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RNA silencing of *South African cassava mosaic virus* in transgenic cassava expressing AC1/AC4 hp- RNA induces tolerance

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ABSTRACT

Cassava mosaic disease (CMD), caused by geminiviruses, is a major hurdle to cassava production. Due to the heterozygous nature of cassava, breeding for virus resistance is difficult, but cassava has been shown to be a good candidate for genetic engineering using RNA interference (RNAi). T This study reports on the ability of a transgene-derived RNA hairpin, homologous to an overlapping region of the SACMV replication associated protein and putative virus suppressor of silencing protein (AC1/AC4), to confer tolerance in the CMD-susceptible model cassava cultivar 60444. Three of the fourteen transgenic lines expressing SACMV AC1/AC4 hairpin-derived siRNAs showed decreased symptoms and viral loads compared to untransformed control plants. Expression of SACMV AC1/AC4 homologous siRNAs showed that this tolerance is most likely associated with post-transcriptional gene silencing of the virus. This is the first report of targeting the overlapping AC1 and AC4 genes of SACMV conferring CMD tolerance in cassava.

1. Introduction

Cassava (*Manihot esculenta* Crantz) is a perennial shrub grown for its tuberous roots, which are a major source of food for almost 700 million people worldwide [1,2]. One of the greatest threats to cassava crop security is cassava mosaic disease (CMD) which causes deformation and chlorotic mosaic in the leaves and is responsible for huge yield losses [3–6]. Although yield losses due to CMD are dependent on the geographical region of cultivation and cultivar susceptibility, in Africa the average annual yield loss losses due to CMD range between 30 and 40% [7]. However losses as great as 90% have been recorded in some areas [8]. In sub-Saharan Africa, CMD is caused by at least 7 cassava mosaic geminivirus (CMG) species including South African cassava mosaic virus (SACMV) [9], and many genetic strains/variants [10–12]. *South African cassava mosaic virus* (genus: *Begomovirus)* is a bipartite circular single stranded DNA (ssDNA) virus transmitted by the whitefly *Bemisia tabaci* (Genn.) in a persistent manner. Its genome consists of two ssDNA molecules, DNA-A and DNA-B, that are separately encapsidated in twin icosahedral particles [13–16]. DNA-A is required for transcription and replication of the virus, while DNA-B is required for cell-to-cell and long distance movement [15,17,18]. DNA-A contains 6 open reading frames (ORF) which encode for 6 proteins namely: AC1 (Replication associated protein/Rep), AC2 (Transcriptional activator protein/TrAP), AC3 (Replication enhancer protein/ REn), AC4, AV1 (coat protein/CP) and AV2 (pathogenicity determinant). Rep is a highly conserved multifunctional protein which is not only essential for viral replication, but also regulates transcription of viral proteins [19,20]. Rep also interacts with several host proteins, and acts as a viral suppressor of host response to geminiviral pathogens by lowering the transcription levels of plant methyltransferases which can methylate viral genomes [21]. The AC4 ORF overlaps the AC1 ORF and encodes a silencing suppressor that plays a role in the suppression of both transcriptional and post-transcriptional gene silencing in plants [22,23]. Additionally, SACMV encodes a putative AC5 ORF, which functions as a virus suppressor of RNA silencing (VSR) in *Mungbean yellow mosaic India virus* [24].

RNA interference (RNAi) is a highly conserved mechanism of gene regulation and plays a role in a variety of biological processes including defence against invading nucleic acids [25,26]. RNAi is triggered by the presence of double stranded RNA (dsRNA), which is processed by several RNase III-like enzymes known as Dicer-like proteins (DCLs) to produce so-called small interfering RNAs (siRNAs) [27]. The siRNAs associate with a number of effector proteins, including ARGONAUTES (AGOs), to form a RNA-induced

Abbreviations: SACMV, *South African cassava mosaic virus*; siRNA, Small interfering RNA; AC1, replication associated protein; AC4, Silencing suppressor protein; CMD, Cassava mosaic disease; hp-RNA, hairpin RNA; VSR, virus suppressor of RNA silencing.

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Silencing complex (RISC) [28,29]. AGOs are responsible for the cleavage of the passenger RNA from the siRNA duplex, which triggers the unwinding of the guide siRNA strand and activates the siRNA-RISC complex [30]. The activated complex then uses the guide siRNA strand to target and mediate cleavage of homologous viral mRNA in a process known as Post-transcriptional gene silencing (PTGS) [31]. siRNA can also mediate transcription gene silencing (TGS), where the siRNA is incorporated into a RITS (RNA-induced initiation of transcriptional silencing) complex [32] and guides target gene and histone methylation [33]. Small interfering RNA has also recently been shown to mediate translational repression in plants [34] and was also demonstrated in *mad6* and *ago1-27 Arabidopsis* mutants [35,36]. AGO1, AGO2 and AGO19 have been implicated in translational repression in plants [35,37,38]. Translational repression of viral mRNA was first observed in association with the defence response activated by a viral elicitor interaction with a resistance gene in *Nicotiana benthamiana* [39], and also in *Tomato ringspot virus* infection of *N. benthamiana* [37].

Geminivirus infection has been shown to induce the production of virus-derived siRNAs, which trigger both PTGS and 24 nt-mediated TGS silencing of the virus that has been linked to resistance and recovery [1,40]. Recovery is phenomenon found in plants, where a plant that is initially symptomatic shows milder symptom in the new growth cycle [41]. Plants which show recovery are referred to as tolerant. West African landrace TME3 displays a tolerant phenotype against *South African cassava mosaic virus* [42]. Symptom recovery in virus-infected plants has been linked to the induction of RNA silencing and generally a concomitant reduction in virus levels [37,43]. However, in contrast, recovery from tomato ringspot virus (ToRSV) in *N. benthamiana* is not associated with viral clearance in spite of active RNA silencing being triggered [44]. Recovered leaf tissues, infected with geminiviruses, have been shown to have lower small RNA (21–23 nt) levels [45], and this was also shown in SACMV-tolerant cassava TME3 [46].

Cassava is heterozygous and has a strong inbreeding depression [47–49], which has made production of CMG resistance cultivars through traditional breeding difficult. However, the genetic modification of cassava using a RNAi-mediated approach has shown great potential for increasing cassava resistance to a number of viruses including African cassava mosaic virus (ACMV) [48,50,51] and Sri-Lankan cassava mosaic virus (SLCMV) [52]. In this approach, transgene constructs are transformed into the plant, which then induce TGS or PTGS of the virus through the production of virus specific siRNAs derived from a hairpin or inverted repeat transgene. While siRNAs are produced from the entire begomovirus bipartite genome [53], several studies have shown hot spots that can be exploited for developing anti-geminiviral resistance [53–55]. Further, AC1 and AC4 on DNA-A of geminiviruses have been identified as hotspots, with higher level of siRNAs associated with these regions, specifically targeting the AC1/AC4 overlapping region [53,56]. In most studies, the AC1 ORF has been used as a preferred target due to its critical multifunctional activities [51,57]. However the overlapping C2/C3 (monocots) and AC2/AC3 (dicots) region has been reported to be effective for induction of resistance [58]. Viral suppressors of gene silencing, such as AC2 and AC4 [23,59], have also been shown to be hotspots for PTGS [55]. In cassava, AC1 and the bidirectional promoter region in the Intergenic region (IR) of ACMV have been targeted [51,60].

While engineering resistance to ACMV and SLCMV in cassava, and to Indian cassava mosaic virus (ICMV) in *Jatropha curcus* [61] has been accomplished, resistance against SACMV, has not yet been achieved by genetic engineering. High throughput sequencing of small RNAs associated with SACMV-infected cassava identified siRNAs targeting ORFs and IR on both sense and antisense DNA-A and B strands [46]. This study reports increased tolerance in genetically modified cassava cv.60444 expressing a hp-RNA from a transgene homologous to the AC1/AC4 overlap sequence from SACMV. Three transformed lines showed a decrease in viral load and symptom severity when compared to infected wild-type (untransformed cv.60444), and similar to the tolerance response of TME3 to SACMV. This increased tolerance was likely associated with enhanced PTGS, as confirmed by the expression of transgene-specific siRNAs.

2. Material and methods

2.1. Plasmid construction and cassava transformation

A double stranded hairpin RNA (hp-RNA) construct targeting a 598bp region over-lapping the AC1/AC4 region of SACMV DNA-A (NC_003803.1), separated by a PDK intron was constructed previously by Taylor et al [62] using the pHannibal system [63] (Fig. 1)*Agrobacterium tumefaciens* LBA4404 was used to transform cassava cultivar 60444 friable embryonic callus (FECs) using the method described in Bull et al. [64].

2.2. Molecular characterisation of transgenic lines

2.2.1. Nucleic acid extraction

Total DNA was extracted from approximately 50 mg of leaf material from 14 putative transgenic lines using the CTAB method described in [65].Total RNA was extracted from approximately 50 mg of leaf tissue, collected from infected and mock inoculated plants and flash frozen in liquid nitrogen. The leaf tissue was ground into a fine powder using the Qiagen TissueLyser II system (Qiagen) and total RNA was extracted using Qiazol lysis reagent (ThermoFisher Scientific), according to the manufacturer's instructions. Total RNA was treated with RiboLock (ThermoFisher Scientific) and stored at −80 °C.

2.2.2. PCR

Putative transgenic cv.60444 lines were screened by PCR for the presence of reporter genes *Hyg* and *Gus* as well as the forward arm of the AC1/AC4 construct with the following primers; HygF and HygR, GusPlusF and GusPlusR, and NBSACMVF and NBSACMVR respectively (Table 1). Purified empty vector control pC-AC1/AC4 vector DNA and wild-type untransformed cv.60444 DNA were included as controls.

2.2.3. Southern blot hybridisation

In order to determine the number of transgene integration events in the 14 transgenic lines, Southern blot hybridisation was carried out using DIG-High Prime DNA Labelling and Detection Starter Kit II (ThermoFisher Scientific) according to the manufacturer's instructions. Twenty micrograms of DNA from each line was digested overnight with HindIII (15053–15059) which cuts once in the cassette, and EcoRI, restriction enzymes (ThermoFisher Scientific) and separated using gel electrophoresis, in a 1% agarose gel in 1X TAE. The DNA was then transferred to positively charged nylon Hybond-N + membrane (Amersham), pre-hybridised at 38 °C, and hybridised at 64 °C overnight with a DIG-labelled *Hyg* gene probe, which was labelled using the DIG-High Prime DNA Labelling and Detection Starter Kit II (Roche). Pre-hybridization (3 h) and hybridization (overnight) were carried out using DIG EasyHyb buffer (ThermoFisher Scientific). Any unbound probe was washed from the membrane by sequential washing with buffers 2xSDS, 0.1% SDS twice, and 0.1xSDS, 0.1% SDS buffer twice (incubated at 40 °C). Signal detection (CDP star) was performed following DIG-High Prime DNA Labelling, and the Detection Starter

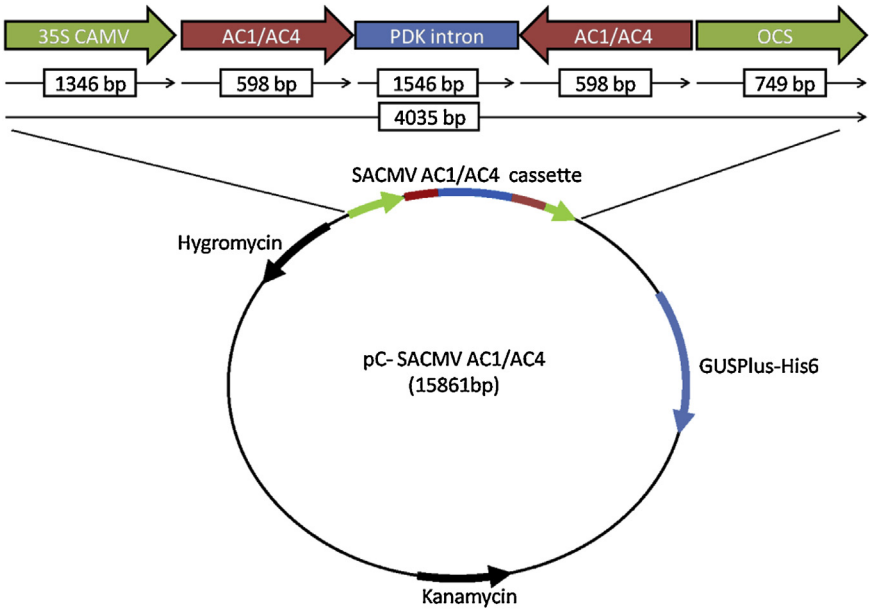

Fig. 1. Schematic representation of the SACMV hp-RNA AC1/AC4 construct in pCambia 1305.1 vector (pC-SACMV AC1/AC4). The construct targets the overlap between the ORFs of the replication-associated protein (AC1) and silencing suppressor AC4. The forward and reverse arms (AC1/AC4) of the hairpin are separated by the PDK intron and are under the control of the CaMV35S promoter (35S promoter) and OCS terminator.

Table 1
Primers used for screening of transgenic cassava cv.60444 transformed with SACMV hpRNA construct AC1/AC4.

Target	Primer	Primer sequence (5'-3')	Tm
Gus reporter gene	GusPlusF	CAACATCCTCGACGACGATAGCA	54 °C
	GusPlusR	GGTCACAACCGAGATCTCCT	
Hyg reporter gene	HygF	TCTCGATGAGCTCATGCTTTGG	56 °C
	HygR	AGTACTTCTACACAGCCATGGG	
AC1/AC4 forward arm	pHANREPXhoI	CCTCGAGGTACTCGGTCTCCATGGCC	56 °C
	pHANREPEcoRI	GGAATTCACTCTCTCGAAAGAAGCGG	
AC1/AC4 Northern probe	NBSACMVF	TAATACGACTCACTATAGGGTACTCGGTCTCCATGGCC	68 °C
	NBSACMVR	AATTAACCCTCACTAAAGGGTCGAAAGAAGCGG	

Kit II (Roche) protocol. Results were visualised using GelDoc XR+ (Biorad) after 10 min.

2.2.4. Expression of Gus, Hyg *and SACMV AC1/AC4 transgene*

In order to determine transgene expression, cDNA was synthesised from 1 μg of total RNA (treated with DNase I) with random hexamer primers, using the RevertAid First strand cDNA synthesis kit (Thermo Fisher Scientific). Two microliters of cDNA product was used as the template for PCR amplification of *Gus, Hyg* and the AC1/AC4 transgene insert using primers GusPlusF and GusPlusR, HygF and HygR and pHANREPXhoI and pHANREPEcoRI, using DreamTaq (ThermoFisher Scientific) according to protocol, with annealing temperatures specified in Table 1. The relative expression of the transgene, compared to 1 μg of purified PCR AC1/AC4 product amplified from pC-AC1/AC4 (reference band), was calculated using LabImage 4.0 (Bio-Rad).

2.3. Northern blot for expression of siRNA

2.3.1. Probe construction

RNA probes were produced from a SACMV AC1/AC4 PCR fragment using the DIG northern Starter kit (Roche) according to manufacturer's instructions. The PCR fragment was produced using Phusion Master mix (ThermoFisher Scientific) with primers NBSACMVF (including T7 promoter)and NBSACMVR (including T3 promoter) (Table 1) using 50 ng of pBS-SACMV DNA A plasmid as a template. Two hundred nanograms of purified PCR product quantified using NanoDropOne (ThermoFisher Scientific), was used to produce the RNA DIG-labelled SACMV AC1/AC4 probe in both the sense and antisense orientation.

2.3.2. Northern hybridisation

Thirty micrograms of total RNA from transgenic lines was separated on 15% polyacrylamide gel (8 M urea, 30% APS, 1x TBE, 0.4% TEMED) and transferred to a positively charged nylon membrane (Amersham, Hybond N+). Hybridisation was performed using DIG northern Starter Kit (ThermoFisher Scientific), according to the manufacturer's instructions. Pre-hybridization (60 min) and hybridization (over-night) were carried out at 60 °C, using DIG EasyHyb hybridisation buffer (Thermo Fisher Scientific). The post-hybridization removal of excess probe and signal detection (CDP star) were performed according to northern Starter Kit instructions (ThermoFisher Scientific). Excess probe was removed by sequential washing of membrane in (2x SSC, 0.1 SDS) and (0.1 × SSC, 0.1% SDS) buffers incubated at 25 °C and 60 °C, respectively. Northern Blots were exposed for 10 min and imaged using BioRad Transluminator. Labelled 21 nt miRNA (mes-miR169) was used as a positive control.

2.3.3. Evaluation of transgenic AC1/AC4 transgenic lines for resistance to SACMV

2.3.3.1. Agro-inoculation of transgenic lines. From the fourteen lines screened for integration events, the ten lines with a single copy of the transgene were screened for resistance to SACMV. From

this initial trial, six single copy transgenic lines (O14-1, O4-1, O12-2, O4-4 O13-5 and O13-8) which displayed lower viral loads and reduced symptom severity, in comparison to the infected untransformed cassava cv.60444 control, as well as untransformed cv.60444 and tolerant landrace TME3 were selected for further virus resistance trials. Twenty to thirty plantlets from each line were micro-propagated in a controlled environment at 28 °C, with 16 h light (8000–10000 lux) and 8 h dark cycles and 50% humidity. After 6 weeks, 12 plants of similar size were selected from each transgenic line. Twelve plants from each line were agro-inoculated with SACMV pBIN-DNA-A and pBIN-DNA-B infectious clones in *A. tumefaciens* Agl1 (Agl1) [9]. The infectious clones were prepared by inoculating 50 ml of YEP broth containing appropriate antibiotics (50 μg/ml carbenicillin, 50 μg/ml kanamycin) with DNA-A and DNA-B components, which was then incubated at 28 °C under constant agitation (200 r.p.m) until the OD600 = 2.0. The bacterial cultures were then pelleted by centrifugation (12,000xg) and re-suspended in fresh YEP supplemented with acetosyringone (200 mM) to a final OD600 = 2.0. DNA-A and DNA- B components were combined in equal amounts and 200 μl of the culture was used to inoculate the petioles and stems of each plant using a fine needle (5 mm). Untransformed *A. tumefaciens* Agl1 was cultured in the same manner and was used to agro-infect 3 plants from each line as a negative control. The trial was repeated twice more on the three lines (O12-2, O13-5 and O13-8) that showed highest SACMV tolerance in the first transgenic trial.

2.3.4. Monitoring of disease progression

At14 days DPI, the 12 plants from each of the transgenic lines as well as untransformed wild-type cv.60444 and TME3 were screened using PCR for the presence of SACMV. Six of the infected plants from each line then were selected at random from each line and were monitored at 32 and 65. The symptom severity of each plant was evaluated by examining the two upper most leaves, below the apical meristem and scoring the symptom severity based on the following system modified from Legg and Fauquet [66] (0= no symptoms, 1= faint mosaic, 2= mosaic with mild curling, 3= severe mosaic with severe curling) (Fig. 2A) then an average was calculated to score each plant. The leaves were harvested and frozen in liquid nitrogen and stored at −70 °C for further evaluation. The transgenic and control plants were phenotyped by measuring height at the beginning (0 DPI) and the end of the trial (65 DPI).

2.3.5. Quantifying viral load in infected lines

Relative real-time quantitative PCR was used to determine the amounts of viral DNA-A in the collected leaf samples in relation to internal control *Ubiquitin 10* (*UBQ10*)*UBQ10* was quantified using primers UBQ10 F (5′ TGCATCTCGTTCTCCGATTG 3′) and UBQ10R (5′ GCGAAGATCAGTCGTTGTTGG 3′).

2.3.6. Statistical analysis

The data on symptom severity, plant height and viral load was subjected to a student *t*-test to determine if there was a statistically significant difference between the transgenic lines and wild-type cv.60444 and TME3. Pearson correlation coefficient (r) test was used to establish if there was any correlation between symptom severity and viral load.

3. Results

3.1. Molecular characterisation of transgenic lines

3.1.1. Presence of Gus, Hyg *and transgene insert*

In order to confirm successful stable transformation of cassava cv.60444 with the SACMV AC1/AC4 hp-RNA construct (pC-AC1/AC4), the transgenic lines were screened for the presence of reporter genes *Gus* and the antibiotic resistance gene *Hyg*, and the forward arm of the AC1/AC4 hairpin construct using PCR. Fourteen

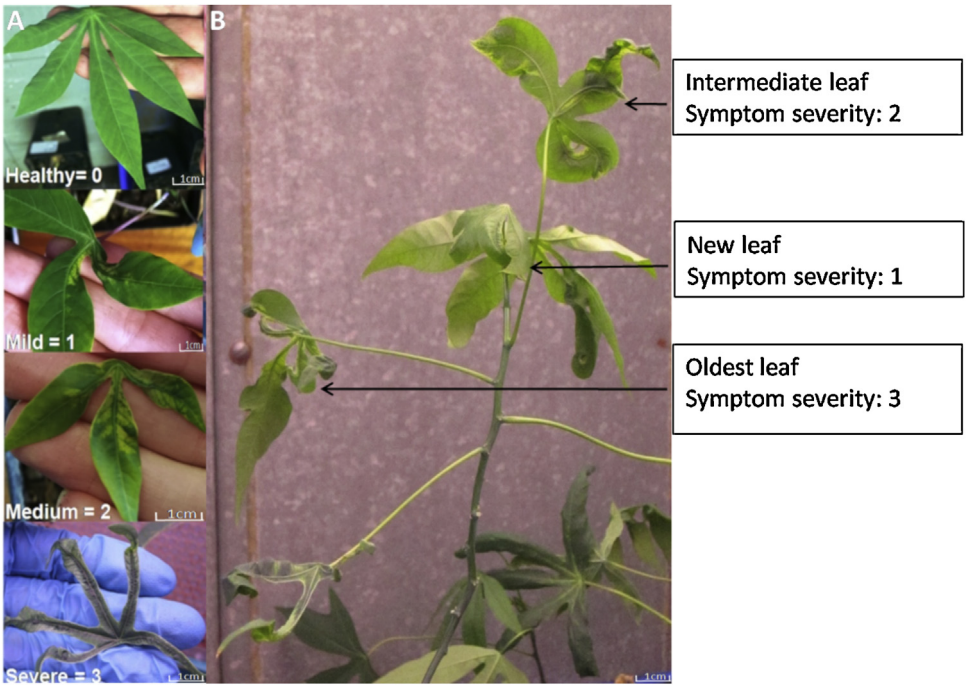

Fig. 2. Symptom severity (A) and recovery (B) transgenic cassava cv.60444 infected with South African cassava mosaic virus. A) modified symptom severity scale used to determine symptom severity score of plants infected with SACMV. On the scale 0: Healthy (no symptoms), 1: light mosaic or curling, 2: Severe mosaic, or curling, 3: Severe mosaic and curling with reduction in leaf size. B) Recovery phenotype in transgenic cv.60444 line O13-8 in infected with SACMV, where oldest leaves show severe symptoms (symptom severity: 3) while youngest leaves show milder symptoms (symptom severity: 1).

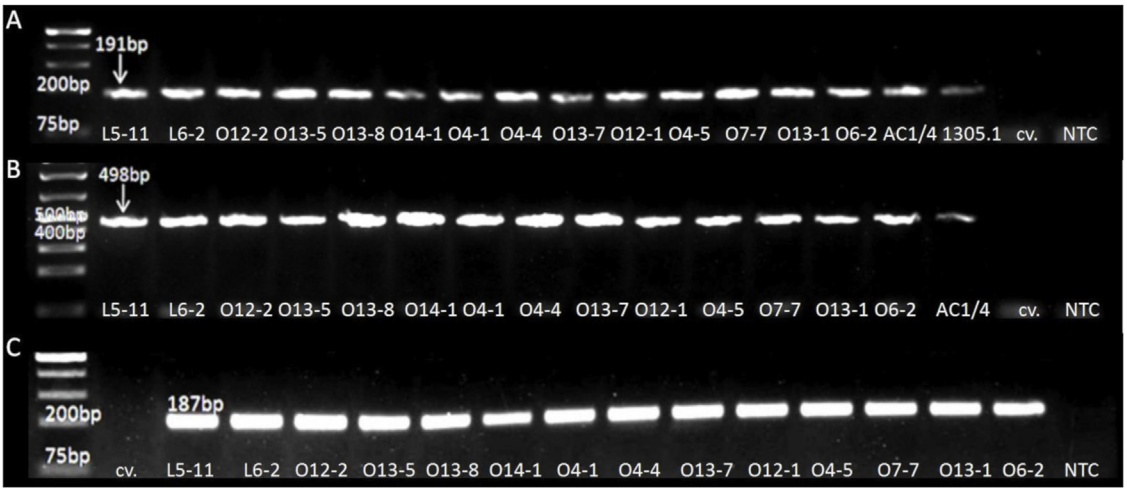

Fig. 3. PCR detection of *Gus* (A), *Hyg* (B) and AC1/AC4 forward arm (C) in genomic DNA extracted from transgenic cv.60444 transformed with pC-AC1/AC4. The GeneRuler 1 kb Plus (Thermo Fisher Scientific) molecular marker was loaded in the first lane of each gel. A: lane 1-14: transgenic cassava lines, lane 15: pCambia-AC1/AC4, lane 16: pCambia 1305.1, lane 17: wild-type untransformed cv.60444 and lane 18: No template control (water). B: lane 1-14: independent transgenic cassava lines, lane 15: pCambia-AC1/AC4, lane 16: wild-type untransformed cv.60444 and lane 17: No template control (water). In C; lane 1: wild-type untransformed cv.60444, lane 2-15: independent transgenic cassava lines, lane 16: No template control (NTC). The AC1/AC4 positive control for the AC1/AC4 insert was run on a separate gel and is not shown here.

lines were confirmed to contain the copies of the *Gus* (Fig. 3A)*Hyg* (Fig. 3B) and pC-AC1/AC4 constructs (Fig. 3C)

3.1.2. Southern blot hybridisation

The total DNA from the transgenic lines was digested with restriction enzymes EcoRI and HindIII, which cuts once within the cassette. Southern blot hybridisation was then carried out using a DNA probe specific to the *Hyg* gene found within the cassette. Purified pCambia 1305.1 containing pC-AC1/AC4 was used as a positive control and wild-type untransformed cv.60444 was included as a negative control (Fig. 4). While a single integration event had occurred in the majority of the transformed lines (10 lines), some lines (6 lines) had multiple integration sites. Six of the lines where one integration site was detected and which displayed increased tolerance to SACMV were selected for further testing.

3.1.3. Expression of transgene in transformed lines

Transgene expression was measured in 6 independent transgenic lines (L5-11, O12-2, O4-1, O4-4, O13-5 and O13-8) by RT-PCR. All transgenic lines showed expression of *Gus*, *Hyg* and the pC-AC1/AC4 insert (Fig. 5**).** In order to infer a rough estimation of the relative expression of the transgenes in each of the transgenic lines, the relative ratio of RT-PCR product in each of the lines was compared to a known amount of DNA (1 μg of purified AC1/AC4 PCR product) AC1/AC4 control (Fig. 5C). The ratio ranged from 0.37-0.81, with line L5-11 (Fig. 5C) having the lowest concentration of pC-AC1/AC4 (relative quantity ratio = 0.37) which indicates it has the lowest relative expression of the transgene. Line O13-8 (Fig. 5C) had the highest concentration (relative quantity ratio = 0.81), which would indicate that it had the highest relative expression.

3.1.4. Evaluation of transgenic AC1/AC4 transgenic lines for resistance to SACMV

3.1.4.1. Symptom severity and plant height. In order to evaluate whether the pC-AC1/AC4 construct confers resistance to the transgenic cassava lines, 6 transgenic lines with a single construct copy, as well as wild-type untransformed cv.60444 and CMD-tolerant TME3, were agro-inoculated with SACMV infectious clones and evaluated for resistance over a period of 65 days for resistance. Three transgenic lines (O12-2, O13-8 and O13-5) which showed decreased viral symptoms and load in the initial screening (Supplementary Data A, B, C) were subjected to further testing. Twelve six week old plants from transgenic lines O12-2, O13-5 and O13-8 were infected with SACMV. After 14 days, leaves were collected from each of the plants, and were screened using coat protein (CP) PCR to establish the success of the infection (Supplementary Data D). Six infected plants were randomly selected for further evaluation and at 32 and 65 days post infection (DPI) the symptom severity, height (Fig. 6) and viral load (Fig. 8) was evaluated. The difference in plant height between 0 DPI and 65 DPI was also measured.

After infection, all plants remained symptomless until 20 DPI and by 32 DPI all infected plants displayed leaf curling and mosaic (Fig. 6A). The average symptom score for wild-type untransformed cv.60444 (1.59 ± 0.12) and tolerant TME3 (1.7 ± 0.08) was similar

Fig. 4. Southern hybridisation of total DNA from transgenic cassava cv.60444 transformed with SACMV AC1AC4 construct, digested with restriction enzymes EcoRI and HindIII, and hybridized with DNA probe specific to *Hyg* gene. Lane M: DIG-labelled molecular weight parker (ThermoFisher Scientific), lane 1-16: independent transgenic cassava lines. Purified binary vector pC1305.1-AC1/AC4 and wild-type untransformed cv.60444 were included as positive (+) and negative (-) controls respectively.

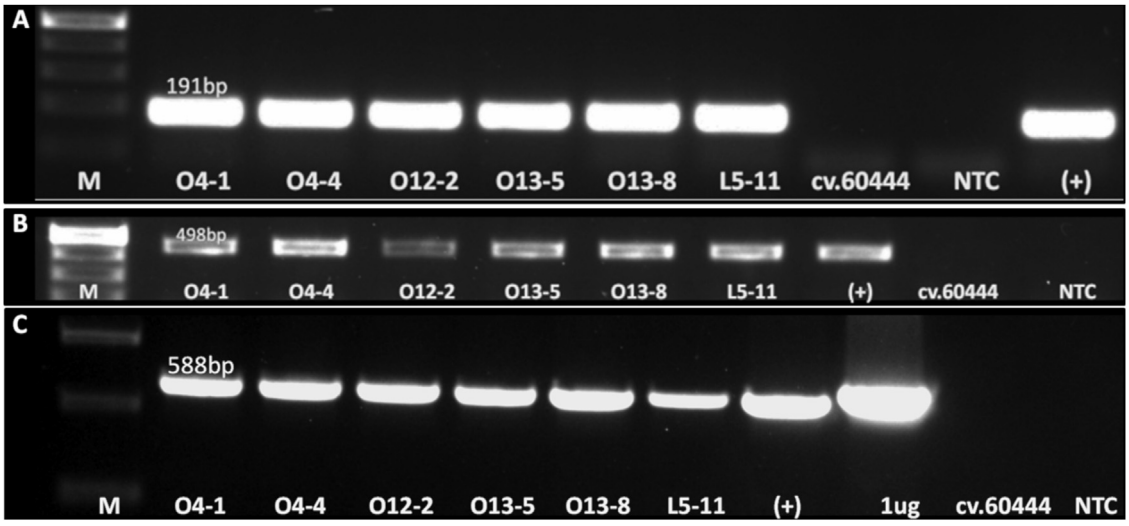

Fig. 5. Expression of *Hyg* (A), *Gus* (B) and AC1/AC4 transgene (C) in transformed cassava cv.60444 lines (O4-1, O4-4, O12-2, O13-5, O13-8 and L5-11). Molecular weight marker was loaded into the first lane of each gel; 1 kb Plus molecular weight marker (Thermo Fisher Scientific) was used for (A) and (B) and 1 kb ladder molecular weight marker (Promega) was used for (C). A: Expression of *Hyg* in transgenic lines. Lane 1: O4-1, lane 2: O4-4, lane 3: O12-2, lane 4: O13-5, lane 5: O13-8, lane 6: L5-11, lane 7: pC-AC1/AC4 (positive control), lane 8: cv.60444, and lane 9: NTC. B: Expression of *Gus* in transgenic lines. Lane 1: O4-1, lane 2: O4-4, lane 3: O12-2, lane 4: O13-5, lane 5: O13-8, lane 6: L5-11, lane 7: cv.60444, lane 8: NTC and lane 9: pC-AC1/AC4. C: Expression of AC1/AC4 transgene in transgenic lines. Lane 1: O4-1, lane 2: O4-4, lane 3: O12-2, lane 4: O13-5, lane 5: O13-8, lane 6: L5-11, lane 7: pC-AC1/AC4, lane 8: purified AC1/AC4 PCR fragment (1 μg) (reference), lane 9: cv.60444 and lane 10: NTC.

at 32 DPI, however at 65 DPI, TME3 displayed recovery (1.36 ± 0.24) which did not occur in wild-type cv.60444 (1.57 ± 0.18) (Fig. 6A). Lines O12-2, O13-5, O13-8 had lower symptom severity at 32 DPI (1.18 ± 0.40, 1.36 ± 0.40 and 1 ± 0.20) when compared to infected wild-type cv.60444. These levels were maintained at 65DPI (1.44 ± 0.28, 1.33 ± 0.23 and 0.85 ± 0.22), with most plants only showing mild curling in the upper leaves. Line O13-8 average symptom score remained low throughout the course of the trials, with very mild curling of the leaves. O13-8 displayed recovery at 65 DPI with new leaves having no visible symptoms (Fig. 7**)**. At 65 DPI, line O12-2 and O13-5, had a similar symptom severity score as tolerant TME3, but did not display any signs of recovery. The difference in symptom severity between the transgenic lines and wild-type cv.60444 was shown to be significant at both 32 and 67 DPI ($p < 0.05$). The symptoms displayed by wild-type cv.60444 and O13-5 at 65 DPI remained similar to those displayed at full systemic infection, with O13-5 symptoms being less severe than that in wild-type cv.60444.

In order to ensure that the expression of the transgene did not affect the growth (height) of the transgenic lines, the height of the healthy transgenic lines was compared to the healthy untransformed cv.60444 at 65 DPI. No statically significant difference between the plant height of the healthy cv.60444 infected and transgenic lines was found (Fig. 6B). In addition, to determine whether infection with SACMV had an effect on the growth of the transgenic lines, the height difference between the healthy and infected transgenic lines (as well as TME3) was calculated. Transgenic lines O13-5 and O13-8 had a reduction in height between infected (14.48 ± 1.87 and 14.45 ± 0.81) and mock inoculated (22.19 ± 6.09 and 19.56 ± 3.29) of 34.7% and 26% respectively. The average height difference between transgenic line O12-2 infected (14.19 ± 7.99) and mock-inoculated (16.78 ± 5.60) lines varied by 15.4%, however this difference was not statistically significant and height does not appear to be affected by the presence of the virus in transgenic line O12-2. There was no statically significant difference between the heights of the infected cv.60444 and the infected transgenic lines (O13-5, O13-8 and O12-2) or landrace TME 3.

3.1.5. Viral load

The relative viral load of each sample was determined by real time qPCR on DNA samples extracted from the plants at 32 and 67 DPI. The data was normalised using internal control gene *UBQ10* [42] (Fig. 8).The viral loads were analysed for statistically significant differences between transgenic lines O12-2, O 13-8 and O13-5 as well as TME 3 and wild-type infected cv.60444 and which showed a statistical significant difference between viral loads in leaf samples at 32 DPI and 67 DPI ($P < 0.05$).

In comparison to non-transgenic cv.60444, the viral loads of transgenic lines O13-5 and O13-8 were significantly lower at 67 DPI ($p < 0.05$). Overall, O13-5 had the lowest viral accumulation (average of 47-fold ± 32) at 32 DPI and the relative viral load remained low at 65DPI. Both O12-2 (180-fold ±90) and O13-8 (219 ± 109) had lower relative viral loads than cv.60444 (480-fold±48) at 32DPI and the viral load in both decreased at 67 DPI (which correlates to the recovery observed in line O13-8). The viral loads in TME3 (264-fold± 200), O12-2 and O13-8 were similar at 32 DPI however this was shown not to be significant. Pearson's correlation test was used to test the correlation between viral load and symptom severity and showed a low positive correlation between the symptom score and the viral load for all samples ($R < 0.3$ in all sample comparisons, $p < 0.05$) except TME3 which showed a negative correlation (R= -0.0135). The relative viral load of O13-5 was also lower than infected wild-type cv.60444, correlating with less severe symptoms. Transgenic line O12-2 symptoms increased over the course of the study (similar to wild-type cv.60444), however its height was not affected by presence of SACMV and its relative viral load decreased at 67 DPI which indicates viral attenuation.

3.1.6. Northern blot analysis for siRNA expression

In order to establish whether the tolerance observed in transgenic lines was linked to the expression of siRNAs, northern blot hybridisations were performed on the three tolerant lines (O12-2, O13-5 and O13-8) as well as the susceptible line L5-11. Cultivar 60444 and TME 3 were also included as susceptible and tolerant controls, respectively (Fig. 9). The uninfected wild-type cv.60444 controls did not produce SACMV-targeted siRNAs, while SACMV infected cv.60444produced low levels of siRNA (relative to

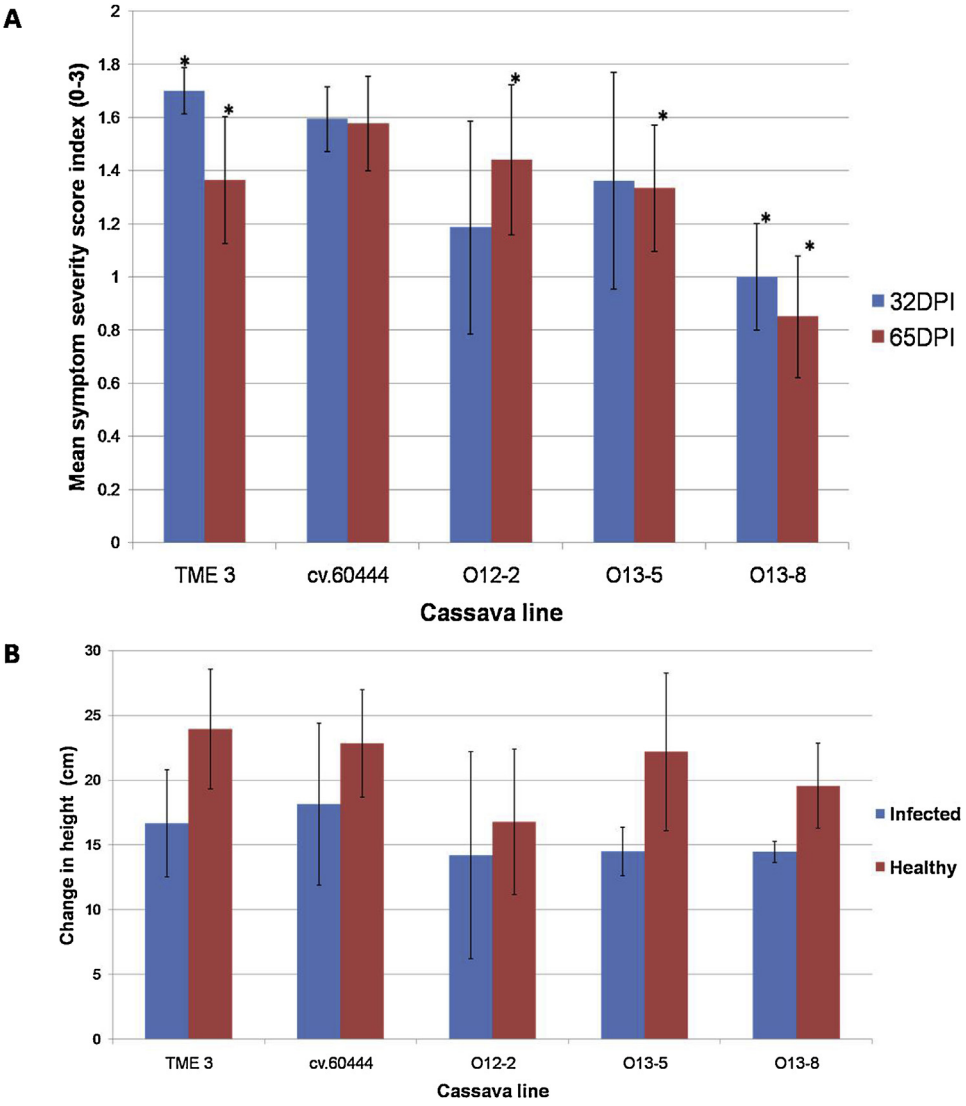

Fig. 6. Average symptom severity scores (A) and change in plant height (B) in SACMV AC1/AC4 transgenic (O12-2, O13-5, and O13-8), wild-type untransformed cv.60444 and tolerant TME3 cassava plants *Agro*-inoculated with South African cassava mosaic virus. Values represent the mean of three independent biological replicates each with 6 plants per treatment and bars indicate standard deviation (SD). Asterisks (*) indicate a statistical significant difference ($p < 0.05$) between the means of the infected transgenic lines (O12-2, O13-5 and O13-8) or TME3 and cv.60444 at the same time point calculated using the student *t*-test. (a) represents a statistically significant difference between the mean of the healthy and infected O13-8 plants.

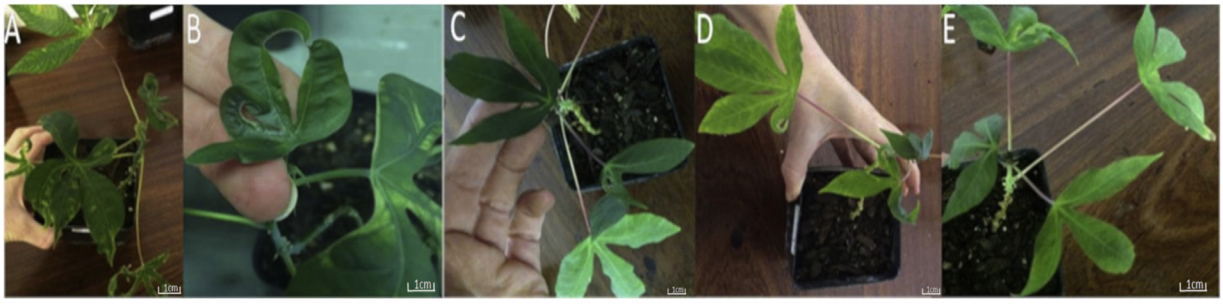

Fig. 7. Symptoms in apical leaf of transgenic lines O12-2, O13-5 and O13-8 as well as TME3 and untransformed cv.60444 infected with SACMV at 68 DPI. A) TME3, B) cv.60444, C) O13-8, D) O13-5 and O12-2. Line O13-8 and TME3 had low symptom severity and showed recovery at 65 DPI. Lines O12-2 and O13-5 had similar symptom severity to tolerant TME3 but did not show any recovery during the course of the trial.

200 ng of the 21 nt marker) targeting SACMV DNA-A AC1/AC4 region (relative quantity ratio: 2.0). Small interfering RNAs specific to AC1/AC4 were detected in mock-inoculated (relative quantity ratio: 1.68) TME3, and SACMV-infected tolerant TME3 landrace (relative quantity ratio: 2.67), but significantly higher relative levels of siRNAs were detected in infected TME3 plants.

Small interfering RNAs targeting the AC1/AC4 region of SACMV were detected in the uninfected transgenic lines O13-5, O13-8 and

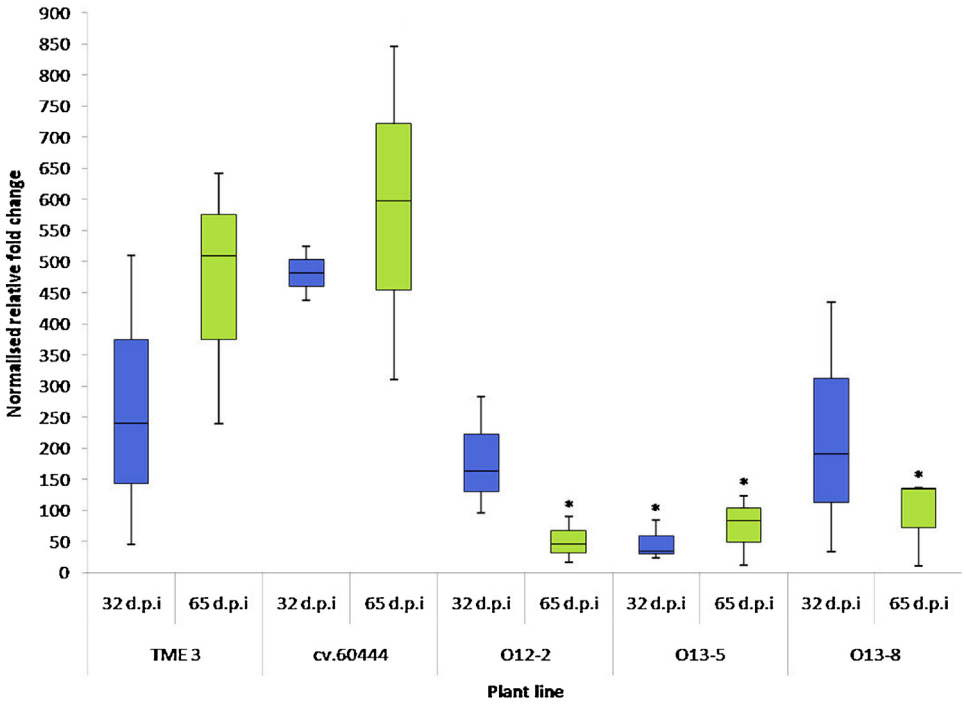

Fig. 8. The viral load of South African cassava mosaic virus relative to reference gene *UBQ10* in SACMV AC1/AC4 transgenic (O12-2, O13-5 and O13-8), wild-type untransformed cv.60444 and TME3 infected with SACMV, at 32 and 67 DPI. Values represent the mean of three independent biological replicates each with 6 plants per treatment and bars indicate standard deviation (SD). Asterisks (*) indicate a statistical significant difference ($p < 0.05$) between the means of the infected transgenic lines (O12-2, O13-5 and O13-8) or TME3 and cv.60444 at the same time point, calculated using the student *t*-test.

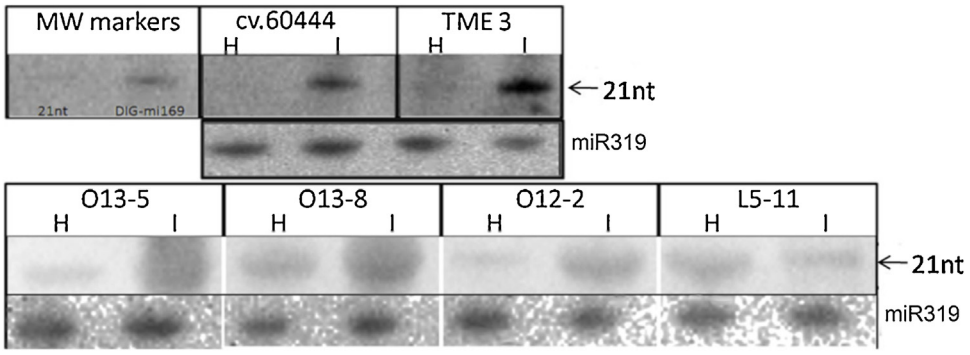

Fig. 9. Northern blot hybridisation of RNA extracted from infected (I) and healthy (H) (non-infected) transgenic lines (L5-11, O12-2, O13-5 and O13-8), wild-type cv.60444 and TME3. A DIG-labelled miRNA marker (MW) (ThermoFisher Scientific) and DIG-labelled miR169 were included as size controls.

O12-2 (Fig. 9), indicating their origin from the transgene-derived hp-RNA. Notably, there was an observed increase in the amount of siRNA produced by the infected tolerant transgenic lines. Uninfected line O13-8 had the highest siRNA level (relative quantity ratio: 2.0), which increased (2.77) in the presence of SACMV. A lower quantity of siRNA was detected in uninfected O13-5 and O12-2 (1.17 and 1.14 respectively) which increased after SACMV challenge, although O12-2 (2.04) siRNA levels increased comparatively less than O13-5 (2.62). In contrast to the tolerant lines, in susceptible line L5-11, although a relatively high level of siRNA was present in uninfected plants (1.81), the siRNA levels decreased (1.57) following infection with SACMV.

4. Discussion

Globally, the threat presented by CMD is one of the greatest hurdles to cassava production. In Southern Africa, SACMV is widely spread in South Africa, Zimbabwe, Mozambique and Madagascar [68]. To date CMD resistance has primarily been engineered against ACMV, using antisense [48,67] or hp-RNA PTGS [69–71]. This study reports, for the first time, SACMV tolerant transgenic cassava. Three transgenic lines (O12-2, O13-5 and O13-8) transformed with a hp-RNA construct targeting the AC1/AC4 region of DNA-A of SACMV displayed less severe symptoms compared with wild-type cv.60444 and tolerant landrace TME3, and had reduced viral loads. Further, one of the lines (O13-8) showed recovery at 65 DPI, demonstrating that the AC1/AC4 transgene was effective at reducing virus infection. Significantly, the tolerance displayed the three transgenic cv.60444 lines (O13-5, O13-8 and O12-2) is similar to CMD resistant wild-type West African landrace TME3 [72]. AC1 and AC4 were chosen as effective targets for RNAi-induced PTGS in this study as AC1 is a critically important protein for viral replication, and interactions with host proteins, and AC4, a viral suppressor, has been shown to inhibit both TGS and PTGS in host plants [22,73]. Hairpin-RNA constructs targeting the AC1/AC4 overlap has been shown to confer resistance to other

geminiviruses, including *Tomato leaf curl virus* [73]. Further, cassava lines transformed with antisense and hp-RNA constructs targeting the AC1 of ACMV-NOg and ACMV-KE have exhibited increased resistance to the virus [48,51,70].

Tolerance and recovery have been shown to be linked to the production of siRNAs which target the virus genome [74]. In this study the SACMV AC1/AC4 transgenic lines O13-5, O13-8 and O12-2 displayed tolerance in response to infection with SACMV. Hairpin-derived siRNAs corresponding to the AC1/AC4 region of SACMV were detected in the three uninfected tolerant transgenic lines, as well as in the susceptible L5-11 transgenic line (Fig. 5) demonstrating that siRNAs are being produced from the transgene in the absence of the virus. A RNAseq study [46] demonstrated that while siRNA targeting SACMV was detected in both infected T200 and TME3, siRNA populations were in general lower in infected TME3 during recovery, compared to susceptible T200. While, in general siRNAs were lower in SACMV-infected TME3, TME3 did produced high levels of siRNAs targeting the AC4 region of SACMV [46], however these results were not confirmed experimentally. AC1 and AC4 genes being have also been reported as siRNA "hot spots" [55,75,76] in other geminivirus studies. Infected susceptible non-transgenic cassava T200 landrace has also been shown to generate virus-derived siRNAs that target SACMV, but these do not confer resistance [46]. Similarly, the highly susceptible transgenic L5-11 line did generate siRNAs, but levels of siRNAs declined when L5-11 plants were inoculated with SACMV. While induction of transgene-derived siRNAs has been linked to decreased viral loads [77], in this transgenic line the presence of AC1/AC4-derived siRNAs did not lead to lower virus loads or resistance. This suggests some interference or suppression of PTGS by SACMV, leading to lower siRNA levels and increase in symptoms and virus load. It is suggested that SACMV may be partially suppressing both natural [78] and transgene-mediated PTGS in transgenic lines, through viral suppressors such as AC4 and AC2 [23].

Tolerance and symptom recovery in virus challenged O13-5, O13-8 and O12-2 transgenic lines may also represent a battle between virus interference and antiviral silencing, but in this case transgene-mediated PTGS appears to be more effective against SACMV than in L5-11. Viral suppressor proteins inhibit RNA-mediated defence using several mechanisms including inhibition of siRNA generation, inhibition of siRNA incorporation into RISC complex and direct interference with the RISC complex [46,79]. Rogans et al. [46] hypothesised that SACMV avoided RNA silencing in the highly CMD-susceptible T200 landrace through inhibition of incorporation of siRNA into RISC complex. It is possible that this inhibition may also occur in the tolerant transgenic lines in this study. Cultivar cv.60444 is also highly susceptible to cassava geminiviruses, but the plants from the three transgenic cv.60444 lines (O13-5, O13-8 and O12-2) displayed lower symptom severity scores than wild-type cv.60444 and some plants showed recovery, a typical response found in SACMV infected TME3 [42].

Tolerance in the three transgenic lines correlated with lower viral loads compared to both cv.60444 and TME3, and northern blots of siRNAs confirmed that is most likely associated with siRNA-mediated PTGS silencing of the virus. Interestingly, at 65 DPI while both wild-type TME3 plants and O13-8 displayed signs of recovery, the viral load in line O13-8 was lower than CMD-tolerant TME3. Although decreased symptom severity correlated with a decrease in viral load in transgenic lines O12-2, O13-5, and O13-8, as shown in other studies [80], there are several reports where no correlation between viral load and symptom severity was found [81]. The most promising SACMV-transgenic cv.60444 line O13-8 warrants further investigation to unravel contributing factors to its tolerance to SACMV. Interestingly, a study in cowpeas (*Vigna unguiculata L. Walp*) transformed with a hp-RNA construct targeting the AC1/AC4 of Mungbean yellow mosaic virus, also showed milder symptoms rather than systemic resistance [82]. Different geminiviral species may also respond differently in similar transgenic plant systems. Hairpin or antisense-AC1 RNA which targeted ACMV AC1 [48,70] conferred resistance in cv.60444 through PTGS, whereas in this study, only tolerance was achieved with the SACMV-AC1/AC4 hairpin construct. These above-mentioned studies, amongst others, demonstrate that several host and virus factors can determine the outcome in PTGS-transgenic crops [74]. The transgenic resistance and tolerance achieved in cassava to ACMV and SACMV, respectively, may reflect different host-virus co-adaptability since SACMV and ACMV evolved in different geographical regions, namely East and West Africa, respectively [1,9,83]. Adaptability or fine tuning of PTGS efficacy between cassava host and geminivirus may be evolutionary specific.

Transgene-induced RNA silencing enhances the natural plant host antiviral defences, but highly pathogenic viruses are able to suppress or evade the production or action of siRNAs [84]. Virus-induced PTGS is highly dependent on plant-virus interactions and well as the intrinsic features of the virus [57]. SACMV is a recombinant virus, with an origin in East Africa/south-west Indian Ocean islands region [85], it also has a AC2 from an unknown virus source, and its IR is closely related to TYLCV-Israel [9]. SACMV is able to induce severe symptoms and as well as high numbers of virus derived siRNAs targeting DNA-A and B components in non-transgenic T200 landrace [75]. A number of studies have shown that in order for a host to display either resistance or recovery, it must produce a high level of siRNAs against the virus [51,57,86]. This has been shown in PTGS-induced resistance studies, where resistance was induced by the constitutive expression of the transgene-derived siRNA. Fuentes et al. [87] and Vanderschuren et al. [51] also demonstrated that the constitutive expression of transgene derived small RNAs, prior to ACMV challenge, was important for engineered virus resistance. Three tolerant transgenic lines in this study were shown to produce transgene-derived siRNAs, however they produced varying levels of tolerance to SACMV, and there did not appear to be a correlation between pre-inoculation siRNAs levels and tolerance. Transgene expression can be affected by several factors including the position of insertion in the genome, which can affect the levels of siRNA produced [51,84]. This may be a factor in this study, but requires further investigation.

A number of other factors can also play a role in the efficiency of siRNA-mediated PTGS silencing in plants. The genetic background of plant cultivar and type of geminivirus can effect effectivity of siRNA-mediated PTGS. For example, CMD resistance was shown to be linked to the ability of different cassava genotypes to induce RNA silencing, evidenced by varying levels of virus-derived siRNA (vsiRNA) [74]. Resistance also depends on the ability of plants to overcome the viral suppressors [88]. The response of Ty resistant tomato to beet curly top Iran virus (BCTIV) is variable and depends on the type of begomovirus [89]. The number of integration events or integration site of the hp-RNA may also affect the efficacy of siRNA-mediated PTGS [90]. In this study the highest relative level of siRNA was detected in transgenic line O13-8, which showed recovery. The variation in tolerance shown by the three lines may also be due to location and expression levels of the transgene within the three lines [51], where hairpin transcription may not necessarily lead to efficient siRNA production and the RNA silencing pathway. A study in Cucumber mosaic virus-infected tobacco showed that the location of transgene insertion played a role in whether 21 nt RNAs were processed [91]. Northern blots using an AC1/AC4 specific probe showed that small interfering RNA targeting the AC1/AC4 region of SACMV was also expressed uninfected non-transgenic tolerant TME3 [Fig. 9]. This finding was also observed in TME3 in a RNAseq study [75] and in TME3 that showed recovery from infection with ACMV-NOg that had high

levels of siRNA targeting the virus [74]. We conclude that the AC1/AC4 transgene may enhance tolerance to SACMV in transgenic cv.60444 lines, similar to natural CMD tolerance in TME3 which showed similar siRNA levels.

The role of diverse RNA degradation pathways and DNA methylation [92] in variable transgene expression and hpRNA-derived siRNA production could also play a role in efficiency of RNA silencing. The lines may additionally require RNA-dependent RNA polymerase 6 (RDR6) mediated secondary siRNA production to accumulate enough siRNA to mount an effective resistance response, and RDR2-mediated DNA methylation to silence geminiviruses [53]. RDR6 contributes to anti-viral immunity by converting viral transcripts to dsRNA precursors of secondary vsRNAs [93]. Efficient resistance to geminiviruses is proposed to rely on both primary and secondary siRNA amplification [53], however cassava RDR2 (cassava v4.1_013755 m and 021122 m) and RDR6 (Manes 16G121400 v6.1) homologs were not found to be up-regulated in TME3 [42] and bisulphite sequencing did not reveal a role for DNA (cytosine) methylation of SACMV at recovery in TME3 [75].Tomato yellow leaf curl virus was also shown to evade host RNAi defence through a population of *de novo* synthesised unmethylated viral DNA [94]. This does not rule out the possibility of histone methylation which needs to be investigated in future. Our results support the suggestion that transgene-induced PTGS was not sufficient to suppress SACMV replication completely in cv.60444.

Additionally, with regards to the most efficient strategy of inducing antiviral RNA silencing in cassava, are considerations with respect to the environment in which RNA silencing operates. Temperature has been shown to influence geminivirus-induced RNA silencing in plants [18,95]. For example, African cassava mosaic virus-induced silencing increased by elevating the temperature from 25oC to 30oC [18]. While SACMV displays non-recovery in wild type cv.60444, and transgenic cv.60444 plants were grown at 28oC and it is possible that this may have influenced resistance efficiency. It is also possible the lower resistance levels displayed by the transgenic lines may be due to high agro-infectious SACMV inoculum pressure. In an ACMV bombardment assay, resistance was shown to be broken when the virus load was increased [48]. Some dsAC1 ACMV-transgenic cassava lines showed an increase in infection rate when the virus pressure was increased from 350 to 700 ng [51]. The needle-mediated agro-inoculation method performed in this study, used because cassava is recalcitrant to mechanical inoculation and leaf-infiltration, delivers high virus titres directly into the plant vascular system. It is possible that due to the high viral load, this direct and rapid method of inoculation could over-come the RNAi engineered resistance. Additionally, the effectiveness of RNAi-mediated response is reliant on a there being a high level of sequence homology between the transgene and the viral target (>90% homology). Fuentes et al. [87] showed that RNAi-mediated resistance in crops can be stably maintained, when the viral population remains stable. However, recently Mehta et al. [96] showed that field cassava geminivirus populations changed in response to RNAi-mediated resistance pressure which could have massive consequences for the development for resistance plants in the field.

In conclusion, this study has shown that a hp-RNA construct targeting the AC1/AC4 region of SACMV DNA-A can confer tolerance to SACMV. This tolerance is most like due to the induction of enhanced PTGS and primary hpRNA-derived siRNAs in lines expressing AC1/AC4 specific siRNA, leading to reduced symptoms and viral load, and recovery in the latter stages of infection. However, this mechanism has not been definitively proven, and some other mechanism may also be involved. It is suggested that total resistance is not achieved as SACMV is able to maintain some level of counter defence via PTGS suppression. This finding, and other studies, show variability in PTGS efficacy related to many factors, which will prove challenging for implementing this control strategy under variable environmental and field conditions. While direct comparisons cannot be made between SACMV and existing resistance studies on two other cassava viruses, namely ACMV and SLCMV, as the exact virus genome target sequences (constructs) and host genome integrations in cv.60444 are not identical, it is notable that distinct cassava viruses behave differently in the same cassava cultivar (cv.60444). Other complementary natural resistance mechanisms need to be explored and manipulated through techniques such as gene editing if robust and enduring resistance to cassava mosaic disease is to be successful. Tolerance may offer some advantages over total resistance, as it is more durable and less likely to break down under high virus pressure in field conditions. It has been suggested that symptom recovery can be regarded as an inducible form of tolerance [97], and is associated with a diversity of mechanisms which could be exploited in developing approaches to control plant virus disease. A balance between virus and host defence mechanisms which reduces the fitness cost of the plants [97,98] more closely resembles durable resistance in wild undomesticated crops. However one disadvantage is that if there are still virions present in the leaves, they could be picked up by the whitefly vector. It has been proposed that developing dual resistance to whitefly and geminiviruses could provide a more stable long term solution to reducing the impact of CMD [99] or combining natural and genetically engineered resistance [100].

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Declaration of Competing Interest

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2019.e00383.

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