here provides encouragement towards the overall aim of producing SACMV resistance cassava.

## 4.2 INTRODUCTION

### 4.2.1 The use of double-stranded RNA invert-repeat duplexes to induce gene silencing in plants

Until 1998, sense or antisense transgene expression was the focus of PTGS studies against plant viruses. However, as more information about the RNA silencing pathways became available so the strategy shifted to one of expression of invert-repeat duplexes. Waterhouse *et al*. (1998) co-expressed sense and antisense protease gene (potato virus Y) mRNA in tobacco and found this to be much more effective at inducing PVY immunity than by transforming plants with only sense or antisense transgenes. Another such comparison was made by Lacomme *et al.* (2003) when engineering 40 to 60 basepair inverted-repeats of a gfp transgene into a tobacco mosaic virus-based vectors. The invert repeats were more efficient at inducing gene silencing than vectors with sense or antisense fragments only. They also showed that these shorter invert-repeat sequences were more effective than 100 to 200 nt inverted-repeat sequences separated by a loop.

There are numerous examples of silencing induced by the expression of hpRNA from invert-repeat transgenes in plants. The length of the arm sequence varies as well as the incorporation of a spliceable intron. The first successful experiment using dsRNA against a DNA virus resulted in the silencing of *Vigna mungo* yellow mosaic virus (VMYMV), by the transient expression of an intron invert-repeat construct of the VMYMV promoter sequence (209bp; Saini, 2003). Resistance to *Tomato yellow leaf curl virus* (TYLCV) has been induced by either a hpRNA consisting of a 726-bp sequence from TYLCV *C1* and the castor bean catalase intron (Fuentes *et al*., 2006) or a hpRNA with 419-bp arms derived from

TYLCV CP and a maize ubiquitin intron (Zrachya *et al*., 2007). Resistance to *African cassava mosaic virus* (ACMV) was achieved by expression of a hpRNA, transcribed from a invert-repeat construct comprised of a 256-bp ACMV promoter sequence in the sense and antisense orientations separated by a 114-bp synthetic intron (Vanderschuren *et al*., 2007).

### 4.2.2 Invert-repeat constructs with mismatched base-pairing

Although invert-repeat transgenes are the basis of silencing studies in plants, this technology has limitations, as described in chapter 3. A cruciform is the structure usually taken on by palindrome sequences, creating barriers to DNA replication or acting as targets for endonucleases (Lobachev *et al*., 2002). In our study, an RNA silencing construct was produced, using a method that eliminated the problems potentially caused by the formation of cruciforms. To realise this, it was necessary to destabilise the DNA secondary structure and this was achieved by introducing sequence modifications along the sense arm of the invert-repeat sequence resulting in G-U wobble base pairs. It has previously been shown that such G-U mismatches have no impact on RNAi knockdown efficacy in animal cells (Akashi *et al*., 2005 and Weinberg *et al*., 2007). The introduction of G-U mismatches into plant virus silencing vectors is novel but it is predicated that the mismatches will be similarly tolerated by plant RNA silencing proteins.

Sodium bisulphite is a mutagen, commonly used to detect and quantify methylation in genomic DNA. Upon treatment with this mutagen, unmethylated cytosine residues are deaminated, converting them to uracil while methylated cytosines remain unchanged. DNA is then analysed by either sequencing or

restriction endonuclease digestion allowing for a methylation profile to be generated. Sodium bisulphite treatment has been shown to assist with sequence analysis of long perfect palindromes (Rattray, 2004). The conversion of unmethylated cytosines to uracil was exploited in this study to introduce base-pair mismatches into the sense arm of an RNA silencing construct. This is the first account of the use of this technique to produce stable invert-repeat constructs against plant viruses (patent application WO 2008/093283).

In brief, a PCR template was mutated by exposure to sodium bisulphite and subsequently PCR amplified. Ligation of the modified and unmodified amplicons resulted in the invert-repeat sequence, which was subsequently cloned between a promoter and terminator sequence to generate an invert-repeat expression cassette. Since bisulphite catalysed deamination only affects cytosine residues, PCR of the mutated double-stranded template results in two species of PCR product with distinct sequences. Therefore, strand-specific PCR amplification of the positive strand of the mutated template DNA is necessary, and PCR primers were designed specifically for this purpose. It is essential to ensure that the antisense strand is not mutated, as this would compromise the binding of subsequently formed siRNAs to target sequences, thereby negatively impacting on the induction of PTGS.

### 4.2.3 Cassava transformation systems

An efficient cassava transformation system requires reliable transformation and regeneration. Organised tissues such as friable embryogenic callus (FEC) and somatic cotyledons are used however their efficacy is cultivar

dependent (Taylor *at al.*, 2004) A friable callus is produced when tissues proliferate in an uncoordinated manner, and consists of thousands of embryogenic units less than 1mm in diameter. The resulting tissues disperse easily and proliferate rapidly in liquid culture to produce high quality embryogenic suspension cultures (Taylor *et al.*, 1996). FEC and cotyledons are derived from somatic embryos, initiated from immature leaf lobes or axillary buds, and can be transformed by particle bombardment or *Agrobacterium*-mediated transfer. Following transformation, explants undergo organogenesis to produce shoots and roots.

### 4.2.4 Target region

A 183-bp region of the SACMV AC1 open reading frame (ORF) was selected as the target site for RNA silencing. This region was selected to coincide with the sequence of a previously made invert-repeat construct for comparative purposes (AC1: m-hp:193; chapter 3). The AC1 ORF encodes the replication-associated protein (Rep) (Hanley-Bowdoin *et al.,* 1999) and overlaps the AC4 ORF, which acts as a silencing suppressor (Vanitharani, *et al.*, 2004). The selected region begins 72-bp from the 3' end of the AC1 ORF and overlaps the 5' end of the AC4 ORF by 99-bp (Figure 4.1). In this way, both the AC1 transcript and the transcript for the AC4 silencing suppressor would be targeted for degradation upon the initiation of RNA silencing. It has been predicted that using a silencing suppressor sequence can inhibit the negative influence of RNA silencing on transgene expression, reducing the variation of transgene expression caused by RNA silencing (Butaye *et al*, 2005).

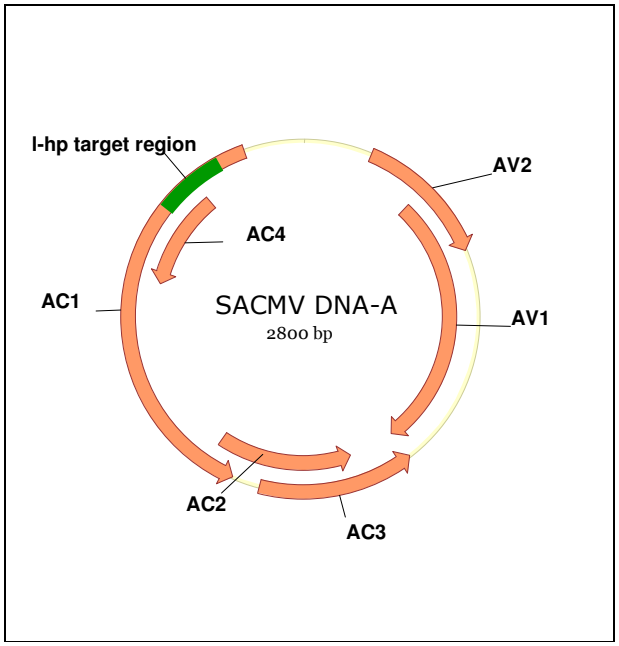


**Figure 4.1:** SACMV DNA-A showing target region for an invert-repeat RNA silencing construct.

### 4.2.5 Objective and aims

The objective of this study was to generate a SACMV AC1 sequence derived RNA silencing invert-repeat construct with sequence modifications to target the SACMV AC1 transcript, and to test the construct for SACMV replication knockdown efficiency. The specific aims were to:

1. produce an invert-repeat construct with basepair mismatches in the arm sequence by introducing mutations in the sense sequence,
2. transform *N. benthamiana* and cassava with the invert-repeat construct,
3. screen putative transformants by PCR and GUS assays,

4. detect siRNA species present in transgenic lines,
5. challenge transgenic lines with SACMV DNA-A and DNA-B and
6. to quantify SACMV DNA-A in challenged lines using absolute real-time PCR

## 4.3 MATERIALS AND METHODS

### 4.3.1 Invert-repeat construct with sequence modifications

**Preparation of sense and antisense arms of the invert-repeat construct**

A 183-bp region of SACMV AC1 ORF at the N-terminus of the gene sequence (Figure 4.1) was amplified by PCR from full-length SACMV DNA-A in pBluescript (kindly donated by L. Berrie). The PCR reaction consisted of the following components:1.6x HighFidelity Buffer with $Mg^{2+}$; 2.5U TripleMaster Polymerase mix (TripleMaster® PCR System, Eppindorf); 0.2mM dNTPs; 0.2µM each primer (F unmodified: 5' CTCGAAAGAAGCGGCATTAG; R unmodified: 5' GACCTGGAAGGGGATATGAGGTCGAAGAATC); 1.5ng template DNA. The primers were annealed to the template DNA at 55°C and a final extension step at 72°C for 15 minutes was included. The PCR product was purified (High Pure PCR Purification kit, Roche Biochemicals) and quantified using The NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). An aliquot of the PCR product was bisulphite-treated (EZ DNA Methylation-Gold Kit ™, Zymo) at 65°C for two and a half hours, according to the manufacturer's recommendations. The bisulphite treated DNA fragment was re-amplified (TripleMaster® PCR System, Eppindorf as previously described) using modified PCR primers (table 4.1). The design of the modified primers ensured the selection of the sense strand in the first round of primer annealing and restriction enzyme recognition sequences were included to facilitate downstream cloning steps.

The unmodified PCR product and the re-amplified bisulphite-treated PCR product were TA cloned into separate pTZ57R cloning vectors (Fermentas). The

ligation reactions (0.54pmol ends insert; 0.18pmol ends vector; 1x ligation buffer; 1U T4 DNA ligase) were incubated at 22°C for one hour, following which competent *E. coli* DH5α cells were transformed with 15µl of the respective ligation reaction. Transformants were selected for on Luria Agar supplemented with 100µg/ml ampicillin. Clones transformed with pTZ harbouring the unmodified AC1 fragment (pTZ:AC1 unmod) were screened by *Bgl*II/*EcoR*I double digestion, whilst clones transformed with pTZ harbouring the modified AC1 fragment (pTZ:AC1 mod) were screened by *EcoR*I/*Xho*I double digestion, allowing the orientation of each insert to be determined (Figure 4.2). Clones harbouring the insert in the correct orientation were sequenced with the M13 reverse primer aligned with the SACMV DNA-A sequence (AF155806) using DNAMAN (Version 4.0; Lynnon BioSoft).

**Table 4.1:** PCR primers designed to re-amplify the bisulphite treated PCR fragment. [1]

| **PCR Primer** | **Sequence** |
|---|---|
| RepF ((*Xho*I/*Spe*I) (modified) | 5' GATCCTCGAGACTAGTCTCGAAAGAAG**T**GGCATTAG |
| RepR(*Bgl*II) (modified) | 5' GATCAGATCTGACCTGGAAG**A**GGATATG |

Green highlighted regions indicate restriction sites; bold letters indicate nucleotides that were changed to ensure selection of the sense strand in the first PCR cycle.

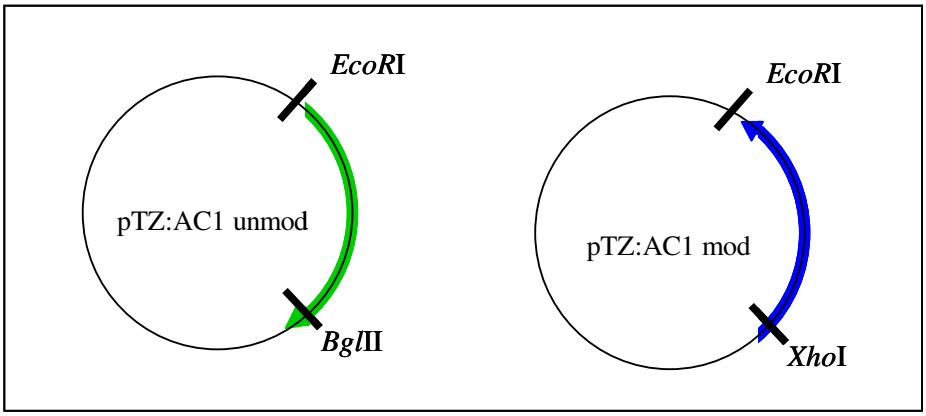


**Figure 4.2:** Modified and unmodified AC1 PCR fragments cloned into pTZ57R

**Invert-repeat construct in pTZ**

A *Bgl*II/*Sca*I double digestion reaction was used to remove the modified AC1 fragment from the pTZ:AC1 mod clone, and a *BamH*II/*Sca*I double digestion reaction was used to remove the AC1 unmodified fragment from the pTZ:AC1 unmod clone. *BamH*I and *Bgl*II have compatible ends allowing for ligation (Figure 4.3). The respective bands were resolved on a 1% agarose gel, visualised with ethidium bromide and gel purified (Gel purification kit, Qiagen). The unmodified and modified fragment was ligated by adding equal molar ratios of each to 1µl T4 DNA ligase and 1x ligase buffer (Fermentas). The reaction was incubated at 22℃ for one hour following which competent *E. coli* DH5α cells were transformed. Transformants were selected for on Luria Agar supplemented with 100µg/ml ampicillin. Putative clones were screened for by *Xba*I/*Xho*I double digestion (Figure 4.3). Clones harbouring the invert-repeat sequence were sequenced (Inqaba Biotech, South Africa) with the M13 reverse primer.

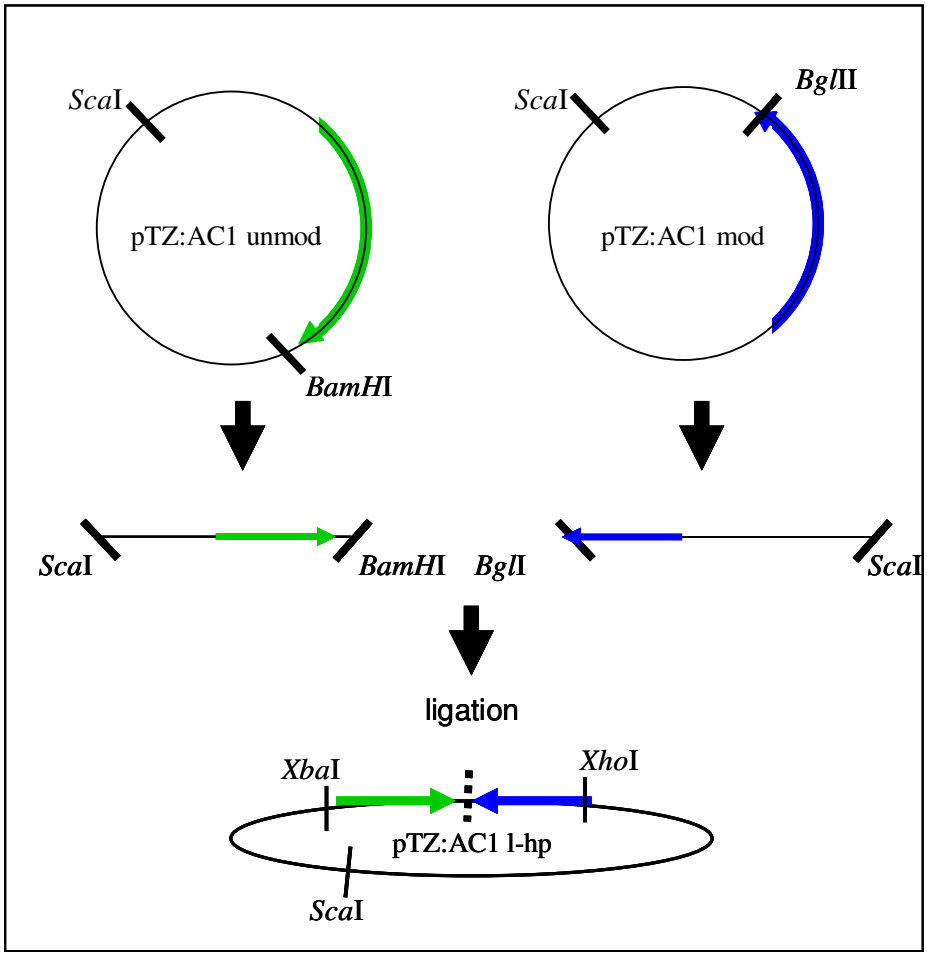


**Figure 4.3:** A schematic representation of procedure used to generate a mismatched invert-repeat construct in pTZ57R

**Sub-cloning of invert-repeat construct into pART7**

An expression cassette was generated by sub-cloning the invert-repeat construct into pART7, between the CaMV 35S and ocs 3' sequences. A pTZ:AC1 l-hp clone and the pART7 vector were subjected to a double restriction digestion with *Xba*I/*Xho*I (Fermentas), thereby releasing the ~400bp invert-repeat sequence and creating ligation ends respectively. The required DNA fragments

were agarose gel purified (Gel purification kit, Qiagen). A 1:3 ratio (pmol ends) of linearised vector DNA to invert-repeat DNA were ligated with T4 DNA ligase (Fermentas) for one hour at 22°C, following which, competent *E. coli* DH5α cells were transformed. Transformants were selected for on Luria Agar supplemented with 100µg/ml ampicillin. Putative clones were screened for by digestion with *Pst*I, resulting in the generation of a 796-bp DNA fragment in clones harbouring the invert-repeat sequence.

**Sub-cloning the invert-repeat expression cassette into a plant transformation vector**

The invert-repeat (IR) expression cassette (CaMV 35S: IR sequence: ocs 3') was amplified from the pART7:AC1 I-hp clone by PCR (1U Taq DNA polymerase (Fermentas), 0.1µm each primer (pART7 cassette F: 5' TTAACGTTTACAATTTCCCATTCGC; pART7 cassette R: 5' GGAATTGTGAGCGGATAAC) 1x Taq buffer with $(NH_4)_2SO_4$, 0.2mM dNTP mix and 2.5mM $MgCl_2$; 35 cycles of PCR with a 55°C annealing temperature at the end of each cycle and a final extension time of 15 minutes at 72°C; BioRad, MyCycler). The PCR product was resolved on a 1% agarose gel and gel purified (Gel purification kit, Qiagen).

To assist with the sub-cloning of the invert-repeat cassette into a pCambia plant transformation vector (CAMBIA, Australia), which has a limited multiple cloning site, the invert-repeat cassette was first TA-cloned into pTZ57R (InsTAclone ™ PCR cloning Kit, Fermentas) according to manufacturer's recommendations. The ligation reaction was incubated at 22°C for one hour, following which competent *E. coli* DH5α cells were transformed. Transformants

were selected for on Luria Agar supplemented with 100µg/ml ampicillin. Putative clones were screened for by digestion with *Pst*I, resulting in the generation of three DNA fragments (3117-bp, 1770-bp and 796-bp or 4639-bp, 796-bp and 278-bp orientation dependent) in clones harbouring the invert-repeat expression cassette.

The invert-repeat expression cassette was released from pTZ with *EcoR*I and *Hind*III and sub-cloned into the *Hind*III/*EcoR*I digested pCambia 1305.2 vector DNA using equal molar amounts of vector and insert DNA. Ligation reactions were incubated at 22℃ for one hour, following which competent *E. coli* DH5α cells were transformed. Transformants were selected for on Luria Agar supplemented with 100µg/ml kanamycin. Putative clones were screened by digestion with *Sca*I.

### 4.3.2 *Agrobacterium* transformation

*Agrobacterium* AGL1 strain was transformed with pCambia1305.2:AC1 I-hp using the freeze-thaw method (Holsters *et al.*, 1978). Transformants were selected for at 30℃ for 2 days on Luria Agar supplemented with 100µg/ml kanamycin and 100µg/ml carbenicillin. Putative clones were screened with PCR using pART7 cassette primers as described in section 4.3 1.

### 4.3.3 *Nicotiana benthamiana* transformation

#### In vitro *N. benthamiana* cultures

*N. benthamiana* seeds were surface sterilized in 0.35% sodium hyperchlorite followed by several rinses in distilled sterile water, and placed on Murashige and Skoog (MS) medium plus 1% sucrose and 0.5% agar. Seeds were geminated and *N. benthamiana* plants were maintained at 25°C for a sixteen hour photoperiod in a tissue culture growth chamber (Binder).

#### *Agrobacterium* preparation

AGL1:1305.2 AC1 l-hp and AGL1:1305.2 (control) clones were grown at 30°C with agitation (220rpm) for sixteen hours in Luria Broth (LB) plus 100µg/ml kanamycin. Overnight cultures were sub-inoculated into LB supplemented with 200µM acetosyringone (Sigma-Aldrich) and incubated for a further 6 hours at 30°C with agitation (220rpm). The bacterial cells were pelleted at 4500rpm for 5 minutes at 4°C, and re-suspended in 30ml liquid Murashige and Skoog (MS) medium plus 1% sucrose, supplemented with 200µM acetosyringone and 500mg/L cefotaxime sodium (Sigma-Aldrich).

***Agro*-mediated transformation of *N. benthamiana* leaf discs**

Leaf discs (5mm) were cut from newly expanded leaves on tissue culture plants and placed on MS medium plus 1% sucrose for eight hours prior to infection. Twenty-five leaf discs were transferred to each AGL1 suspension (preparation described above) and subjected to vacuum infiltration at 85kPa for 10 minutes. Excess suspension was removed and the leaf discs were co-cultivated in the dark at 22℃ for four days in a tissue culture growth chamber (Binder). The leaf discs were rinsed in liquid MS medium plus 1% sucrose, supplemented with 500mg/L cefotaxime sodium (Sigma-Aldrich), followed by shaking incubation (50 rpm) in liquid MS medium plus 1% sucrose and supplemented with 500mg/L cefotaxime sodium at 22℃ for one hour.

### 4.3.4 *N. benthamiana* leaf-disc regeneration and acclimatization

**Selection and regeneration of transgenic tobacco shoots**

The transformed leaf discs were transferred to solid selection and shooting media (MS medium plus 1% sucrose, supplemented with 0.1mg\L NAA, 1mg\L BAP, 30 mg/L hygromycin and 500mg/L cefotaxime sodium) in petri-dishes and incubated at 25℃ in a plant tissue culture chamber (Binder) for a sixteen hour photoperiod until shoots appeared.

**Rooting of transgenic shoots**

Shoots of 1-2cm in length were cut from the leaf discs approximately four weeks after transformation and placed on MS medium plus 1% sucrose and 0.5% agar, supplemented with 500mg/L cefotaxime sodium in tissue culture bottles for rooting. An ethylene scrubber was made by saturating Perlite in a 7.5% (w/v) potassium permanganate solution. Plastic caps (25mm diameter) were filled with saturated perlite and sterilised by autoclaving. One sterile perlite filled cap was placed in each culture bottle. Rooting shoots were transferred to peat pellets (Jiffy), covered with plastic wrap and maintained in a temperature controlled growth chamber at 22°C for a sixteen hour photoperiod for two weeks. During this time period, the humidity was gradually reduced by adding holes in the plastic wrap. After acclimatisation, the plants were transferred to soil in larger pots and grown under standard conditions.

### 4.3.5 Confirmation of transformation of $T_0$ plants

$T_O$ acclimatised plants were screened to confirm transformation. A region of the hygromycin resistance gene sequence was amplified by PCR (BioRad, MyCycler) using Taq polymerase (Fermentas). The primers (*hpt*II F: 5' ctatttctttgccctcgg; *hpt*II R: 5' ttcgatgatgcagcttgg) were annealed to the template DNA at 55°C and the PCR amplicon was resolved on 1% agarose gels stained with ethidium bromide. $T_0$ plants that were confirmed to be transformed were selfed and seed was germinated in soil to produce $T_1$ lines.

### 4.3.6 Analysis of $T_1$ lines

**Histochemical detection of β -glucoronidase**

GUS assays were performed on $T_1$ unchallenged leaf material from AGL1:1305.2 AC1 I-hp transformed plants. Leaves were placed in staining buffer (1M sodium phosphate (pH 7.0); 0.5M EDTA; 10% Triton X-100; 50mM $K_3Fe(CN)_6$; 0.1M X-Gluc (Fermantas)) and incubated at 37ºC for upto 24 hours followed by several washes in 50% ethanol. Leaf tissue was viewed under a Olympus dissecting microscope and photographed with a Nikon DXM1200 digital camera.

**siRNA detection in unchallenged $T_1$ transgenic lines**

A highly sensitive solution hybridisation and RNA-protection-based assay was used to detect small RNA species in unchallenged $T_1$ lines. The *mir*Vana™ miRNA probe construction kit (Ambion®) was used to generate a $_{32}$P-labelled antisense RNA probes from an oligonucleotide DNA template (5' attgtacttgccctcgaactgaatga**cctgtctc**; bold nucleotides represent T7 promoter sequence). The target-specific oligonucleotide was annealed to T7 promoter primer and a dsDNA transcription template was generated using Exo-Klenow enzyme (Ambion®). A complementary 5' to 3' RNA transcript was transcribed *in vitro* by T7 phage RNA polymerase, using the 3' to 5' strand of the dsDNA as a template. [$\alpha_{32}$P] UTP was incorporated into the transcript during the transcription reaction. All reactions were performed according to manufacturer's instructions. The full-length labelled transcript was resolved on a 15% denaturing polyacrylamide gel, visualised by autoradiography and gel-purified to separate

full-length transcript from prematurely terminated transcription products and unincorporated nucleotides. The specific activity of the probe was determined (TriCarb Liquid Scintillation counter, Packard).

Total RNA was isolated from 100mg leaf tissue from $T_1$ plants using Trizol (Sigma-Aldrich) according to manufacturer's instructions and checked for integrity on 1% TAE agarose gels (80V for 1 hour) stained with ethidium bromide. RNA was quantified with a spectrophotometer (The NanoDrop® ND-1000, NanoDrop Technologies, Inc.). Hybridisation reactions were carried out according to manufacturer's recommendations (*mir*Vana™ miRNA detection kit, Ambion®). Sample RNA (5µg per reaction) was mixed with 5.0 x $10^4$ cpm radiolabelled antisense RNA probe. The RNA was heat denatured and incubated to 42℃ for 16 hours to allow hybridisation of the probe to the target RNA. After hybridisation, single stranded RNA, including the unhybridised sample RNA and excess RNA probe was digested with ribonuclease and the remaining dsRNA was precipitated. Radiolabelled protection RNA probe fragments were resolved on 12% denaturing polyacrylamide gels (20mA) and visualised by autoradiography.

### 4.3.7 Inoculation with SACMV DNA-A and DNA-B

Six week old *N. benthamiana* $T_1$ seedlings (four replicate plants per line) grown in soil at 25ºC with a 16 hour photoperiod, were challenged with SACMV DNA-A and DNA–B dimers in pBIN19 by agroinoculation, using pBINSA-A and pBINSA-B transformed *Agrobacterium tumefaciens* (kindly donated by L. Berrie). A Hamliton syringe was used to deliver a mixture of pBINSA-A and pBINSA-B transformed *A. tumefaciens* (O.D = 0.6 each; equal volumes mixed) along the

stem of the seedlings. Twenty microliters of the mixture was injected at three different positions along the stem. *N. benthamiana* control plants transformed with pCambia 1035.2 only were inoculated in the same way. Inoculated plants were maintained at 25°C with a 16 hour photoperiod and viral symptoms were scored every seven days until 28 dpi using the six point symptom severity score (Fauquet and Fargette, 1988) where 0 is no symptoms, 1 is mild chlorosis without deformation, 2 is clear mosaic with or without slight leaf deformation, 3 is strong mosaic all over the leaflets with leaf deformation, 4 is same as score 3 except with severe leaf deformation and 5 is severe mosaic and severe reduction of leaf size.

### 4.3.8 Quantification of DNA-A in SACMV challenged $T_1$ plants

The amount of viral DNA present in leaf material 28 days post-infection (dpi) was quantified in duplicate using absolute real-time PCR. Leaf tissue (50mg per plant) was collected from six replicate plants per line. DNA was isolated using the CTAB method (Doyle and Doyle, 1987) and was quantified using The NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). A region of the AV1 ORF of SACMV DNA-A was amplified, in duplicate, by real-time PCR using SYBRGreen fluorescent dye (LightCycler FastStart DNA Master$^{PLUS}$ SYBR Green I kit, Roche Biochemicals) with the LightCycler 1.5 Instrument (Roche). Reactions were set up according to manufacturer's instructions to which 2µl of 1:10 diluted DNA and 2µM of each PCR primer (F: 5' gcacaagcgtcgat; R: 5' ctgccagtatgcttaacgtca) was added. The following PCR conditions were used to amplify the 154-bp fragment: 5 min at 95ºC; 40 cycles of

5 sec at 95ºC, 10 sec at 60ºC and 10 sec at 72ºC. For quantification purposes, a standard curve was generated by amplification of SACMV DNA-A in pBluescript (kindly donated by L.Berrie) as described in chapter 2 section 2.3.3. Data was analysed using Microsoft Excel and GraphPad InStat 3.01 software (GraphPad Software, Inc., San Diego, CA).

### 4.3.9 Cassava transformation

**Plant material**

*In vitro* cassava cultivars TMS60444 (model cultivar), T200 (local cultivar) and SM 1433-4 (a high starch producing cultivar) were maintained on Murashige and Skoog (MS) medium supplemented with 20g/L sucrose under a 16 hour light regime at 25℃ in a plant growth chamber (Binder).

**Production of plant tissue for transformation**

Friable embryogenic callus (FEC) and cotyledons are ideal tissues for transformation of cassava. Attempts were made to initiate FEC from cassava cultivars TMS60444, T200 and SM 1433-4. Immature leaf lobes were excised from *in vitro* plantlets, placed on MS medium supplemented with 50µM picloram (Sigma) with the abaxial surface in contact with the medium, and sub-cultured every three weeks removing any non-embryogenic tissue. Organised embryogenic structures (OES) appeared after three to four sub-culturing events and were transferred to Gresshoff and Doy (GD) medium plus 2% sucrose and

0.5% agar, supplemented with 50 µM picloram (Sigma-Aldrich). OES was sub-cultured every three weeks, carefully removing callus with every sub-culturing event. FEC started to appear from twelve weeks. FEC clusters were maintained on GD medium plus 2% sucrose and supplemented with 50 µM picloram (Sigma-Aldrich).

**Preparation of Agrobacterium clones for transformation**

A single colony of *Agrobacterium* AGL1:1305.2 AC1 l-hp was isolated by streaking on Luria agar supplemented with 100µg/ml kanamycin and 100 µg/ml carbenicillin. One colony was subsequently inoculated into Luria broth (LB) supplemented with kanamycin and carbenicillin (100 µg/ml each) and grown at 28℃ for 16 hours on an orbital shaker (220rpm). Acetosyringone (200µM) was added and the culture was incubated under the same conditions for a further five hours. The bacterial cells were obtained by centrifugation and resuspended in liquid GD medium plus 2% sucrose, supplemented with 50µM picloram (Sigma-Aldrich) and 200µM acetosyringone (Sigma-Aldrich) for FEC transformation or liquid MS medium plus 4% sucrose supplemented with 200µM acetosyringone (Sigma-Aldrich) for cotyledon transformation.

**Agrobacterium-mediated transformation of cassava**

FEC initiation from OES was unsuccessful for SM1433-4 and T200 cultivars. TMS60444 FEC clusters (twelve to fifteen weeks from OES) and MTAI 16 cotyledons initiated from immature leaf lobes (kindly donated by C. Rossin) were transformed with *Agro*bacterium AGL1:1305.2 AC1 l-hp. Twenty FEC

clusters (5mm diameter each) were placed on GD plus 2% sucrose and 0.5% agar, supplemented with 50µM picloram (Sigma-Aldrich) and 200µM acetosyringone (Sigma-Aldrich) and incubated for three days at 28°C for a twelve hour photoperiod in a plant growth chamber (Binder). Cotyledons were prepared for transformation by placing twenty clusters on MS plus 4% sucrose, supplemented with 200µM acetosyringone (Sigma-Aldrich) and incubated for three days at 28°C for a twelve hour photoperiod in a growth chamber (Binder).

Each explant (FEC cluster or cotyledon) was inoculated with 20µl freshly prepared bacteria. Co-culture took place for three days at 21°C in the dark. Following co-culture, FEC clusters were washed by shaking (90rpm) in liquid GD medium supplemented with 50µM picloram (Sigma-Aldrich) and 500mg/L cefotaxime (Sigma-Aldrich) at 28°C for a twelve hour photoperiod for seven days. The washing regime was continued for a further eight days in freshly prepared liquid medium with the addition of 20mg/L hygromycin. FEC clusters were placed in solid GD medium plus 2% sucrose supplemented with 50µM picloram, 500mg/L cefotaxime and hygromycin (10mg/L). Excess bacteria were removed from cotyledons by blotting with sterile filter paper and immediately transferred to solid MS medium plus 4% sucrose and 50mg/L cefotaxime (Sigma-Aldrich) for seven days. FEC clusters and cotyledons were then transferred to organogenesis medium for regeneration.

**Regeneration of transformed cassava explants**

Cotyledons were placed on solid MS medium supplemented with IBA (0.5mg/L), BAP (0.5mg/L) and 10mg/L hygromycin (Sigma-Aldrich) for shoot

induction. FEC clusters were placed on solid GD medium with 2% sucrose, supplemented with 50µM picloram, 500mg/L cefotaxime and 10mg/L hygromycin to initiate growth of the transformed clusters. After one month, clusters were placed on MS medium plus 2% sucrose, supplemented with IBA (0.5mg/L), BAP (0.5mg/L) and 10mg/L hygromycin (Sigma-Aldrich) for shoot induction.

**Histochemical detection of β -glucoronidase**

GUS assays were performed on selected FEC clusters and cotyledons, after eight weeks on shoot induction medium. Explants were placed in staining buffer [1M sodium phosphate (pH 7.0); 0.5M EDTA; 10% Triton X-100; 50mM $K_3Fe(CN)_6$; 0.1M X-Gluc (Fermantas)] and incubated at 37ºC for upto 24 hours followed by several washes in 50% ethanol. Explants were viewed under an Olympus dissecting microscope and photographed with a Nikon DXM1200 digital camera.

**PCR amplification of the GUSPlus region of the transgene**

DNA was isolated from selected FEC clusters and cotyledons using the CTAB method (Doyle and Doyle, 1987). DNA was quantified using The NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). A region of the GUSPlus gene sequence was amplified by PCR (BioRad, MyCycler) using Taq polymerase (Fermentas). Four hundred nanograms of DNA was used in each PCR reaction. PCR primers (F: 5'ggtcacaaccgagatgtcct; R: 5' caacatcctcgacgatagca) were annealed to the template DNA at 55°C. The PCR amplicon was resolved on 1% agarose gels stained with ethidium bromide.

## 4.4 RESULTS

### 4.4.1 Invert-repeat construct with sequence modifications

To alleviate the problems encountered when cloning with perfectly matched invert-repeat sequences (chapter 3), a strategy was used in this study to incorporate basepair mismatches into the arm sequence of the invert-repeat (IR) construct. A 183-bp region of the SACMV AC1 ORF was PCR amplified from full-length SACMV DNA-A and treated with sodium bisulphite. Selection of the positive strand for PCR amplification of the mutated DNA molecule and the successful conversion of the cytosine nucleotides into thymine nucleotides within the target region was confirmed by DNA sequencing (Figure 4.4). Untreated PCR product was TA cloned into pTZ57R to generate the pTZ:AC1 unmod clone. For head-to-head directional cloning of the modified and unmodified sequences, a pTZ:AC1 unmod clone with insert in the positive orientation was screened for by double restriction digests with *Bgl*II and *EcoR*I. A clone that generated a 148-bp fragment was selected (Figure 4.5A). Similarly, pTZ:AC1 mod clone with insert in the negative orientation was screened by *EcoR*I/*Xho*I double digestion yielding a 240-bp fragment (Figure 4.5B).

GATCCTCGAGACTAGTCTCGAAAGAAGTGGCATTAGAT
TAATTTTGATAATTTTAAATTTTAATAAATAAATTGTTTAT
TAAGATTTGTAGAGAATTTTATGAAAATGGGGAATTTTA
TTTGTATGTTTTTATTTAGTTTGAGGGTAAGTATAATTGT
ATTAATTAATGATTCTTCGACTTCATATCCTCTTCCAGG
TCAGATCTGAT

**Figure 4.4:** pTZ:AC1 mod clone sequence. Grey highlighted region indicates the PCR amplified region excluding primer binding. All cytosine nucleotides were converted to thymine nucleotides (underlined)

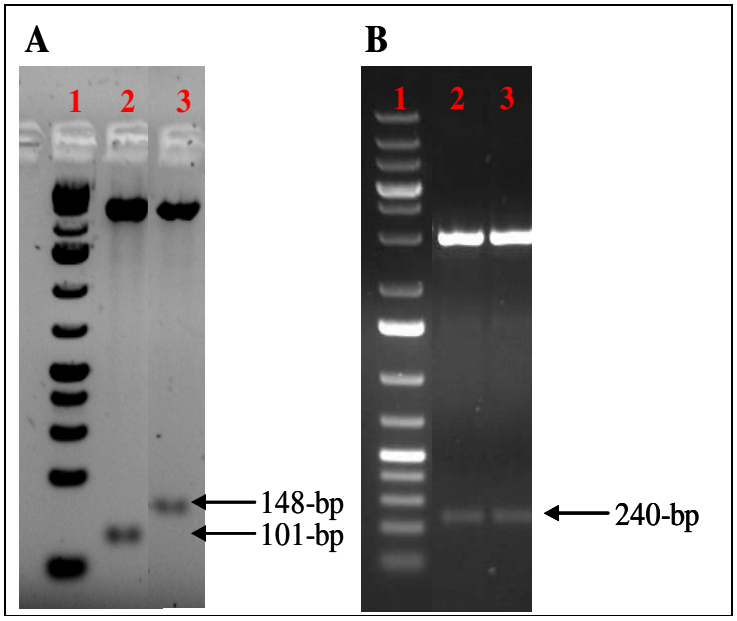


**Figure 4.5:** Screening of pTZ:AC1 clones for use in head-to-head directional cloning to generate a invert-repeat construct. A: pTZ:AC1 unmod clones digested with *Bgl*II and *EcoR*I resulting in a 101-bp or a 148-bp fragment for negative orientation and positive orientation inserts respectively (lanes 2 and 3). Lane 1: Fermentas 1kb MWM. B: pTZ:AC1 mod clones digested with *EcoR*I and *Xho*I resulting in a 240-bp fragment for negative orientation inserts (lanes 2 and 3). Lane 1: Fermentas 1kb MWM.

A pTZ:AC1 mod clone was digested with *Bgl*II and *Sca*I and the resulting 1081-bp fragment (Figure 4.6A) was gel purified and ligated to the 1263-bp fragment generated from the *BamH*I/*Sca*I digestion of a pTZ:AC1 unmod clone (Figure 4.6B). In this way a pTZ:AC1 I-hp clone was generated. Clones harbouring the AC1 I-hp were identified by *Xba*I/*Xho*I digestion and sequenced with the M13 reverse primer (results not shown). The SACMV DNA sequence of the AC1 invert-repeat construct was converted to RNA sequence and submitted to the RNA MFOLD server (Rensselaer Polytechnic Institute) for secondary structure prediction at 25°C. It was confirmed that a stable hairpin structure

containing a long dsRNA stem was formed from the SACMV AC1 invert-repeat construct (results not shown).

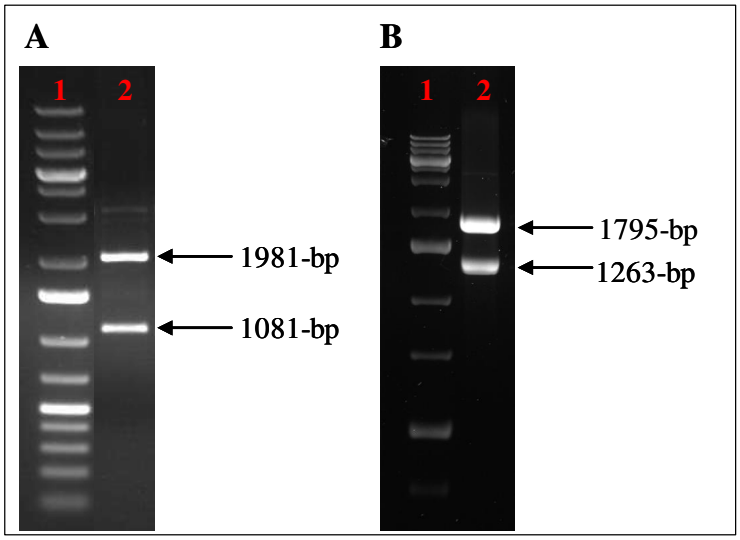


**Figure 4.6:** Restriction digests to produce fragments for head-to-head directional cloning to generate an invert-repeat construct. A: pTZ:AC1 mod clone *Bgl*II/*Sca*I digestion. The 1081-bp fragment contains the AC1 insert. B: pTZ:AC1 unmod clone *BamH*I/*Sca*I digestion. The 1263-bp fragment contains the AC1 insert.

The AC1 invert-repeat sequence was released from the pTZ vector (*Xba*I/*Xho*I) and successfully sub-cloned into pART7 to generate an expression cassette (Figure 4.7).

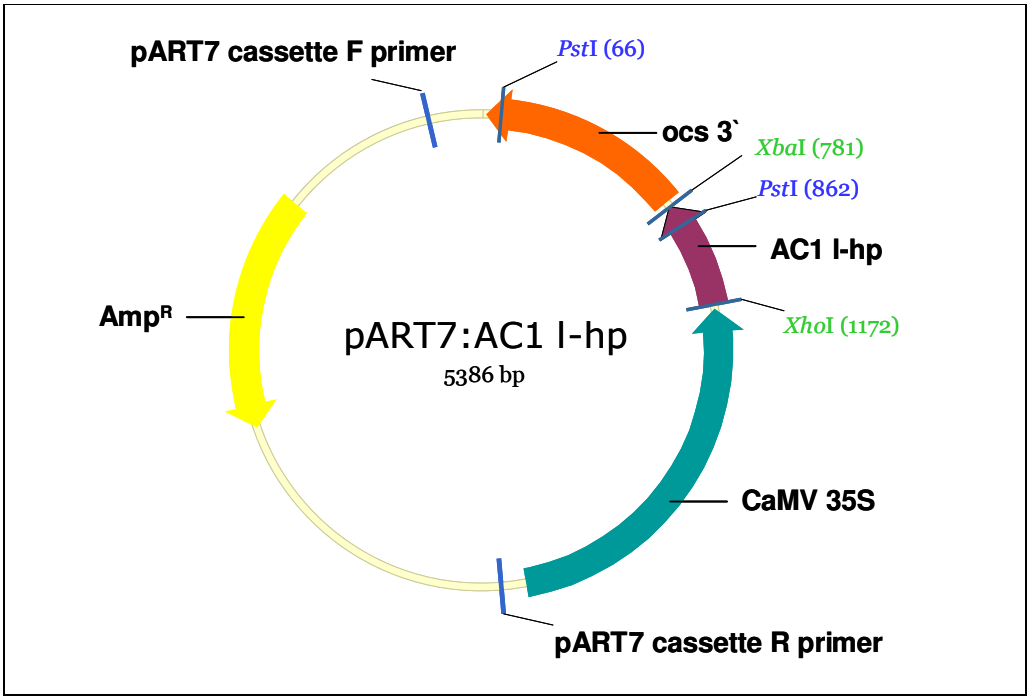


**Figure 4.7:** pART7:AC1 I-hp vector diagram illustrating the expression cassette comprising the CaMV35S promoter, AC1 invert-repeat sequence and ocs 3' terminator.

The AC1 invert-repeat cassette was successfully amplified from a pART7:AC1 I-hp clone by PCR (pART7 cassette R and F primers; Figure 4.7). To assist with the sub-cloning of the invert-repeat cassette into pCambia binary vector 1305.2, which has a limited multiple cloning sites, the AC1 invert-repeat cassette was first TA-cloned into the pTZ57R vector (pTZ:AC1 I-hp). Positive clones were identified by *Pst*I digestion, resulting in the generation of three DNA fragments, orientation dependent (3117, 1770 and 796-bp or 4639, 796 and 278-bp; results not shown). The AC1 I-hp cassette was released from the pTZ:AC1 I-hp clone with *EcoR*I and *Hind*III and sub-cloned into the respective *EcoR*I and *Hind*III digested pCambia vector (Figure 4.8). Putative clones were screened by

restriction with *Sca*I, which resulted in a 3167-bp fragment unique to positive pCambia 1305.2 clones (Figure 4.9).

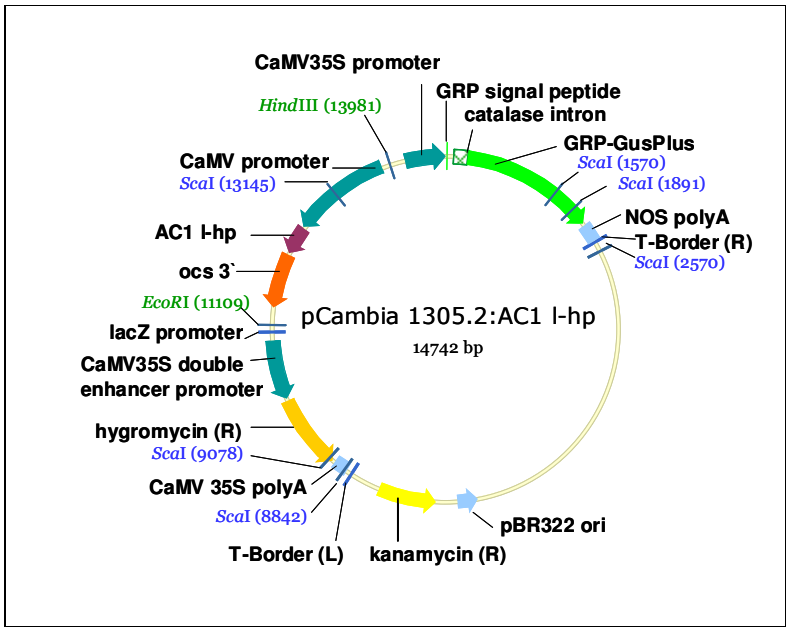


**Figure 4.8**: Vector diagram of pCambia 1305.2:AC1 I-hp. The AC1 invert-repeat sequence was inserted in the *Eco*RI and *Hind*III sites. *Sca*I restriction was used to screen for positive clones.

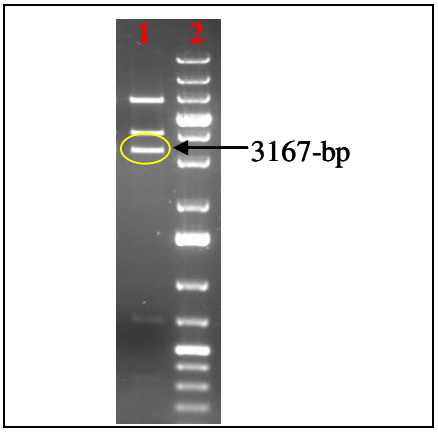


**Figure 4.9:** Screening pCambia:AC1 I-hp clones. Clones were digested with *Sca*I to a unique 3167-bp fragment in positive pCambia 1305.2 clones (lane 1). Lane 2: Fermentas 1kb MWM.

### 4.4.2 *N. benthamiana* transformation with AC1 invert-repeat construct

*Agrobacterium* AGL1 strain was transformed with pCambia 1305.2:AC1 l-hp. Transformants were selected for at 30℃ for 2 days on Luria Agar supplemented with 100µg/ml kanamycin and 100µg/ml carbenicillin. Putative clones were screened by PCR (pART7 cassette F and R primers; Figure 4.7). A 2827-bp amplicon was obtained for positive clones (Figure 4.10).

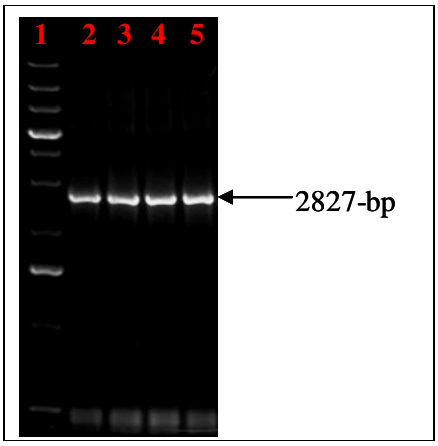


**Figure 4.10:** AGL1:1305.2 AC1 l-hp clones screened by PCR with pART7 cassette primers. Lanes 2 to 5: A 2827-bp amplicon was obtained for positive clones. Lane 1: Fermentas 1kb MWM.

Sixteen $T_0$ plants were confirmed to be transformed by PCR of the *hpt*II selection marker gene (not shown). GUS assays of selected leaves of 1305.2:AC1 l-hp lines showed strong GUS activity, and no chimaeric expression was observed (Figure 4.11; three representative lines shown).

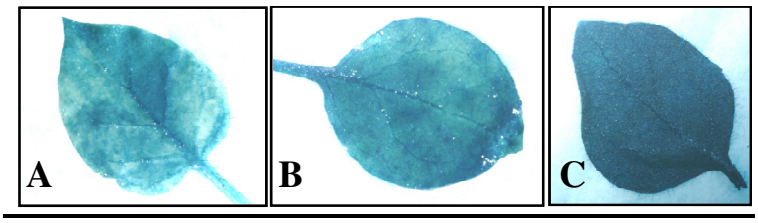


**Figure 4.11:** Gus expression in leaves from $T_0$ *N. benthamiana* 1305.2:AC1 l-hp transformants. A, B and C: representative leaf from three lines.

### 4.4.3 siRNA detection in unchallenged $T_1$ lines

Small interfering RNAs (siRNAs) were detected in ten unchallenged AC1 l-hp $T_1$ *N. benthamiana* lines. Using a $^{32}$P-labelled 36-mer RNA probe with 26-nt sequence complementary to the SACMV AC1 sequence, siRNAs of between 20-nt and 26-nt were detected by autoradiography in the ten lines (Figure 4.12; four representative lines shown).

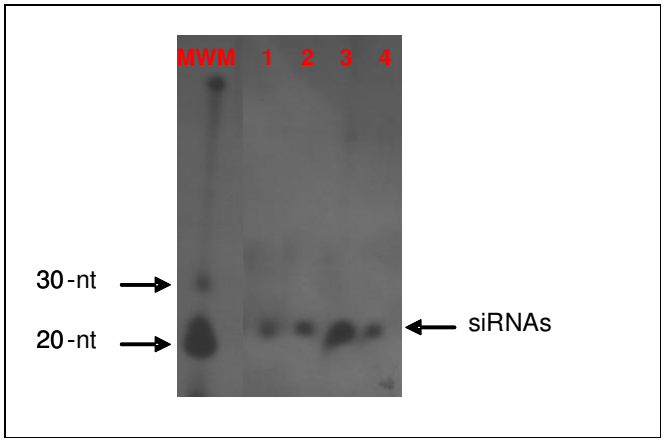


**Figure 4.12:** siRNAs detected in AC1 l-hp $T_1$ *N. benthamiana* using an RNAse-protection assay. A siRNA of between 20-nt and 26-nt complementary to AC1 SACMV sequence was visualised in each of ten lines. Lanes 1 to 4: Lines A2, A3, A4 and A11. MWM: Decade™ Marker, Ambion.

### 4.4.4 Quantification of DNA-A in SACMV challenged $T_1$ lines

Ten $T_1$ lines were challenged with SACMV DNA-A and DNA-B by *Agro*-inoculation (four replicates per line). Plants were scored for symptom severity twenty-eight days post infection (dpi). No symptoms were observed in any challenged plants from lines A2, A12, A16, B4 and B6 at 28 dpi. The remaining five lines had a least one plant with mild symptoms at 28dpi (Table 4.2). Viral load was quantified by real-time PCR at 28 dpi. Lines A16 ($p<0.05$), A2, A3, A12, B4 and B6 ($p<0.01$) show significantly less viral DNA than the control (GraphPad InStat 3.01; GraphPad Software, Inc., San Diego, CA). The reduction in SACMV replication was between 98% and 99.9% (Figure 4.13)

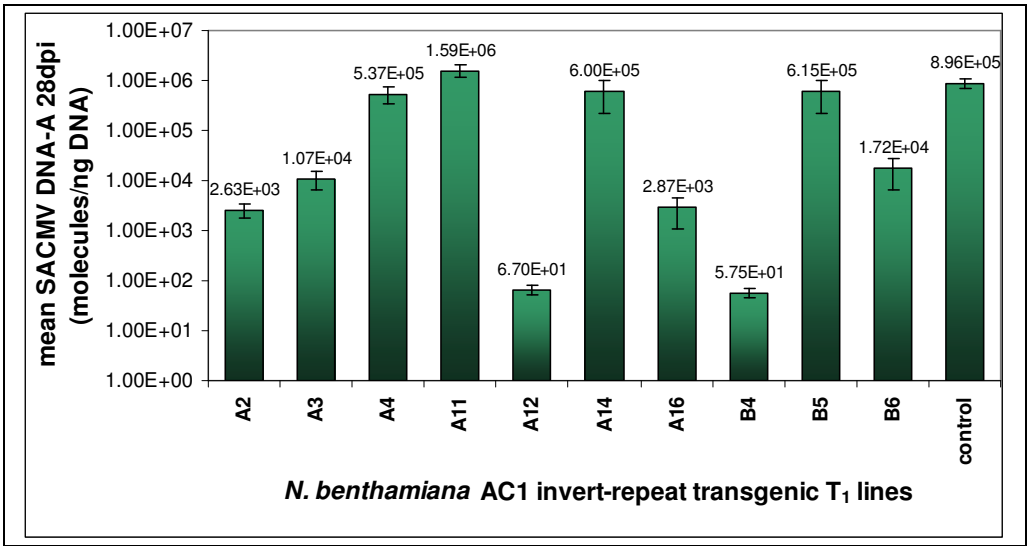


**Figure 4.13:** SACMV DNA-A quantified at 28dpi in SACMV-challenged *N. benthamiana* AC1 I-hp lines (n=4). Control – pCambia 1305.2 SACMV-challenged *N. benthamiana*. The amount of SACMV DNA-A is expressed as molecules/ng DNA and is the mean of four replicates per line. Error bars represent standard error of two quantification reactions. Y-axis is logarithmic scale.

**Table 4.2:** AC1 l-hp transgenic $T_1$ challenged plants showing SACMV symptoms 28 dpi.[1]

| Plant | SACMV DNA-A 28dpi (molecules/ng DNA) | Symptom score 28dpi |
|---|---|---|
| A3 rep 2 | (1.33E+06) | 2 |
| A4 rep 3 | (1.14E +06) | 2 |
| A4 rep 6 | (1.00E+06) | 2 |
| A11 rep 1 | (1.80E+06) | 2 |
| A11 rep3 | (1.20E+06) | 2 |
| A11 rep6 | (3.37E+06) | 2 (slight curling and mosaic on upper leaves indicated below)  |
| A14 rep1 | (2.09E+06) | 2 |
| A14 rep 4 | (1.80E+06) | 2 |
| B5 rep 3 | (1.83E+06) | 2 |

[1] Symptom severity was scored as by Fauquet and Fargette (1988).

### 4.4.5 Cassava transformation

#### Agrobacterium elimination

*Agrobacterium* AGL1 strain was used to transform cassava TMS60444 FEC and MTAI 16 cotyledons with the invert-repeat construct (AC1 l-hp). This is a highly virulent strain and is not recommended for cassava cotyledon transformation. The less virulent LBA4404 strain is preferable since it requires a less rigorous washing regime (*pers comm*. P. Chavarriaga). The virulence of the strain did not pose a problem in this study. The co-cultured cotyledon explants

were blotted dry with filter paper as recommended and washed in MS medium sucrose and 50mg/L cefotaxime for seven days. No subsequent bacterial growth was noted.

**Regeneration**

Shoot initiation was unsuccessful in all putative transformed cassava explants. The twenty cotyledon explants that were co-cultured with *Agrobacterium* remained green whilst on shooting medium but did not produce new shoots. Similarly, FEC clusters did not grow, nor begin to produce shoots. After several months, all FEC and cotyledons were sacrificed for the transformation confirmation experiments.

**Confirmation of transformation**

Successful transformation of cassava cotyledons and FEC was confirmed by GUS assays and PCR. Since shoot induction was unsuccessful, all co-cultured tissues were used in the GUS assay and subsequently in PCR. GUS was not detected in any of the FEC clusters (Figure 4.14a) however transformation was confirmed by PCR (Figure 4.15). Twenty cotyledons showed strong GUS expression (Figure 4.14b) and the region of the GUSPlus gene was amplified by PCR (Figure 4.15).

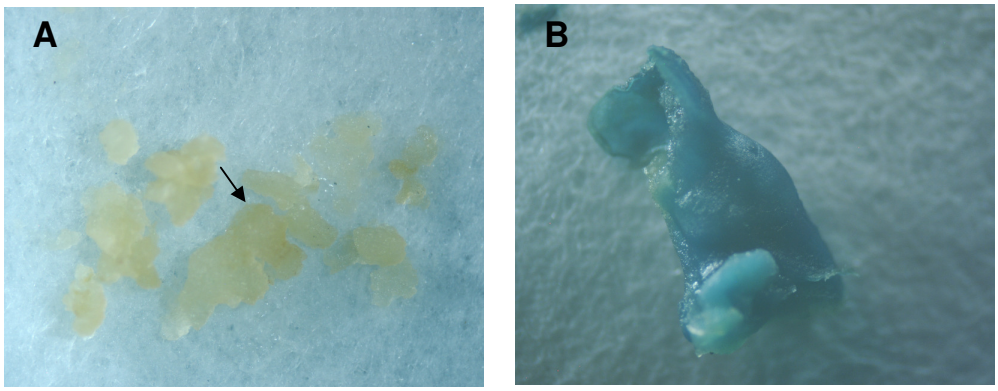


**Figure 4.14:** GUS expression of ACI l-hp transformed cassava explants. A: GUS expression was not evident in TMS 60444 FEC. B: Strong expression was detected in all MTAI 16 cotyledons.

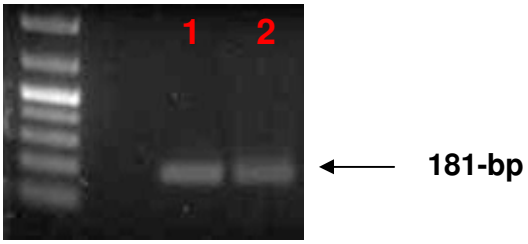


**Figure 4.15:** PCR confirmation of AC1 l-hp transformed cassava tissues by amplification of a 181-bp region of the GUSPlus sequence. Lane 1: TMS60444 FEC; lane 2: MTAI 16 cotyledon.

## 4.5 DISCUSSION

In this study, an invert-repeat construct (AC1 I-hp) was designed for the induction of RNA silencing of *South African cassava mosaic virus* (SACMV). A novel application of sodium bisulphite was adopted which alleviated the cloning difficulties that were previously encountered (chapter 3). The efficiency of the construct to effectively reduce SACMV DNA-A replication in *Nicotiana benthamiana* was tested and encouraging SACMV replication knockdown results were obtained. Cassava explants were successfully transformed with the invert-repeat construct. However, the transformed tissues were not regenerated and therefore knockdown efficiency of the invert-repeat (IR) construct in cassava was not established.

Previous attempts to produce invert-repeat constructs with SACMV DNA proved challenging. We found the extensive secondary structure that results from long stretches of invert repeat DNA makes cloning, sequencing and transformation almost impossible (discussed in chapter 3). Therefore a technique was adopted in this study whereby basepair mismatches were introduced into the sense arm of the invert-repeat sequence thereby stabilising the construct. As a result, all subsequent cloning steps were easily performed. Deamination of cytosine by the mutagen sodium bisulphite resulted in cytosine to thymine mutations in the sense arm of the IR sequence. Sequence analysis revealed 37 cytosine nucleotides (100%) were mutated after 2.5 hours of treatment with sodium bisulphite. This resulted in twenty-eight percent of the nucleotides making up the arm of the IR being mismatched. Using MFOLD, it was predicted that a stable hairpin structure containing a long (~180 bp) dsRNA stem would be formed from the SACMV AC1 invert-repeat construct at 25℃.

Previously in our laboratory, it has been shown that there is no significant difference in the number of mutations that occur after five minutes of treatment with sodium bisulphite (55-80%) and it was noted that 10% mutation efficiency was found after 2.5 hour treatment (Harmse, unpublished). The efficiency of sodium bisulphite to deaminate cytosine residues was not in the scope of this study, however it does seem as if deamination efficiency is variable. In this study several clones were screened before a clone was obtained in which 100% of the cytosine residues had been deaminated.

*Nicotiana benthamiana* leaf discs from *in vitro* germinated plantlets were transformed with *Agrobacterium* harbouring the invert-repeat construct in a plant transformation vector. Shooting and rooting was successfully induced on hygromycin-selection medium. An ethylene-scrubber was used to reduce excessive ethylene accumulation in the sealed tissue culture vessels. Ethylene accumulation has been associated with hyperhydricity, leading to the vetrification of *in vitro* plants (Parka *et al*., 2004). This phenomenon has been encountered in our laboratory, and it is now standard practice to include the ethylene scrubber when regenerating *in vitro* plants. Potassium permanganate saturated perlite granules were used to absorb excess ethylene from the *in vitro* system.

Ten $T_1$ lines were established under greenhouse conditions and challenged with SACMV DNA-A and DNA-B. Symptom development was delayed with mild symptoms in newly emerged leaves being recorded in some plants at 28dpi. Six lines showed SACMV resistance by a significant reduction in replicating viral DNA-A (98 to 99.9%) in the newly emerged leaves. Similarly, in a recent study in which RNA silencing against *Tomato chlorotic mottle virus* was induced, five of eleven *N. benthamiana* $T_1$ lines expressing transgene-derived

double-stranded RNA showed enhanced resistance with a significant delay in symptom development in most plants. The two best performing lines had 50% and 30% virus free plants at 45dpi (Ribeiro *et al*., 2007). In our study, all of the plants in five resistant lines were symptom free at 28dpi. Ingelbrecht *et al*. (1999) obtained immunity (no symptoms and virus free; 9 of 220 plants) to SrMV-SCH using an untranslatable sorghum mosaic potyvirus strain coat protein gene and showed undetectable transgene mRNA, but active transcription of the transgene in three immune plants. Immunity was not achieved by the invert-repeat construct used in our study since some viral DNA-A was detected in symptom free plants.

In some cases high standard deviation within lines was obtained when quantifying the amount of replicating SACMV DNA-A in challenged AC1 transgenic lines (data not shown). Similarly Zrachya *et al.* (2007) found differences in transgenic tomato expressing an intron-hpRNA against TYLCV CP with high standard deviation values within resistant lines. The agro-inoculation method used in our study makes it difficult to control the amount of viral DNA being delivered to the plant and some plants may receive more viral DNA than others. This could account for the within line variation observed. Also resistance to SACMV infection at different viral load pressures was not assessed in the AC1 l-hp transgenic lines, due to the lack of controlled delivery of viral DNA. We could achieve this by using a transient assay system of leaf disc inoculation as reported by Zhang *et al.* (2005).

The presence of siRNAs of SACMV sequence origin in the ten $T_1$ lines confirmed the action of an RNA silencing mechanism induced by transgene derived-hpRNA. One size class of siRNA was detected in unchallenged transgenic lines. This size class corresponds to the smallest size class of siRNA

that was detected in sense and antisense transgenic lines in chapter 2. It is unlikely that only one size class is generated by the processing of this invert-repeat transgene transcript, since it has been hypothesized that the production of different sized siRNAs may vary along the length of the hairpin. There was no correlation between the level of siRNA detected and resistance phenotype as has been demonstrated in ACMV (Kumria *et al*., 2008). This is in disagreement with the independent observations that a correlation exists between high siRNA levels and resistant phenotypes (Chellappan *et al*., 2004, Zhang *et al*., 2005; Fuentes *et al*., 2006, Vanderschuren *et al*., 2007 and Ribeiro *et al*., 2007). It is expected that other size classes would have been detected in challenged AC1 l-hp transgenics since cassava-infecting geminiviruses have been shown to produce siRNAs probably from illegitimate read-through transcripts (Chellappan *et al*., 2004). Three size classes of siRNAs (transgene and virus-derived) were detected in ACMV infected transgenic cassava (Vanderschuren *et al*., 2007).

This study adds to the list of successful reduction of viral replication by siRNA induced RNA silencing of plant viruses, particularly geminiviruses. More so however, the prospects towards developing SAMCV resistant cassava using an RNA silencing induction mechanism are promising. Here we have developed a technique (patent WO 2008/093283) that allows for the rapid generation of invert-repeat expression cassettes through the stabilization of the invert-repeat construct. In this study the invert-repeat expression cassette transcript was shown to be effectively processed into siRNAs. These siRNAs in turn effectively induce a RNA silencing pathway that resulted in highly resistant *N. benthamiana* lines.

Following this success of an invert-repeat RNA silencing construct to alleviate SACMV replication in *N.* benthamiana, three cassava cultivars were selected for transformation with the construct. TMS60444 is a widely used model cultivar and as expected, this was the cultivar from which FEC was the most prolifically obtained. Very little FEC was obtained from SM 1433-4 and T200 cultivars in the time frame of this study, therefore these cultivars were not used. The MTAI 16 cotyledons were a kind donation and so these were included in this study. Transformation of TMS60444 FEC and MTAI 16 cotyledons was successful as confirmed by PCR. Although the presence of the GUSPlus gene was confirmed by PCR, GUS expression was not detected in X-Gluc stained FEC. This phenomenon is likely to be due to tissue age since the callus had been on regeneration medium for several months. It is encouraging that 100% transformation efficiency was obtained for the cotyledons but it is disappointing that the transformed explants could not be regenerated. Low regeneration efficiency reported by Vanderschuren *et al.* (2007) was suggested to be due to transgene-derived siRNAs affecting the regeneration of transformed tissues. Other cassava transformation and regeneration methods are currently being explored in our laboratory.

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# CHAPTER 5 - Final conclusions

The first account of transgene induced RNA silencing of *South African cassava mosaic virus* (SACMV) was reported in this study resulting from the expression of sense and invert-repeat constructs. RNA silencing of other cassava mosaic geminiviruses, especially strains of *African cassava mosaic virus* and *East African cassava mosaic virus* can be expected due to sequence identity of 90 to 93% within the AC1 target region of these constructs. A sense transgene construct expressing a truncated SAMCV AC1 sequence in *Nicotiana benthamiana* induced the production of siRNAs and resulted in the sequence specific degradation of almost 100% of the incoming SACMV AC1 transcript in two lines. It is believed that an RNA-dependent RNA polymerase converts ssRNAs transcribed by sense transgenes to dsRNA molecules, which induce RNA silencing (Dalmay *et al*., 2000; Brodersen *et al*., 2006). Before it was learnt that dsRNA plays a crucial role in a number of RNA silencing pathways, pathogen resistance strategies in plants focused on the use of sense and antisense transgenes. One proposed antisense silencing mechanism was that the antisense transgene transcript, through RNA:RNA hybridization, blocked the translation of a homologous gene transcript (Bourque, 1995). Successful viral suppression using antisense RNA has been reported (examples include Day *et al*., 1991; Bejarani *et al*., 1994; Bendahmane *et al*., 1997). More recently Praveen *et al.* (2005), Mubin *et al.* (2007) and Zhang *et al.* (2005) obtained resistance lines, expressing an antisense transgene, to *Tomato leaf curl virus*, *Tomato leaf curl New Delhi virus* and *African cassava mosaic virus* respectively. No SACMV

resistant lines were obtained in our study using an SAMCV AC1 antisense construct, and it is speculated that this is due to the low number of lines tested.

Although almost 100% viral replication knockdown was achieved with a sense SAMCV AC1 transgene, only two resistant lines were obtained. To improve silencing efficiency, invert-repeat (IR) transgene constructs were developed. The expression of hpRNA from an IR transgene is a more efficient initiator of RNA silencing (Waterhouse *et al.*, 1998). The pHannibal gene-silencing vector was used to generate two RNA silencing constructs containing sense and antisense arms of 572- or 193-bp separated by a spliceable intron. Intron-containing invert-repeat constructs have been shown to result in 100% silencing efficiency (Smith *et al.*, 2000). Secondary structure and predicted formation of cruciform-junctions in the perfectly matched invert-repeat sequences introduced problems when cloning, sequencing and transforming the constructs. Therefore the constructs were tested for efficacy of SACMV replication reduction in a transient expression system. Minimal gene expression cassettes were produced and bombarded together with SACMV DNA-A and DNA-B into *N. benthamiana* callus tissue and a 60% to 80% reduction in SACMV DNA-A replication was observed with the IR construct with the shortest arm length providing the greatest reduction in viral replication. RNA silencing of geminiviruses has been successfully induced by dsRNAs of various arm lengths. Some examples include 209-nts against *Vigna mungo* yellow mosaic virus; (Pooggin *et al.*, 2003); 19-nts under the expression of *Arabidopsis 75L* RNA gene promoter in tobacco (Lu et al, 2004); 726-nts of *Tomato yellow leaf curl virus* C1 sequence origin (Fuentes et al, 2006); 938-nts to induce the degradation of the *Tomato chlorotic mottle virus* AC1 transcript (Ribeiro et al, 2007) and 419nts of TYLCV coat-protein derived sequence (Zarchya et al, 2007).

RNA silencing constructs need to be produced and tested in the laboratory in the shortest possible time, prior to lengthy greenhouse and field testing. It is therefore not ideal to encounter cloning and transformation complications as described in chapter 3. To improve on this situation, sequence mismatches were introduced into the sense arm of an invert-repeat sequence, using a novel application of sodium bisulphite, which stabilized the construct and facilitate downstream cloning and transformation steps. This resulted in a considerable reduction in the time taken from construct design to testing *in planta*. The expression cassette containing the sequence modified invert-repeat sequence was stably expressed in *N. benthamiana.* Six transgenic lines exhibited significant reduction (approximately 99%) in SACMV DNA-A replication. In addition the invert-repeat construct was tested in a transient system whereby *N. benthamiana* callus was co-bombarded with the minimal gene expression cassette (35S promoter: invert-repeat sequence: terminator) and SACMV DNA-A and DNA-B, resulting in a 93% reduction in SACMV DNA-A replication compared to the control (data not shown). This illustrates a correlation between the quantification of viral DNA replication in a transient system and in stably transformed *N. benthamiana* lines. The transient callus assay used in this study negates the need for *Agrobacterium,* compared to the *in vivo* transient leaf assay based on *Agro*–infiltration with a gene construct that has been developed in cassava (Yadav *et al.*, 2008).

The application of absolute real-time PCR used in this study, allowed for the quantification of transgene RNA in unchallenged plants and SACMV DNA-A in challenged plants for comparative purposes. Current methods to quantify viral load in transgenic lines include dot-blot hybridisation, as in a recent study of transgenic tomato expressing the coat protein for TYLCV (*Tomato yellow leaf curl*

*virus*) (Zrachya *et. al*., 2007), or southern blots as used by Chellappan *et. al.* (2004) to quantify ACMV infection in transgenic cassava. Both these methods require large quantities of nucleic acid and radioactive probes. Mason *et al.* (2008) proposed a method for relative and absolute viral load quantification using real-time PCR. This method requires the use of TaqMan probes and internal references in the form of endogenous genes. The absolute quantification method described here using SybrGreen chemistry is less time consuming and requires less starting material than either Southern or Northern blotting and is more cost efficient than relative quantification or absolute quantification using TaqMan chemistry. Recently, Beltrán *et. al.* (2008) developed a real-time PCR-based method to quantify transgene expression using relative quantification which requires the use of a reference gene. Certain precautionary measures are however always required when performing real-time PCR experiments, including careful experimental design, precise quantification, and careful sample preparation (Freeman *et al*., 1999). It was important in this study to obtain a viral load or transgene RNA value that could be compared between transgenic lines. It was therefore decided that the most accurate way to express these values was as a representative of sample added to each PCR reaction (DNA or cDNA). This ensured that any variation between samples in the nucleic acid extraction procedures or cDNA synthesis reactions was not incorporated into the final result.

With the technologies developed in this study, the realization of obtaining cassava mosaic geminivirus resistant cassava is progressing. An efficient construct design and production technique is fundamental and is now in place in our laboratory. The minimal cassette technology-based method applied in this study to test the efficacy of the constructs allows for an initial and rapid screen. Using real-time PCR, viral DNA replication in transformants can be quantified

with the purposes of comparing lines and constructs. Reliable and efficient cassava transformation and regeneration, especially of African cultivars, remains an obstacle in the passage towards virus resistant cassava in laboratories throughout Africa (Schlegel *et al.*, 2008). The quality of embryonic tissue used as the primary tissue for cotyledon and FEC induction is critical for successful transformation and regeneration. In a recent study on African cultivars, it was determined that larger leaf lobes, mid-rib wounding and crushing the embryonic structures through 1mm mesh increased quality of FEC produced (Jayatilleke *et al.*, 2008). Studies are continuing in our laboratory in South Africa to optimise transformation and regeneration of somatic cotyledons and friable embryogenic callus of selected local cultivars. It has been determined that the optimal conditions for somatic embryo production in six cultivars including the model cultivar (TMS60444) and the local cultivar (T200) are axillary buds on Murashige and Skoog medium supplemented with 50µM picloram (Rossin, unpublished).

The commercialization of genetically modified cassava in southern Africa requires proof of concept at the levels of laboratory and greenhouse testing, small-scale confined field trails and large-scale field trials (*pers. comm.* J. Thome). Furthermore, fundamental to making the commercialization of GM cassava a success is communication with the public sector. Public confidence in the safety of the crop must be gained prior to commercialization. Demand requires there to be no negative environmental impact, toxicological effects nor allergenic effects of the genetically modified product and thinking towards this end must begin at the construct design phase. The integration of vector backbone sequences is undesirable as it could lead to illegitimate recombination events, transgene rearrangement and plasmid multimerization (Kumar *et al.*, 2006; Vidal *et al.*, 2006). Biosafety regulations are jeopardized when commercial

transgenic plants are produced that contain unwanted bacterial sequences, including antibiotic resistance genes (Lange *et al*., 2006). Minimal cassette technology was explored in this research and it provides a way of excluding unwanted bacterial sequences and most importantly, antibiotic resistances genes from the constructs that enter the plant tissue.

Furthermore, minimal cassette technology can play a role in the quest to improve the transformation of cassava. Cassava transformation by particle bombardment is an efficient way of transferring DNA in to intact tissues (Zhang *et. al*., 2000). Cassava friable embryogenic callus and cotyledon-derived callus tissue have successfully been transformed using particle bombardment, however instances of somoclonal variation have been reported (Taylor *et al*., 2004). In addition, particle bombardment usually results in multiple transgene integration and this has been reported to induce transgene silencing in subsequent generations. Simple integration events and low transgene copy number (one to two copies) are associated with minimal cassette technology (Fu *et. al.,* 2000). Future research should focus on the optimization of cassava transformation by the delivery of minimal cassette DNA into friable embryogenic callus by particle bombardment.

Several construct designs can be explored in conjunction with minimal cassette technology. Stable intron-containing invert repeat constructs can be produced using the pHannibal vector system by cloning sequence modified sense fragments. Direct repeats have been shown to induce RNA silencing in plants (Ma *et. al.,* 2002; Xu *et. al.,* 2008) and are easier to make than invert repeat constructs (Xu *et. al*., 2008). Multiple gene targeting, as well as multiple

virus complex resistance can be achieved by cloning three or four repeats of each gene fragment.

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