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Susceptibility to South African cassava mosaic virus is Associated with a RING Finger E3 Ubiquitin Ligase

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Abstract

Background

The ubiquitylation of proteins is reprogrammed by plant geminiviruses which alter the ubiquitin proteasome system (UPS) to fully infect the host. A RING Finger E3 Ubiquitin Ligase (*MeE3L*) is located on a major cassava mosaic disease resistance-associated quantitative trait locus. Here, we examine the genetic structure and relative expression of *MeE3L* (native and gene-edited mutant), and determine how *MeE3L* affects geminivirus South African cassava mosaic virus (SACMV) DNA accumulation.

Methods

Cassava protoplasts of model, susceptible and tolerant genotypes were transformed with SACMV infectious clones and/or a CRISPR-editing construct targeting the *MeE3L* using PEG4000-mediated transfection. DNA and RNA were extracted from transformed protoplasts at 24 hours post-transfection. Relative SACMV load quantitation was determined using *DpnI*-digested total DNA via qPCR and *MeE3L* relative expression was determined via reverse transcriptase qPCR, and results were analysed using the $2^{-\Delta\Delta}$ method. The *MeE3L* exonic region was sequenced on the ABI 3500XL Genetic Analyzer platform; and sequences were analysed for mutations and for construction of a phylogenetic tree using the Maximum Likelihood method and Tamura-Nei model.

Results

Results show that SACMV DNA accumulation is cassava genotype-dependent. The study also reveals that native and mutant *MeE3L* is differentially expressed during SACMV infection in protoplasts of susceptible and tolerant cassava landraces. The susceptible cassava landrace encodes a RINGless *MeE3L* and the *MeE3L* base sequence is a determinant of cassava's response to SACMV. Results further show that SACMV silences the *MeE3L* RING domain in the susceptible and tolerant landraces; and specifically targets the tolerant *MeE3L* gene homolog for silencing.

Conclusions

These findings suggest that *MeE3L* is a target of SACMV, contributing to susceptibility in cassava. The *MeE3L* base sequence is a determinant of cassava's response to SACMV. The *MeE3L* RING domain is actively silenced by SACMV and therefore may be essential for host defence against geminiviruses. The study provides further evidence, in addition to existing literature, that plant E3 ligases are exploited by geminiviruses to enhance pathogenicity.

Background

The ubiquitin proteasome system (UPS) plays contrasting roles in plant immunity during viral infection. This post-translational modification system may boost innate immunity or enhance viral activity (Verchot,

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which the E1 ubiquitin-activating enzyme presents an

ubiquitin molecule to the E2 ubiquitin-conjugating enzyme which complexes with the E3 ubiquitin ligase for transfer of the ubiquitin molecule to the proteins destined for degradation, or to another ubiquitin molecule (Komander, 2009). The E3 ligase recognises and binds to the substrate before covalently ligating at least one ubiquitin to the substrate (Pickart, 2001). Plant E3 ligases are subdivided into 11 classes: the RING (Really Interesting New Gene), the CRL (Cullin RING), the RBR (RING-between-RING RING), the HECT (Homologous to E6-associated protein C-terminus) (Morreale and Walden, 2016), the U-Box (Hatakeyama et al., 2001), the F-box (Lee et al., 2016), the ASK (Arabidopsis SKP1-related), the BTB (bric a brac, tramtrack and broad complex), the APC (anaphase-promoting complex), the CUL4-DDB (cullin4-damaged DNA-binding protein) and the cyclin F (CCNF) proteins (Craig et al., 2009). E3 ligases can function independently as a single component or as part of modular multi-subunit complexes containing a RING module (e.g. RBX1), an assembly platform module (e.g. APC2) or a substrate recognition module (e.g. DDB1) (Haglund, 2005).

RING E3 ligases function in innate and adaptive immunity to restrict viruses and can be responsible for particular regulation of specific substrate proteins based on their ability to act as a molecular bridge between the substrate and the ubiquitin-carrying E2 enzyme (Barry and Früh, 2006). Plant and animal viruses may encode RING E3 ligases which ubiquitinate host proteins to downregulate host immune responses or they may hijack host RING E3 ligases to prevent ubiquitination of viral proteins and promote viral replication (Zhang et al., 2018). Some viruses encode RING proteins that mediate degradation of undesirable host proteins. It has been suggested, based on experimental evidence, that some viral factors recruit viral F-box proteins which usurp the function of host F-box proteins and direct E3 ligase complexes from the immune response to phytohormone signalling, or to interfere with the RNA silencing process by targeting Argonaute proteins for degradation (Verchot, 2016). Some R proteins recognise specific viral domains and relay them to E3 ligase complexes and the 26S proteasome for degradation (Liu et al., 2002), hence blocking needless activation of defense signalling (Duplan and Rivas, 2014) by controlling their accumulation (Gou *et al*., 2012). Degradation of viral movement proteins by the plant UPS directly diminishes viral mobility and thus, infection (Shen et al., 2016). Viral-encoded DUBs may reverse ubiquitination processes to improve viral replication (Alcaide-Loridan and Jupin, 2012). Geminiviruses, in particular, hamper the SCF complex functions by removing the ubiquitin-like molecule from neddylated cullins (Lozano-Duran and Bejarano, 2011). More recently, it has been demonstrated that the replication initiator protein (Rep) of the geminivirus, *Chilli leaf curl virus*, interacts with the host UPS and promotes transcription of the viral genome (Kushwaha et al., 2017).

Geminiviruses are circular, single-stranded plant DNA viruses that are transmitted by the whitefly, *Bemisia tabaci* Genn., and infect a variety of crops globally. They belong to the family *Geminiviridae* which comprises of 9 genera, of which the *Begomovirus* genus is the largest (Zerbini et al., 2017). Begomoviruses significantly contribute to reduced production of economically and nutritionally important crops by causing diseases such as Pumpkin yellow mosaic disease, Tomato yellow vein streak disease, Bean dwarf mosaic disease, Sweet potato leaf curl disease, Squash mild leaf curl disease, and Cassava mosaic disease (Ramesh et al., 2017; Rey and Vanderschuren, 2017; Yang *et al*., 2019). Geminiviruses

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ocesses for their own benefit (Voinnet et al., 1999; Hanley-

Bowdoin et al., 2004; Hanley-Bowdoin et al., 2013; Wu et al., 2019), and one such process is ubiquitination (Kumar, 2019). They interfere with host UPSs to redirect protein metabolism, alter defense processes (Ramesh et al., 2017) and even suppress the plant's response to insect vectors (Li et al., 2019). Geminiviruses have been known to inhibit proteasomal degradation of the viral silencing suppressor C2 protein, simultaneously target multiple E3 ligases, alter SUMOylation when the Rep protein interacts with SUMO-conjugating enzyme E1, and degrade of the movement protein (MP) (Alcaide-Loridan and Jupin, 2012).

One of the important geminivirus-induced diseases is Cassava Mosaic Disease (CMD), which is caused by a number of cassava mosaic begomovirus species, and produces foliar symptoms that include reduced leaf size, causing production of few or no tubers by the cassava (*Manihot esculenta* Crantz) plant (Alabi *et al*., 2011). CMD can result in yield losses of up to 100% in Africa alone (FAO, 2013), with major regional pandemics causing major food security destabilisation such as occurred in East and Central Africa in the 1990s (Legg and Thresh, 2000; Legg et al., 2006). Certain cassava genotypes exhibit resistance or tolerance to cassava mosaic begomoviruses, such as tropical *M. esculenta* 3 (TME3) and Tropical Manihot Series (TMS) 96/0023, whereas others (T200, TMS 8017) are susceptible to the virus and do not recover from infection (Rogans et al., 2016; Fondong and Rey, 2018). CMD resistance can be polygenic as is the case for CMD1 in wild cassava (*Manihot glaziovii*), reportedly monogenic dominant as in CMD2 in certain West African cassava landraces (Akano et al., 2002), or a cross of CMD1 and CMD2 to produce CMD3 (Lokko et al., 2006). Traditionally, CMD-resistant cultivars have been bred by introgression and marker-assisted breeding of resistant landraces with preferred cassava cultivars (Jennings, 1994; Legg and Thresh, 2000; Okogbenin et al., 2007; Ceballos et al., 2015; Nzuki et al., 2017). Genetic modification of cassava for introduction of resistance traits has been hampered by the plant's recalcitrance to transformation (Zainuddin et al., 2012; Lentz et al., 2018). It has been posited that the use of 21st century genetic engineering (GE) or editing techniques such as artificial miRNA, transactivating siRNA, and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), to improve native antiviral mechanisms, can confer virus resistance without adverse effects on the plant (Vanderschuren *et al*., 2006; Fondong & Rey 2018). However, this will depend on an intimate understanding of the molecular processes of existing innate antiviral mechanisms, such as the degradation of viral proteins that is directed by the UPS. In this respect, CRISPR has the upper hand over other modern GE techniques because of its efficiency in silencing genes, which simplifies the process of identifying key genes involved in resistance/tolerance by analysing gene expression during virus infection (Doudna and Charpentier, 2014; Mao et al., 2018).

Transcriptome studies during infection have shown that the geminivirus, *South African cassava mosaic virus*, and *Cassava brown streak virus* (ssRNA ipomovirus) induce differential expression of UPS proteins, including four E3 ligases (Allie et al., 2014; Maruthi et al., 2014). A genome-wide association study found one E3 ligase (*Manes.12G069400/cassava4.1_026906 m.g*) among 105 gene candidates associated with the CMD2 locus (Wolfe et al., 2016). This E3 ligase (hereafter designated *Me*E3L) is located on a major CMD resistance-associated QTL (Lozano et al., 2015) and has been implicated in cassava's

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2016; Patanun *et al*., 2016). To test whether *Me*E3L is involved

in the response of cassava to South African cassava mosaic virus (SACMV), we set out to determine how the transient expression of *MeE3L* and SACMV replication in cassava protoplasts from model cultivar (cv.60444), susceptible (T200) and tolerant (TME3) landraces (Kuria et al., 2016) are affected by CRISPR-mediated gene editing of *MeE3L*.

Methods And Materials

1. CRISPR-Cas9 vector construction

Two genomic gRNA targets were identified using the CRISPOR version 4.7 web-based program (www.crispor.tefor.net; Haeussler et al., 2016) and used in designing duplex sgRNA that included an *Arabidopsis thaliana* U6-26 promoter, the sgRNA scaffold and terminator for each gRNA sequence. Synthesis of the duplex sgRNA was outsourced to Inqaba Biotech (Pretoria, South Africa) and the duplex was cloned into the pCambia1380 vector. A *Cas9* insert (*Tobacco mosaic virus* promoter + *Cas9* gene + *eGFP* gene + *Hsp* terminator) from the pl1m-f2-p35s-cas9-egfp-nucleo-thsp Golden Gate vector was cloned into the pCambia1380-gRNA construct. The construct was confirmed by restriction digestion and sequencing.

2. Protoplast isolation

Sterile nodal cultures of cassava (cv.60444, T200 and TME3 genotypes) were grown for 4 weeks at 28 °C (3,000 lux; 12/12 h light/darkness) on ½ Murashige and Skoog (MS) medium (2.2 g Murashige and Skoog Basal Medium, 2% sucrose, 0.002 mM $CuSO_4$, 0.78% plant tissue culture agar) (Murashige and Skoog, 1962) to provide 3 biological replicates of each genotype. For each treatment, 0.3 g of fully expanded leaves were transversely sliced into 2–3 mm strips, which were plasmolysed by immersion in CPW9M medium (0.5 M mannitol, 27.2 mg KH_2PO_4, 100 mg KNO_3, 150 mg $CaCl_2$, 250 mg $MgSO_4$, 2.5 mg $Fe_2(SO_4)_3.6H_20$, 0.6 mg KI, 0.00025 mg $CuSO_4$ per litre; pH 5.8) for 1 h (Anthony et al., 1995). The strips were vacuum-infiltrated in enzyme digestion solution (5 mM morpholinoethanesulphonic acid (MES), 0.4% cellulase, 0.2% macerozyme, CPW9M medium) for 30 min and then incubated at 25 °C in the dark at 40 rpm for 16 h. Protoplasts were released by shaking the digested tissue at 80 rpm for 5 min and purified by filtering through a 75 µm sieve. The filtrate was centrifuged at 100 *g* and the protoplast pellet washed twice in CPW9M medium. Protoplast integrity was checked using the Olympus BX 63 OM/FM microscope (Olympus Scientific Solutions, Massachusetts, USA), viability was determined by Evans' Blue Dye staining (Huang et al., 1986), and quantification conducted via flow cytometry using the BD Accuri™ C6 flow cytometer (BD Biosciences, New Jersey, USA). Flow cytometric data were analysed using FCS Express 7 Research Edition software (Treestar, Inc, Oregon, USA). Protoplasts were resuspended in MMg solution (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES; pH 5.8) (Wu et al., 2017) to a concentration of 10^4 cells per ml.

3. Protoplast transfection

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Approximately 15 µg of the CRISPR construct and 4 µg each of pBIN19-SACMV-DNA-A and pBIN19-SACMV-DNA-B infectious clones (Berrie et al., 2001) were mixed with 1 mL of protoplasts and 25% polyethylene glycol 4000 (PEG 4000), and incubated at room temperature for 20 min. The mixture was gently diluted with 3 volumes of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.8) and centrifuged twice at 100 *g* for 2 min. Protoplasts were resuspended in 300 µL of WI solution (4 mM MES, 0.5 M mannitol, 20 mM KCl, pH 5.8) (Yoo et al., 2007) and incubated overnight in the dark at room temperature to induce gene expression. The expression of eGFP was checked using fluorescence microscopy to confirm protoplast transformation. Protoplasts were washed with CPW9M medium at 24 h post-transfection.

4. Mutagenesis, viral load and gene expression assays

DNA was extracted from the transformed protoplasts 24 h post-transfection (hpt) using QIAzol Lysis Reagent according to a user-developed protocol (www.qiagen.com/it/resources/) (Qiagen, Maryland, USA). Quantitative PCR (qPCR) for SACMV relative viral load quantitation using *Dpn*I-digested (ThermoFisher Scientific, Massachusetts, USA) DNA as template was performed in triplicate using forward (5$GGCTAG \top \mathbb{C}CGGA \top ACAT3$) and reverse (5$GAC \forall GGACGGAGACA\mathbb{C}3$) primers, and 18S rRNA as the reference gene. The exonic region of *MeE3L* was amplified using the Phusion U Green Hot Start DNA Polymerase (ThermoFisher Scientific) with forward (5$CGCGCAGA \top C \forall GC3$) and reverse (5$TGT\mathbb{C}ACATGG \forall TG \forall AG3$) primers. Sequencing of amplicons on the ABI 3500XL Genetic Analyzer was outsourced to Inqaba Biotec (Pretoria, South Africa), and sequencing results were analysed for variation and alignment using TIDE web tool (https://tide.deskgen.com/; Brinkman et al., 2014) and MAFTT version 7 (https://mafft.cbrc.jp/alignment/software/; Rozewicki et al., 2019) before being employed as query terms for protein structure and binding prediction using the I-TASSER On-line Server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/; Yang and Zhang, 2015) and protein similarity search in the Protein Data Bank (Berman et al., 2000). Analysed sequences were used as query terms for BLAST (https://blast.ncbi.nlm.nih.gov/; Zhang et al., 2000) searches of the SACMV genome (AF155806/7) and to construct a phylogenetic tree using the MEGA X software based on the Maximum Likelihood method and Jones-Taylor-Thornton (JTT) matrix-based model (Kumar et al., 2018).

RNA was extracted from the transformed protoplasts 24 hpt using QIAzol and according to the manufacturer's (Qiagen, Maryland, USA) protocol. First strand cDNA synthesis using RNA as template was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Massachusetts, USA). Reverse transcriptase qPCR for *Me*E3L relative expression quantitation was performed in triplicate with cDNA as template using the Maxima SYBR Green/ROX qPCR Master Mix (2X) (ThermoFisher Scientific, Massachusetts, USA) with forward (5$CGCGCAGA \top C \forall GC3$) and reverse (5$TGT\mathbb{C}ACATGG \forall TG \forall AG3$) primers according to the manufacturer's protocol using 18S rRNA was the reference gene for qPCR. Relative expression and viral load of *MeE3L* and SACMV, respectively, were determined using the $2^{-\Delta\Delta T}$ method (Livak and Schmittgen, 2001).

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1. Isolation and transformation of cassava protoplasts

The *in vitro* growth of the cassava plantlets was conducted under controlled, sterile conditions and only young, expanded leaves were used as donors (Fig. 1A). Different sizes (~ 15–35 µm) of cassava protoplasts of round and irregular shape, containing one vacuole and chloroplasts, were observed (Fig. 1B-D). The viability of protoplasts was at least 85% as shown by staining with Evans' Blue Dye (Fig. 2A-C). Protoplast yields were 4.90–6.36 × 10^6/g fresh weight (FW) (Additional File 1). The integrity of isolated protoplasts was analysed by flow cytometry prior to transfection (Fig. 2D-F). The presence of protoplasts as well as irregularly-shaped debris outside the gated area was detected. Approximately 10^4 protoplasts of each cassava genotype were transfected with 15 µg eGFP-tagged CRISPR construct and/or 4 µg SACMV infectious clones using PEG-mediated transformation. Stability of transient expression was verified by detection of eGFP expression 24 hpt, showing that at least 90% of protoplasts had been successfully transformed (Fig. 2G-H).

Cassava protoplast isolation from leaf mesophyll cells by 16 h-long enzymatic digestion. (A)M. esculenta**4-week old donor plants cultured on ½ Murashige and Skoog medium. (B) Protoplasts from**M. esculenta**cv.60444 (C) Protoplasts from susceptible**M. esculenta**T200 (D) Protoplasts from tolerant**M. esculenta**TME3. Spherical protoplasts with chloroplasts around the edge of the cell membrane and central vacuole were observed (shown by red arrows). Protoplasts were visualised under bright field microscopy.**

Analyses of viability, quality and transformation of cassava protoplasts. Viability of freshly isolated protoplasts was determined by Evans' Blue Dye staining and visualisation under bright field microscopy. Analysis of protoplast quality was done by flow cytometric density measurement where events are discriminated by size and granularity, represented in log scale density plots. The size and shape of cassava protoplasts are measured by their effect on the forward scatter (FSC-A) and side scatter (SSC-A) of the laser. Stable transformation with the CRISPR construct was determined by fluorescence microscopy visualisation of eGFP fluorescence through the GFP filter and/or bright field. (A) Protoplasts fromM. esculenta**cv.60444; (B) Protoplasts from**M. esculenta**T200 (C); Protoplasts from**M. esculenta**TME3. Non-viable cells are stained blue. (D) Plot of**M. esculenta**cv.60444 protoplast density (E) Plot of**M. esculenta**T200 protoplast density (F) Plot of**M. esculenta**TME3 protoplast density. Circled regions correspond to desirable protoplasts. (G)**M. esculenta**T200 protoplasts visualised through the GFP filter (H)**M. esculenta**T200 protoplasts visualised through both the bright field and GFP filters.**

2. Structure and phylogenetic analysis ofMeE3Lin model, susceptible and tolerant cassava genotypes

PCR amplification and sequencing of the *MeE3L* partial transcript from leaves revealed that the T200 landrace homolog is slightly longer than the TME3 homolog (Fig. 3B). Sequencing of the exonic region of

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bp insertion mutation of 9 TGAGAA nucleotide repeats that

are absent in cv.60444 and TME3 (Fig. 3A). The resulting frameshift introduces a stop codon at amino acid 141 (Fig C-D). Computational analysis revealed that the truncated T200 MeE3L homolog is structurally distinct from the reference (AM560-2), cv.60444 and TME3 MeE3L homologs (Fig. 3E). It is also not significantly similar in structure to any protein in the Protein Data Bank (Berman et al., 2000), and it is not confidently predicted to bind any ligands. The other three (AM560-2, cv.60444 and TME3) homologs are structurally closest to the cIAP1 inhibitor of apoptosis protein which contains a RING domain with E3 ligase activity for autoubiquitination and modulates cell death (Dueber et al., 2011). An analysis of the phylogeny of E3 ligase homologs in plants (Fig. 3F) reveals that although the MeE3Ls share *Hevea brasiliensis* as a common ancestor, the T200 homolog is significantly more evolutionarily distant from AM560-2 than the TME3 and cv.60444 homologs.

Primary structure, secondary structure and phylogenetic analysis of MeE3L **and/or its protein product. Sequence alignment and agarose gel resolution of** MeE3L **partial gene and partial transcript respectively show a 53 bp insertion mutation in the lengthier T200 homolog that is absent in the AM560-2, cv.60444 and TME3 homologs. Computational prediction of secondary, molecular and zinc-binding structures of MeE3L homologs shows significant differences between T200 structure and the other structures. N→C. Phylogenetic analysis shows significant evolutionary distance between T200 MeE3L and other plant MeE3L homologs. (A) Genomic nucleotide sequence alignment showing insertion mutation between nucleotides 397–398 and 422–423 in the T200** MeE3L **homolog (B) Agarose gel resolution of the PCR-amplified T200 and TME3 partial transcripts of** MeE3L **(C) Amino acid sequence alignment showing premature stop mutation at amino acid residue 141 in T200 MeE3L protein homolog (D) The reference (**M. esculenta **AM560-2) MeE3L amino acid sequence. Asterisks denote stop codons in T200 (amino acid residue 141) and AM560-2/cv.60444/TME3 (amino acid residue 200) homologs respectively. Red letters denote the first T200 MeE3L stop mutation at amino acid residue 141. Underlined letters denote the sequence adhering to the RING finger domain consensus sequence** $[CX_2CX_{(9-39)}CX_{(1-3)}HX_{(2-3)}CX_2CX_{(4-48)}CX_2X]$**.(Ei) AM560-2 MeE3L homolog predicted secondary structure (Eii) AM560-2 MeE3L homolog predicted tertiary molecular structure (Eiii) Zinc binding in RING domain of AM560-2 MeE3L (Eiv) cv.60444 MeE3L homolog predicted secondary structure (Ev) cv.60444 MeE3L predicted tertiary molecular structure (Evi) Zinc binding in RING domain of cv.60444 MeE3L (Evii) T200 MeE3L homolog predicted secondary structure (Eviii) T200 MeE3L predicted tertiary molecular structure (Eix) Predicted ligand binding structure of T200 MeE3L (Ex) TME3 MeE3L homolog predicted secondary structure (Exi) TME3 MeE3L predicted tertiary molecular structure (Exii) Zinc binding in RING domain of TME3 MeE3L [Predictions were run on the I-TASSER On-line Server (**https://zhanglab.ccmb.med.umich.edu/I-TASSER/; **Yang and Zhang, 2015)] (F) Evolutionary analysis of plant MeE3L homologs using Maximum Likelihood method and Jones-Taylor-Thornton (JTT) matrix-based model in MEGA X (**Kumar et al., 2018). **Bootstrap support was calculated from 1000 replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.**

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3. Relative accumulation of SACMV DNA and relativeMeE3Lexpression in transformed cassava protoplasts

Relative SACMV DNA accumulation in wild-type protoplasts was highest in T200 at 8-fold, compared to 3-fold in cv.60444 and TME3 at 24 hpt. Gene editing of *MeE3L* by CRISPR (Additional File 1) resulted in a substantial increase in accumulation of SACMV in T200 and TME3 (12-fold and 4-fold respectively) whereas only a 1-fold reduction was seen in cv.60444 (Fig. 4A). *MeE3L* expression is significantly upregulated with a log fold change of 1 and 9 in T200 and TME3 SACMV-infected protoplasts, respectively. Gene editing of *MeE3L* significantly upregulated expression of the MeE3L variant in TME3 but had no significant effect on T200 *MeE3L* expression under the different transformation conditions. No significant change in *MeE3L* expression was observed under all conditions in cv.60444 (Fig. 4B).

4. Predicted MeE3L primary structure in transformed cassava protoplasts

Predicted MeE3L protein primary structures based on the *MeE3L* genomic sequence in SACMV-infected protoplasts showed virus-induced stop mutations (amino acid residues 133 and 99 respectively) (Fig. 4C-D) upstream of the RING domain in cassava T200 and TME3 variants, compared to uninfected controls and reference sequence AM560-2. Sequencing of MeE3L from SACMV-infected and/or gene-edited TME3 protoplasts revealed multiple random single base mutations along the length of MeE3L which translate to substitutions by altered amino acids (Fig. 4E).

Assessment of viral DNA accumulation, relative MeE3L**expression, and predicted MeE3L primary structure in transformed cassava protoplasts. (A) Relative DNA accumulation of SACMV- and SACMV + CRISPR-Cas9-transformed cassava protoplasts under different transformation conditions. Δ**MeE3L **= mutant CRISPR-edited**MeE3L. **Real-time qPCR was performed using**DpnI**-treated total DNA extracted from cassava protoplasts 24 hpt as template. (B)**MeE3L**relative expression levels in transformed cassava protoplasts. V = SACMV-transformed. Δ**MeE3L **= gene-edited**MeE3L. **RT-qPCR was performed using total mRNA as template. (C) Stop mutation induced in SACMV-infected T200**MeE3L. **(D) Stop mutation induced in SACMV-infected TME3**MeE3L. **(E) The predicted amino acid sequence of TME3 MeE3L at reference sequence positions 2-148 showing multiple mutations in SACMV-infected variant. V = SACMV-infected. C = gene-edited. Sequence alignment was conducted in MEGA-X (**Kumar et al., 2018).

Discussion

1. African cassava landraces susceptible and tolerant to SACMV are amenable to enzymatic protoplast isolation and PEG-mediated transformation

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Protoplasts were chosen for this transient gene expression study because they can conveniently and efficiently be transformed with several DNA constructs simultaneously, and they allow higher resolution imaging compared to cells in intact tissue (Faraco *et al*., 2011). Additionally, they can be used for high throughput efficient screening of many candidate genes, and those that show an effect can then be silenced by CRISPR or virus induced gene silencing (VIGS) *in planta*, which are considerably longer (3-4 months) and more complicated procedures for the non-model host cassava. Leaf mesophyll was used as the source for protoplasts because it provides functional information about proteins expressed in other plant organs (Faraco *et al*., 2011). Round and irregularly-shaped protoplasts of different sizes were observed (Fig.1B-D), although generally leaf mesophyll protoplasts should be uniformly spherical (Wu *et al*., 2017).

A previously determined enzyme concentration (1.6% cellulase, 0.8% macerozyme) that is suitable for obtaining the optimum number of viable protoplasts was used for leaf digestion (Wu *et al*., 2017). The viability of protoplasts in this study was at least 85% although cassava protoplast viability of up to 95% has been reported (International Plant Research Institute, 1984). The long digestion period (16 h) was ruled out as the cause of death for ~15% of protoplasts as this reportedly does not induce serious damage in protoplasts (Tang, 1982). It has been reported that micro-propagated plants grown *in vitro* lack epicuticular wax and thus allow rapid enzyme penetration (Kumar and Rao, 2018). Cassava, however, has a thick epicuticular layer which necessitates the long digestion period of 16h compared to 0.3-1h for Arabidopsis (Wu *et al*., 2009). Macerating enzymes such as macerase are known to cause wound reactions in protoplasts because of their degradation of the cell wall, which may lead to necrosis (Ishii, 1988), while cellulase is known to exert inadequate enzymatic activity at low concentrations and higher concentrations have no benefit or detriment (Uchimiya and Murashige, 1974). Therefore, a balance between digestion enzyme concentration and viability is essential in order to obtain the optimum yield of viable good quality protoplasts. The difference in cassava protoplast viability from previously reported percentages possibly may vary according to cassava variety, and in this case could be due to the particular physiological characteristics of the African cassava landraces from which protoplasts were derived in this study.

Although lower than the previously reported yields of $4.4x10^7$ protoplasts/g FW leaves from *M. esculenta* cv. South China 8 (Wu *et al*., 2017) and $1.9x10^7$ protoplasts/g FW leaves from *M. esculenta* cv. M. Thai 8 (Anthony *et al*., 1995), the protoplast yields in this present study ($4.90\text{-}6.36x10^6$/g FW) were sufficient to provide the recommended number of protoplasts ($10^4\text{-}10^7$) required for each transfection (Yoo *et al*., 2007). Both pre-treatment of leaves in the dark (24-72 h) (Shahin and Shepard, 1980) and vacuum infiltration before enzyme digestion (Nanjareddy, 2016) were shown to intensify enzyme penetration in bean leaves. We found that pre-treatment resulted in release of much undesirable plant debris alongside protoplasts, and that vacuum infiltration did indeed help increase protoplast yield. Flow cytometry indicated a high concentration of protoplasts compared to irregularly-shaped debris. Based on microscopy images of purified protoplasts (Fig.1B-D), the irregular debris outside the gates was deemed

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to be free chloroplasts, plasmolysed cells, undigested cell wall fragments and other aggregates arising from the long digestion period of leaf material.

PEG-mediated plant protoplast transfection with plasmid DNA is a well-established procedure (Hayashimoto *et al*., 1990; Locatelli *et al*., 2003) and a popular protocol uses 10 µg DNA to transfect $2x10^4$ Arabidopsis protoplasts (Yoo *et al*., 2007). At least 5 µg of plasmid DNA have previously been used to transform 10^6 tobacco protoplasts with African cassava mosaic virus (ACMV) (Ermak *et al*., 1993) and *Cowpea mosaic comovirus* (Wellink *et al*., 1993). Highly efficient co-expression of multiple constructs has been reported (Chen *et al*., 2006; Walter *et al*., 2004). Virus infectious clones have also been used in conjunction with other plasmid constructs for co-inoculation of plant protoplasts. *Nicotiana tabacum* protoplasts have been co-transformed with 5 µg eGFP construct, 3 µg siRNA and 4 µg each of ACMV or East African cassava mosaic virus (EACMV) with DNA/RNA extraction at 36 and 48 hpt (Vanitharani *et al*., 2003). To our knowledge, the present study is the first report of co-transformation of cassava protoplasts with a CRISPR construct and geminivirus infectious clones.

2. The *M. esculenta* T200 *MeE3L* encodes a truncated RING-less protein due to a nonsense mutation

The frameshift resulting from the insertion mutation in T200 *MeE3L* introduces a stop codon upstream of the RING domain, thus encoding a truncated protein in which the C3HC4-type RING finger motif is absent. Essentially, the E3 ligase domain in the T200 *MeE3L* would not be translated because of this mutation, making its potential protein product non-functional with respect to this E3 ligase activity. There is evidence that intronic GAA repeats impede transcription via the assembly of an inaccessible chromatin structure (Punga and Buhler, 2010). However, we amplified the T200 *MeE3L* exon from cDNA, showing that this gene is transcribed. It is more likely that the premature translation termination instruction makes the T200 *MeE3L* a possible candidate for nonsense-mediated mRNA decay (NMD) whereby mRNAs with long 3'-untranslated regions (UTRs) are targeted as substrates for degradation (Hori and Watanabe, 2005). In Arabidopsis, for example, evolutionarily conserved protein factors are deployed by the NMD system where the 3'-UTR is less than 300 nucleotides (Hori and Watanabe, 2007).

It is proposed that the loss of a substantial portion of the T200 MeE3L C-terminal region would alter the spatial chemical conformation necessary for any other interactions (such as binding the ubiquitin-conjugated E2 and substrate) to occur. Furthermore, the possible non-functionality of the T200 MeE3L due to the nonsense mutation would suggest that the susceptibility of T200 to SACMV might be linked the loss of the RING domain since this is where the E3 ligase activity resides. However, susceptible cv.60444 encodes a functional RING domain in its MeE3L, suggesting that susceptibility to the virus is subject to at least another pathway besides that in which *MeE3L* is involved, or is under polygenic control.

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The structural similarity between between MeE3L and cIAP1 is significant because IAPs are known for contributing to disease initiation, maintenance or progression by controlling protein stability (Silke and Meier, 2013). Binding of a substrate to cIAP1 leads to activation of the E3 ligase, leading to activation of downstream caspases which set cell death in motion (Berger *et al*., 2006). Viruses tend to inhibit host apoptotic responses in a bid to prolong cell viability and facilitate replication by targeting caspases for suppression. However, because caspases also play a role in cell proliferation and differentiation, some viruses stabilise caspase activity to maintain cell survival of the cell at a particular differentiation stage, or to facilitate replication by suppressing early apoptosis (Lee, Liao and Lin, 2005; Best, 2008). Reduced viral yield has been reported in a case where specific caspases were inhibited, suggesting that these caspases expedite viral replication (Best, Wolfinbarger and Bloom, 2002). It is suggested that silencing of the *MeE3L* RING domain would promote caspase activity in the susceptible T200 genotype, thereby enhancing pathogenicity by aiding SACMV replication.

The AM560-2, TME3, and cv.60444 cassava genotypes have previously been shown to cluster together under their nearest ancestor, *Hevea brasiliensis* (Bredeson *et al*., 2016). The evolutionary history of T200, a southern African landrace, is unknown and the phylogenetic analysis suggests that the T200 *MeE3L* evolved after the cv.60444 and TME3 variants. While it is known that wild plants in natural ecosystems may co-evolve with their virus partners, information regarding how viruses apply selective pressure to increase plant susceptibility is not known. A study of *Drosophila* and its host-specific viruses found that coevolution may cause sustained genetic variation in susceptibility. This may explain why a southern African cassava landrace is highly susceptible to SACMV that appears to have migrated south from its origin, suspected to be in east Africa or the south-west Indian Ocean islands such as Madagascar (De Bruyn *et al*., 2016) that were once geographically attached to the African continent (Lefeuvre *et al*. 2007). South African cassava mosaic virus is a recombinant between *East African cassava mosaic virus* and two other unknown geminiviruses which contributed the AC4 and IR regions (Berrie *et al*., 2001), and moved southwards into Mozambique, Zimbabwe and South Africa where it may have encountered the T200 landrace. Subsequent to its first discovery in South Africa, it has been reported in Zimbabwe (Briddon *et al*. 2004) and Madagascar (De Bruyn *et al*., 2016). It is suggested that the T200 landrace and SACMV may still be in the process of co-adaption, which would be why T200 exhibits extreme susceptibility to SACMV. It is known that infection with a new recombinant begomovirus requires hosts to adjust to minor or major differences in virus-host interactions (Montes *et al*., 2019). We speculate that maybe the *MeE3L* is either a paralog in T200 or it was introgressed from a wild relative in southern Africa.

3. *MeE3L* affects SACMV DNA accumulation in a genotype-dependent manner

Quantitative PCR is a well-established method for precise quantitation of viral DNA amount in infected tissue and it requires a host reference gene with stable expression patterns under experimental conditions as the internal control for correct data normalisation (Moreno *et al*., 2011). Quantitative detection of *African cassava mosaic virus* and *East African cassava mosaic virus* using real time -qPCR has been

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reported (Otti *et al*., 2013) and SACMV load, in particular, has been assayed *in planta* in Arabidopsis (Pierce and Rey, 2013) and cassava (Allie, 2014). Replication of the geminivirus, *Cassava brown streak virus,* in cassava leaf mesophyll protoplasts has been assayed at 6 hpt (Anjanappa *et al*., 2016) and it has been reported that there was significant viral DNA accumulation in tobacco BY-2 protoplasts 36 and 48 hpt by co-inoculating with infectious ACMV and EACMV clones and siRNA (Vanitharani *et al*., 2003). Our finding that SACMV DNA accumulates in cassava protoplasts correlates well with these reports.

It was expected that viral DNA accumulation would be significantly lower in TME3 than in the susceptible cv.60444 and T200 if MeE3L was involved in tolerance since the TME3 landrace is known to have the CMD2 locus. Evidently, CRISPR-induced mutations in *MeE3L* in cassava protoplasts differentially affect viral DNA accumulation based on the cassava genotype. CRISPR-associated modification of the MeE3L structure and subsequent disruption of its activity may enhance SACMV DNA accumulation and pathogenicity in T200 and TME3 by interfering with the UPS-dependent tolerance/resistance response mechanisms of cassava.

4. Viral activity and gene editing of *MeE3L* affect the expression of *MeE3L*

Geminiviruses elude plant defense mechanisms by hijacking and redirecting ubiquitination, and interfering with responses regulated by ubiquitin E3 ligases (including responses to jasmonates, auxins, gibberellins, ethylene,abscisic acid) (Lozano-Duran *et al*., 2011). It follows then that alterations to E3 ligase genomic sequences may alter E3 ligase expression patterns during viral infection, as viruses are known to modulate RNA levels to enhance infection (Verchot, 2016). Both plant viruses and CRISPR systems are known to induce mutations in the genome (Machida *et al*., 2004; Cougot *et al*., 2005; Sander and Joung, 2014), and the employment of both against the *MeE3L* would provide an indication of how this gene is involved in the plant's response to SACMV.

Results indicate that in TME3 protoplasts, *MeE3L* expression is upregulated during SACMV infection. The concurrent CRISPR-mediated gene editing of *MeE3L* and infection with SACMV appears to induce increased expression of the *MeE3L*, suggesting that the interaction between the virus and *MeE3L* is based on recognition of the *MeE3L*'s specific base sequence. Previously, plant E3 ligases have been shown to be induced by viral infection (Lai *et al*., 2009; Czosnek *et al*., 2013; Chen *et al*., 2018) and plant defence elicitors (Libault *et al*., 2007; Sadanandom *et al*., 2012). The muted response of the T200 *MeE3L* to all treatments was expected given its nonsense mutation which silences the RING domain responsible for E3 ligase activity. However, the muted response of susceptible cv.60444 *MeE3L* was unexpected and suggests that this *MeE3L* sequence variant is not responsive to SACMV infection. It is possible that downstream plant hormone responses may be linked to MeE3L activity. It is known that geminiviruses interact with plant E3 ligases and induce their up- or down-regulation to promote infection or undergo degradation. *Beet severe curly top virus* (BSCTV) C4 induces activity of RING E3 ligase, RKP, to interfere with the cell cycle, increase susceptibility and promote infection (Lai, 2009). The C2 protein interacts with

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CSN5, a component of the SCF E3 ligase complex, to alter CUL1's regulation of plant hormones (Lozano-Duran *et al*., 2011). An F-box protein adaptor is triggered to overexpress by *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and hence the virus co-opts SCF E3 ligase activity (Lozano-Duran and Bejarano, 2011). The geminiviral βC1 protein is ubiquitinated by E3 ligase, NtRFP1, for degradation and resultant attenuation of symptoms. Conversely, βC1 is able to interact with NbSKP1 to impair the SCF complex integrity and hence enhance symptom induction by interfering with jasmonic acid and gibberellic acid signalling. Evidently, geminiviruses can usurp the UPS by preventing E3 ligases from targeting viral proteins; or they can redirect the UPS to degrade host resistance-related proteins.

The *MeE3L* homolog sequences in SACMV-infected protoplasts show that SACMV is actively inducing silencing of the RING domain in order to achieve full infection of the host. The concomitant increase in SACMV DNA accumulation and CRISPR-mediated editing of *MeE3L* in TME3 shows that the interaction of *MeE3L* with SACMV is more directed at advancing susceptibility. One other plant E3 ligase, RHF2A, is silenced by TYLCSV to promote infection (Lozano-Duran *et al*., 2011). It has also been shown that inhibition of a RING E3 ligase impairs plant defence during Cabbage leaf curl virus (CaLCuV) infection (Sahu *et al*., 2013) and that silencing of tobacco RING E3 ligase, NtRFP1, leads to development of severe symptoms during *Tomato yellow leaf curl China virus* (TYLCCV) (Shen *et al*., 2016). The present study provides further evidence that geminiviruses interfere with expression of a plant E3 ligase in order to establish infection, and demonstrates for the first time that *MeE3L* in cassava is responsive to a cassava-infecting geminivirus, SACMV.

5. SACMV's interaction with a tolerant cassava genotype induces numerous mutations in *MeE3L*

Functions of E3 ligases in regulating immunity systems are orchestrated at the interface of host-virus interactions (Zhang *et al*., 2018) and some of these interactions occur in the nucleus (Kushwaha *et al*., 2017). Sequencing of *MeE3L* from SACMV-infected TME3 protoplasts revealed multiple random single base mutations along the length of *MeE3L*, which translate to amino acid substitution (Fig. 4E). While these mutations do not alter the reading frame, they are apparently aimed at silencing the whole protein and not just the RING domain. The resulting disordered protein would presumably not only lack RING E3 ligase activity, but also the E2 and substrate binding activity.

Several viral proteins reportedly hinder E3 ligase function by encoding these E3 ligases in their viral genomes (Lai *et al*., 2009; Rosa-Diaz *et al*., 2013; Wang *et al*., 2013; Chen *et al*., 2018; Lozano-Duran and Bejarano, 2018). However, a blastn search for *MeE3L* homologs in the SACMV genome did not return any hits, thus ruling out the possibility that SACMV encodes a protein with E3 ligase activity as a strategy to target host proteins for degradation via the RNA silencing pathway. Since E3 ligases are frequently hijacked by different viruses (Jackson and Xiong, 2009), we suggest, based on the *MeE3L* mutations induced during SACMV infection, that *MeE3L* is one of the targets of SACMV-induced host reprogramming and plays a crucial strategic role in enhancing susceptibility. This is not a case of viral

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arrogation of *MeE3L* by SACMV to redirect the UPS to new targets but rather to inhibit its function. Interestingly, the discovery of these mutations in genomic DNA possibly point to a yet unknown geminivirus-induced host mechanism for genome editing. Some E3 ligases and viral proteins can localise to the nucleus, such as the tobacco E3 ligase, NtHUB1 which has a nuclear localisation sequence, is recruited by geminiviral Rep protein, and co-localises and interacts with the Rep protein to monoubiquitinate cellular chromatin and thus enable infection (Kushwaha *et al*., 2017). The viral coat protein, CP, also has a nuclear localisation signal, can localise in the nucleolus and nucleoplasm, and facilitates entry of ssDNA into the nucleus (Wang *et al*., 2017; Kumar, 2019). However, the mechanisms for these processes are yet to be investigated. Geminiviruses are known to induce the expression of genes related to repair of double-stranded breaks (DSBs) and DNA synthesis (Lozano *et al*., 2015), and to promote somatic homologous recombination (Richter *et al*., 2014). In spite of this evidence for geminiviral activity at the host genomic level, the mechanisms for these processes have not been fully investigated. However, this evidence provides a measure of support for our finding of gene mutation induction by SACMV in TME3.

6. The response of MeE3L to SACMV is virus- and host-specific

Ubiquitin ligases are abundant in plants and provide substrate specificity to target particular proteins. In Arabidopsis alone, RING E3 ligases make up 499 out of over 1,500 E3 ligases (Mazucotelli *et al*., 2006). A comparison of E3 ligase and E3 ligase complex-associated gene expression during other plant geminivirus infection studies (Additional File 2) was conducted to determine whether *MeE3L*'s response to SACMV is geminivirus-specific or host-dependent.

In susceptible cassava, E3 ligase expression is downregulated during SACMV infection at early, middle and late time points (12, 32 and 64 days post infection (dpi)) but there is no differential expression of E3 ligases in tolerant cassava at any time point (Allie *et al*., 2014). However, no differential expression of E3 ligases is recorded during SACMV infection of Arabidopsis which is susceptible (Pierce and Rey, 2013). *A study of transcriptomic responses to geminivirus Tomato leaf curl New Delhi virus (ToLCNDV) infection in potato found that five E3 ligases in the susceptible cultivar and two in the tolerant cultivar are upregulated at 30 dpi (Jeevalatha et al., 2017) while the geminivirus Tomato yellow leaf curl virus (TYLCSV) has been shown to induce upregulation of E3 ligases in susceptible tomato at 42 dpi, except in the case of a CUL1 which is downregulated (Miozzi et al., 2014).* A transcriptome study of Arabidopsis during geminivirus CaLCuV infection found that, out of 1570 E3 ligases, 149 were up-regulated and 23 were downregulated (Ascencio-Ibanez *et al*., 2008; Lozano-Duran *et al*., 2013). The *CaLCuV AC2 protein, in particular, induces downregulation of two E3 ligases in Arabidopsis (Liu et al., 2014). These findings, together with the current study, prove that plant E3 ligase responses to geminivirus infection are neither uniform nor similar, but they vary according to the specific geminivirus and host involved in the interaction.*

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Responses of cassava to the ssRNA potyviruses Cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV) with respect to E3 ligase expression variably show both downregulation and upregulation in the susceptible varieties. Interestingly, there is no differential expression of E3 ligases in resistant cassava varieties except in Kaleso where a CUL1 is upregulated and a RZPF34 is downregulated (Maruthi et al, 2014; Anjanappa et al., 2016; Amuge et al., 2017). This variable expression of E3 ligases with respect to the virus in the same host suggests that while responses to viral infection are host-dependent, they are also modulated according to the particular virus infecting the plant.

It appears that MeE3L, like other plant E3 ligases, is reprogrammed by SACMV in a manner that is virus-specific and host-dependent. Moreover, current evidence indicates that E3 ligase responses are more pronounced in the virus-susceptible plant varieties. SACMV infection *in planta* is associated with occurrence of severe symptoms leading to persistent severe infection in susceptible T200 and mild symptoms with recovery at 67 days post infection (dpi) in tolerant TME3 (Allie *et al*., 2014). The SACMV-induced genetic mutations and differential expression of *MeE3L* post-infection in TME3 and T200 indicate that it is one of the significant genes involved in the plant's response to the virus, and the data shown herein suggest that the *MeE3L*-SACMV interaction is aimed at silencing the gene to promote viral activity. Although the mechanism for silencing of *MeE3L* by SACMV is not clear, we propose that the silencing of *MeE3L* is part of the SACMV-induced reprogramming of the UPS to inhibit ubiquitination and trigger a decrease in global ubiquitinated proteins. The subsequent developmental disturbance linked to reduced caspase activity would contribute to increased symptom severity, which is visible at 32 dpi. At 67 dpi in TME3, the homeostasis of ubiquitinated versus non-ubiquitinated proteins would be restored and the effects of SACMV on the UPS would be counteracted, leading to symptom decrease and plant recovery. Alternatively, it is possible that SACMV suppressors of RNA silencing (VSRs) such as C2 and C4 target E3 ligases, such as *MeE3L* in T200 and TME3, for silencing, thereby usurping the UPS and contributing to disease symptoms. In TME3, other defence mechanisms such as RNA silencing at 67 dpi (Rogans *et al*., 2016) and resistance (NLR) proteins (Louis and Rey, 2015) persist, which interfere and reduce the activity and replication of SACMV.

Conclusions

We have developed a simple and fast protocol for CRISPR-mediated transient gene expression assay in cassava protoplasts. While gene editing/knockout in cassava plants takes 6–8 months, our protoplast-based method takes 6 weeks to provide experimental data that is suitable for informing *in planta* functional genomics studies. The findings of this present study show that the *MeE3L* base sequence is a determinant of cassava's response to SACMV, via a mechanism for virus-mediated recognition and/or interaction with *MeE3L* at the host genomic level. The response of the RINGless T200 *MeE3L* to virus infection suggests that the RING domain in the C-terminal region of *MeE3L* may be essential for defence against SACMV and this is why it is actively silenced by the virus, thus contributing to pathogenicity. In the case of *MeE3L* in susceptible cassava protoplasts, it appears that SACMV conducts a delicate ation by the *MeE3L* RING domain and to inhibit expression

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compared to the tolerant landrace in order to stabilise caspase activity for cell survival. Most importantly, this study reinforces previously reported evidence that E3 ligases are an integral component of how plants respond to geminivirus infection and provides further evidence of how E3 ligases may be exploited by geminiviruses to enhance susceptibility.

Sequencing and expression assaying of *MeE3L* in other CMD-resistant/tolerant cassava genotypes would help further unravel *MeE3L*'s role in the response presented by cassava against SACMV. CRISPR multiplexing for high throughput targeting of candidate genes implicated in the SACMV-cassava interaction would assist in creating functional networks to further map the molecular mechanisms underlying cassava's response to SACMV.

Abbreviations

AC4
post-transcriptional gene silencing suppressor protein
ACMV
African cassava mosaic virus
APC
anaphase-promoting complex
APC2
APC Regulator of WNT Signaling Pathway 2
ASK
Arabidopsis SKP1-related
BLAST
Basic local alignment search tool
bp
base pair
BSCTV
Beet severe curly top virus
BTB
bric a brac, tramtrack and broad complex
C2
Transcriptional activator protein
C2
Transcriptional activator protein
C4
post-transcriptional gene silencing suppressor protein
CaLCuV
Cabbage leaf curl virus
CCNF

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cIAP1
Cellular inhibitor of apoptosis protein 1
CMD
Cassava mosaic disease
CPW9M
Cell and Protoplast Washing 9 Mannitol
CRISPR
Clustered regularly interspaced short palindromic repeats
CRL
Cullin ring
CUL1
Cullin 1
CUL4-DDB
cullin4-damaged DNA-binding protein
cv
cultivar
DBS
double-stranded break
DDB1
DNA damage-binding protein 1
DNA
Deoxyribonucleic acid
dpi
days post infection
DUB
Deubiquitinating enzyme
EACMV
East African cassava mosaic virus
eGFP
Enhanced Green Fluorescent Protein
FAO
Food and Agriculture Organisation
FW
fresh weight
g
gram
g
standard acceleration due to gravity
GFP

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h
hour
HECT
Homologous to E6-associated protein C-terminus
hpt
hours post-transfection
Hsp
Heat shock protein
IAP
Inhibitor of apoptosis protein
IR
intergenic region
I-TASSER
Iterative Threading ASSEmbly Refinement
JTT
Jones-Taylor-Thornton
µg
microgram
µM
micromolar
M
molar
MeE3L
Manihot esculenta E3 Ligase
MEGA X
Molecular Evolutionary Genetics Analysis X
mg
milligram
min
minute
ml
millilitre
mM
millimolar
MMg
mannitol magnesium
N→C
amino terminus to carboxy terminus
NbSKP1
ociated protein 1

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NLR
nucleotide-binding leucine-rich repeat
NMD
nonsense-mediated mRNA decay
NtHUB1
Nicotiana benthamiana homologous to ubiquitin protein 1
PCR
Polymerase chain reaction
PEG
polyethylene glycol
qPCR
Quantitative polymerase chain reaction
QTL
quantitative trait locus
R protein
resistance protein
RBR
RING-between-RING RING
RBX1
RING-box protein 1
Rep
Replication-associated protein
RHF2A
RING-H2 finger E3 ubiquitin ligase protein
RING
Really Interesting New Gene
RNA
Ribonucleic acid
rpm
revolutions per minute
rRNA
ribosomal RNA
RZPF34
RING zinc finger protein 34
SACMV
South African cassava mosaic virus
SCF
Skp, Cullin, F-box containing complex
sgRNA

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siRNA
small interfering RNA
ssRNA
single stranded RNA
T200
Tropical *Manihot esculenta* 200
TIDE
Tracking of indels by decomposition
TME3
Tropical *Manihot esculenta* 3
ToLCNDV
Tomato leaf curl New Delhi virus
TYLCCV
Tomato yellow leaf curl China virus
TYLCSV
Tomato yellow leaf curl Sardinia virus
UCBSV
Ugandan cassava brown streak virus
UPS
ubiquitin proteasome system
UTR
untranslated region
βC1
movement protein

Declarations

Ethics approval and consent to participate

Not applicable

Consent to publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Competing Interests

The authors declare that they have no competing interests.

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Authors' contributions

CR conceptualised the study. PC performed experiments, analysed results and wrote the manuscript. Both authors interpreted data, and read and approved the final manuscript.

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Figures

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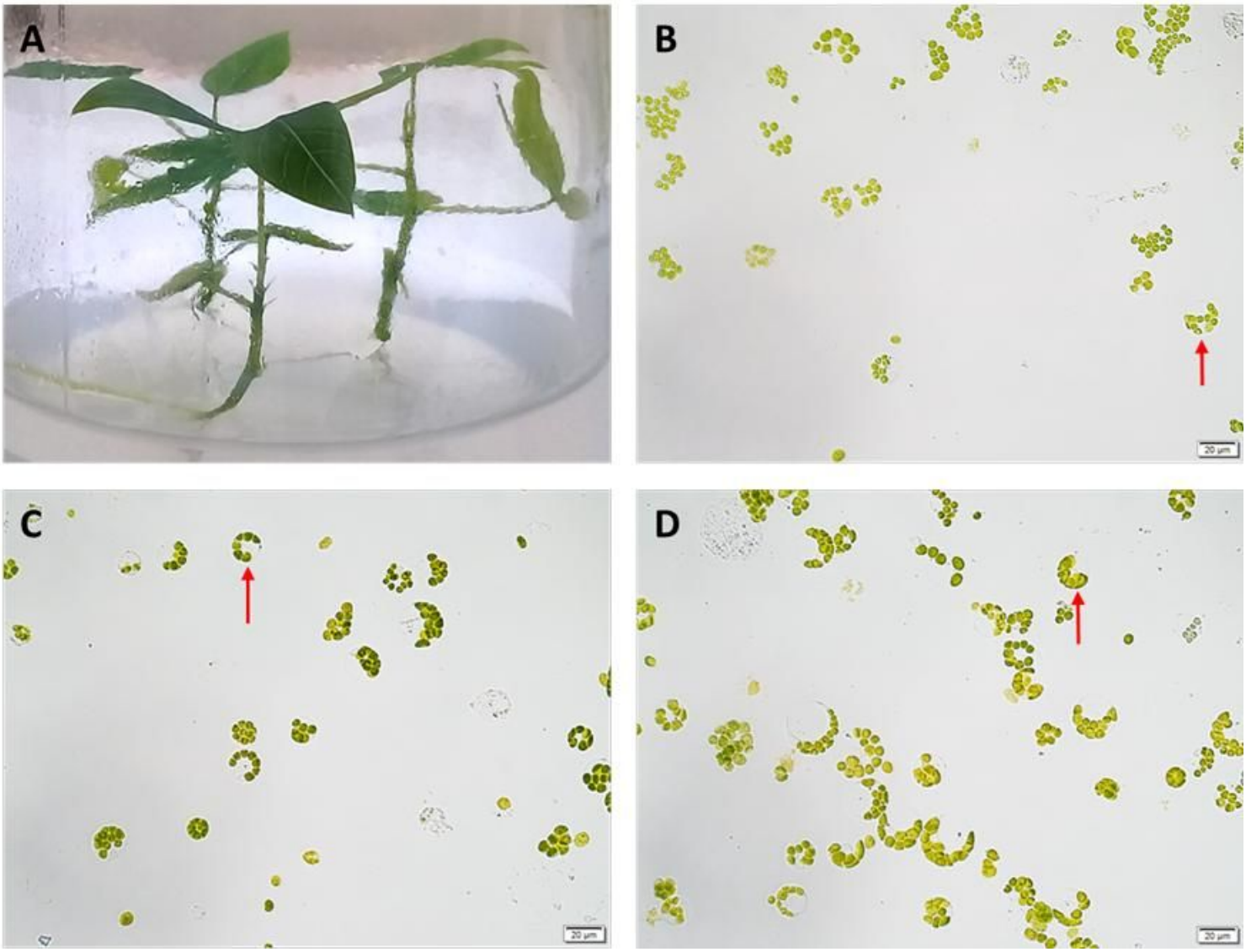

Figure 1

Cassava protoplast isolation from leaf mesophyll cells by 16 h-long enzymatic digestion. (A) M. esculenta 4-week old donor plants cultured on ½ Murashige and Skoog medium. (B) Protoplasts from M. esculenta cv.60444 (C) Protoplasts from susceptible M. esculenta T200 (D) Protoplasts from tolerant M. esculenta TME3. Spherical protoplasts with chloroplasts around the edge of the cell membrane and central vacuole were observed (shown by red arrows). Protoplasts were visualised under bright field microscopy.

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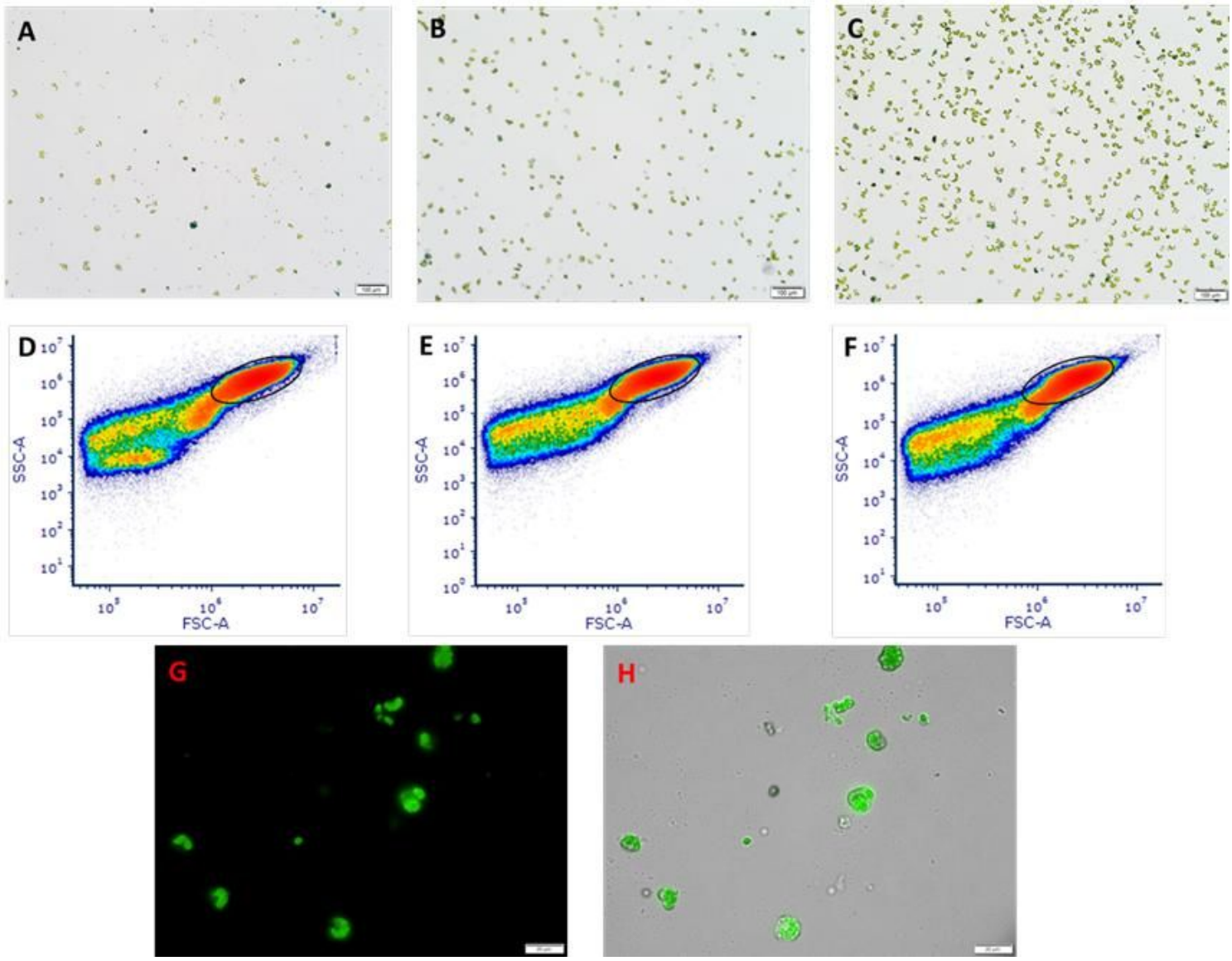

Figure 2

Analyses of viability, quality and transformation of cassava protoplasts. Viability of freshly isolated protoplasts was determined by Evans' Blue Dye staining and visualisation under bright field microscopy. Analysis of protoplast quality was done by flow cytometric density measurement where events are discriminated by size and granularity, represented in log scale density plots. The size and shape of cassava protoplasts are measured by their effect on the forward scatter (FSC-A) and side scatter (SSC-A) of the laser. Stable transformation with the CRISPR construct was determined by fluorescence microscopy visualisation of eGFP fluorescence through the GFP filter and/or bright field. (A) Protoplasts from M. esculenta cv.60444; (B) Protoplasts from M. esculenta T200 (C); Protoplasts from M. esculenta TME3. Non-viable cells are stained blue. (D) Plot of M. esculenta cv.60444 protoplast density (E) Plot of M. esculenta T200 protoplast density (F) Plot of M. esculenta TME3 protoplast density. Circled regions correspond to desirable protoplasts. (G) M. esculenta T200 protoplasts visualised through the GFP filter (H) M. esculenta T200 protoplasts visualised through both the bright field and GFP filters.

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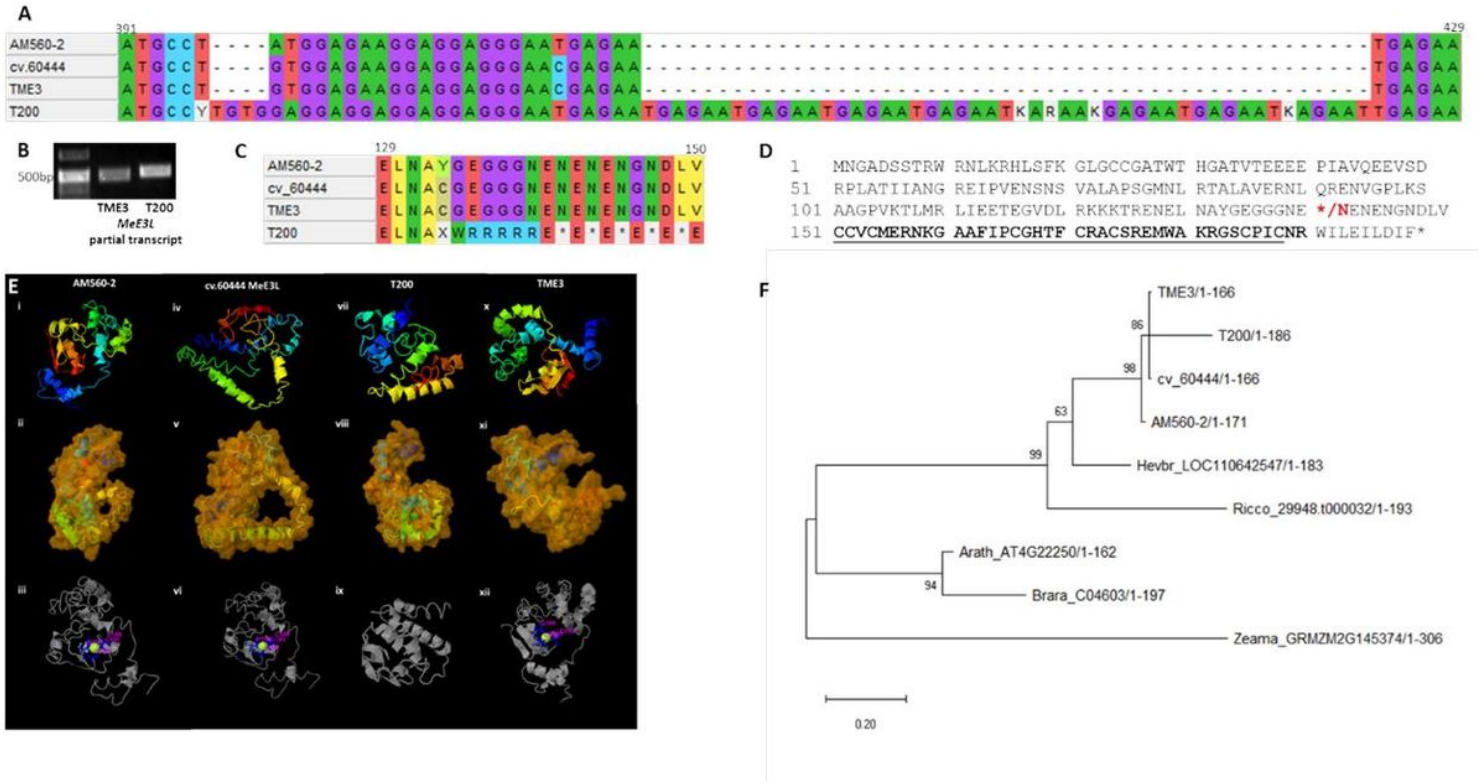

Figure 3

Primary structure, secondary structure and phylogenetic analysis of MeE3L and/or its protein product. Sequence alignment and agarose gel resolution of MeE3L partial gene and partial transcript respectively show a 53 bp insertion mutation in the lengthier T200 homolog that is absent in the AM560-2, cv.60444 and TME3 homologs. Computational prediction of secondary, molecular and zinc-binding structures of MeE3L homologs shows significant differences between T200 structure and the other structures. N→C. Phylogenetic analysis shows significant evolutionary distance between T200 MeE3L and other plant MeE3L homologs. (A) Genomic nucleotide sequence alignment showing insertion mutation between nucleotides 397-398 and 422-423 in the T200 MeE3L homolog (B) Agarose gel resolution of the PCR-amplified T200 and TME3 partial transcripts of MeE3L (C) Amino acid sequence alignment showing premature stop mutation at amino acid residue 141 in T200 MeE3L protein homolog (D) The reference (M. esculenta AM560-2) MeE3L amino acid sequence. Asterisks denote stop codons in T200 (amino acid residue 141) and AM560-2/cv.60444/TME3 (amino acid residue 200) homologs respectively. Red letters denote the first T200 MeE3L stop mutation at amino acid residue 141. Underlined letters denote the sequence adhering to the RING finger domain consensus sequence [CX2CX(9-39)CX(1-3)HX(2-3)CX2CX(4-48)CX2X].(Ei) AM560-2 MeE3L homolog predicted secondary structure (Eii) AM560-2 MeE3L homolog predicted tertiary molecular structure (Eiii) Zinc binding in RING domain of AM560-2 MeE3L (Eiv) cv.60444 MeE3L homolog predicted secondary structure (Ev) cv.60444 MeE3L predicted tertiary molecular structure (Evi) Zinc binding in RING domain of cv.60444 MeE3L (Evii) T200 MeE3L homolog predicted secondary structure (Eviii) T200 MeE3L predicted tertiary molecular structure (Eix) Predicted ligand binding structure of T200 MeE3L (Ex) TME3 MeE3L homolog predicted secondary structure (Exi) ructure (Exii) Zinc binding in RING domain of TME3 MeE3L

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[Predictions were run on the I-TASSER On-line Server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/; Yang and Zhang, 2015)] (F) Evolutionary analysis of plant MeE3L homologs using Maximum Likelihood method and Jones-Taylor-Thornton (JTT) matrix-based model in MEGA X (Kumar et al., 2018). Bootstrap support was calculated from 1000 replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

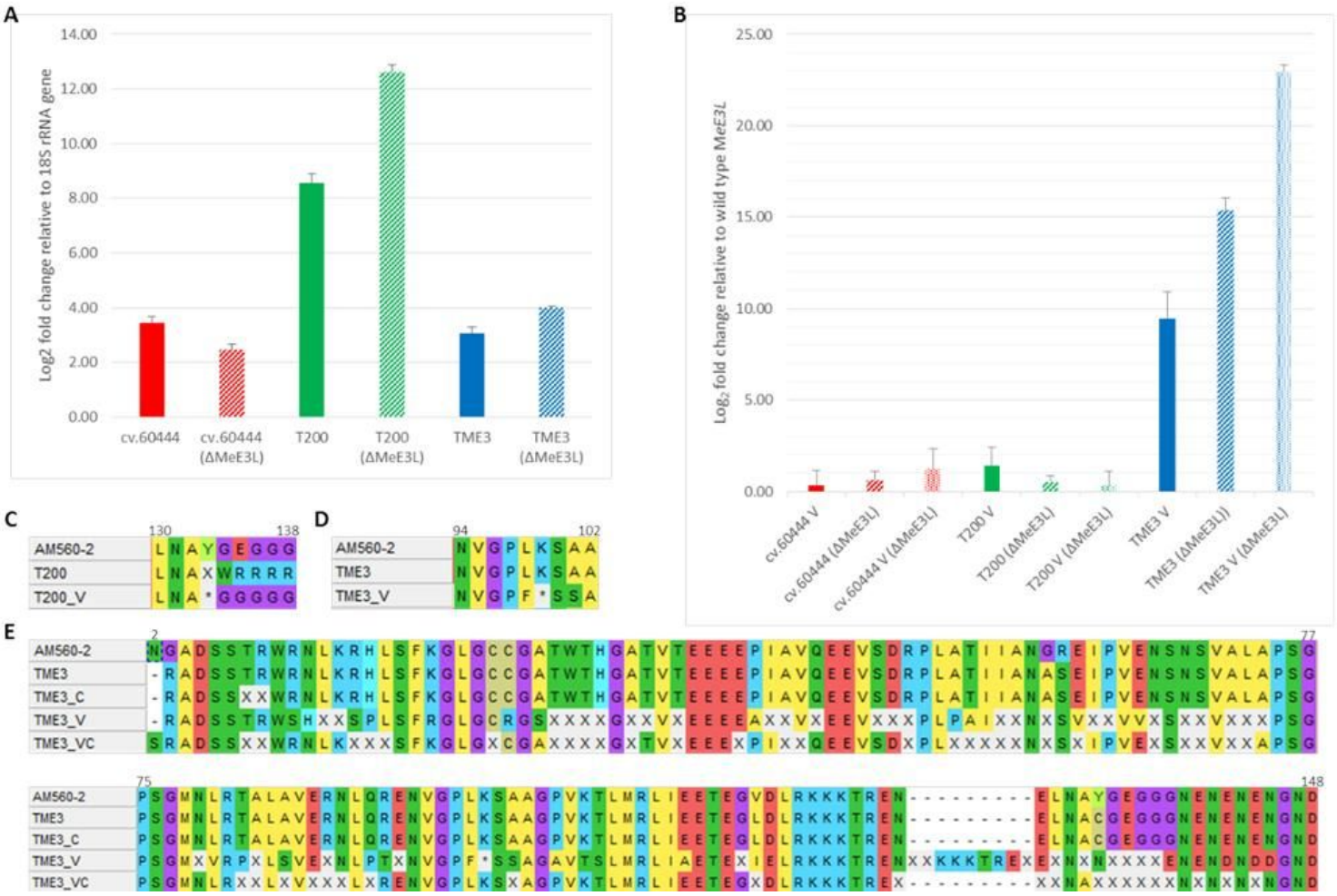

Figure 4

Assessment of viral DNA accumulation, relative MeE3L expression, and predicted MeE3L primary structure in transformed cassava protoplasts. (A) Relative DNA accumulation of SACMV- and SACMV+CRISPR-Cas9-transformed cassava protoplasts under different transformation conditions. ΔMeE3L = mutant CRISPR-edited MeE3L. Real-time qPCR was performed using DpnI-treated total DNA extracted from cassava protoplasts 24 hpt as template. (B) MeE3L relative expression levels in transformed cassava protoplasts. V = SACMV-transformed. ΔMeE3L = gene-edited MeE3L. RT-qPCR was performed using total mRNA as template. (C) Stop mutation induced in SACMV-infected T200 MeE3L. (D) Stop mutation induced in SACMV-infected TME3 MeE3L. (E) The predicted amino acid sequence of TME3 MeE3L at reference sequence positions 2-148 showing multiple mutations in SACMV-infected variant. V = SACMV-infected. C = gene-edited. Sequence alignment was conducted in MEGA-X (Kumar et al., 2018).

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