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Optimisation of regeneration systems for a range of Cassava (Manihot Esculenta Crantz) cultivars suitable for growth in South Africa and transformation with SACMV N-REP gene

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Chapter 5

OPTIMIZATION OF SACMV N-REP TRANSFORMATION EFFICACY USING TOBACCO LEAF DISKS AND CASSAVA FEC

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5.1 INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is the most important root crop in sub-Saharan Africa. It is cultivated for its starchy tuberous roots and its leaves are relatively rich in protein and can be consumed. It terms of caloric production, it ranks fourth after rice, wheat and maize in developing countries (Schöpke *et al*., 1993; FAO 2003). It provides food for over 600 million people, mostly small-scale and subsistence farmers in developing countries (Taylor *et al*. 2004). Cassava mosaic disease (CMD) is the most important disease of cassava in Africa, causing an estimated loss of yield of 1200 to 2300 million US dollars a year (Thresh *et al*., 1997; Hillocks and Thresh, 2000). CMD is caused by whitefly-borne viruses of the genus *Begomovirus* (family *Geminivridae*) of which *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV) and *South African cassava mosaic virus* (SACMV) have been identified (Berrie *et al*., 1998; Hillocks and Thresh, 2003; Ogbe, 2003). Berrie *et al*. (1998) characterized SACMV as a begomovirus possessing two DNA molecules called DNA-A and DNA-B. Component A of the genome encodes *AC1* gene on the virion strand, the coat protein (*AV1*) gene and three overlapping genes on the complementary strand (Sangaré *et al*. 1999). Of the latter, the *AC1* gene (Rep gene), replication-associated protein gene, is responsible for the replication of both genomic components, *AC2* is thought to be involved in the *trans*-activation of sense gene transcription from both A and B components, and *AC3* and the B component is implicated in efficient replication of the virus while the function of *AC4* remains elusive (Sangaré *et al*.1999). The component encodes one gene in the sense

orientation (*BV1*) and another one in the complementary sense (*BC1*) and both are indispensable for virus movement (von Arnim *et al*. 1993).

Several strategies have been used to engineer plants resistant to viral pathogens. Genetically engineered expression of viral gene sequences has been proposed as an efficient system to confer protection against virus diseases by eliciting protection mechanisms in the plant (Noris *et al*. 1996; Brunetti *et al*. 1997; Chatterji *et al*. 1999; Sangaré *et al*. 1999; Sivamani *et al*. 2000). These are one of the examples of pathogen derived resistance (PDR) where sequences from a pathogen are used to protect the host from the effects of pathogen.

Rep protein-mediated resistance against a virus in transgenic plants was first shown in tobacco against TMV in plants containing the 54 kDa putative Rep gene (Golemboski *et al*. 1990). The study showed that transgenic tobacco plants expressing the Rep gene were highly resistant to challenge inoculation by TMV. Gene constructs of Rep genes that have been used for resistance include full-length, truncated or mutated genes. Hong *et al*. (1996) showed that transient expression of *AC1* or the truncated N-terminal portion of the protein caused significant reduction in the level of viral DNA replication in *N*. *tabacum* protoplasts. In another study by Noris *et al*. (1996), a truncated form of the Rep gene, capable of expressing the N-terminal 210 amino acids (aas), was cloned under the control of the CaMV 35S promoter and introduced into *N*. *benthamiana* using *Agrobacterium tumefaciens*. When self-pollinated progeny of 19 primary transformants were tested for resistance to TYLCV by agroinoculation, some plants proved to be resistant, particularly in the sense lines.

Recently, Zhang *et al*. (2003) developed an alternative resistance strategy in which a hypersensitive reaction is mimicked using the bacterial *barnase* and *barstar* genes from *Bacillus amyloliquefaciens* under control of the ACMV DNA-A bidirectional promoter. In un-induced conditions, this promoter has a low basal activity in both directions. Upon infection, the virion-sense (AV) promoter is up-regulated by the ACMV TrAP protein, while the complementary sense (AC) promoter is down-regulated. In their study, six out of sixty transgenic plant lines recovered, integration of both *barnase* and *barstar* genes was detected by PCR and Southern analyses. Expression of *barnase* and *barstar* at the RNA level was detected by RT-PCR in five of these lines. A viral replication assay with leaves of untransformed and transgenic plants showed a reduction of viral replication activity in transgenic leaves by 86% to 99% (Zhang *et al*. 2003).

In South Africa (SA), cassava is grown by subsistence farmers and commercially for starch production. Cassava transformation capability is still underdeveloped for SA grown and highly desirable cultivars, especially those susceptible to virus infection by SACMV, leading to a decline in yields. In order for the cassava farmers to benefit from cassava biotechnology advances already achieved by well established overseas laboratories such as ILTAB, Wageningen University, ETH and CIAT, there has to be a strong technology transfer programme that will support local scientists in the transfer of already existing cassava transformation systems. Through collaboration with ILTAB cassava transformation techniques were accessed in this study to allow development cassava transformation and regeneration systems for a local commercially-grown

cassava landrace T200. This cultivar was shown to be responsive in the generation of organised embryogenic structures (86%) (see results in Chapter 3), a requisite step for the subsequent production of friable embryogenic structures which are considered ideal for gene insertion. In order to engineer resistance against SACMV, a N-terminus truncated replicase (N-Rep) was isolated by PCR. We report results of transformation of tobacco leaf disks and cassava FEC tissues with two truncated SACMV-N-Rep sequences of different sizes.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

Cassava plants infected with SACMV and healthy plants were obtained from Mpumalanga Province situated in the eastern part of S.A. and maintained in a greenhouse at 25 ºC with a day length of 16h and a dark period of 8h.

5.2.2 Preparation of the first 600bp N-Rep construct

Polymerase chain reaction primers pAL1v1979 (5´GCATCTCTGCAGGCCA CATYGTCTTYCCNGT3´) and pAR1c496 (5´AATACTGCAGGGCTTYCTRT ACATRGG3´) were used to amplify a fragment of *AC1* region of SACMV, approximately 1400bp. This fragment contained an internal *PstI* site, producing two fragments of approximately 600bp and 793bp respectively. The truncated N-terminus 600bp was then cloned into p*Bluescript* (clone pACMV7). This fragment was ligated into pART7 intermediate vector at the *SmaI* site. Transformed colonies were selected on LB medium containing 100µg/ml streptomycin and

incubated at 37 ºC for 16h. Colonies were screened for the desired recombinants. Both pART7-NRep and pBI121 were digested with *NotI* and then transformed into *E.coli* HB101. Digested plasmids were electrophoresed in a 1% low melting temperature agarose gel and the bands of interest extracted. Sticky-end ligation of *NotI* fragments was performed. Putative clones of pBI121-N-Rep were digested with *NotI* in order to determine whether the correct sized fragment had been cloned. Triparental mating was performed with *A*. *tumefaciens* LB4404, pRK2013 and a control pBI121 without N-Rep and LB4404, pRK2013 and pBI121-N-Rep as described by Semsey *et al*. (2001) (Fig 5.2)

5.2.3 Preparation of the second 621bp N-Rep construct

Since transformation results of the 600bp N-Rep proved unsuccessful due to its toxicity, another N-terminus truncation (621bp) of the *AC1* gene from SACMV was produced. The primers PN-REPC2611 (5´AATGAATTCCTCA CGTATCCG3´) and PN-REPV1990 (5´CGATGAGGATCCTACTCGG3´) were designed and a 621bp N-Rep was first cloned into pMON999 vector to fuse it to the 35S promoter and the nos 3´ stop sequence, prior to subsequent cloning into pCAMBIA2301,the plant transformation vector, which also contains the GUS visual marker gene driven by the 35S promoter. Successful cloning of N-Rep was confirmed by restriction analysis and PCR. The newly prepared N-Rep gene in the pCAMBIA2301 vector was then used to transform both S.A. cassava cultivar T200 FEC tissue by microparticle bombardment and tobacco leaf disks by *Agrobacterium* co-cultivation (Fig 5.3). Tobacco was employed as a model plant

species to allow rapid determination of the efficacy of the N-Rep transgene in controlling SACMV infections.

5.2.4 Transformation into *A. tumefaciens*

Competent *A. tumefaciens* cells from strain C58C1 were prepared as described earlier (Koo, 1999). Binary vector constructs pCAMBIA2301 and pCAMBIA2301-anti-N-Rep were individually transformed into C58C1 by heat shock and plated on Luria Broth (LB) media supplemented with 0.1% glucose plates containing kanamycin (50 mg/l) and rifampicin (30 mg/l). Two hundred µl were plated out and plates were inverted for two days at 28ºC. Several bacterial colonies were chosen for plasmid extraction. *Agrobacteria* were multiplied in YEP media (10g peptone, 10g yeast extract, 5g NaCl) containing glucose, kanamycin and rifampicin as above, for 24h at 28ºC with shaking at 180rpm. Plasmids were extracted using the Wizard Plus SV minipreps from Promega. Plasmids were sequenced by Inqaba Biotechnologies to confirm the presence of the insert in the plasmids. All C58C1 *Agrobacteria* strains containing the binary vector constructs described in the previous section 5.2.3 were used for tobacco transformation experiments.

5.2.5 Transformation of *N.tabacum* leaf disks

Leaves of 4 weeks-old tobacco plants from the greenhouse were pre-cultured by firstly surface sterilising in 10% sodium hypochlorite supplemented with 2 drops Tween20 for 15 min, followed by three rinses in sterile distilled water. Leaves

were cut into 0.5 cm^{-2} and the 10 leaf pieces were placed upside down on MS104 (MSO + BA 1 mg/l + NAA 0.1 mg/l + Difco-bacto agar 9 g/l, pH5.8) plate supplemented with 2 mls 4COO5K liquid medium (MS salts + B5 vitamins 4.4 g/l + sucrose 30 g/l + DL-p-chlorophenylalanine 4 mg/l, pH5.8) and two sterile filter discs in contact with the medium. Leaf disks were allowed to pre-culture for two days. Explants were then inoculated with an overnight *Agrobacterium* C58C1 pCAMBIA2301and C58C1 pCAMBIA2301-anti-NRep suspension that has been adjusted to 1.2 x 10^9 bac/ml with 4COO5K liquid medium (1/5 dilution). Two mls 1/5 agro culture was added directly to each plate of pre-cultured explants. Plates were allowed to incubate for 10 min and then *Agrobacterium* was pipetted off leaving explants as dry as possible. Plates were blot dried with sterile filter disc and allowed to co-culture for 3 days at 25 ºC. Explants were transferred to MS104 medium supplemented with kanamycin (300 mg/ml), carbenicillin (500 mg/ml) for selection phase. Explants were removed from filter paper and distributed evenly onto selection plates (10 explants/plate). After 4 weeks, shoots were cut from callus and placed on MSO medium (MS salts + B5 vitamins 4.4 g/l sucrose 30 g/l + Difco-bacto agar 9 g/l, pH5.8) supplemented with kanamycin (100 mg/ml), carbenicillin (500 mg/ml) rooting medium.

5.2.6 Hardening-off

After 5 weeks shoots with well developed roots were transferred into potting mix for hardening off. Plantlets were placed in containers covered with a transparent

lid which was uncovered gradually until plants were well acclimatised into the greenhouse conditions.

5.2.7 Transformation of cassava FEC tissues

Friable embryogenic tissue of about 21 days of age (since last subculture) was taken, selecting the best quality tissues. Selected FEC tissues from various mother plates were placed into an empty Petri dish and mixed together. Circular colonies of about 1.5 cm in diameter were made on GD2 50P medium (GD 20X Macro solution 40 ml + GD 1000X Micro solution 1 ml + FeEDTA solution 5 ml + GD 1000X Vitamin solution 1 ml + 1000 mM picloram 50 ml + sucrose 20g/l + Agar 7.9g/l, pH5.8) containing 100 µM acetosyrongone at a pH of 5.2-5.4. Colonies were bombarded with gold particles coated with pCAMBIA2301-anti-N-Rep DNA as described earlier in section 3.2.3.

Bombarded FEC tissues were transferred into baby jars with liquid SH2 50P medium (SH salts + vitamins + 1000 mM picloram 50 ml + sucrose 20g/l + pH5.8). After 5 days in this medium, the old medium was drawn off with a sterile pipette and replaced with 15 ml of fresh SH2 50P containing 500 mg/l carbenicillin and 25 µM paramomycin. Four days later tissues were transferred to Petri dishes containing semi-solid GD2 50P medium supplemented with 500 mg/l carbenicillin and 30 µM paramomycin. Fec tissues were drawn into a 10 ml pipette and transferred to GD agar plate. Excess liquid medium was removed using a 200 µl Pipetteman so that tissues are spread evenly over the agar surface. Ten days later, depending on the rate of the tissue growth, larger yellow

units were picked from the GD plate and transferred to a new plate of GD2 50P 500 mg/l carbenicillin and 30 µM paramomycin. The picking process was repeated every 7 days thereafter for a total of five pickings. After 3 weeks growth on the new selection medium, growing FEC colonies were transferred to the third selection stage. FEC tissues that remained growing and healthy were deemed as putatively transgenic. Such tissue was tested for marker gene expression by GUS assay as described by Jefferson (1987).

5.2.8 Molecular analysis of the second 600bp N-Rep construct

Putative transgenic tobacco plant lines were analysed using PCR and Southern blots for the integration and expression of N-Rep gene as described by Saambrook *et al* (1989).

5.3 RESULTS AND DISCUSSION

5.3.1 Cloning of the first 600bp N-Rep construct

N-Rep gene (600bp) of SACMV was PCR amplified using specific forward and reverse primers pAL1v1979 and pAR1c496. The truncated N-terminal region of *AC1* 600bp was initially cloned into p*Bluescript* as *PstI* fragment and later subcloned as *SmaI* fragment downstream of the 35S promoter of the intermediate vector pART7. From pART7-N-Rep, the truncated N-Rep cassette was further subcloned as a *NotI* fragment into the binary vector pBI121. The right clone was confirmed by restriction analysis and was mobilised into LBA4404 by performing triparental mating for transformation of tobacco leaf disks. Right

LBA4404-pBI121-N-Rep clones were confirmed successfully confirmed by means of PCR using primers pAC1v2078 and pAC1c2557 amplifying the truncated N-Rep.

5.3.2 Expression of the GUS gene in 600bp N-Rep transgenic tobacco plants and pCAMBIA2301-anti-N-Rep (621bp) transformed TMS60444 FEC tissues

A total of five hundred transgenic tobacco plants transformed with control LBA4404-pBI121, LBA4404-pBI121-N-Rep (600bp) and LBA4404-anti-N-Rep (600bp) were obtained (Fig 5.2). Different tissues of all these transformants were stained in X-gluc for histochemical GUS assay. Histological analysis of GUS expression proved negative in all lines. It was therefore deduced that n-rep (600bp) gene either cloned in sense or anti-sense, provided it is fused to the *uid*A gene, exerts toxic effects switching off the expression of GUS in tissues.

This led into the design of a second truncated N-Rep (621bp). The 621bp N-Rep was successfully cloned by first cloning its PCR product into pGEM-Teasy plasmid. The ligated N-Rep was released by digesting pGEM-N-Rep with *Eco*RI. The released *Eco*RI N-Rep fragment was further cloned into pMON999 expression cassette also digested with *Eco*RI so that the truncated N-Rep (621bp) is driven by the 35S promoter and terminated by nopaline terminator. The selected pMON999-anti-NRep clone and pMON999 expression cassette were digested with *Pvu*II whereas pCAMBIA2301 was digested with *Sma*I. Both the pMON999-anti-NRep clone and pMON999 expression cassette were ligated

into digested pCAMBIA2301 plant transformation vector to generate pCAMBIA2301-pMON999 and pCAMBIA2301-anti-NRep (621bp). These were later used to transform *Agrobacterium* C58C1 strain by heat shock method.



Fig 5.1 PCR analysis of *Agrobaterium* C58C1- pCAMBIA2301-pMON999 and C58C1- pCAMBIA2301-anti-N-Rep using NRep primers. Lane M Marker DNA, lane1-5 –ve control C58C1- pCAMBIA2301-pMON999 DNA, +ve control SACMV DNA, lanes 7-11 C58C1- pCAMBIA2301-anti-N-Rep DNA.

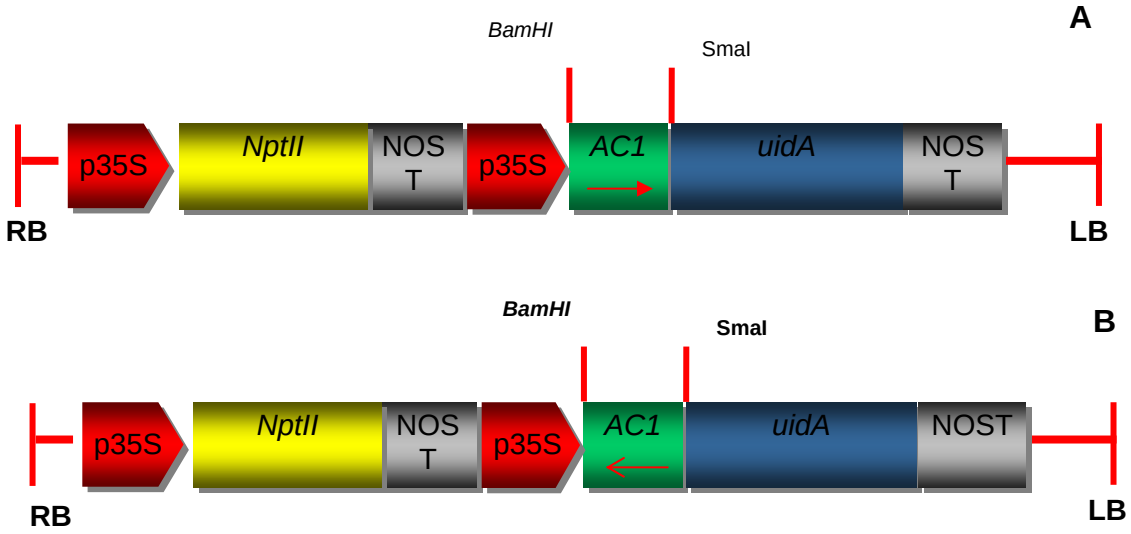


Fig 5.2 Diagrams represent constructs containing fusions between the GUS coding sequence and the AC1 sequence in the sense orientation **(**pBI121**-**Nrep) or in the anti orientation (pBI121-anti-Nrep). The truncated N-Rep is driven by the CaMV-35S promoter (p35S) and terminated by the nopaline synthetase terminator (NOS!). Arrows in the AC1 truncated N-Rep indicate its orientation. The *NptII* gene is also driven by the CaMV-35S promoter (p35S) and terminated by the nopaline synthetase terminator (NOS!). Abbreviations: RB and LB, right and left T-DNA borders of from *A*. *tumefaciens*.

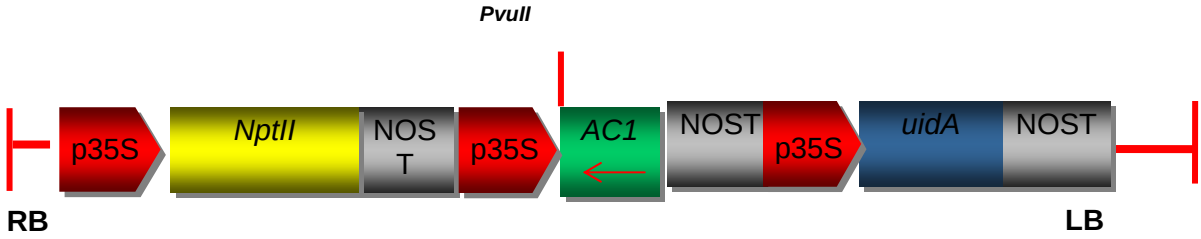


Fig. 5.3 Schematic representation of the T-DNA of pCAMBIA2301-anti-N-Rep containing the *NptII*, anti N-Rep and *uidA* genes. The truncated N-Rep is driven by the the CaMV-35S promoter (p35S) and terminated by the nopaline synthetase terminator (NOS!). No other *PvuII* sites are present on pCAMBIA2301

DNA isolated from putative C58C1-pCAMBIA2301-anti-NRep transformed colonies was isolated and sequenced by Inqaba Biotechnologies. The sequence was later sent to BLAST for sequence alignment and the results proved 98% homology with SACMV sequence (AF155806). Before transforming FEC tissues with pCAMBIA2301-anti-N-Rep by particle bombardment, three histochemical staining parameters had to be optimised. These were pre-treatment of FEC tissues before staining, selection pressure, and light culture conditions on putative transgenic FEC tissues. The results of these optimisation steps indicated that pre-treatment of bombarded FEC tissues before staining with X-gluc in GD2 solution supplemented with 6% sucrose for 24h gave relatively most blue spots (Fig. 5.4). This optimised pre-treatment step was used during the bombardment of TMS60444 FEC tissue with pCAMBIA2301-anti-NRep. After such a pre-treatment step, putative transformed FEC tissues were subjected to

various selection pressures in GD2 medium supplemented with various concentrations of a selection agent, in this case paramomycin. Concentrations range of 0, 20, 25 and 35 µM was tested. Different light regimes of dark, low light (0.44µmol.m-2.s-1) and high light (7.1µmol.m-2.s-1) were also tested as it was shown in a previous study that induction of OES was influenced by the amount of light available (Akano *et al*., 2001). The results of the latter two parameters indicated that during the first three OES transfer phases, 25 µM paramomycin under high light conditions was ideal in the multiplication of FEC units (Data not shown). Multiplied fec colonies were later matured in MS2 medium supplemented with 5 µM NAA and the resulting embryos regenerated in MS2 medium supplemented in 2 µM BAP (Fig 5.5). Although the capability of producing FEC tissue from OES was achieved and transferred from ILTAB to our laboratory in the model cv. TMS60444 and it must be stated that at this stage, our laboratory could only optimise the production of OES step from various SA landraces as reported earlier in chapter 4. However, the production of FEC and regeneration into plantlets is still proving to be difficult to optimise and further studies are currently underway to address the situation.

5.3.3 Molecular characterization of control and anti-N-Rep (621bp) transgenic tobacco plants

Hundred and thirty eight tobacco plants transformed with the plasmid pCAMBIA2301 and eighty four tobacco plants transformed with pCAMBIA2301-anti-NRep under the transcriptional control of 35S promoter were obtained from

twenty five and thirty three independent leaf disk explants, respectively (Fig 5.6). The phenotype of these plants was observed as normal. When transformants were analysed by polymerase chain reaction (PCR) for the presence of the truncated N-Rep transgene, fragments of predicted size were amplified from all plants (Fig. 5.1). Plants that yielded DNA products of expected molecular size were considered PCR positive. Transformed tobacco plants from each original independent explants were transferred to soil for further analysis at a molecular level. A Southern blot of genomic DNA of eleven transgenic plants, collected using the FTA® Classic Card method (Ndunguru *et al*., 2005) and hybridized with AC1-specific probe, is shown in Fig. 5.7. The results of southern blot hybridization analysis of nine transgenic tobacco lines confirmed stable integration of the introduced DNA.

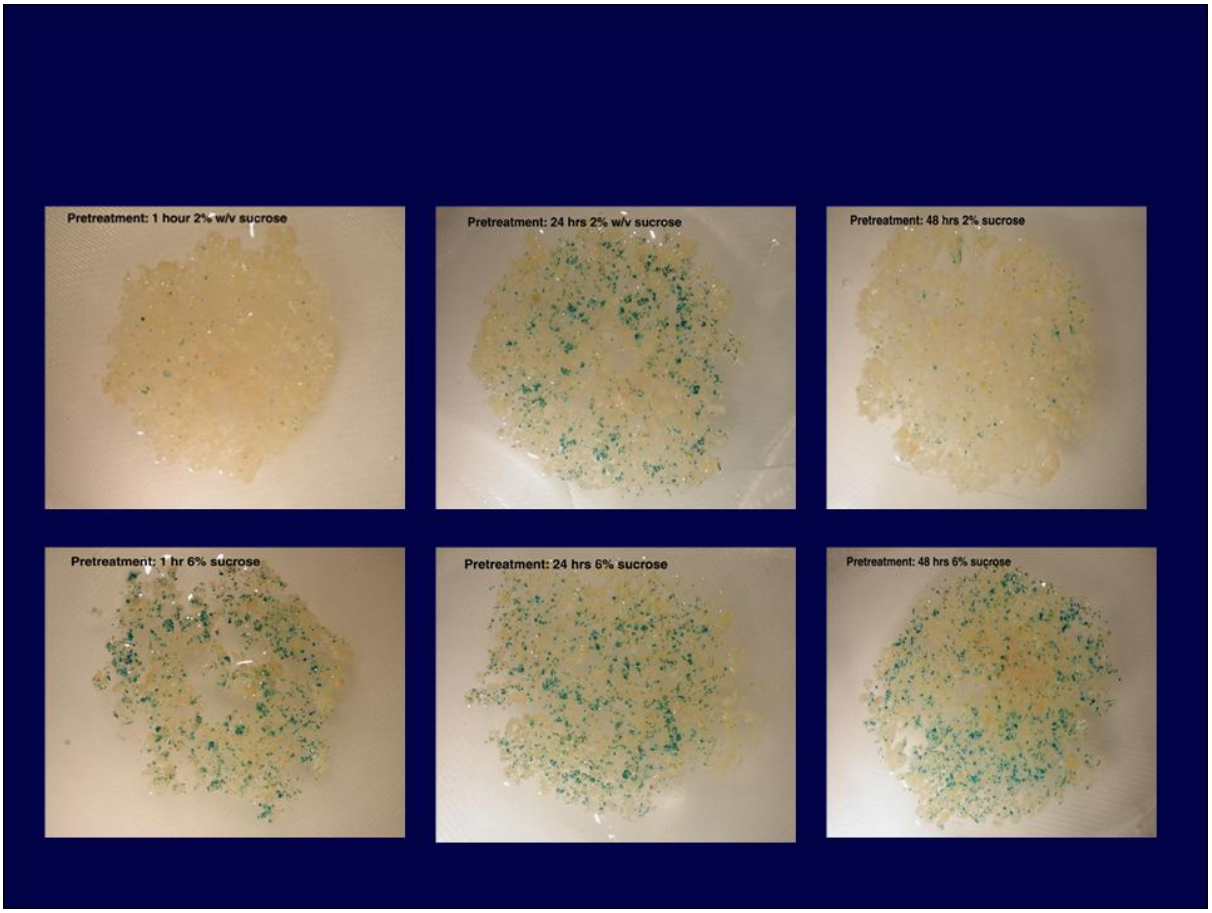


Fig 5.4 Histochemical GUS assay of FEC tissues. Blue spots represent putative transformed FEC units

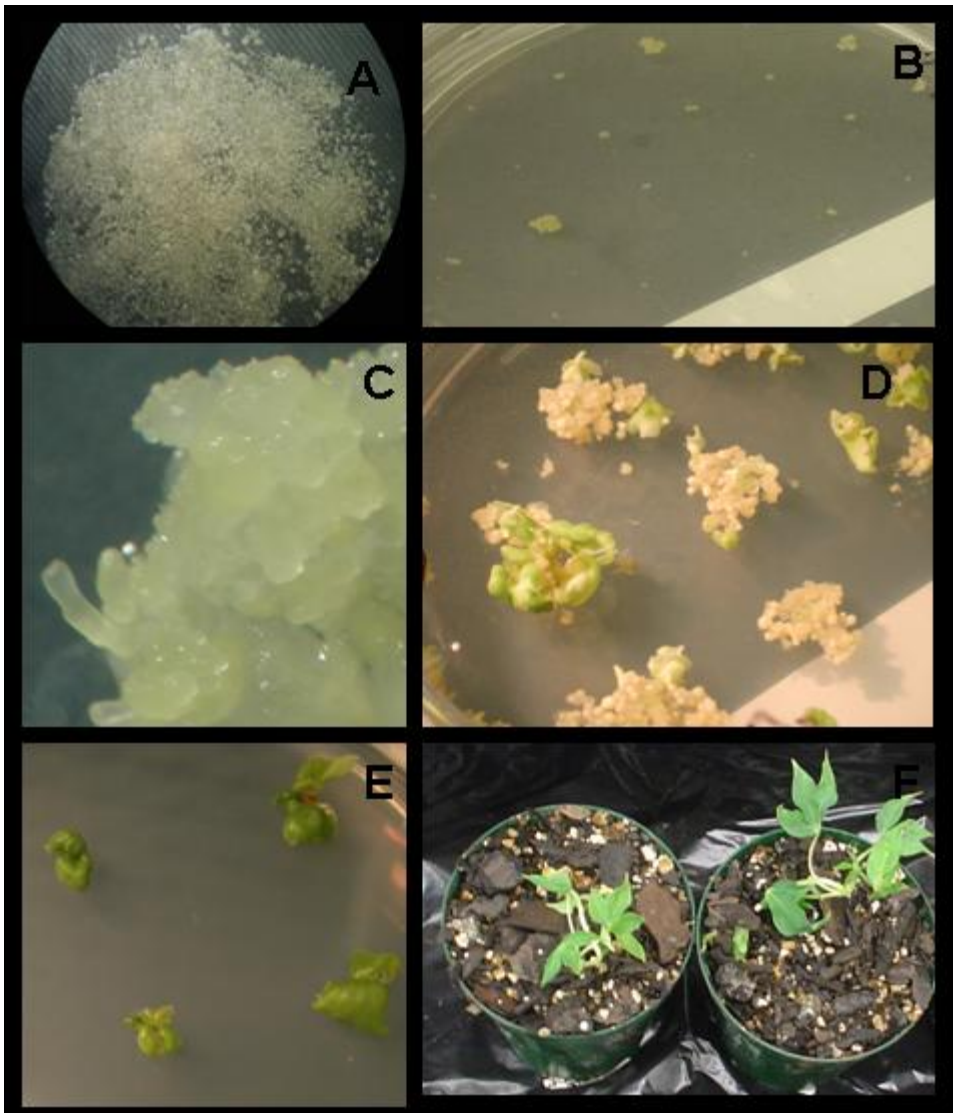


Fig 5.5 Transformation of TMS60444 FEC tissues with *Agrobacterium* C58C1-pCAMBIA2301-anti-NRep. **A** Circular FEC colonies of about 1.5 cm in diameter 3 days after co-cultivation with *Agrobacterium* **B** FEC units in GD2 selection medium supplemented with 25 µM paramomycin **C** Torpedo stage embryos developing from mature embryos in MS2 supplemented with 5 µM NAA **D** Coteledonary stage embryos **E** Emerging shoots in MS2 supplemented with 2 BAP **F** Regenerated putative TMS60444 plants 4 weeks after transfer to soil.

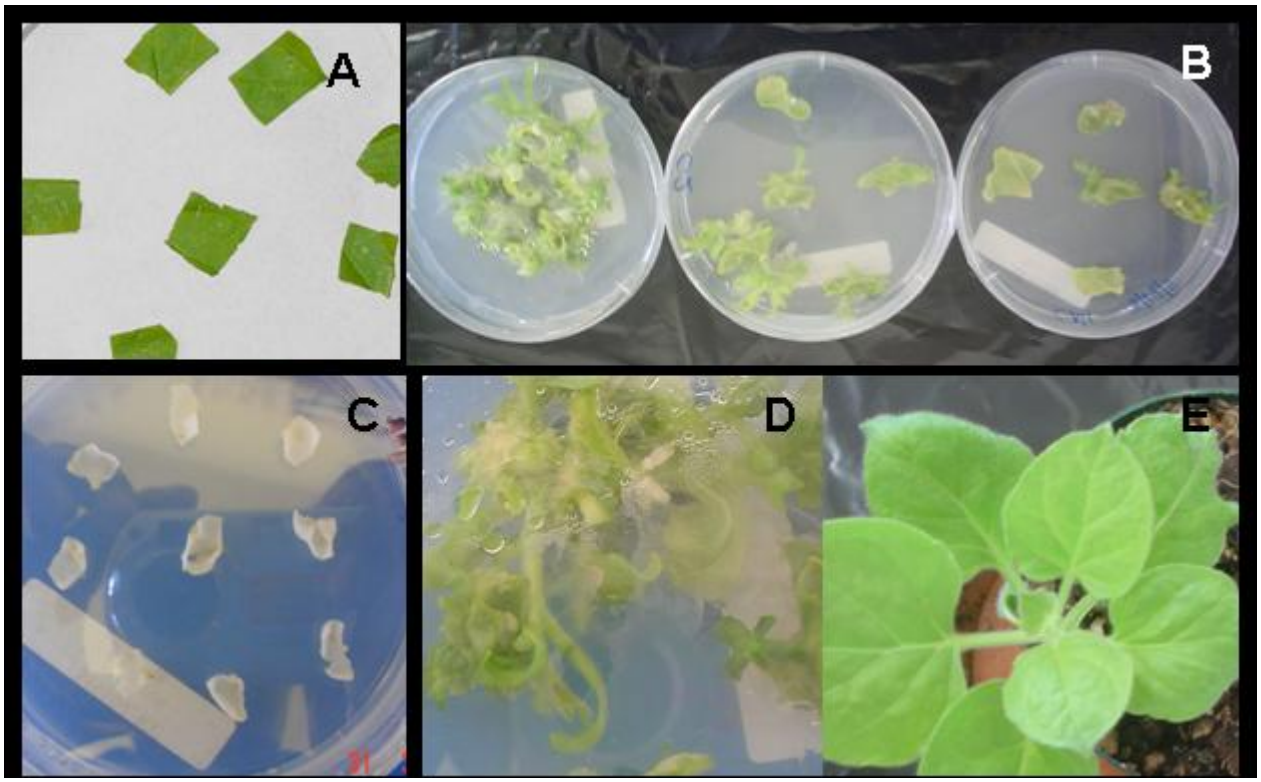


Fig 5.6 Transformation of *N.tabacum* leaf disks with *Agrobacterium* C58C1-pCAMBIA2301-anti-NRep. **A** leaf disks, **B** disks from three transformation events with plate on the left representing negative control of untransformed plants, middle plate represents positive control of transformed disks with C58C1-pCAMBIA2301-pMON999 and the right plate representing transformed disks with C58C1- pCAMBIA2301-anti-N-Rep. **C** Untransformed disks on selection medium **D** Four weeks old shoots before transplanting into soil and **E** three weeks old tobacco plants acclimatised in greenhouse conditions

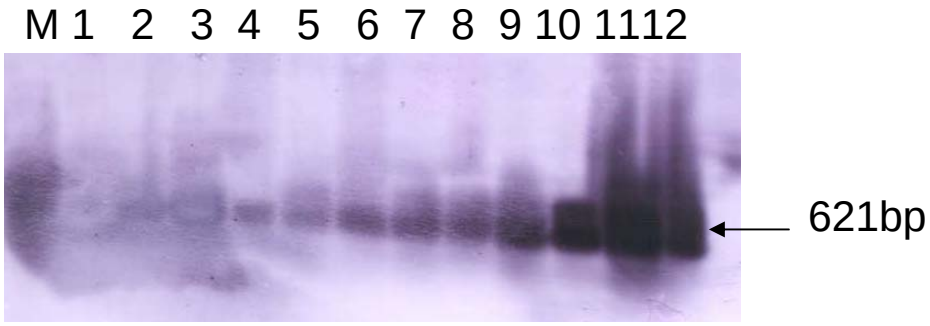


Fig. 5.7 Southern blot analysis of genomic DNA of 2 selected transgenic C58C1-pCAMBIA2301-pMON999 and 9 selected C58C1- pCAMBIA2301-anti-N-Rep tobacco plants. M Marker DNA, lane 1 +ve untransformed control, lanes 2-3 –ve transformed C58C1- pCAMBIA2301-pMON999 and lanes 4-12 transformed C58C1- pCAMBIA2301-anti-N-Rep tobacco plants

5.3.4 Transient viral replication assay

Leaf disks were prepared from selected transgenic tobacco lines and from nontransgenic *N.bethamiana* control as decribed earlier (Elmer *et al*., 1988) and agroinoculated with *A.tumefaciens* strain C58C1-pCAMBIA2301/SACMV A+B. Four days after transfer to selective medium, total DNA was extracted using the FTA® Classic Card system as described before by Ndunguru *et al*. (2005) and analysed for replication of SACMV DNA by Southern blotting using an SACMV truncated AC1 gene-specific probe.

Following agroinoculation, SACMV replicating DNA was detected in leaf disks obtained from transgenic plants in varying amounts whereas higher levels became apparent in all plants that did not express truncated N-Rep. Our results show that it is possible to introduce resistance to SACMV by transiently

expressing the truncated N-terminus Rep gene. This study is the first to report on potential resistance that the truncated SACMV AC1 N-Rep may have on transgenic plants. From the results depicted in Southern blot of transgenic tobacco lines, it was clear that expression levels of the truncated N-Rep varied from one transgenic to another. This could mean that expression of the truncated N-Rep is not standard and therefore not all transformed plants transcribe and express the gene in the same way. These results are in line with previous studies involving viral replicase-mediated resistance in transgenic plants as reported in study conducted by Sivamani *et al*. (2000). Brunetti *et al*. (1997) showed in their results that it was possible to obtain resistance to TYLCV-Sr by expressing a truncated C1 gene in transgenic plants, however, the resistance was only present in a few plants and was not inherited and expressed in a Mendelian manner. Futhermore, not all plants with detectable transgenic Rep at the time of infection were resistant, suggesting that a threshold level of expression may be necessary to confer resistance.

Various mechanisms for replicase-mediated resistance have been proposed and it is highly likely that in many cases there are multiple mechanisms induced by a single construct (Rovere *et al*. 2001). In some instances, protein expression appears to be required (Carr *et al*. 1992; Mac Farlane and Davies, 1992) while in other cases protection could be RNA-mediated (Baulcombe, 1996; Sijen *et al*., 1995). These studies tend to correlate with the findings of the current study elucidated by variation in Southern blot of TNA of selected transgenic tobacco lines expressing pCAMBIA2301-anti-NRep. It therefore would mean the

same construct has different effects on replication of SACMV within the cells of the transgenic plant.

Geminivirus Rep genes are multifunctional, participating in both viral DNA replication and the control of gene expression. SACMV AC1 binds with high specificity to motifs located between the concensus TATA box and the initiation site for complementary-sense transcription (Fontes *et al*. 1992; 1994) prior to introducing a nick in the virion-sense strand and binding covalently to the exposed 5´ terminus during the initiation of DNA replication (Laufs *et al*. 1995; Stanley 1995). Presumably, binding at this position is responsible for the observed down-regulation of complementary-sense gene expression for both TGMV (Sunter *et al*. 1993; Eagle *et al*. 1994) and ACMV (Haley *et al*. 1992; Hong and Stanley 1995). How SACMV truncated N-Rep confers resistance to SACMV infected plants is yet to be well understood. The proposed roles of geminivirus Rep suggest two possible explanations of the varied levels of expression in transgenic tobacco plants. Firstly, truncated N-Rep could act as a dominant-negative mutant by competing with wild-type SACMV Rep by competing with the wild-type Rep for binding to viral DNA and thus impairing its replication capacity, or, if Rep acts as a multimer, as speculated before, it is possible that it could interact with wild-type Rerp to generate a dysfunctional product (Herkowitz 1987; Brunetti *et al.* 1997). Secondly, there is a possibility that truncated N-Rep could repress transcription of the viral AC1 gene through interaction with its upstream recognition sequence (Hong and Stanley, 1995) and thus limiting wild-type Rep accumulation (Brunetti *et al*. 1997).

On the strength of these preliminary results, our laboratory is currently investigating the transformation of cassava explants using the same pCAMBIA-2301-anti-N-Rep truncated fragment. At this stage, it is too early to declare AC1 truncated N-Rep to be the pathogen-derived resistance strategy of choice in cassava, although several studies have by now proved to a certain degree the efficacy of the truncated AC1 gene in conferring resistance against several geminiviruses (Sangaré *et al*. 1999; Chatterji *et al*. 2001). Another alternative strategy that our laboratory is currently investigating is that one of RNA silencing as a possible mechanism. The essence of RNA interference technology is the delivery of dsRNA as a potent activator of RNA silencing into a cell, with the purpose of triggering sequence sequence-specific degradation of homologous target RNAs. The dsRNA can be delivered by stably transforming plants with transgenes that express a self-complementary RNA. The resulting transcript hybridizes with itself to form a hairpin structure that comprises a single-stranded loop region and a base-paired stem, which mimics the dsRNA structure that induces RNA interference (Tenllado *et al*., 2004).

Our laboratory is currently optimizing the challenge of transgenic cassava plants as we use a home-made gun which at most, yield varied results as parameters can not be easily controlled. After a period of technology development in the 1990s, a significant progress has been made, with several research groups reporting the production of cassava plants genetically modified for enhanced value to producers and consumers (Taylor *et al.* 2004). Field trials are already underway in Kenya and CIAT in Colombia and currently (November

2005) other field trials are being planned at ARC Roodeplaat in South Africa in collaboration with ILTAB and Wageningen University. All these trials are eventually aimed at integrating desired traits into farmer-preferred germplasm intended for commercial deployment.

In conclusion, although the results of this study has made a contribution in our laboratory, it is strongly recommended that effective partnerships with the Cassava Advanced Transformation Group be maintained in order for less advanced developing laboratories in southern Africa share technologies and techniques towards geminivirus resistance in cassava that are forever changing.

It is considered that cassava transformation technology can be transferred to the African environment when suitable institutes and individuals have been identified in the continent. One such laboratory, ILTAB has established a program by which African scientists are trained in cassava biotechnology and return to their home institutions to help transfer skills and capacity building. However, the issue of staff turnover due to lack of resources and proper funding can frustrate such a program Currently, USAID through CIAT and ILTAB are funding a program that will enable ARC scientists in South Africa train elsewhere in developed laboratories on cassava transformation aiming at improved starch qualities and resistance against CMD. South Africa is also a good country to test for efficacy of transgenic cassava as issues concerning Intellectual property rights (IPR) and biosafety implications are already legislated and in place. These issues can be a bottleneck in many African countries as recently experienced by ILTAB while trying to deploy a field test on their cassava replicase transgenic

event. For African farmers to benefit from this technology, developed laboratories must start forming collaborations with identified African laboratories to establish a critical mass of scientists and deployment of transgenics in the farmer's fields.

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