



FUNCTIONAL CHARACTERISATION OF RESISTANCE GENE ORTHOLOGUES IN
CASSAVA USING GENE EDITING

by

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Thesis

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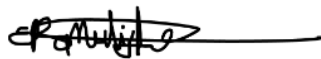
Declaration

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Abstract

Cassava (*Manihot esculenta*, Crantz) is the 3rd largest source of carbohydrates in Africa and feeds millions globally. Although cassava can thrive in drought prone regions, no genotype has overcome the threat by the geminivirus South African cassava mosaic virus (SACMV), which causes cassava mosaic disease (CMD) and can lead to 82% cassava production losses. Cassava landrace TME3 shows tolerance to SACMV while T200 landrace is highly susceptible. Disease resistance genes (R genes) encoded NBS-LRR proteins are key players in host defence mechanisms because of their role as receptors that recognise pathogen effectors and trigger plant effector-triggered immunity (ETI). The first objective of this study was aimed to analyse the amino acid sequences of two NBS-LRR resistance gene analogs (RGAs) *MeTNL* and *MeRPPL1*, and to determine their putative role in tolerance or susceptibility to the South African cassava mosaic virus *in planta*, using virus induced gene silencing (VIGS) and transiently in protoplasts, using clustered regular interspaced short palindromic repeats-CRISPR-associated protein 9 (CRISPR-Cas9) genome editing technologies. Cassava mosaic disease-tolerant cassava landrace TME3 plantlets were inoculated with SACMV DNA A-derived VIGS vector and challenged with SACMV. Relative qPCR results demonstrated that the *MeRPPL1* and *MeTNL*-VIGS-silencing vectors decreased expression of *MeRPPL1* and *MeTNL*, respectively in TME3 compared to GFP-vector only control. This consequently led to increased SACMV DNA A accumulation in *MeRPPL1* and *MeTNL*-silenced plants compared to SACMV infected plants not treated with the silencing vector. CRISPR-silencing vectors and SACMV were used to transfect TME3 protoplasts isolated from leaf mesophyll cells. In contrast to VIGS, CRISPR-Cas silencing vectors with/without SACMV co-infection resulted in increased expression of both *MeRPPL1* and *MeTNL* in TME3 protoplasts compared to the CRISPR-Cas vector only control. Furthermore, mutagenesis studies revealed that CRISPR-Cas vector and SACMV induced mutations randomly in *MeRPPL1*, resulting in total replacement of the highly conserved MHD motif of the *MeRPPL1* translated polypeptide with other amino acids. This would abolish the regulatory role for the MHD motif in the control of R protein activity and could account for the increase in SACMV DNA A accumulation observed in *MeRPPL1*-silenced protoplasts. *MeTNL* could not be sequenced due to primers not binding to the target regions and no conclusions could be drawn as to the role of the resultant mutations from CRISPR-Cas silencing and SACMV transfections. Results have shown the involvement of the two R genes in TME3 tolerance and response to SACMV. The second objective of this study was aimed to identify the leaf proteome involved in anti-viral defence. Liquid chromatography mass spectrometry (LC-MS) identified 2682 (54 differentially expressed) and 2817 (206 differentially expressed) proteins in both landraces at systemic infection (32 dpi) and symptom recovery (67 dpi), respectively. KEGG and STRING analysis identified differences in DEPs

between the two landraces, with metabolic pathways highly enriched at both timepoints, and defence-associated pathways such as the chloroplast, proteasome and ribosome enriched at the recovery stage (67 dpi) in TME3. At 67 dpi, a large number of over-expressed proteins (56%) in TME3 were localized in the chloroplast, whereas 31% of these chloroplast proteins were under-expressed in T200. A chloroplast protein (Manes.15G029400) was over-expressed in TME3 (1.25 ratio) at symptom recovery stage (67 dpi). The expression of the Arabidopsis homolog (AT4G14880) of this TME3 chloroplast protein is mediated by abscisic acid (ABA), which is synthesized in the chloroplast. We propose that chloroplast proteins and ABA-mediated defence mechanisms play a role in the reduction of SACMV virus replication and symptoms in TME3 at 67 dpi. RPT2 has been linked with PAMP triggered immunity (PTI) and effector triggered immunity (ETI). RPT proteins were associated with the reduction in systemic infection in TME3 landrace at 67 dpi. Regulatory particle triple-ATPase (RPT) subunits 2a/b of the 26S Proteasome complex were shown to directly interact with a Proteasome component family (Manes.01G032500) and a Holliday junction DNA helicase RuvB P-loop family (Manes.05G155000) that were under-expressed in TME3. In TME3 at recovery, a PCI (Proteasome, COP9 signalosome, and initiation factor 3) domain-containing protein (Manes.01G032500) was also under-expressed in TME3 compared to over-expression (7-fold ratio) in T200. These findings suggest that under-expression of proteasome associated components may contribute to SACMV tolerance in TME3. Sac52, also known as the ribosomal protein L10 (RPL10), was found to interact with Ribosomal_L7Ae domain-containing protein homolog (Manes.12G139100, AT1G77940), which was over-expressed uniquely in TME3 only at 67 dpi. Since L10 is a known key player in the NIK1-mediated antiviral immunity defence response to geminivirus infection triggering ETI, we propose that Sac52 is associated with the reduction in systemic infection in TME3 landrace. In conclusion, the differences in proteome responses between SACMV challenged susceptible and tolerant cassava landraces and these above-mentioned proteins warrant further investigation.

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Dedication

-TO PAPA & THE RAMULIFHO 6. I DID IT!

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Abbreviations

ABA	Abscisic acid
ACMV	<i>African cassava mosaic virus</i>
ACN	Acetonitrile
amiRNA	Artificial microRNA
Avr	Avirulence
bHLH	Basic-helix-loop-helix
bZIP	Basic domain leucine zipper
BaMV	<i>Bamboo mosaic virus</i>
BSCTV	<i>Beet severe curly top virus</i>
CaLCuV	<i>Cabbage leaf curl virus</i>
CaM	Calmodulin
CAMTAs	Calmodulin-binding transcription activators
CaMV	<i>Cauliflower mosaic virus</i>
Cas	CRISPR associated
CBL	Calcineurin B-like
CC	Coiled coil
CDPKs	Calcium-dependent protein kinases
ChI	Magnesium chelatase subunits I
CLCuKoV	<i>Cotton leaf curl Kokhran virus</i>
CMD	Cassava mosaic disease
CMGs	Cassava mosaic geminiviruses
CMMGV	<i>Cassava mosaic Madagascar virus</i>
CMV	<i>Cucumber mosaic virus</i>
CNL	CC-NBS-LRR
CP	Coat protein
CPSMV	<i>Cowpea severe mosaic virus</i>
CR/IR	Common region/intergenic region
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CSN	COP9 signalosome
CWMV	<i>Chinese wheat mosaic virus</i>
DAMPs	Damage-associated molecular patterns

DEGs	Differential expressed genes
DIGE	Differential in-gel electrophoresis
Dpi	Days post infection
dsDNA	Double stranded deoxyribonucleic acid
EACMV	<i>East African cassava mosaic virus</i>
EACMKV	<i>East African cassava mosaic Kenya virus</i>
EACMMV	<i>East African cassava mosaic Malawi virus</i>
EACMZV	<i>East African cassava mosaic Zanzibar virus</i>
ECS	Endocytosis cell signalling
eIF4E	Eukaryotic initiation factor 4E
eLRR	Extracellular leucine rich repeat
ESI	Electrospray ionisation
ET	Ethylene
ETI	Effector triggered immunity
FA	Formic acid
FSC	Forward scatter
gRNA	Guide RNA
HDR	Homology-directed repair
HR	Hypersensitive response
HSP90	Heat shock protein 90
ICAT	Isotope-coded affinity tag
ICMV	<i>Indian cassava mosaic virus</i>
IITA	International Institute of Tropical Agriculture
IRGs	Immunity-related genes
iTRAQ	Isobaric tagging for relative and absolute quantification
JA	Jasmonic acid
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LEA	Late embryogenesis abundant
LIMYB	L10-interacting MYB domain-containing protein
LRR	Leucine rich repeat
MALDI	Matrix-assisted laser desorption-ionisation
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase

MAPKKK	MAPKK kinase
MKP1	MAPK phosphatase 1
MMTS	Methyl-methane-thiosulphonate
MP	Movement protein
MS	Mass spectrometry
MS/MS	Triple-quadruple tandem mass spectrometry
MYMIV	<i>Mungbean yellow mosaic Indian virus</i>
<i>m/z</i>	Mass/charge ratio
NBS	Nucleotide-binding site
NHEJ	Non-homologous end joining
NLRs	Nucleotide-binding domains and leucine-rich repeats
NLS	Nuclear localisation signal
NRIP1	N RECEPTOR-INTERACTING PROTEIN1
NSP	Nuclear-shuttle protein
ODM	Oligonucleotide-directed mutagenesis
ORFs	Open reading frames
PAMPs	Pathogen-associated molecular patterns
PCI	Proteasome, COP9 signalosome, and initiation factor 3
PCNA	Proliferating cellular nuclear antigen
PD	Plasmodesmata
PDS	Phytoene desaturase
PEST	Pro-Glu-Ser-Thr
PFK	Phosphofructokinase
PGIPs	Polygalacturonase-inhibiting proteins
PGK	Phosphoglycerate kinase
PR	Pathogenesis-related
PRGdb	Pathogen receptor genes database
PRRs	Pathogen recognition receptors
PSbMV	<i>Pea seed-borne mosaic virus</i>
PSI	Photosystem I
PTI	Pathogen triggered immunity
PTGS	Post-transcriptional gene silencing
PVPP	Polyvinylpyrrolidone
PVX	<i>Potato virus X</i>

QTL	Quantitative trait locus
R	Resistance
RAR1	REQUIRED FOR MLA12 RESISTANCE 1
RBOHD	Respiratory burst oxidase homologue D
RCR	Rolling circle replication
REn	Replication-enhancer protein
Rep	Replication associated protein
RGAs	Resistance gene analogs
RGS1	Regulator of G protein signalling 1
RLCKs	Receptor-like cytoplasmic kinases
RLKs	Receptor-like kinases
RLPs	Receptor-like proteins
RNBS-A	Resistance nucleotide-binding site A
ROS	Reactive oxygen species
RPL10	Ribosomal protein L10
RPPL1	RPP13-like protein 1
RPT	Regulatory particle triple-ATPase
RSV	<i>Rice stripe virus</i>
SA	Salicylic acid
SACMV	<i>South African cassava mosaic virus</i>
SAR	Systemic acquired resistance
SCE	SUMO E2 conjugation enzyme
SDS	Sodium dodecyl sulphate
SEGS	Sequences enhancing geminivirus symptoms
sgRNA	Single guide ribonucleic acid
SGT1	Suppresor of the G2 allele of <i>skp1</i>
siRNAs	Short interfering RNAs
SKP1	S-phase kinase-associated protein 1
SLCMV	<i>Sri Lankan cassava mosaic virus</i>
SSC	Side Scatter
ssDNA	Single stranded deoxyribonucleic acid
T7E1	T7 endonuclease 1
TALENs	Transcriptional activator-like effector nucleases
TCEP	Tris (2-carboxyethyl) phosphine

TCV	<i>Turnip crinkle virus</i>
TEAB	Triethylammonium bicarbonate
TF	Transcription factor
TIR	Toll-Interleukin-1-receptor
TISS	Type III secretion system
TLPs	Thaumatococcus-like proteins
TMV	<i>Tobacco mosaic virus</i>
TMS	Tropical <i>Manihot</i> species
TME	Tropical <i>Manihot esculenta</i>
TNA	Total nucleic acid
TNL	TIR-NBS-LRR
TOF	Time of flight
ToCMoV	<i>Tomato chlorotic mottle virus</i>
ToLCV	<i>Tomato leaf curl virus</i>
tracrRNA	Trans-activating crRNA
TrAP	Transcriptional activation protein
TrD	Transmembrane domain
TuMV	<i>Turnip crinkle virus</i>
TRV	<i>Tobacco rattle virus</i>
TTP	Thiamine pyrophosphate enzyme
TYLCSV	<i>Tomato yellow leaf curl Sardinia virus</i>
TYLCV	<i>Tomato yellow leaf curl virus</i>
UPS	Ubiquitin proteasome system
VIGS	Viral induced gene silencing
VSR	Viral suppressors of RNA silencing
WAKs	Wall-associated kinases
WMD3	Web MicroRNA Designer 3
WMV	<i>Watermelon mosaic virus</i>
WRKY	Tryptophan-arginine-lysine-tyrosine
YEP	Yeast extract phosphate
ZFNs	Zinc-finger nucleases
2-DE	Two-dimensional polyacrylamide gel electrophoresis

Outputs from this study

1. Results of Chapter 3 have been published in a peer review journal as follows:

Ramulifho, E.; Rey, M.E.C. Proteome mapping of South African cassava mosaic virus-infected susceptible and tolerant landraces of cassava. *Proteomes* **2021**, *9*, 41.
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2. Elelwani Ramulifho and Chrissie Rey. (2021). Proteome mapping of tolerant TME3 and susceptible T200 cassava landraces infected with *South African cassava mosaic virus*. Oral presentation presented at a SASM 2021 virtual conference, 4 - 6 May 2021.

Chapter 1: Literature review

1.1. Cassava cultivation and economic importance

Cassava (*Manihot esculenta* Crantz) is an exotic perennial shrub and root crop belonging to the *Euphorbiaceae* family. It is native to South America and it is believed to have been introduced to Africa by the Portuguese traders in the 16th century. Cassava can be grown from seeds, however; the standard cultivation method involves the use of stem cuttings for vegetative propagation and establishing new crops. Cassava plants can grow up to 1-4 m in height, while its tubers may stay in the ground for up to 4 years after planting [1]. Due to its ability to withstand and thrive under unfavourable environmental conditions, cassava is considered a plant of last resort for tropical and subtropical rural smallholder farmers. This is mainly because its cultivation does not require expensive or specialised external inputs such as herbicides [2] and it can grow in drought-prone and poor soils areas. As such, cassava has become the major source of both dietary food energy and income for the majority of the people living in the drought-prone areas. In the year 2019, the world cassava production was just over 303 million tonnes, the highest in the past decade. Africa is the biggest contributor with 63.3% cassava production followed by Asia (28%), Americas (8.6%), and lastly, Oceania, producing 0.1% of the world's cassava (Figure 1-1; www.fao.org/faostat/).

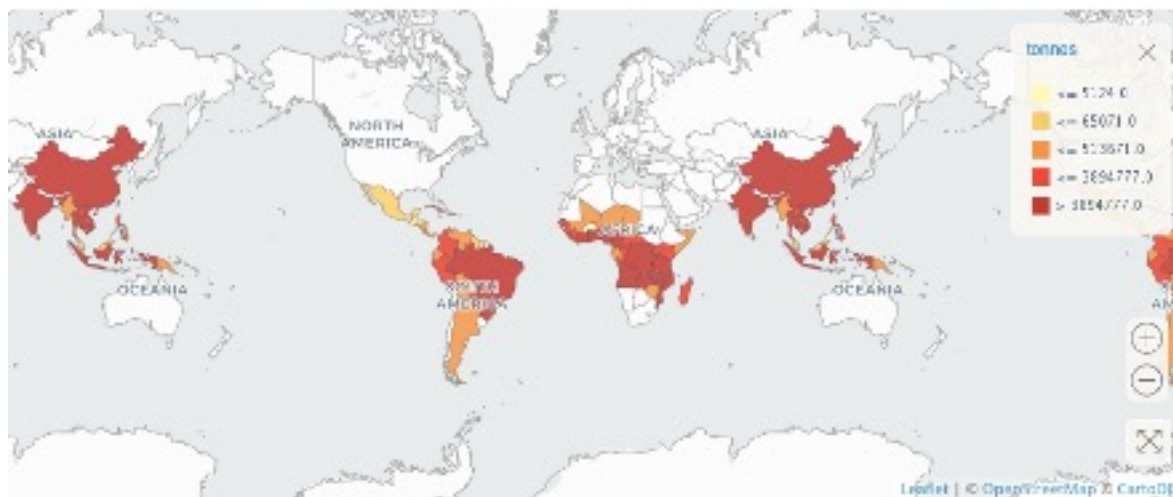


Figure 1-1. The world distribution of production quantities of cassava in the year 2019. Source: (www.fao.org/faostat/).

Cassava is the third biggest source of carbohydrates after rice and maize (www.fao.org) in Africa, Asia, and Latin America, feeding millions of people. Cassava roots and leaves are the two nutritionally valuable parts of the cassava plant, each part constituting about 50% and 6% of the mature cassava

plant, respectively. Cassava roots are high in carbohydrates, whereas its leaves bridge the nutritional gap by being an excellent source of both proteins and vitamins [3]. Different cultivars of cassava produce varying amounts of cyanide, making them toxic to both humans and animals. This brings in the subject of cassava processing, which aids in reducing the amount of cyanide and antinutrients to levels that are acceptable for consumption. However, the downside of processing is that it results in reduced nutritional value [4]. Cassava-derived foods include fresh roots, dried roots, pasty products, granulated products and cassava leaves. Cassava uses has diversified into industry within the last few years, where its uses include starch, animal feed, ethanol, and flour [5]. The increased use of cassava for commercial purposes has in turn resulted in the increase in the production and yield throughout Africa.

1.2. Cassava mosaic disease

The production of cassava worldwide is threatened by a broad range of viruses belonging to several families including *Comoviridae*, *Potyviridae*, *Flexiviridae*, *Caulimoviridae*, *Rhabdoviridae*, and *Geminiviridae* [6,7]. These viruses cause a spectrum of diseases, but the most severe and widespread amongst them is the cassava mosaic disease (CMD). Figure 1-2 shows a representation of CMD characteristic symptoms displayed as yellow and/or pale green chlorotic mosaic of leaves accompanied by distortion, crumbling and overall reduction in leaf size [8]. CMD causes detrimental damage to the root yield and production by significantly reducing the number and size of tubers. However, the severity of the damage that CMD may cause is determined by the strain of the virus, cassava variety and the age of the plant, sensitivity of the host, and environmental factors such as soil fertility [9]. In susceptible cultivars, CMD can result in yield losses of up to 82% [10]. Since its first report in the late 19th century, CMD has resulted in major cassava production and economic losses to the value of US\$1300-2300 million in Africa [11]. In Africa, the first CMD epidemic was recorded in Uganda in the late 1980s [12] and this resulted in cassava production losses of about US\$60 million per year in the duration of this epidemic [13]. At one-point, farmers got frustrated and temporarily abandoned cassava cultivation as a result of CMD [14], and to date, CMD remains a major constraint to cassava production worldwide and a threat to food security [1,15].



Figure 1-2. Cassava mosaic disease characteristic symptoms on cassava leaves. Yellow/pale green chlorotic mosaic, distortion and size reduction.

1.2.1. Control measures for CMD

As far as the control of CMD is concerned, there is no one size fits all strategy but rather a series of different approaches that can be applied in conjunction, in order to increase the success of controlling the disease. These include phytosanitation, the use of disease-resistant varieties, cultural practices, and vector control. Looking at Africa and the fact that cassava is mostly produced by smallholders' farmers with limited resources, for the above-mentioned approaches to be successfully implemented, it is advisable that they be simple, affordable, sustainable, and also limit the use of pesticides for health concerns. Phytosanitation aims to control the spread of the disease by insuring that healthy disease-free stem cuttings are used for planting possibly in a location that is not prone to the introduction of the inoculum either via short-distance or long-distance travel by the insect vector, the removal of any infected cassava that could serve as the source of inoculum for new infections, and lastly, roguing [1].

The use of virus-resistant cassava varieties is highly encouraged to delay and prevent infection. This approach has long been used around Africa, where cassava growers were required to replace cassava varieties that were susceptible to the CMD virus with varieties that showed some level of resistance to the virus. The advantages of using the latter include delaying and reducing the infection, and subsequently, reducing yield losses greatly [16]. Some of the resistant varieties that gave higher yields were bred at the International Institute of Tropical Agriculture (IITA) in Nigeria and these include the polygenic tropical Manihot series (TMS) associated with *CMD1* gene derived from *M. glaziovii* [17] and the tropical *M. esculenta* (TME) associated with the *CMD2* dominant locus [18].

Cultural practices aim to control infection by looking at factors that can greatly influence the vector populations and how they can spread the virus. These factors mostly require proper planning before there can be any planting, although this might prove difficult for smallholders' farmers, who can only grow cassava in small plots in their backyards. This is especially true when it comes to field size and shape, crop disposition, and crop spacing, where it is advisable to plant in large areas that are possibly in remote location far from any possible source of infection. In addition, the latter also looks at crop spacing and even encourages that different crops, preferably taller than cassava be planted on the edges of the field to divert and obstruct incoming whitefly vector that might be carrying the virus [16]. Cultural practices also take into account planting date to avoid exposing vulnerable plants when the whitefly vector, *Bemisia tabaci* is abundant, intercropping to reduce the spread of CMD and the fertility of the soil, which will affect how the plant response to the virus.

CMD can also be controlled by controlling its vector from transmitting the virus and directly feeding on cassava through pesticides, biological control, and/or by using vector-resistant cassava varieties. The use of pesticides is discouraged because of the health hazards to humans, environment and other dwellers in nature, and for this reason, they are hardly used for controlling CMD. Other reasons for their limited use are that their effectiveness is not long-lasting since whitefly develop resistance to them over time [19] and the fact that they are not affordable to most smallholders' farmers. Biological control involves introducing natural enemies of the whitefly vector to control it. Although this method has been used successfully in conjunction with others [20], it cannot be deemed as a long-term control method due to the antagonistic relationship other methods such as using pesticides and resistance varieties might have with the introduced natural enemies [21]. There is an ongoing search for cassava varieties that will be resistant to both the virus and its whitefly vector, however this has proven difficult with varieties found to be resistant to the whitefly vector being found to be susceptible to CMD [16,22].

1.3. Cassava mosaic geminiviruses (CMGs)

1.3.1. The causal agent of cassava mosaic disease

Molecular typing and sequencing have revealed that CMD is caused by seven related species of cassava mosaic begomoviruses (CMVs; family *Geminiiviridae*), occurring in single or mixed infections: African cassava mosaic virus (ACMV), Cassava mosaic Madagascar virus (CMMGV), East African cassava mosaic Malawi virus (EACMMV), East African cassava mosaic Kenya virus (EACMKV), East African cassava mosaic virus (EACMV), East African cassava mosaic Zanzibar virus (EACMZV), and South African cassava mosaic virus (SACMV) [23,24].

These viruses have resulted in major economic losses in the production of cassava in different regions of Africa. Their symptoms can range from mild to severe, depending on the strain of the virus and on whether the plant is infected by a single virus or there is a concurrent infection of two or more of these CMGs. A case has been reported, where there are two CMGs infecting one cassava plant that resulted in synergistic interaction between ACMV and EACMV [25,26]. CMGs are primarily transmitted across regions via the movement of cuttings from an infected cassava plant, and once planted, the virus replicates in the newly developed plants parts, which consequently become the host. Transmission of CMGs within and between fields is aided by the whitefly *Bemisia tabaci*, a phloem-feeder and the sole vector of CMGs [27]. It is recorded in the literature that there is a long-term association of up to 9 days between CMGs and its vector [1]. This consequently means that the virus can be carried over long distances of up to 35 km in a year [28].

1.3.2. Genome structure

Cassava mosaic geminiviruses are bipartite single-stranded DNA (ssDNA) genome viruses, enclosing two circular ssDNA molecules designated as DNA-A and DNA-B (Figure 1-3). Both the DNA components are required for systemic infection, even though DNA-A alone can induce CMD symptoms [29]. DNA-A and DNA-B components are each enclosed within a twin geometry particle formed by two incomplete icosahedral capsids [30]. In addition, they vary in sizes ranging between 2.7 - 2.8 kb for each molecule, depending on virus species. Both these DNA components share a common conserved noncoding region known as the common region/intergenic region (CR/IR) of about 200 nucleotides, and this contains a hairpin structure comprising the key sequence (TATAATT|AC) for initiating rolling cycle replication [31]. DNA-A is necessary for transcription and replication of the virus, while DNA-B is crucial for cell-to-cell and long-distance movement [32]. DNA-A has six open reading frames (ORFs); AC1, AC2, AC3, AC4, AV1, and AV2, encoding the replication initiator protein (Rep), the transcriptional activator protein (TrAP), the replication enhancer protein (REn), the RNA-silencing suppressor, the coat protein (CP), and the pre-coat protein, respectively. DNA-B has two ORFs; BV1 encoding the nuclear-shuttle protein (NSP) and BC1 encoding the movement protein (MP) [7,33]. These encoded proteins not only function as outlined above, but also influence defense responses, hormonal responses and cell cycle regulation, ubiquitin-proteasomal pathways, and protein-signalling cascades [34].

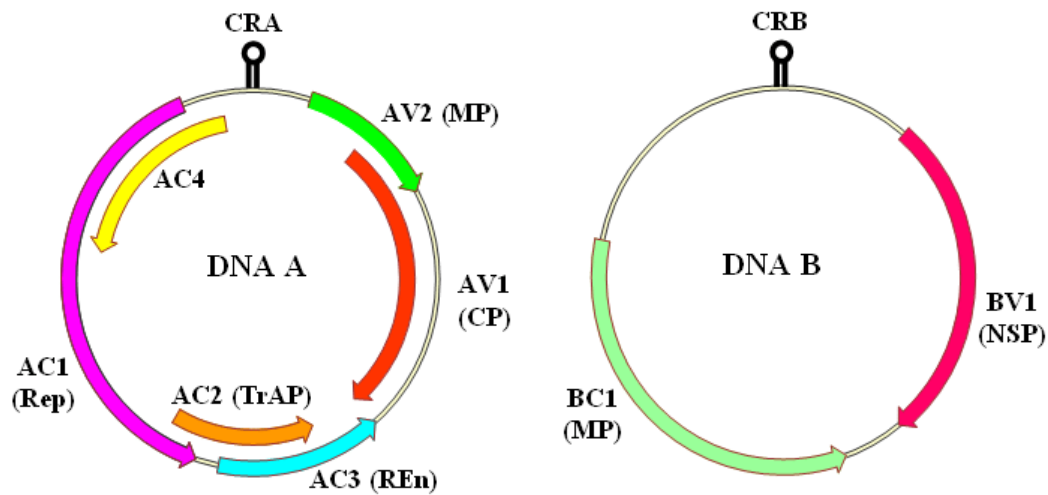


Figure 1-3. Bipartite genome organisation of cassava infecting geminiviruses. Source: Alabi et al. 2011 [7].

1.3.3. Virus-host interactions and replication

In general, plant viruses possess very limited coding capacity, which makes them susceptible to defective replication. As such, these viruses heavily depend on the host machinery for their survival, replication and spread [35]. The genome replication of CMGs occurs through double-stranded DNA (dsDNA) intermediates via rolling circle replication (RCR), occurring in the host plant nucleus. Viral infection is initiated when a whitefly carrying CMGs feeds on the sap transported through the phloem of a healthy leaf and transmits virions to phloem-associated cells. Thereafter, viral ssDNA is released from the capsid in the plant cell and transported to the nucleus. Here, the DNA can either bind to movement related proteins (BC1 and host chaperones) for transportation to nearby uninfected cells via the plasmodesmata, be encapsulated into infectious virions for long-distance virus transmission, or converted into dsDNA (with the help of cellular host factors), which then serves as a template for transcription or replication [32]. The transcripts are exported to the cytoplasm for translation into viral proteins. Subsequently, proteins needed for carrying out replication, transcription of movement of viral DNA enter the host nucleus via nuclear pores, allowing other cycles of replications to occur [36]. Viral protein Rep, in association with REn protein and host factors, recognise a conserved sequence within the CR/IR (Section 1.3.2) within the replicative dsDNA, introduces a nick and give rise to the rolling circle replication fork. The replication fork is then elongated until replication is terminated and the genome-long viral ssDNA is released and immediately circularized by Rep [36]. Rep protein functions in transcriptional activation and repression of viral genes [37], interacting with host factors [38], [39] and ATP/GTPase activity [40], all of which results in successful replication of the viral genome. REn protein stimulates DNA replication by interacting with Rep via high order oligomerization to enhance the ATPase activity of Rep protein [41].

1.4. South African cassava mosaic virus (SACMV)

South African cassava mosaic virus is one of the CMGs, originally identified in the eastern part of South Africa as an isolate of ACMV [42], however, it was later characterized as an individual species due to the differences in CP and common region (CR) nucleotide sequences which revealed differences to those of other known CMGs [42,43]. Although it is a bipartite virus, Berrie et al. 1997; 1998 [42], [43] showed SACMV clustering with a monopartite Tomato yellow leaf curl virus (TYLCV), but still showing distinct characters for it to be identified as a distinct species. SACMV DNA-A and DNA-B components are 2800 and 2760 bp, respectively. Complete nucleotide sequence studies revealed SACMV to be most closely related to EACMV, with its DNA-A component having 85% similarity to that of EACMMV (MH and MK) isolates, and its DNA-B having 90% similarity to the DNA-B of EACMV Uganda 2 mild (Mld) and severe (Svr) isolates [44]. Outside the South African border, SACMV has been recorded in Swaziland, Madagascar and Zimbabwe [45].

1.5. Disease resistance mechanisms in plants

Plants are sessile, as such they need to defend themselves against attack from pathogens for their survival. To do that, plants have developed a complex yet effective mechanisms to perceive and recognize pathogens molecules, carry out signal transduction, and respond to these attacks by activating a chain of reactions involving a number of genes that will bring about a defense response [46]. Pathogens however, actively attempt to elude and interfere with the defense response mechanisms so that they can continue with their infection cycle. Stages of plant disease resistance mechanisms and their components are discussed here.

1.5.1. Pathogen detection

The most critical step in plant defense is the detection of foreign pathogen molecules. Host plants use pattern recognition receptors (PRRs) localised within the cell membrane to detect pathogen-associated molecular patterns (PAMPs) [47]; wall-associated kinases (WAKs), transmembrane proteins with a cytoplasmic Ser/Thr kinase domain and an extracellular domain to detect damage-associated molecular patterns (DAMPs) [48]; and nucleotide-binding domains and leucine-rich repeats (NLRs) receptors for the detection of pathogen effectors [49].

Plants PRRs are known to be surface localised and can either be receptor-like kinases (RLKs) or receptor-like proteins (RLPs). The cytoplasmic kinase domain found in RLKs plays a role in signal transduction which is brought about through phosphorylation, while RLPs have leucine-rich repeats, involved in plasma membrane localisation. These receptors contain different domains, which enables

them to bind to bacterial proteins such as flagellin and EF-Tu [50]; carbohydrate-containing molecules including fungal chitin, bacterial peptidoglycans, extracellular ATP and plant-cell-wall-derived oligogalacturonides [51–53]; and viral nucleic acids [54]. In *Arabidopsis* receptor kinase FLS2 perceives and binds to flg22, inducing immune responses by interacting with BAK1, also known as SERK3 [50]. Three PRRs in wheat (TaLRK10, TaRLP1.1 and TaRLK-R1-1) were linked to resistance to fungal wheat rust [55,56].

In viruses, it is believed that viral dsRNA are PAMPs, which in turn activate Dicers as PRRs [57,58]. These PRRs then are perceived by transmembrane co-receptors SERK1, SERKs and BAK1, which then induce PAMP-Triggered Immunity (PTI)-based antiviral responses [59]. The evidence comes from studies involving plant viruses from different genus; turnip crinkle virus (TuMV) and TMV [60], *Soybean mosaic virus* [61], *Alfalfa mosaic virus* [62], cucumber mosaic virus (CMV) [63] and *Cauliflower mosaic virus* [64]. Begomoviruses DNA, on the other hand, is perceived by co-receptor NIK1, which in turns activates NIK1-mediated signalling and antiviral PTI in the form of translation suppression [65,66]. Unlike PRRs, WAK1 receptors perceive a change in cellular components resulting from damage caused by pathogenic enzymes as was shown in *Arabidopsis* [53,67] that WAKs have two domains that enable them to trigger defense signalling to fight off pathogens. One domain (extracellular galacturonan) is on the N-terminal of WAKs and it interacts with cell wall pectin, while the other domain is a cytoplasmic kinase with structure similar to that of PRRs [46].

To counteract PTI, pathogens have evolved a mechanism in which they obstruct the plasma membrane recognition process by delivering effector proteins into plant cell cytosol to suppress PTI response and interfere with host defence signalling [68]. Subsequently, plants defend themselves from the latter by targeting effector proteins delivered into the plant's cytosol with R proteins, thereby inducing Effector-Triggered Immune (ETI), also known as race-specific or gene-for-gene resistance [69]. NLRs are cytoplasmic localised R genes, made up of at least an evolutionary conserved nucleotide-binding site present in apoptotic protease-activating factor, R proteins, and *Caenorhabditis elegans* death-4 protein (NB-ARC) and a highly variable leucine-rich repeat (LRR) at the C-terminus [46]. Proteins containing these two functional domains play pivotal roles in perceiving and binding to effectors, leading to signalling pathway activation and subsequently ETI. In addition to NBS and LRR domains, R genes can have extra domains such as the prominent Toll/interleukin-1 receptor-like (TIR) or a putative coiled-coil (CC) domain on their N-terminus, while other R genes may lack NBS and/or LRR but have extra domains (Figure 1-4) [70]. CC-NBS-LRR (CNL) genes have been found in both monocots and dicots, whereas TIR-NBS-LRR (TNL) genes are only found in dicots [71]. The LRR motif is highly

variable and as such, gives R protein receptors an advantage to recognize effectors with different structures. The NB-ARC domain is made up of different motifs with conserved amino acids present for that respective motif. These motifs are named P-loop/Walker-A, resistance nucleotide-binding site A (RNBS-A), kinase-2/Walker-B, RNBS-C, GLPL, RNBS-D and MHDV, and are not all required for R gene to be fully functional [72], also noting that NBS-LRR genes mostly function in networks consisting of numerous genes [73,74].

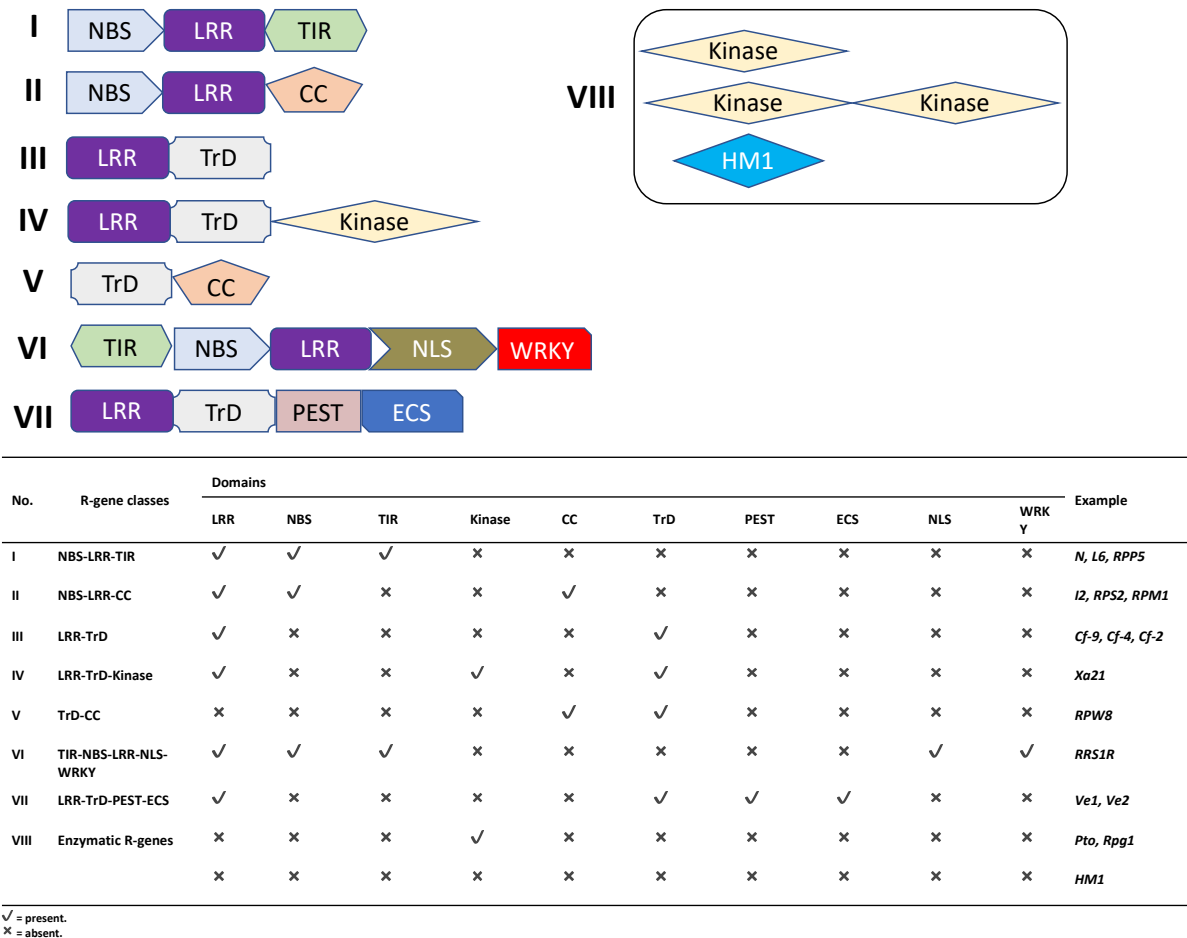


Figure 1-4. Major classes of R genes. LRR, Leucine-rich repeats; NBS, Nucleotide-binding site; TIR, Toll/Interleukin-1- receptors; C-C, Coiled coil; TrD, Transmembrane domain; PEST, Protein degradation domain (proline-glycine-serine-threonine); ECS, Endocytosis cell signaling domain; NLS, Nuclear localization signal; WRKY, Amino acid domain; HM1, *Helminthosporium carbonum* toxin reductase enzyme. Adapted from Gururani et al. 2012 [70].

The most characterised effectors to date are from bacterial pathogens, as opposed to fungal and viral effectors. Since bacteria have evolved a Type III secretion system, it became possible for them to directly deliver effector proteins into plant cells, and thus enabling them to suppress the PTI response [75]. When plants are infected with viruses, the primary response is RNA silencing [76], which limits

viral replication and systemic movement. However, viruses have evolved a way to suppress this response, and for this reason, there are virus-specific R proteins belonging to several structural classes [70] that targets virion components or virus-encoded proteins such as MP, Rep and CP [77]. SACMV virus infection of cassava has resulted in the alterations in R gene expression [78], which could indicate that SACMV possibly has avirulence (*Avr*) proteins targeted by these R proteins. Generally, effectors have a catalytic activity which results in alteration of the host plant to increase pathogen virulence and growth inside the host [68].

Viruses were not thought in the past to have effector proteins and as such, R proteins-triggered antiviral immune responses were not classified as ETI response. However, this is until more recently that progress in identifying R genes and their corresponding viral *Avr* genes has been achieved [70]. The majority of these characterised viral R genes from plants are from the NBS-LRR class, which suggests monogenic dominant resistance, however, recessive resistance genes have also been identified in viral systems [79]. Dominant resistance is often associated with hypersensitive response (HR), but this is not the case for some plants, including cassava. HR-mediated resistance is common for viruses and other pathogens [80]. Several plant R genes associated with dominant resistance to plant-infecting viruses has been cloned [70]. However, there may be a case where some R gene-encoded proteins have highly similar amino acid sequences, but confer resistance to different viruses. For example, *Arabidopsis RCY1* and *HRT* R genes encode proteins that are 91% similar, but confer resistance to CMV and TuMV, respectively [81].

Examples of dominant resistance R gene-encoded proteins include tobacco N protein, which recognises Tobacco mosaic virus (TMV) replicase p50 with the help of N RECEPTOR-INTERACTING PROTEIN1 (NRIP1) protein. The presence of p50 results in the translocation of NRIP1 from the chloroplast to the cytosol and nucleus where it interacts with the N-terminal TIR domain of N protein and enables it to recognise p50 and triggers resistance [82]. A similar interaction is observed in potato where proteins such as Rx and Rx2 interact with RanGAP2 via the CC domain of Rx and related proteins to recognise the Potato virus X (PVX) coat protein and confer resistance against PVX [83]. RanGAP2 enables the translocation of Rx protein from the nucleus to the cytosol [84]. In cassava, several potential R genes containing NBS-LRR domain have been identified and some of these genes may be linked to the monogenic dominant resistance response that may be the reason behind cassava TME3 landrace tolerance phenotype when infected with begomovirus SACMV [85]. These genes, however, need further characterisation to ascertain their roles in SACMV resistance. Other dominant resistance proteins such as tomato *Tm-1*, *Tm-2* and *Tm-22*, citrus *Ctv-R* and common bean *RT4-4* R genes are

discussed by Gururani et al. 2012 [70].

There have been several recessive R genes that have been characterised and cloned in different plant hosts. Recessive resistance occurs as a result of the loss of function in host proteins crucial for the establishment of disease [70,86] and seems to function at the single-cell level or affect cell-to-cell movement [79]. For examples, protein amino acid mutations in translation initiation factors, eukaryotic initiation factor 4E (eIF4E), and eIF4G, lead to resistance to potyviruses. The *eIF4E* gene plays a crucial role in conferring resistance to a large number of *Potyviridae* family viruses infecting pepper [87], lettuce [88], tomato [89], and barley [90]. On the other hand, *eIF4G* gene confers resistance to *Yellow mottle virus* in rice [91], and CMV and TMV in *Arabidopsis* [92]. These mutations in eIF result in reduced efficiency of viral protein translation and subsequently, viral movement and replication [70].

In addition to R genes, there are several other host proteins such as lectins which are important for pathogen perception [77]. Examples of these lectins include soybean β -glucan binding protein, which binds with high affinity to a PAMP (1,3-branched heptaglucoside) found in the cell walls of *Phytophthora sojae* [93], resulting in a defence response triggered. In viruses, Jacalin-type lectin, JAX1, was demonstrated to be involved in conferring resistance against *White clover mosaic virus*, *Asparagus virus 3*, PVX, and *Plantago asiatica mosaic virus* [94]. Apart from these proteins, immunity-related genes (IRGs) also play an important role in plant disease resistance. Genomic studies have led to the identification and mapping of IRGs in *Arabidopsis* and cassava [95,96]. Some of *Arabidopsis* IRGs obtained from NCBI are listed on a study by Leal et al. [95], and these include *RPS2* and *RPM1* which confer resistance to *P. syringae* strains carrying *avrRpt2*, and *avrB* and *avrRpm1* genes, respectively. Physical mapping of IRGs into a cassava genetic map has been developed based on the presence of conserved domains [96].

Plant-pathogen resistance requires combinatorial interactions among multiple host and pathogen factors such as R genes, *Avr* genes, IRGs, and other genes encoding proteins that are involved in plant-pathogen resistance. As a result, all these genes possessing varying biological functions, molecular processes, and cellular components, do not act individually, but rather as networks in different KEGG pathways to bring about plant disease resistance [95,97]. Gene co-expression or protein-protein networks enable gene functional studies. Today, many plants have their genome sequenced and assembled [98,99], and genomic and protein data can be used to gain an understanding of different

molecular mechanisms resulting in different traits such as resistance/tolerance by studying gene networks [97].

1.5.2. Signal transduction

Resistance involves multiple signalling mechanisms such as mitogen-activated protein kinase (MAPK), G-proteins, ubiquitin, and calcium influx activated by different receptors. MAPK activation plays important role in the establishment of disease resistance [100]. When PRRs are perceived, two MAPK cascades are activated promptly through a cascade of phosphorylation reactions to further activate immunity response. One cascade involves MAPK kinase kinase (MAPKKK) MEKK1, MAPK kinases (MAPKKs) MKK1 and MKK2 (MKK1/2), and MAPK MPK4 [101]. The second cascade starts with a phosphorylated MAPKKK3/5, which then phosphorylates MKK4/5, which further phosphorylates and activates MPK3/6 [102]. In *Arabidopsis* receptor-like cytoplasmic kinases (RLCKs), RLCK VII-4, and RLCK VII-8 act downstream of flg22/elf18, chitin, and Peps PRR, respectively to facilitate the activation of the two MAPKs cascades which then confer disease resistance [103,104]. One study proved that there are additional MAPKKKs that are involved in regulating the activation of MAPK3/6 cascade by silencing both *mapkkk3* and *mapkkk5* and still observing some level of MAPK3/6 activation in *Arabidopsis* [105]. Similarly, MAPK phosphatase 1 (MKP1) was found to have a positive regulatory role in MAPK cascades, which led to reduced replication of the bamboo mosaic virus (BaMV) in *N. benthamiana* [106].

In plants, heterotrimeric G-proteins are molecular switches that are activated by receptor kinases following activation by different PRRs. Regulator of G protein signalling 1 (RGS1) keeps the alpha subunit of the G protein inactive by keeping it in a GDP-bound form by enhancing GTP hydrolysis and by also interacting with PRR complexes [100]. After an effector is perceived, RGS1 is phosphorylated by activated RLCKs and this in turn frees RGS1 from the complexes, leaving G proteins free to auto-activate and further activate immune signalling [107]. Ubiquitin proteasome system (UPS) also plays a key role in defense signalling. This system works by targeting proteins for degradation through ubiquitination and thus repressing pathogen infection [108]. The UPS involves three enzymes; E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligase), which act sequentially to bring about protein degradation. In *Arabidopsis*, a RING-finger E3 ligase related to Kip1 ubiquitylation-promoting complex1 (RKP) was induced by geminivirus C4 protein. RKP plays a role in the degradation of cyclin-dependent kinase inhibitor Kip-Related Protein1 that functions in the G1-S transition of the cell cycle, meaning that the observed C4-induced abnormal cell division in *Arabidopsis* may be due to the expression of RKP and that this environment may instead favour viral replication [109]. In a recent study, CRISPR knockdown of a E3L in cassava protoplasts resulted in an increase in SACMV replication,

suggesting that E3L targeted proteins may play a role in tolerance in TME3 [110]. Ubiquitination of geminiviral proteins can contribute to plant defence and geminivirus often subvert or hijack ubiquitin-proteasomal pathways [34]. Further *in planta* studies on the role of the UPS in cassava in response to SACMV would prove invaluable.

A rapid and transient calcium ions (Ca^{2+}) influx is crucial for in a number of immune responses in the cell including reactive oxygen species (ROS) production and stomal immunity, amongst other cellular responses [111]. Ca^{2+} influx is activated subsequent to the activation of PRRs and the influx returns to normal concentrations within minutes in an activated PTI [112], while it can last for hours in an ETI-activated response [113]. Plants have three groups of Ca^{2+} sensors, such as calcineurin B-like (CBL), calmodulin (CaM) and calcium-dependent protein kinases (CDPKs), that leads to the activation of a range of transcription factors, such as calmodulin-binding transcription activators (CAMTAs) [114,115]. CaM modifies its interaction with CaM-binding proteins, some of which have been reported to function in salicylic acid (SA) homeostasis regulation [116], which is involved in ROS production through MAPK cascade initiation [117]. CDPKs play both a positive and negative regulatory role in plant immune responses. For example, overexpression of rice calcium-dependent protein kinase CPK4 led to production of ROS, callose deposition, SA and defence genes expression, which improved disease resistance against rice blast disease [118]. An example where CDPK plays a negative regulatory role is in rice, where an overexpression of CPK12 resulted in suppressed ROS production, which led to increased susceptibility to blast fungus [119].

Plant hormones systematically initiate resistance to pathogens. SA, jasmonic acid (JA), and ethylene (ET) plant hormones are key players in signal transduction pathways and plant immune responses regulation. Other hormones such as auxins, abscisic acid, cytokinin, gibberellins, and brassinosteroids have been long known to mainly function in plant growth and development, but their roles in regulation of plant immunity have been reported, although not fully understood yet. These hormones mostly operate in networks amongst themselves and also with other genes downstream after the pathogen has been perceived. For example, SA can trigger the activation of MAPK cascades [120] and also transfers signal to transcription factors upon pathogen detection. In addition, SA can also regulate systemic acquired resistance (SAR) induced resistance. Plants overexpressing SA have been shown to have protection against geminiviruses due to reduced symptoms [121]. The latter was true also in *Arabidopsis*, which showed enhanced resistance to pathogenic bacteria, following overexpression of SA [122]. JA and ET are known to function in plant response against necrotrophes and herbivorous

insects by emitting volatile compounds to deter insects [123,124]. JA is also known for its role in geminivirus infection where it reduces virus transmissibility by their whitefly vector [125].

Transcription factors (TFs) play a key role in transcription reprogramming, which is required at different levels of resistance from the expression of immunity mechanism components such as receptors and kinases to the downstream activation of immune responses [126]. TFs function by binding to genes at specific target sites and either positively or negatively regulate the expression of the gene. APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF), NAC, basic-helix-loop-helix (bHLH), basic domain leucine zipper (bZIP) and the tryptophan-arginine-lysine-tyrosine (WRKY) are families of TFs that play prominent regulatory roles in plant immunity responses [127]. Members of the AP2/ERF family such as ERF1, ORA59 and ERF6 are involved in regulating the transcriptome. In *Arabidopsis*, the over expression of *ERF96* induced resistance to *Botrytis cinerea* and *Pectobacterium carotovorum* [128]. Similar results were also observed in rice [129] and *Arabidopsis* [130], where *ERF6* activated plant defensin 1.1 and 1.2 post MAPK cascade activation. Overexpression of *Arabidopsis* TF ANAC032 improved resistance to *Pseudomonas syringae* DC3000, while silencing of the same TF resulted in susceptibility [131].

The bHLH family has few members known to be essential in plant immunity, however, one key TF in this family is AtMYC2/JAI1/JIN1, which functions in close relation with AtMYC3 and AtMYC4 in regulating JA-mediated defense response and in facilitating the crosstalk with other hormones [132]. TFs belonging to the bZIP family and play a role mostly in SA-mediated resistance, while some are more active in the activation of SAR [133]. WRKY TFs family has more than 70 and 100 members in *Arabidopsis* and rice, respectively. Their roles are not limited to the critical role they play in plant immunity response by regulating hormone signalling pathways, but are also involved in plant growth and development, senescence, and metabolism [134]. Extensive research has been done on this TF family to understand its functional roles in plant resistance and susceptibility in response to different stimuli including pathogens. WRKY TFs involved in plant response against abiotic stresses have been identified [135,136]. In biotic stress, WRKY TFs have also been identified in response to bacteria [137], fungi [138], nematodes and aphids [139,140], oomycetes [97], and viruses [141].

1.5.3. Defense response

Downstream of transcriptional regulations, is a complex network of defense mechanisms that are activated to fight off pathogens. It is also possible for some of these defense mechanisms such as some toxic secondary metabolites, to be stored and get ready as soon as pathogens are detected. But

the greater part of defense response is only induced and activated following pathogen detection. Defense responses that are activated include; HR, ROS, and cell wall modifications which mainly function to inhibit pathogen infection; enzymes and enzyme inhibitors to counteract the effect of effectors and facilitate detection; defensins and thaumatin-like proteins to offensively inhibit pathogen infection; and phytoalexins and symbionts as chemical and biological plant weapons [46].

Hypersensitive response is a type of defense mechanism found in most higher plants, characterised by the rapid death of cells at a point of pathogen infection and surroundings to prevent further spread of the infection [80]. HR response has been described to be a result of gene-for-gene relationship between the plant host and the pathogen. This is loosely explained by the relationship between a dominant avirulence (*Avr*) gene and the host's corresponding *R* gene in which their encoded protein interaction brings resistance in the form of HR. *Avr* proteins are effector proteins that are secreted into the host cell and perceived by specific *R* proteins and through tight regulation, result in the activation of ETI [142]. The expression and accumulation of NLR proteins are crucial in the control of HR and proteins such as SGT1, RAR1 and their molecular chaperone HSP90 that are important as they regulate the accumulation of *R* proteins [143]. HR has been reported in different hosts [144,145] and although it does not occur in some hosts, resistance was still conferred [146,147].

Pathogen infection induces the production of peroxidases in order to generate ROS. Depending on concentration levels and where they are produced, ROS can cause irreversible DNA damage and cell death but also play a crucial role in regulating cell oxidative homeostasis for normal plant growth and defense responses [148]. ROS production is mainly in the apoplast, but can also be produced in other cellular compartments such as the chloroplast, peroxisomes and mitochondria. A plasma membrane-localised NADPH oxidase respiratory burst oxidase homologue D (RBOHD) is important for ROS production as its interaction with PRRs EFR and FLS2 leads to its activation by multiple kinases and subsequent ROS production [149]. RBOHD activity is further regulated by binding to calcium and phosphatidic acid, and interaction with G proteins [150,151]. ROS helps in facilitating HR response and also create an environment that is not conducive for a pathogen to thrive under [152]. ROS production has led to increased pathogen resistance [153–155]. ROS also play a role in preventing some pathogens from penetrating the cell wall and thus depriving them of nutrients required for reproduction and also denying them entry into the cell. This occurs because ROS mediate glycoprotein crosslinking which strengthens cell walls [156,157].

Some plant pathogens such as fungi, viruses, oomycetes and nematodes, need to breach the host plant's cell wall barrier in order for them to gain entry into the plant cells and initiate infection. The latter is attained through the use of specific enzymes such as cellulases to degrade the cell wall of the host in a controlled manner that will keep the host cells alive and the pathogens still obtaining nutrients beneficial for their growth [158]. Other pathogens such *Fusarium culmorum* also secrete pectinases and xylanases to degrade plant carbohydrates as a way to get entry into the cells [159]. Plants have however evolved ways in which they retaliate against these pathogens. Plants produce enzyme inhibitors upon pathogen detection, to suppress cellulases and also release callose and lignin to reinforce its cell wall. In addition, plants also counteract by degrading fungal carbohydrates with chitinases and β -1-3-glucanases, leading to reduced pathogen growth and also the detection of PAMPs by plant's PRRs and subsequent PTI activation [160,161]. Similar to any other defense mechanism, pathogens are always evolving ways to escape the plant's retaliation and this is an ongoing battle in plant-pathogen interactions. Fungal spore germination was inhibited in wheat as a result of chitinases that degraded components of fungal cell walls [162,163]. Interestingly, wheat chitinases have been shown to function further than in just wheat pathogenic fungi [164]. Different classes of chitinases have been induced in different plant hosts as defense response against a number of pathogens [165]. Lipids also play a role by forming a barrier to keep pathogens from entering plant host cells and initiating infection, as seen in *Arabidopsis* and barley [166,167].

Plant defensins play an important role in plant defense response by directly attaching and inhibiting pathogens. Other functions include regulatory role in signal transduction pathways, wound healing, controlled proliferation and chemotaxis [168]. Defensins act by interfering with the synthesis of pathogen proteins and enzyme function, thus limiting pathogen replication and infection. This was first observed in barley, where two defensins (γ -hordothionin and ω -hordothionin) inhibited protein translation [169,170]. Defensins suppress proteases which reduces the efficacy of catalytic proteins [171], triggers ROS production [172], and blocks calcium ion signalling [173]. Plant defensins are antifungal and many have been isolated from different plants such as pea, chili, alfalfa, *Medicago sativa*, and also human [174]. These defensins were overexpressed in transgenic plants to enhance resistance against fungal pathogens. For example, human β -defensin 2 (hBD-2) was expressed in *Arabidopsis* which then showed resistance to *Botrytis cinerea* [175]. MsDef1, caDef1, DEF2 and wasabi defensins were separately overexpressed in tomato and conferred resistance against *Fusarium* species and *B. cinerea* [176,177]. Other overexpressed defensins include BrD1 [178], WjAMP-1 [179], NaD1 [180], to mention but a few.

Thaumatin-like proteins (TLPs) are classified as a pathogenesis-related-5 (PR-5) protein family; a family of highly complex proteins that contribute to plant development and defense mechanisms [181]. TLPs have predicted functions under drought and osmotic stresses but are mostly associated with responses to biotic stimuli, specialised in SAR [182]. The expression of PR-1, PR-2 and PR-5 induced SA-dependent SAR in *Arabidopsis* roots and leaves following infection with beet-cyst nematode. Similar results were observed when *Arabidopsis* was infected with root-knot nematode, but only in roots [182]. TLPs have also been reported to induce antifungal activity in different plant hosts [183]. This was for example seen in tobacco and potato plants, where overexpression of *Pyrus pyrifolia* TLP and *Camellia sinensis* TLPs, respectively resulted in enhanced fungal resistance [184,185]. Evidence of TLPs functioning in viral defense is very limited but there have been studies that noted interaction of TLPs with β -1,3-glucanase, known to function in viral immunity. But there has been inconsistency on how this interaction works since in one instance the mutants that were deficient of β -1,3-glucanase were observed to have reduced viral susceptibility [186], while in another instance, the expression of β -1,3-glucanase enhanced increased TMV infection [187]. Another study reported the interaction of NtTLP1 with CMV coat and movement proteins and proposed that NtTLP1 may affect entry of the virus into cells, possibly delaying or inhibiting infection [188].

Phytoalexins are low molecular weight secondary compounds that are produced by plants as a defense mechanisms to fight off invading pests and pathogens, especially necrotrophs. The discovery of these compounds dates back to just over 80 years ago as they were first recorded in potato tubers where infection by *Phytophthora infestans* was greatly inhibited as a result [189]. There are different types of phytoalexins and these have been identified in plants from different families [190]. The regulation of phytoalexin production includes many components such as phytohormones, signal transcription, defense-related genes and phosphorylation cascades [191,192]. Camalexin is one of the most studied phytoalexins produced in *Arabidopsis* [192,193]. It has been shown to be regulated by MAPK cascades and WRKY TFs [191,194]. *B. cinerea* has however overcome the effect of camalexin [195]. Phytoalexin 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) was reported to deter aphids from feeding from yellow dwarf virus infected barley [196]. When *isoflavonoid-7-O-methyltransferase*, involved in the phytoalexin maackian production, was overexpressed in alfalfa plants, resistance levels to *Phoma medicaginis* was heightened [197]. Similar results were seen in soybean expressing genes involved in phytoalexins resulting in increased resistance against *Rhizoctonia solani* [198,199]. Although many phytoalexins may have been identified, mechanisms leading to their productions are not well understood for some of them. Plants are also able to defend themselves by producing compounds that attract natural predators of herbivorous insects, triggered

by their attackers [200]. In addition, they can also produce sticky substances such as latex that trap insects and prevent them from feeding [201].

1.6. Cassava mosaic disease resistance

Geminiviruses continue to be a challenge to the world crop production due to a spectrum of diseases they cause, CMD included. As it stands, there are many attempts to control geminivirus-induced diseases such as controlling the insect vector using pesticides or genetic insect resistance [202] and the generation of geminivirus-resistant varieties. Here we discuss efforts that have been made to control CMD. There are three naturally occurring cassava markers that have been associated with resistance to CMD, namely CMD1, CMD2 and CMD3. CMD1 locus provides quantitative polygenic recessive resistance and it was derived from *M. glaziovii* [18,203,204]. Cultivars that have acquired the CMD1-type resistance have been deployed in different regions of the Eastern and Western Africa as tropical *Manihot* species (TMS) series [204]. CMD2 confers monogenic dominant resistance derived from a single genetic locus and was discovered in related tropical *Manihot esculenta* (TME) landraces that were collected from farmers from Nigeria and other West African regions in the 1980s and 1990s [18,205,206]. CMD2-type resistance landraces were shown to display near-immunity/tolerant phenotypes against most of the CMG species [204,207]. In addition, this germplasm has become breeders' favourite due to the ease of transferring the monogenic dominant locus between germplasm through simple crosses [205]. This, however, makes this locus more susceptible to viruses overcoming this resistance and thus needs constant innovation to keep ahead of viral evolution [121].

The last and more recent marker is CMD3 [204], which resulted from crossbreeding TMS97/2205 elite cultivar derived from CMD1-type resistant TMS30572 and CMD2-type resistant TME6 and appears to provide the highest level of resistance [208]. A less than 1% disease incidence on CMD3-cultivars under field conditions and high disease pressure was recorded [204]. This indicates that cultivars possessing the CMD3-type resistance are highly resistant with little or no CMD expression on leaves. In contrast, CMD1- and CMD2-type plants do become infected with CMD and show severe symptoms in the initial stages of infection, however, recover from the disease with reduced or no symptoms on newly developed leaves [204]. A loss of CMD2-related resistance has been recorded in TME204, TME3 and TME7 grown from somatic embryogenesis and infected with EACMV, while CMD1- and CMD3-type plants retained their resistance levels [209,210]. Loss of resistance can be linked to virus mutation which leads to breaking resistance [211], epigenetic changes that occur during tissue culture [212], and lastly the involvement of sequences enhancing geminivirus symptoms (SEGS) in the duration of

cassava mosaic infection [213]. Two SEGs encoded in the genome of cassava resulted in the enhanced appearance of CMD symptoms and viral DNA accumulation in resistance cultivars [213].

There other resistance mechanisms such as reverse genetics and also engineered resistance, although they may not only be direct to CMD but rather geminiviruses. Researchers are constantly seeking understanding of geminivirus-plant interactions and how host resistance is manifested and as such plant host genes that are directly linked to geminivirus resistance have been identified. The *CchGLP* gene found in resistant peppers is involved in apoptosis [214]. *SnRK1* phosphorylates geminivirus proteins TrAP and β C1, leading to delayed symptoms and reduction in viral DNA accumulation [215,216]. Whereas SnRK1 interaction with Rep interfered with viral DNA replication and reduced disease symptoms [217]. Another gene is the receptor kinase, NIK which is a key player in what is known as NIK-mediated antiviral immunity brought about by translational inhibition of host and viral transcripts activated by the ribosomal protein Sac52 [65]. Begomoviruses have however, evolved a defense mechanism to counteract NIK-mediated antiviral immunity by binding their NSP to NIK and suppress its phosphorylation function [218]. Furthermore, GTPase that interacts with NSP can be of interest to suppress NSP from binding to NIK [219]. Lastly, an E3 ligase gene, *MeE3L* has been linked to resistance in SACMV-infected cassava [110].

Some plants have been engineered with resistance that works by interfering with viral proteins and thus interfering with the infection cycle. For example, engineered transgenic plants expressing antibodies against specific viral proteins have resulted in resistance [220,221]. Small peptide sequences known as peptide aptamers can also be engineered and expressed in host to interfere with viral proteins and leads to reduction in viral DNA accumulation [222,223]. Plants can also be engineered to produce antiviral siRNAs that targets the viral transcript for degradation and thus resulting in reduced disease symptoms [34,224]. Geminivirus counteracts siRNA silencing by what is known as viral suppressors of RNA silencing which interacts with siRNAs produced by the host, and thus hindering silencing [225,226]. Although there is measurable progress in discovering ways to protect plant hosts against geminivirus, it is important to always innovate new ways to keep up with the virus evolution and overcoming resistance built against them.

1.7. Genome editing in plants

The global population is estimated to pass nine billion by the year 2050. This puts more pressure on food producers to come up with ways that will result in the production of enough food, considering the current food insecurity crisis [227]. The uncertainty of environmental factors and the global

climate change are also factored in this global crisis and consequently, makes it hard to ascertain how the fight against food security will be overcome. Efforts and progress have been made with breeding for varieties that will be well adapted to the environment and show tolerance and/or resistance to different abiotic and biotic stresses. However, with breeding it takes years before a particular variety can be deemed safe for commercial growing with regards to health and environmental safety regulations [228].

Genome editing technology allows for precise and efficient targeted modification of the genome either by insertion, knockout, or replacing DNA bases within a short turn-around time [229]. The risks associated with using genome editing tools to alter the genome are significantly lower since they involve altering a few bases unlike the changes that come in naturally occurring populations [230]. Genome editing technologies have been used in different plants for functional characterisation studies, to improve agricultural traits, and targeting multiple genes/loci simultaneously by multiplexing made possible the study of pathways [231]. Different genome editing tools are available and these include meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9 (CRISPR-Cas9) and oligonucleotide-directed mutagenesis (ODM). A summary comparison of these tools is shown in Table 1-1 below. CRISPR-Cas9 technique was used to knockoff genes in this study and thus the technology is discussed further.

Table 1-1. Comparison of plant genome editing techniques*.

	Meganucleases	ZFNs	TALENs	CRISPR-Cas9	ODM
Specificity module	Target recognition domain	Zinc-finger domains	TALE domains	crRNA	Exogenous polynucleotide (chimeraplast)
Cleavage module	Nuclease domain (E.g., I-CreI, I-OnuI)	FokI nuclease	FokI nuclease	Cas9 protein	Not required
Length of target sequence (bp)	14-40	24-36	24-59	19-22	68-88
Target recognition efficiency	Low	High	High	High	High
Cleavage efficiency	High	High	High	High	High
Experimental setup	Complicated	Complicated	Relatively easy	Easy and very fast	Easy
Multiplexing	Difficult	Difficult	Difficult	Possible	Difficult
Mode of action	Double-strand breaks in target DNA	Double-strand breaks in target DNA	Double-strand breaks in target DNA	Double-strand breaks or single-strand nicks in target DNA	Information strand directs conversion(s) within target region
Off-targets	Detected	Detected	Detected	Detected	Detected
Reference	[232]	[233]	[234]	[235]	[236]

*Adapted with modifications from Kumar and Jain, 2014 [237].

1.7.1. CRISPR-Cas9 system

A CRISPR-Cas9 system is a form of defence mechanism that targets the DNA or RNA of invading viral and plasmid DNAs [238]. The CRISPR loci have been observed in bacteria and archaeal genomes. However, the Cas9 RNA-guided type II system of the CRISPR system, mostly used for genome editing studies is only found in bacteria. When compared to other genome editing tools (Table 1-1), the CRISPR-Cas9 system is robust, more efficient, high specific, less time-consuming and has minimal off-targets [239], making it a great genome editing tool. The CRISPR-Cas9 system has many applications in genome editing including introducing point mutations, integrating foreign gene for gene pyramiding purposes, gene knockouts, epigenome editing, and repression/activation of gene expression.

Cas9 protein forms a complex with single guide RNA (sgRNA) which consists of CRISPR RNA (crRNA) and *trans*-activating crRNA (tracrRNA) and guides it to the recognition motif, the protospacer-adjacent motif (PAM) on the 3' end of the target DNA fragment (also known as spacer). This is immediately followed by sequence specific nuclease on the spacer, resulting in double-strand breaks. The double-strand breaks are repaired by the cell's endogenous mechanisms, primarily non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ repairs the breaks using DNA ligase IV, while HDR uses a template for repair [240]. The assembled sgRNA and Cas9 infused in an expression vector can be delivered to the cell via protoplast transformation, *Agrobacterium* transformation, and shotgun methods [237,240].

The CRISPR-Cas9 system is a powerful gene-editing tool that has brought major transformation in the field of molecular biology. Since the validation of the CRISPR-Cas9 system as genomic cutters [241], there has been an exponential increase in the use of this system for genome editing purposes in bacteria, archaea, animals, plants and even human embryo. Tyagi et al. 2020 [242] list plants that have had their genomes edited using the CRISPR-cas9 system to insert, delete, knockout and also replace genes. The system has been used to successfully edit genomes ranging from monocots such as rice, maize, wheat and barley [243]–[245] and dicots such as *Arabidopsis*, tomato, potato, nicotiana, cotton, citrus and soybean [225,246,247]. The highest number of genes to be targeted simultaneously with the CRISPR system was five genes (*PDS3*, *AtFLS2*, *CYCD3*, *1-AtRACK1b* and *AtRACK1c*) in *Arabidopsis* [246].

Multiplex gene editing targeting genes that function in the negative regulation of grain weight (*GW2*, *GW5* and *TGW6*) resulted in improved grain size and number in rice [248]. When *FAD2* and *CsDGAT1* were knocked out simultaneously, a *Camelina sativa* variety with improved fatty acid composition was developed [249,250]. Gomez and co-workers [251] studied the knockout of nCBP-1 and nCBP-2 isoforms associated with cassava brown streak disease *in planta* in transgenic cassava cv.60444 and observed reduced viral symptoms and enhanced resistance. The CRISPR system was also used to knockout *MeE3L* in protoplasts from cassava landraces cv.60444, T200 and TME3 in response to SACMV [110]. This was the first study to successfully use the CRISPR system on cassava protoplasts. It was concluded that the accumulation of SACMV as well as the expression of the knocked-out gene varied between genotypes. Non-coding/coding regions of the viral genome responsible for viral RNA replication and synthesis and AGO2 were targeted for knockout in *N. benthamiana* infected with different viruses such as CMV, TMV, Tomato leaf curl virus (ToLCV) and Beet severe curly top virus (BSCTV) [252–254]. Durable and inheritable resistance was observed for all the above-mentioned

viruses when the viral genome region was targeted. Inheritable resistance was observed when regions of the viral genome with genes responsible viral RNA synthesis were targeted for knockout in *Arabidopsis* infected with TMV, BSCTV, CMV and TuMV [255–257].

There are few things to consider in order to increase the efficiency and success of the CRISPR-Cas9 system in plant genome editing studies. These include the selection of suitable expression vector, optimal promoters for the expression of both Cas9 and sgRNA, as well as the expression vector delivery method. In addition, the use of Cas9 mutant and the concentration and ratio of Cas9 and sgRNA delivered into the cell must be considered to keep off-target silencing or mutations, etc to a minimum [237].

1.8. Viral induced gene silencing (VIGS)

1.8.1. VIGS: a functional genomics tool

The first report of VIGS as a gene silencing method was on a model plant *Nicotiana benthamiana* where the phytoene desaturase (PDS) gene was targeted and successfully silenced [258]. The resulting photobleaching phenotype on the plant as a consequence of the silenced *PDS* gene enabled the identification of the function of photoprotective carotenoid pigments that were lacking as a result of silencing. VIGS has been adapted as a high-throughput technique due to it being cost effective, fast, simple and reliable [259]. In addition, VIGS carries more advantages over some genome editing techniques, including inducing tissue-specific as well as whole plant gene silencing and silencing individual or multiple genes in a family [260]. To date, many VIGS vectors have been developed for functional genomics studies in both monocotyledonous [261–263] and dicotyledonous plants [264–267].

Many of VIGS applications have been done on *N. benthamiana* due to its high susceptibility to a wide range of viruses. This model plant has also been found to display more pronounced and persistent symptoms than other plants. However, with the use of VIGS over the years, there has been improvement in the development of VIGS vectors for application in other plants species including maize, barley, tomato, rice, cassava and *A. thaliana*, just to mention but a few [268]. Tobacco rattle virus (TRV) VIGS vector was used to silence *CTR1* gene in tomato. The gene is a negative regulator of the ethylene pathway and a loss of its function resulted in a constitutive ethylene response [269]. TRV VIGS has also been widely used to silence many genes, including candidate R genes *R1* and *Rx* in potato and *RB* in potato wild relative *Solanum bulbocastanum*. The successful silencing of these genes gave rise to susceptible phenotypes as a result of weakened R gene facilitated disease resistance [270]. In a more recent study, TRV VIGS was used to successfully induce silencing of phytoene desaturase

(*ApPDS*) in an economically important medicinal plant *Andrographis paniculate* [271], showing the diversity of TRV host and also the effectiveness of this VIGS vector.

Geminiviruses VIGS vectors have been developed and used for functional studies in their different host plants. ACMV and SACMV VIGS vectors were used to silence *N. tabacum su* gene encoding a chloroplast enzyme magnesium chelatase unit in cassava and *N. benthamiana* [264,272]. Loss of function gave rise to phenotypes with yellow-white leaf bleaching. In *N. benthamiana*, there was elevation in SACMV viral accumulation when a disease resistance related gene *NOA1* was silenced using SACMV VIGS [266]. Cabbage leaf curl virus (CaLCuV) VIGS was used to silence an *Arabidopsis CH-42* gene encoding a subunit of magnesium chelatase which is required for chlorophyll biosynthesis [273]. The resulting phenotypes were susceptible to the virus with severity varying based on different lines used. Beyene and co-workers [274] used an EACMV VIGS to silence *M. esculenta SPY* gene, resulting in shoot necrosis and subsequent death of the whole plant in cassava lines susceptible to CMD. ACMV VIGS was also used recently to silence genes in *N. benthamiana* and cassava leaves and roots [275].

VIGS has over the years, proven to be a powerful tool for functional genomics studies *in planta*, silencing genes in different organs including leaves and roots. This is because this system possesses high efficiency target recognition and cleavage. In addition, it can be used for both forward and reverse genetics [268]. VIGS technology is also being applied to study stress responsive genes in crops under abiotic stress [276]. Suppression of late embryogenesis abundant (LEA) protein as a result of silencing *lea4* gene using TRV VIGS resulted in increased susceptibility of tomato plants to drought stress [277]. Other abiotic stress related genes that have been silenced in different plant hosts using a range of VIGS vectors are *TdAtg8* (Autophagy related), *GmRPA3* (replication protein), *CaPO2* (peroxidase), and ethylene receptor gene *RhETR3*, amongst others [260,278].

1.8.2. Molecular mechanism of VIGS

When a plant is invaded by a virus, an RNA-mediated post-transcriptional gene silencing (PTGS) defense mechanism targeting viral RNAs (or transgene RNAs inserted into it) is induced to counteract viral proliferation inside the cells and movement between the cells by degradation of viral transcripts [279]. Targeted gene silencing by VIGS exploits this latter innate antiviral defence system to silence or knock down gene expression levels. VIGS involves engineering the viral vector to incorporate DNA fragment of the host target gene to be silenced into the multiple cloning site of the viral vector and infecting the host plant with the engineered VIGS vector (Figure 1-5A). VIGS can be delivered to the plants through viral sap inoculation, biolistic transformation, and *Agrobacterium*-mediated

transformation via different plant inoculation methods such as infiltration, toothpick, agroinfiltration, vacuum infiltration, spraying and plant dipping [280].

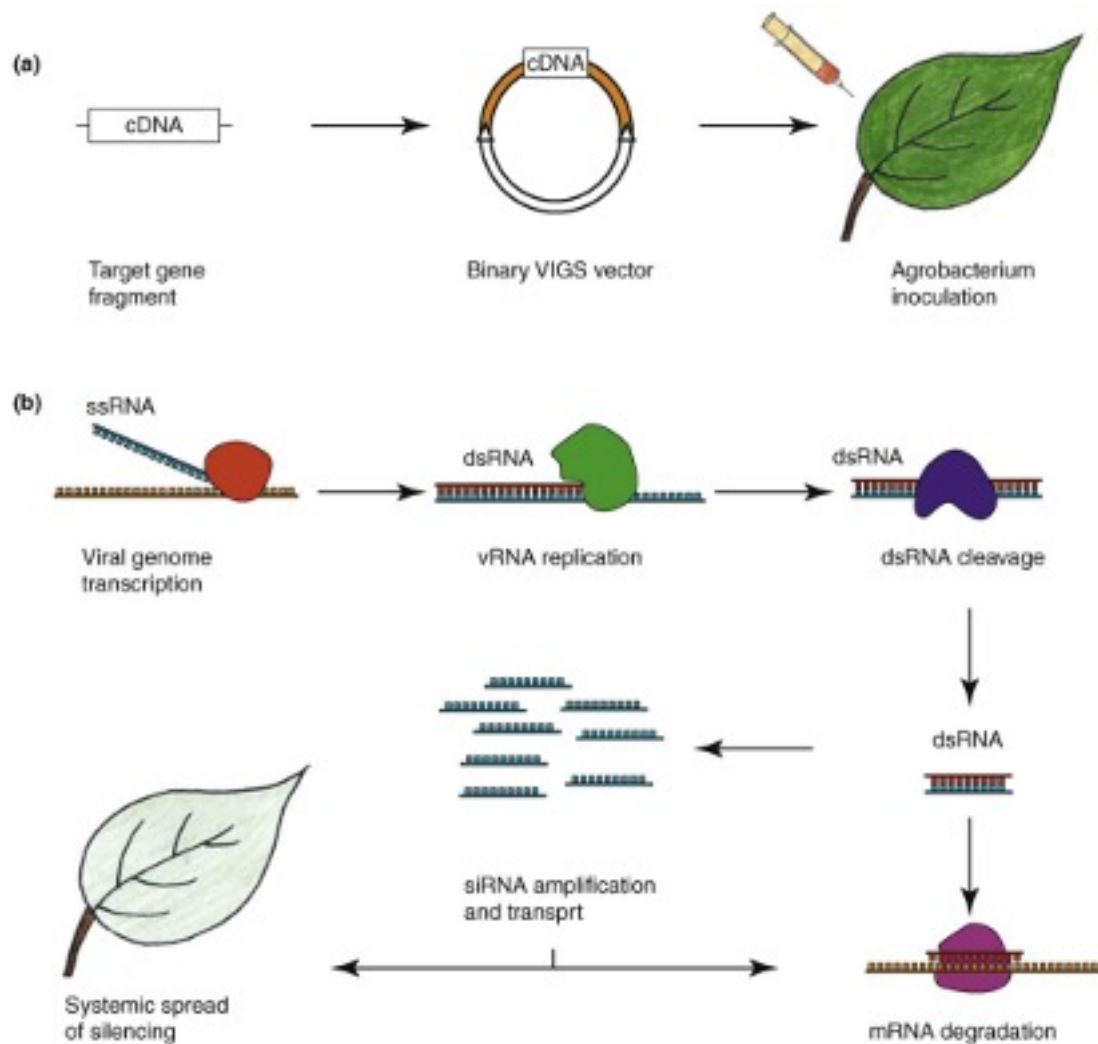


Figure 1-5. A flowchart of VIGS in plants. (A) shows the engineering of the target gene into the VIGS vector and its delivery to the plant via *Agrobacterium* inoculation. (B) shows how the process of gene silencing occurs downstream plant infection with the VIGS vector. Source: Becker and Lange, 2010 [281].

Following successful delivery of the VIGS vector into the plant, RNA silencing is induced downstream, which then leads to the silencing of the target gene in the immediate cells and also systemic gene silencing in distant cells (Figure 1-5B). The mechanisms start with the transcription of the viral genome resulting in single stranded RNA transcript which is then replicated into dsRNA molecules either by an endogenous or a viral RNA dependent RNA polymerase. Successively, Dicer-like protein is triggered by the dsRNA molecules and consequently cleaves them to produce short interfering RNAs (siRNAs) duplexes ranging from 21-24 bp [282]. An argonaute 1-containing RNAi silencing complex interacts with the antisense strand of the siRNAs and directs it to the homologous RNA sequence where

endonucleolytic cleavage and translational inhibition of cognate RNAs occurs, leading to PTGS [283, 284].

It is important to note that although the target DNA fragment inserted into the VIGS vector should be at least 23 bp for silencing to occur, it does not guarantee gene silencing. As such, it is advisable to use longer homologous target gene fragments of up to 350 bp [277]. However, longer fragments require a further look into the composition of nucleotides, as well as the thermodynamic properties of both siRNA and target sequence to ensure efficient silencing. It is also believed that the orientation of the inserted target sequence influences the efficiency of the VIGS as reverse orientated inserts were found to significantly enhance the silencing efficiency [260]. This is also true for target sequences that are constructed to form hairpin structures.

1.8.3. Challenges and limitations of VIGS

Over the years VIGS has proven to be a high throughput tool for functional genomics studies. However, there are challenges and limitations such as off-target silencing, low silencing efficiency, which are being continuously improved to maximize the efficiency of this technology [285]. For VIGS-induced silencing to work, the virus vector needs to infect the host plant and this has proven difficult in some plant species. These include plants such as beans, cassava, pepper and cucumber, to mention but a few, with resistance genes that confer resistance against certain viruses, and therefore making them ineffective as VIGS vectors [278].

Small siRNAs are known to be transferable from one host to another and although it is still unclear if this happens in VIGS, the possibility of it should not be taken lightly and proper procedures should be followed to discard any VIGS products to eliminate the latter possibility. VIGS delivery method can be a challenging factor to plant species that are hard to transform. Even though *Agrobacterium*-mediated transformation is mostly used, for plants that are *Agrobacterium* recalcitrant, viral RNA transcript or DNA can also be bombarded onto the host plant or do a virus sap inoculation, making the possibility of transfer more possible although not through the traditional transformation methods [286].

When working with VIGS, silencing of unintended gene targets can easily occur based on the homology of the target gene sequence. When this happens, more than a single gene can be silenced consequently resulting in ambiguity of the function of the targeted gene. It is crucial that the target sequence selected to be engineered into a VIGS vector has minimal off-targets and one way to do this is the careful selection of target gene region using software such as Web MicroRNA Designer 3 (WMD3) [287]. This free public software designs target sequence miRNAs, taking into considerations

different aspects such as the position of the target sequence on the gene, potential off-targets, hybridization energy for efficient binding, amongst others.

Most of the challenges mentioned above have been overcome, but unfortunately, some challenges are inherent and continue to be a challenge to this day. VIGS vectors are conventionally derived from viruses-based vectors and as such their presence in the plant will automatically trigger innate immune response and interfere with the metabolism of the plant which may in turn affect results [259]. It is therefore important to have appropriate controls and also to confirm VIGS results using other gene disruption methods.

Some viral vectors are excluded from the host meristems and the surrounding cells, resulting in the incomplete silencing of the target gene localized in those cells/tissues [280]. In addition, silencing some genes may not result in a characteristic phenotype change in the plant making it difficult to ascertain if the gene was silenced. For this reason, VIGS would be more commendable to study gene functions that will result in lethal phenotypes [260]. Finally, it is stipulated that insertion of genes in VIGS vector can in some cases, hinder virus multiplication and many viruses are known to delete the insert during multiplication and systemic spread of the virus, meaning that silencing may only occur at one localisation and not be systemic.

1.9. Plant proteomics

Proteomics can be defined as a systemic analysis to identify and quantitatively analyse a complete set of proteins expressed in an organism, tissue, cell, or organelle at a specific time under certain conditions [288]. Understanding plant defense mechanisms that result in tolerance or resistance to different stress inducing factors is of utmost importance when developing crops. Stress-induced changes in gene expression modulate metabolic processes through alteration of cellular protein abundance and function. It is therefore, important to study the changes in host proteomics and their functions in response to different stresses in order to elucidate the molecular mechanisms that they trigger. Focussing on the genome for functional characterisation of genes as opposed to proteins has disadvantages. The genome is not stable and it is prone to many modifications and regulations, resulting in no correlation between mRNAs expression levels and their proteins abundance [289]. As such, a combinatorial approach using proteomics data together with available sequenced genome and other omics data, and present bioinformatics, is a powerful tool effective for identifying novel proteins and changes in protein abundance following biotic and abiotic stresses.

1.9.1. Proteomics techniques

There are two techniques that are used to perform proteomics analysis in plants. First one is the gel-based technique, which involves separating proteins by polyacrylamide gel electrophoresis, quantification, and protein spot digestion first before protein identification through mass spectrometry (MS) [290]. For proteomics studies, proteins are mostly separated by two-dimensional polyacrylamide gel electrophoresis (2-DE) and differential in-gel electrophoresis (DIGE), but 1-DE may also be used. In 2-DE, hundreds of proteins are separated based on their isoelectric points first, followed by molecular mass separation and staining of protein spots using dyes such as Coomassie brilliant blue for easy viewing [291]. DIGE allows separation of multiple samples in a single gel by labelling each protein sample with fluorophores that emit different coloured lights upon excitation [292]. This method was to address the issue of reproducibility and gel-to-gel variation, which 2-DE does not take into account [293]. A selected protein spot from the gel is then subjected to trypsin digestion and followed by MS analysis and identification after database search for matching peptides. Mass spectrometers used for proteomic analysis use matrix-assisted laser desorption-ionisation (MALDI) and electrospray ionisation (ESI) to ionise samples before peptides are separated according to their mass/charge ratio (m/z) using either time of flight (TOF), ion trap, triple-quadrupole tandem MS (MS/MS), and Fourier transform ion cyclotron resonance [294]. Gel-based methods have been used to study proteomics in different plant tissues or organs under a range of stresses [295–297].

The second technique involves methods that are gel-free and were developed mainly to fill the gaps left by gel-based techniques [298,299]. These methods involve protease degradation of protein mixtures followed by liquid chromatography (LC) separation and MS identification. Gel-free methods involve tagging-based labelling, metabolic labelling, and label-free methods. Tagging-based labelling uses tags such as isobaric tagging for relative and absolute quantification (iTRAQ) and isotope-coded affinity tag (ICAT) to label proteins and peptides, while metabolic labelling uses stable isotope to label amino acids in living cells [300]. Label-free methods identify and quantify relative changes in two or more biological samples using multi-dimensional capillary LC coupled with nanoESI tandem MS [301]. Gel-free methods have been proven to be more efficient, identifying 100% more proteins than the gel-based methods [302,303]. Gel-free techniques have mostly been used in recent proteomics studies in plant hosts [304–306] compared to gel-based techniques.

1.9.2. Limitations of proteomics tools

Gel-based methods have been successfully used to study proteomics over the years, however, like many other techniques it suffers some limitations. Although the DIGE method has reduced the issue of reproducibility, gel-based techniques are generally still considered difficult to reproduce same

protein profile between two replicates of the same experiment due to some degree of gel-to-gel and run-to-run variations [299]. In addition, this technique is fairly insensitive to low copy proteins, cannot be used to study certain classes of proteins, and cannot be used to study the entire proteome as it can only cover 30-50% of the whole proteome [289,307].

Gel-free techniques are more accurate and robust compared to gel-based techniques; however, label-based methods are more costly and as such making these methods not affordable to many plant-based research labs. Peptides that are similar between a number of proteins may make it difficult to identify some proteins as compared to gel-based methods where this uncertainty can be eliminated because proteins mass and isoelectric point are known before the identification [308].

1.9.3. Proteomics in the investigations of biotic stress tolerance mechanisms

Proteomics studies have become an exceptional tool to dissect and decipher plant-pathogen interactions as proteomes are major drivers of the defence response triggered upon pathogen perception [309]. Although it has proven difficult to elucidate all the mechanisms involved in virus tolerance and resistance, some progress has been made to explain different pathways and cascades, which in turn contribute to the large objective. Proteomics have been extensively used to understand abiotic and biotic stress responses but herein, we looked at how proteomics has contributed to the investigation of virus tolerance in some plant hosts. All the proteins identified in these studies moved us a bit closer to understanding how plant defense mechanisms work. Diseases caused by viruses are a considerable limiting factor to a number of economically important crops worldwide. Viruses are difficult to control as it was mainly dependent on natural and engineered resistance. Viruses are obligate parasites and are unable to replicate and move inside the host on their own, for this reason, they require the host's proteins and other resources [310]. As such it is important to understand the molecular and biochemical changes that occur during infection in order to get an understanding of how host tolerance and resistance was attained as this will help in crop production programs.

Viruses cause dysfunction to the photosynthetic machinery, resulting in photosynthesis being shut off and symptoms development induced [311]. Chlorophyll biosynthesis proteins such as magnesium chelatase subunits I (ChII) and D, protoporphyrinogen oxide, and magnesium-protoporphyrin IX monomethyl ester were under-expressed in rice infected with rice stripe virus (RSV) [312]. These proteins are responsible for Mg^{2+} insertion into a protoporphyrin IX molecule, an important step in chlorophyll biosynthesis [313]. Di carli et al. 2010 [314] has also reported reduction in magnesium chelatase in a proteomics study of tomato plants infected with CMV. In addition, other studies also reported on the effects of inhibiting chlorophyll production as a result of silencing *ChII* in tobacco

infected CMV-YsatRNA [315], increasing the expression of genes that facilitate chlorophyll degradation in cassava infected with ACMV [316], and the reduction in chlorophyll a/b proteins in cowpea infected with cowpea severe mosaic virus (CPSMV) [317] and *N. benthamiana* infected with Chinese wheat mosaic virus (CWMV) [306]. Furthermore, other proteomic studies have revealed that viruses target photosynthetic electron transport chain by causing a reduction in proteins involved in light harvesting complex (example, chlorophyll a/b binding proteins), photosystem I (PSI) and PSII and oxygen-evolving complex (example, oxygen-evolving enhancer proteins, PSII core proteins D1 and D2), and ferredoxin NAD(P)⁺ reductase (example, ferredoxin-NADP reductase) [312,318,319]. This further confirms the importance of a functional photosynthetic complex so that the plant can be provided with enough energy to initiate defence response as this process is costly.

Carbon fixation is an important process where organic compounds are produced for storing energy and building blocks for other biomolecules. This process has been linked with the production of metabolites that function in plant defense response. For example, phosphoglycerate kinase (PGK) protein has been identified and differentially expressed in many proteomics studies involving viral infection [320,321]. This protein has been over-expressed in some studies and under-expressed in some, making it difficult to cement its exact contribution in virus defence. However, a study linked PGK to virus replication by noting an interaction between PGK and the 3' UTR region of the BaMV RNA which led to transportation of the viral RNA into the chloroplast where replication occurs [322,323]. This interaction was confirmed with the reduction of BaMV RNA in PGK silenced mutants. To further confirm this, *Arabidopsis* Cvi-0 plants with *rwm1* gene responsible for natural recessive resistance against watermelon mosaic virus (WMV) encodes cPGK2, mutated PGK which resulted in the reduction of the virus infection [324].

Photorespiration-related proteins such as glycolate oxidase, T-protein, and alanine-2-oxoglutarate aminotransferase were under-expressed by infection with CMV [314]. Photorespiration is known to be crucial for cellular redox homeostasis and photoinhibition [325]. For viruses to thrive inside the host, they need to shut-off photosynthesis and photorespiration pathways as a way to suppress some of the plant defence mechanisms such as ROS burst [311]. ROS are involved in several routes of organelle retrograde signalling and consequently, leading to the expression of defense-related genes [148] and by under-expressing photorespiration enzymes, CMV was able to prevent the ROS-activated defence response. Pea seed-borne mosaic virus (PSbMV) infection triggered differential expression of 116 proteins in *Pisum sativum* L. These proteins included nuclear proteins, mitochondrial carrier proteins, chloroplast proteins, and proteins involved in JA biosynthesis and redox homeostasis [326],

of which their involvement in plant defence responses has been recorded previously. Cowpea severe mosaic virus (CPSMV) infection of cowpea led to a 76% suppression of differentially expressed proteins involved in redox homeostasis, signalling, protein synthesis, nucleic acid metabolism and defense two days after infection, which promoted viral invasion and systemic movement [327]. Most recently, a proteomics study in maize infected with *Maize chlorotic mottle virus* reported an increase in a number of proteins involved in redox homeostasis to be over-expressed and attributed this to plant tolerance or resistance to the virus [328].

A proteomics study in *N. benthamiana* infected with TuMV done using the gel-free method resulted in 148 significantly differentially expressed proteins [329]. Amongst these proteins was a cell wall-loosening protein, NbEXPA1 that is specific to the plasmodesmata and was under-expressed in response to TuMV infection. This study showed that NbEXPA1 helps aiding virus replication via interaction with RNA replicase Nib and cell-to-cell movement. They further demonstrated that silencing of *NbEXPA1* gene inhibited TuMV infection whereas its overexpression promoted viral infection. Papaya latex proteomics study revealed nine under-expressed cysteine proteases proteins in response to *Papaya meleira virus* [330]. This reduction in cysteine proteases was linked to inhibition of latex coagulation, suggesting virus flow through the laticifers and subsequently, spreading within the plant.

Several more proteins involved in plant defense have been reported to be differentially expressed following infection with different viruses. Tomato plants infected with CMV, reported 14% of differentially expressed proteins to be involved in defense [314]. Proteins involved in signalling and disease resistance such as NBS-LRR resistance protein and ascorbate peroxidase were altered by CMV [297]. Six proteins involved in signal transduction were differentially expressed in resistant and susceptible tomato plants infected with TYLCV [331]. MAPK signalling proteins were identified and changed by CWMV in infected *N. benthamiana* [306].

1.10. Rationale for study

Cassava is an economically important staple crop and the 3rd largest source of carbohydrates after rice and maize in Africa, and responsible for feeding more than 800 million people worldwide. The production of cassava is threatened by cassava mosaic geminiviruses such as South African cassava mosaic virus (SACMV), which causes cassava mosaic disease, previously blameable for up to 100% production losses. The conventional strategies of controlling CMD such as phytosanitation, cultural practices, and vector control have failed over the years [1]. Breeding programs, on the other hand,

have successfully generated cassava genotypes that have natural tolerance/resistance against CMD, and have effectively given higher yields than susceptible genotypes [9]. Although the specific genes involved in the mechanisms behind tolerance/resistance genotypes is not fully understood yet, it is known to be associated with three naturally occurring cassava QTL markers that have been proven to confer resistance to CMD. This study aimed to gain more understanding into disease response mechanisms by functionally characterizing resistance genes and also mapping and identifying proteins that may be involved in, with the bigger purpose of producing cassava varieties that will be able to tolerate or resist viruses. This would further lead to the increase in production and contribute to alleviating food insecurities in the regions that depend on cassava as a staple crop or as a source of income.

1.11. Study aims and objectives

1.11.1 Study aim

The aim of this study was to use genome editing technology, such as virus induced gene silencing (VIGS) and clustered regularly interspaced short palindromic repeats (CRISPR) to identify resistance genes, other immunity-related genes, and interacting partners associated with SACMV-triggered resistance/tolerance to cassava mosaic disease.

1.11.2. General objectives

The overall objectives of the study were:

1. To identify and data mine potential CMD-associated resistance/tolerance candidate genes and their interacting partners in tolerant TME3 from existing data.

Potential CMD-associated resistance/tolerance candidate genes were sourced from transcriptome data of SACMV-infected TME3 [78, 85]. Two cassava resistance gene analogs (RGAs) were selected based on their potential roles in cassava-geminivirus recovery, TIR-NBS-LRR Manes.12G149700 (*MeTNL*) and NBS-LRR Manes.12G091600 (*MeRPPL1*). A number of online software such as TAIR, Phytozome, pathogen receptor genes database (PRGdb), plant geneset enrichment analysis (PlantGSEA), and STRING were used for bioinformatics analyses and datamining of the selected silencing target genes.

2. To validate selected resistance genes in tolerant TME3 *in planta* and in protoplasts by VIGS- and CRISPR-mediated knockdown, respectively.

Web MicroRNA Designer (WMD3) was used to identify multiple artificial microRNAs target sites for VIGS-mediated silencing and these were put under the control of cauliflower mosaic virus 35S promoter and terminator. The construct containing the promoter, sense sequence of the target sites, an intron, antisense sequence, and the terminator was cloned into a SACMV DNA A vector, pC8A-CP and agro-inoculated into cassava TME3 landrace plantlets together with SACMV. CRISPOR was used to generate gRNA target regions for CRISPR-mediated silencing which were integrated into a construct made up of *Arabidopsis* U6-26 promoter, pre-tRNA, gRNA, scaffold, and a terminator. The latter construct was cloned into a pCambia 1380 vector which was then used to transfect TME3 leaf mesophyll protoplasts together with Cas9 and SACMV.

3. To map the leaf proteome of tolerant cassava TME3 and susceptible cassava T200 landraces infected with SACMV.

A HEPES-based buffer and 10% TCA/acetone were used to extract and precipitate proteins, which were further analysed using a non-gel based liquid chromatography-mass spectrometry (LC-MS) technique. Bioinformatics analyses were done on identified proteins with at least two unique peptides.

Chapter 2: Functional characterisation of cassava resistance genes using gene silencing

2.1. Introduction

Cassava (*Manihot esculenta* Crantz) is both an industrial crop and a staple food, responsible for feeding more than 800 million people worldwide [333]. However, its production is threatened by viruses such as *South African cassava mosaic virus* from the *Geminiviridae* family that causes cassava mosaic disease (CMD) [334,335]. Cassava mosaic begomoviruses are amongst the most damaging viruses causing losses of at least 25 million tons per year in Africa, India, and Sri Lanka [1,10]. CMD has effectively resulted to yield losses of up to 82% [338] by significantly reducing the number and size of tubers, however this depends on factors such as virulence, susceptibility or tolerance of the host, and other environmental factors [7]. South African mosaic virus (SACMV) is a bipartite circular ssDNA geminivirus [337] and its infection presents itself with misshapen leaves with yellow/pale green mosaic and general reduced plant growth.

The conventional strategies of controlling CMD such as phytosanitation, cultural practices, and vector control have failed over the years [1]. Breeding programs, on the other hand have successfully generated cassava genotypes that have natural tolerance/resistance against CMD, and have effectively given higher yields than susceptible genotypes [332]. Although the specific genes involved in the mechanisms behind tolerance/resistance genotypes are not fully understood yet, they are known to be associated with three naturally occurring cassava QTL markers that have been proven to confer resistance to CMD. The genotypes are tropical *Manihot* series (TMS96/0023) [17] with CMD1-related resistance and tropical *M. esculenta* (TME) [18,338] with CMD2-related resistance. The CMD1 locus, derived from the wild relative Ceara rubber species *M. glaziovii* provides quantitative polygenic recessive resistance [18,203,204], while CMD2 confers monogenic dominant resistance and landraces with this marker display near-immunity phenotypes against most cassava geminiviruses [204,207]. More recently, an additional source of resistance (CMD3) was identified in the elite Nigerian germplasm TMS97/2205, developed in 2012 by the IITA [204].

Over the years CMD2 has become the favourite for breeders, making CMD2 more susceptible to viruses overcoming the resistance and thus constantly needing innovation to keep ahead of the viral evolution [121]. A loss of CMD2-related resistance has been recorded in TME204, TME3 and TME7 grown from somatic embryogenesis and infected with EACMV, while CMD1- and CMD3-type plants

retained their resistance levels [209,210]. Loss of resistance can be linked to viral evolving and breaking resistance [211], epigenetic changes that occur during tissue culture [212], and lastly the involvement of sequences enhancing geminivirus symptoms (SEGS) in the duration of cassava mosaic infection [213]. Although, breeding works, it is tedious, time consuming and may take years for another tolerance or resistance genotype to be produced, as such researchers are now moving to engineering resistance using modern gene editing technologies such as viral induced gene silencing (VIGS) and clustered regularly interspaced short palindromic repeats (CRISPR) that produce results speedily.

In an effort to understand the CMD2-associated resistance, Rabbi et al. 2014 [338] mapped the dominant monogenic resistance CMD2 locus and reported that all markers linked to qualitative resistance occur in the same chromosome region. Genome-wide association studies (GWAS) further predicted the location of genes associated with CMD resistance on chromosome 8, and also identified two possible epistatic loci and/or multiple resistance alleles at the major CMD2 QTL [207]. Taken together with available transcriptomic data [78,85,339], proteome data [340], and sequenced cassava genome [341,342], we can dissect, select and characterise genes with possible roles in cassava-SACMV interaction. Update of the cassava genome assembly from version *Manihot esculenta* v4.1 to v8.1 relocated the CMD2 QTL on chromosome 12 (<https://phytozome.jgi.doe.gov>).

Other resistance mechanisms have used reverse genetics to identify genes that are directly linked to geminivirus resistance, such as germin-like protein (*GLP*) gene involved in apoptosis in resistant peppers [214]. *SnRK1* phosphorylates geminivirus proteins TrAP and β C1, leading to delayed symptoms and reduction in viral DNA accumulation [215,216], whereas *SnRK1* interaction with Rep interferes with viral DNA replication and reduced disease symptoms [217]. Another gene is the receptor kinase, NSP-interacting kinase (NIK), which is a key player in what is known as NIK-mediated antiviral immunity brought about by translational inhibition of host and viral transcripts activated by the ribosomal protein Sac52 [65]. Begomoviruses have, however, evolved a defense mechanism to counteract NIK-mediated antiviral immunity by binding their NSP to NIK and suppress its phosphorylation function [218]. Furthermore, GTPase that interacts with NSP, suppresses the nuclear shuttle protein (NSP) from binding to NIK [219]. Additionally, an E3 ligase gene, *MeE3L*, has recently been linked to resistance in SACMV-infected cassava [110].

Plants can also be engineered to produce antiviral siRNAs that targets the viral transcript for degradation, thus resulting in reduced disease symptoms [34,224]. Geminivirus counteracts siRNA

silencing by what is known as viral suppressors of RNA silencing (VSRs) which interacts with siRNAs produced by the host, and thus hindering silencing [225,226]. Although there is measurable progress in discovering ways to protect plant hosts against geminivirus, it is important to innovate new ways to keep up with virus evolution overcoming resistance mounted against them by plants.

This study used both the VIGS and CRISPR-cas9 gene editing technology to functionally characterise two resistance gene analogs (RGAs) suggested to be involved in CMD tolerance in cassava landrace TME3 [85]. NBS-LRR resistance genes (*R* genes)-encoded proteins play a crucial role as pattern recognition receptors (PRPs) responsible for perceiving effectors and triggering the activation of signal transduction cascades which resulted in effector-triggered immunity (ETI), suggesting their key role in defense response mechanisms.

2.2. Methods

2.2.1. Target resistance genes selection

Manes.12G091600 (herein referred to as *MeRPPL1*) and Manes.12G149700 (herein referred to as *MeTNL*) were selected as targets for silencing after they were placed on chromosome 12 scaffold, known to be associated with *CMD2* dominant resistance in cassava. The selection was based on cassava transcriptomic data [78] follow-up study by Louis and Rey, 2015 [85], which identified resistance gene analogs (RGAs) involved in cassava-geminivirus tolerance phenotype. Phytozome version 12.1.6 (<https://phytozome.jgi.doe.gov/pz/portal.html>) was used to retrieve sequences of the two genes from *M. esculenta* v6.1 genome.

2.2.2. Selected target genes datamining

Selected genes were mined using different open access bioinformatics tools. The Arabidopsis Information Resource (TAIR; <https://www.arabidopsis.org/>) was used to retrieve gene homolog sequences and annotations. A Pathogen Receptor Genes (PRGs) database (PRGdb) version 3.0 (<http://www.prgdb.org/prgdb/plants/?id=375>) was used to search and confirm that the selected target genes were cassava putative resistance genes. Search Tool for the Retrieval of Interacting Genes (STRING) version 11.0 (<https://string-db.org/>) was used to predict protein-protein interaction networks of the selected target genes. Basic local alignment search tools, Clustal Omega (https://www.ebi.ac.uk/Tools/services/web_clustalo/) was used to align selected target gene amino acids according to default settings, while MEGA 7 (<https://www.megasoftware.net/>) was used to construct a Maximum Likelihood Tree of aligned amino acids. KEGG pathway analysis for the selected

R genes were conducted on an online webserver, the Plant GeneSet Enrichment Analysis (PlantGSEA [343]; <http://structuralbiology.cau.edu.cn/PlantGSEA>).

2.2.3. Silencing constructs design and synthesis

2.2.3.1. CRISPR-mediated silencing in cassava protoplasts

Two gRNA targets were identified for each of the selected two gene accessions from genomic sequence using an online program called CRISPOR version 4.7 (<http://crispor.org>, [344]). The gRNAs were used to design a duplex single gRNA (sgRNA) in the following arrangement: *Arabidopsis thaliana* U6-26 promoter + pre-tRNA + gRNA target 1 + SpCas9 tracrRNA + pre-tRNA + gRNA target 2 + SpCas9 tracrRNA + terminator (Appendix 1-Figure 1). The sgRNAs were synthesized in a pBluescript II SK(+) plasmid at Inqaba Biotech (Pretoria, South Africa).

2.2.3.2. Virus induced silencing (VIGS) in planta

Web MicroRNA Designer (WMD3) (<http://wmd3.weigelworld.org>, [287]) was used to identify multiple artificial microRNA (amiRNA) targets for the two selected gene accessions. Between 5 – 6 amiRNAs were selected for each gene and these were stacked together to make a sense strand between 100-130 bp. A loop forming intron was inserted between the sense and antisense strand to form a hairpin construct arranged as follows: Cauliflower mosaic virus (CaMV) 35S promoter + amiRNA sense strand + intron + amiRNA antisense strand + CaMV 35S terminator to produce VIGS construct (Appendix-Figure 2). The amiRNA constructs were synthesised in pUC57 plasmid by GenScript (Jiangsu, China).

2.2.4. Cloning silencing constructs into silencing vectors

2.2.4.1. CRISPR silencing constructs

CRISPR silencing constructs were digested out from pBluescript II SK(+) plasmid using restriction enzymes HindIII, BglII, and Eco72I and cloned into pCambia 1380 vector produced pC1380-MeRPPL1 and pC1380-MeTNL. A Cas9 insert consisting of *Tobacco mosaic virus* promoter + Cas9 + eGFP + Hsp terminator cloned from the pL1m-f2-p35s-cas9-egfp-nucleo-thsp Golden Gate vector into pCambia 1380 vector produced pC1380-Cas9 [110].

2.2.4.2. VIGS silencing constructs

VIGS silencing constructs were digested out from pUC57 plasmid using restriction enzymes XhoI, XbaI, and SacI and cloned into SACMV-DNA A (pC8A-CP, herein referred to as pC8A) VIGS vector [345] to produce the pC8A-MeRPPL1 and pC8A-MeTNL construct. A control VIGS vector was constructed by cloning the GFP gene sequence in pC8A to produce pC8A-GFP. Cloning was performed using a 5:1

(insert:vector) molar ratio. Constructs were ligated into silencing vectors using T4 DNA ligase enzyme (ThermoFisher Scientific) according to manufacturer's manual.

2.2.5. Silencing vector transformations

2.2.5.1. CRISPR-Cas silencing vectors

CRISPR-Cas silencing vectors were transformed into chemically competent *E. coli* DH5-alpha cells. Competent cells were left to thaw in ice before 5 µl of the ligation mixture was added by gently pipetting. The cells and DNA were mixed by flicking the tube 4 times and the mixture was placed on ice for 30 min without further mixing. Samples were then heat shocked at exactly 42°C for exactly 30 sec without mixing and immediately placed back on ice for 2 min without mixing. A total of 750 µl of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄·7H₂O, 20 mM glucose, pH 6.8) was added to the mixture and incubated at 37°C for 1 h, with shaking at 250 rpm. Between 50-100 µl cells were then spread on solid LB medium (10% tryptone, 10% NaCl, 5% yeast extract, 7.8% bacterial agar, pH 7.0) supplemented with 100 mg/ml kanamycin and incubated overnight at 37°C.

2.2.5.2. VIGS silencing vectors

VIGS silencing vectors were transformed into *Agrobacterium* strain *Ag1* using the snap freeze method. Briefly, 100 ng plasmid was added to 200 µl of CaCl₂ chemically treated Ag1 competent cells, gently flicked to mix and immediately snap frozen in liquid nitrogen for 5 min followed by heat shock at 37°C for 5 min. A total of 500 µl yeast extract phosphate (YEP) medium (10% Bacto-tryptone, 5% NaCl, 10% yeast extract, pH 7.2) was added and samples incubated at 28°C for 2 h with gentle shaking at 200 rpm. Transformed cells were collected by centrifuging for 60 sec at maximum speed, then resuspended in 100 µl YEP and spread on YEP agar plate supplemented with 100 mg/ml Kanamycin. Plates were incubated at 28°C for 1-2 days until colonies appeared. Positive colonies were grown in LB and YEP broth, respectively and glycerol stocks made and stored at -80°C. Restriction enzyme digestions and sequencing were performed to confirm positive ligation of silencing constructs into the vectors.

2.2.6. Knockdown of *MeRPPL1* and *MeTNL*

2.2.6.1. CRISPR knockdown of *MeRPPL1* and *MeTNL*: protoplast isolation and transfection

Protoplasts were isolated from four weeks old TME3 sterile nodal cultures grown on Murashige and Skoog (MS) medium [346] supplemented with 20 g/l sucrose, 2 mM CuSO₄, 7.8 g/l plant tissue culture agar, pH of 5.8 and grown at 28°C as outlined by Chatukuta and Rey, 2020 [110]. For each of the three

independent biological replicates, 300 mg of fully expanded leaves were cut transversely into 2-3 mm strips. CPW9M medium (0.5 M mannitol, 27.2 mg/l KH_2PO_4 , 100 mg/l KNO_3 , 150 mg/l CaCl_2 , 250 mg/l MgSO_4 , 2.5 mg/l $\text{Fe}_2(\text{SO}_4)_3 \cdot 6\text{H}_2\text{O}$, 0.6 mg/l KI, 0.00025 mg/l CuSO_4 , pH 5.8) was used to plasmolyse the leaf strips for 1 h, followed by vacuum infiltration in enzyme digestion solution (5 mM MES, 1.6% cellulase, 0.8% macerozyme, in CPW9M medium) at 80 kPa for 30 min. Samples were incubated in the dark at 25°C for 16 h with gentle shaking at 40 rpm. Protoplasts were then released by shaking at 100 x g for 5 min, filtered through a 75 μm sieve, and washed twice in CPW9M medium by centrifuging at 100 x g for 10 min and re-suspended in MMg solution (0.4 M mannitol, 15 mM MgCl_2 , 4 mM MES, pH 5.8). The integrity and viability of protoplasts was checked using the Olympus BX 63 OM/FM microscope (Olympus Scientific Solutions, Massachusetts, USA) and Evans blue dye staining, respectively. Protoplasts were quantified using the BD Accuri™ C6 flow cytometer (BD Biosciences, New Jersey, USA).

A total of 10^4 cells per ml were transfected with 15 μg CRISPR vector (pC1380-MeRPPL1, pC1380-MeTNL); and controls (pC1380-Cas9, pC1380 vector) and 4 μg of each of pBIN19-SACMV-DNA A and pBIN19-SACMV-DNA B infectious clones. Polyethylene glycol 4000 (PEG4000) to the final concentration of 25% was immediately added to the transfected protoplasts and incubated at 28°C for 20 min. The transfection mixture was then diluted with three volumes of W5 solution (154 mM NaCl, 125 mM CaCl_2 , 5 mM KCl, 2 mM MES, pH 5.8) and washed twice by centrifuging at 100 x g for 2 min. Protoplasts were re-suspended in 300 μl of WI solution (4 mM MES, 0.5 M mannitol, 20 mM KCl, pH 5.8) and incubated in the dark overnight at 28°C to induce gene expression. Protoplasts were collected by centrifuging at 100 x g for 5 min and carefully pipetting out the supernatant. Samples were then stored at -80°C until nucleic acid extractions.

2.2.6.2. VIGS silencing of *MeRPPL1* and *MeTNL*: Plant growth and inoculation of VIGS clones

Nodal cutting of cassava TME3 landrace were micro-propagated on full Murashige and Skoog (MS) medium [346] supplemented with 2% sucrose, 0.78% plant tissue culture agar, 0.002 mM CuSO_4 and a pH of 5.8. To induce roots growth, the nodal cultures were incubated in a temperature-controlled growth room at 28°C under a 12-hr photoperiod at a light intensity of 150 $\mu\text{Em}^{-2}/\text{s}$. Once the roots appeared (approximately 3 weeks), the plantlets were transplanted into 40 mm x 45 mm Jiffy® pellets (Jiffy International AS), placed in a plastic container and covered with a plastic film for acclimatization. Two slits were made to the plastic film to gradually acclimatize plantlets until they reached a 4-6 leaf stage.

VIGS vector transformed *Agrobacterium Agl1* glycerol stocks were grown on 0.05% YEP medium supplemented with 100 mg/ml kanamycin at 28°C overnight with shaking at 200 rpm, followed by sub-culturing in 0.5% YEP medium until OD₆₀₀ between 1.8 – 2.0 was reached. Agl1 cells were then collected by centrifuging at 12 500 x g for 15 min, followed by a wash with double distilled water for 10 min. Cells were then resuspended in 0.1% fresh YEP medium before inoculating plants. Plants from three independent biological replicates were inoculated along the stem with 100 µl VIGS vector transformed (pC8A-MeRPPL1, pC8A-MeTNL, pC8A-GFP) Agl1 plus SACMV-DNA B infectious clone (1:1 VIGS vector:DNA A ratio) using a hypodermic needle. Each biological replicate comprised of eight groups (pC8A-MeRPPL1 + DNA B, pC8A-MeRPPL1 + SACMV, pC8A-MeTNL + DNA B, pC8A-MeTNL + SACMV, pC8A-GFP + DNA B, pC8A-GFP + SACMV, SACMV-only, and Agl1 mock) and each group comprising of nine plants. Plants were then covered with plastic film for re-acclimatization. Seven days post VIGS inoculation, plants were challenged by agro-inoculation with SACMV-DNA-A and DNA-B infectious clones [337] and re-acclimatized further. Plants were then monitored for symptoms and two leaves below the apex were collected at three time points; early, middle and late, representing 16, 28 and over 75 days post SACMV challenge (dpi), respectively.

2.2.7. DNA extraction and viral load

Genomic DNA was extracted from SACMV-VIGS-infected leaf tissue from three biological replicates, each comprising of three technical replicates using a CTAB-based method [347] with modifications. CTAB extraction buffer was supplemented with 40 mg/ml polyvinylpyrrolidone (PVPP) and 0.2% 2-mercaptoethanol before the buffer was preheated at 65°C for 10 min. A total of 500 µl of CTAB buffer was added to 0.5 g ground leaf tissue and incubated at 65°C for 45 min. Chloroform-isoamyl alcohol (24:1 ratio) was added to the samples, followed by gentle mixing and centrifuging at full speed for 10 min. The top aqueous phase was transferred into a new tube and the latter step repeated. Ice cold isopropanol (1:1 ratio) was added, samples thoroughly mixed and incubated in the freezer for 45 min. Samples were then centrifuged at full speed for 10 min and the DNA pellet was washed with ice cold 70% and 95% ethanol, respectively and air-dried. The DNA was resuspended in nuclease free water with 1% RNase and incubated at 37°C for 1 h to allow digestion. Thereafter, the DNA was stored at 4°C overnight, then quantified and stored at -20°C.

For CRISPR, DNA was extracted from transfected protoplasts using a user-developed protocol to isolate genomic DNA from the interphase and organic (phenol) phase of fatty tissue samples treated with QIAzol Lysis Reagent (www.qiagen.com/it/resources/, QIAGEN, Maryland, USA). In short, any RNA aqueous phase residues were removed first, then 0.3 ml of 100% ethanol was added to the

remaining phases and mixed by inversion and incubated at room temperature for 2 min. Samples were centrifuged to sediment DNA at 4°C for 2 min at 2000 x *g* and supernatant removed before 1 ml 0.1 M sodium citrate in 10% ethanol was added to the DNA pellet, incubated for 30 min at room temperature with mixing every 5 min. Samples were centrifuged as above, supernatant removed and step repeated two more times. The DNA pellet was washed with 2 ml of 75% ethanol by incubation at room temperature for 20 min, mixing every 5 min, and centrifuging for 5 min. The pellet was air-dried and redissolved in 8 mM NaOH, centrifuged at full speed for 10 min at room temperature, and neutralized by adding 60 µl 0.1 M HEPES and 5.5 µl 100 mM EDTA per 500 µl 8 mM NaOH. Thereafter, Thermo Scientific GeneJET Gel Extraction and DNA Cleanup Micro Kit was used to clean up the DNA according to manufacturer's protocol. The DNA was then fast digested with restriction enzyme DpnI (ThermoFisher Scientific, Massachusetts, USA) before use in qPCR.

DNA (25 ng) was used as template for SACMV relative viral load quantification using qPCR. Quantification was done in triplicates using Maxima SYBR Green/ROX qPCR Master Mix (2X) (ThermoFisher Scientific) with SACMV CP primers (Fwd: 5' acgtccgtcgcaagtac 3', Rev: 5' attgtcatgtcgaatagtacg 3'). *UBQ10* and *18S rRNA* were used as reference genes for VIGS silencing and CRISPR knockdown qPCR quantifications, respectively. SACMV viral load were calculated using the log $2^{(\Delta\Delta CT)}$ method [348]. Student's t-test was used to access statistical differences between samples.

2.2.8. RNA extraction and RT-qPCR gene expression analysis

RNA was extracted from both transfected protoplasts and VIGS ground leaf tissue using QIAzol Lysis Reagent (QIAGEN) according to manufacturer's protocol. RNA from protoplasts was then cleaned up using GeneJET RNA Cleanup and Concentration Micro Kit. RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific) was used to synthesise first strand cDNA using Oligo(dT)18 primer according to manufacturer's protocol. For relative gene expression, RT-qPCR was done in triplicates with cDNA as template using Maxima SYBR Green/ROX qPCR Master Mix (2X) with MeRPPL1 (Fwd: 5' agctcttgtgtatgggtgcc 3', Rev: 5' atggtgcccataatggaggc 3') and MeTNL (Fwd: 5' acttgaagggcattcgagg 3', Rev: 5' atcatccaagtgccaccag 3') primers. *UBQ10* and *18S rRNA* were used as reference genes for VIGS silencing and CRISPR knockdown qPCR quantifications, respectively. Gene expression fold change was calculated using the log $2^{(\Delta\Delta CT)}$ method [348]. Student's t-test was used to access statistical differences genes.

2.2.9. Sequencing and mutagenesis analysis

DpnI-digested DNA was sent for sequencing of *MeRPPL1* and *MeTNL* gene regions inclusive of the two gRNAs at Inqaba Biotech (Pretoria, South Africa) using the ABI 3500XL Genetic Analyzer. SnapGene software was used to assess variation (<https://www.snapgene.com/>), while MEGAX software was used for multiple sequence alignment using ClustalW algorithm [349]. Target gene homologs from Wild-type AM560-2 (Accession NC_035172.1) and *Manihot esculenta* cultivar TME3 (RefSeq ID RSFT01000007, GenBank ID GCA_003957995.1, unpublished data) were used as references for the alignments. Frequency of clones with altered sequence was obtained by expressing a number of amplicons from polyclonal mix with sequence alteration as a ration of 10 amplicons sequenced.

2.3. Results

2.3.1. Datamining and characterization of selected target resistance gene analogs (RGAs)

Two RGAs with accessions Manes.12G149700 (*MeTNL*) and Manes.12G091600 (*MeRPPL1*) were selected as target genes for silencing. These genes are located on chromosome 12, known to be associated with the CMD2 QTL for dominant CMD resistance in cassava. *MeTNL* is a TIR-NBS-LRR class disease resistance protein encoding gene found on the reverse strand of chromosome 12 (locus position 30792584...30797830). It is involved in signal transduction (GO:0007165), ADP binding (GO:0043531) and protein binding (GO:0005515). Its *A. thaliana* homolog AT5G36930.2 is annotated to be involved in defense response and signal transduction. *MeRPPL1* is a NB-ARC domain-disease resistance protein encoding gene found on the reverse strand of chromosome 12, locus position 13894519...13896410. Manes.12G091600 contains two domains namely NBS and LRR (Table 2-1). The *A. thaliana* ortholog, AT3G14470.1, Putative disease resistance RPP13-like protein 1 (RPPL1; Source: UniProtKB/Swiss-Prot; Acc:Q9LRR4) is involved in defense response (GO:0006952), salicylic acid biosynthesis (GO:0009697) and systemic acquired resistance (GO:0009627) and located extracellularly on the cell membrane (GO:0005576). Unexpectedly, Manes.12G091600 peptide sequence was found to be highly similar to other six *M. esculenta* peptides. The latter, referred to as paralogs are listed in Table 2-1. Paralogous genes are believed to be a result of gene duplication within the genome of one species. Amino acid sequence percentage identity matrix showed that the paralogs that have at least 90% amino acid sequence homology to *MeRPPL1* (Appendix 2-Table 1), and they are located on chromosome 9.

Table 2-1. Description of cassava *MeTNL* (Manes.12G149700) & *MeRPPL1* (Manes.12G091600) and its paralogs (underlined).

Gene ID	Genomic (bp)	Transcript (bp)	CDS (bp)	Peptide (aa)	Biological Process	Molecular Function	Cellular Component	Domains Present [#]	Domain Position ^{#+} (bp)
Manes.12G149700	5247	4150	3432	1143	Signal Transduction	ADP Binding	None Predicted	TIR	12-150
								NB-ARC	190-410
								LRR	779-800; 804-825; 827-849
Manes.12G091600	1892	1746	1434	477	None Predicted	ADP Binding	None Predicted	NB-ARC	12-99
								LRR	241-428
<u>Manes.09G023800</u>	4807	2587	2544	847	None Predicted	ADP Binding	None Predicted	Rx-N	13-102
								CC	14-136
								NB-ARC	173-421
								LRR	494-747; 748-822
<u>Manes.09G023200</u>	4266	2572	2541	846	None Predicted	ADP Binding	None Predicted	Rx-N	13-102
								CC	14-136
								NB-ARC	173-421
								LRR	505-833
<u>Manes.09G023900</u>	3811	2538	2538	845	None Predicted	ADP Binding	None Predicted	Rx-N	13-102
								CC	14-136
								NB-ARC	172-419
								LRR	562-743; 744-820
<u>Manes.09G023300</u>	4335	2751	2538	845	None Predicted	ADP Binding	None Predicted	Rx-N	13-101
								CC	14-136
								NB-ARC	173-420
								LRR	496-744; 745-818
<u>Manes.09G022600</u>	2428	2244	2244	747	None Predicted	ADP Binding	None Predicted	NB-ARC	54-301
								LRR	428-626
<u>Manes.09G022500</u>	10411	2891	2571	856	None Predicted	ADP Binding; Protein Binding	None Predicted	Rx-N	13-102
								CC	14-136
								NB-ARC	173-420
								LRR	580-602; 603-625; 627-647

[#] Domains and domain positions within genes were retrieved from the European Bioinformatics Institute (EMBL-EBI) protein sequence analysis and classification resource, InterPro (<https://www.ebi.ac.uk/interpro/protein>).

⁺ More than one domain positions indicate domain repeats.

The NB-ARC domain region of MeTNL, and MeRPPL1 and its six paralogs was assessed by aligning their amino acid sequences and screening for five different conserved motifs. An NB-ARC containing human Apaf1 (GenBank accession ABQ59028.1) protein and two *Populus trichocarpa* TIR-NBS-LRR resistance proteins ABF81419.1 (GenBank accession ABF81419.1), and ABF81465.1 (GenBank accession ABF81465.1) were used for the alignments. The P-loop motif is defined by the consensus sequence GXXXXGKS/T, where X represents any amino acid. Resistance protein alignments demonstrated a P-loop consensus sequence of GIGGLGKT (Figure 2-1). Paralog Manes.09G022500.1 has a single amino acid mutation of the first glycine (G) to an alanine (A), whereas the other paralogs and MeTNL had sequences that were not different from the P-loop consensus. MeRPPL1 does not have motifs P-loop, Walker B and RNBS-B found in the NBS region of the NB-ARC domain. Walker B is recognised by the consensus sequence hhhhDD/E, where h represents any hydrophobic residue. The consensus sequence LLILDD was deduced from the alignment and all the proteins followed this consensus although there were some differences in the hydrophobic residues in the TIR-NBS-LRR proteins (Figure 2-1). The conserved aspartic acid (D) in the 5th position was present in all the proteins.

Paralog Manes.09G023200 has five out of ten mutations on amino acid position 2,3,5,6 and 10 of the RNBS-B motif consensus sequence GSRIITTRD, while the other five paralogs had a consensus sequence GSKILVTTRS with four mutations at positions 3, 5, 6 and 10 (Figure 2-1). MeTNL does not have any mutations on this motif, whereas the other two *P. trichocarpa* TIR-NBS-LRR proteins have each a single amino acid mutation at position 4 where an isoleucine (I) was replaced with a valine (V). Human Apaf1 protein had four out of ten amino acid similarity to the motif's consensus sequence. The GxP motif found on the ARC1 region of the NB-ARC domain has a conserved consensus sequence of CGGLPLA. MeRPPL1 and its paralogs have two mutations at positions 2 and 4, where the first G and leucine (L) are replaced with lysine (K) and V, respectively. MeTNL has mutations of the first two amino acids; cysteine (C) and G replaced with A and arginine (R), respectively. Apaf1 has the sequence CKGSPLV and three mutations at positions 2, 4, and 7 (Figure 2-1). The MHD motif found on the ARC2 region of the NB-ARC domain has the consensus sequence hxhHD, with histidine and D amino acids highly conserved. Only MeTNL has a mutation in the conserved region, where aspartic acid was replaced with a hydrophobic residue A.

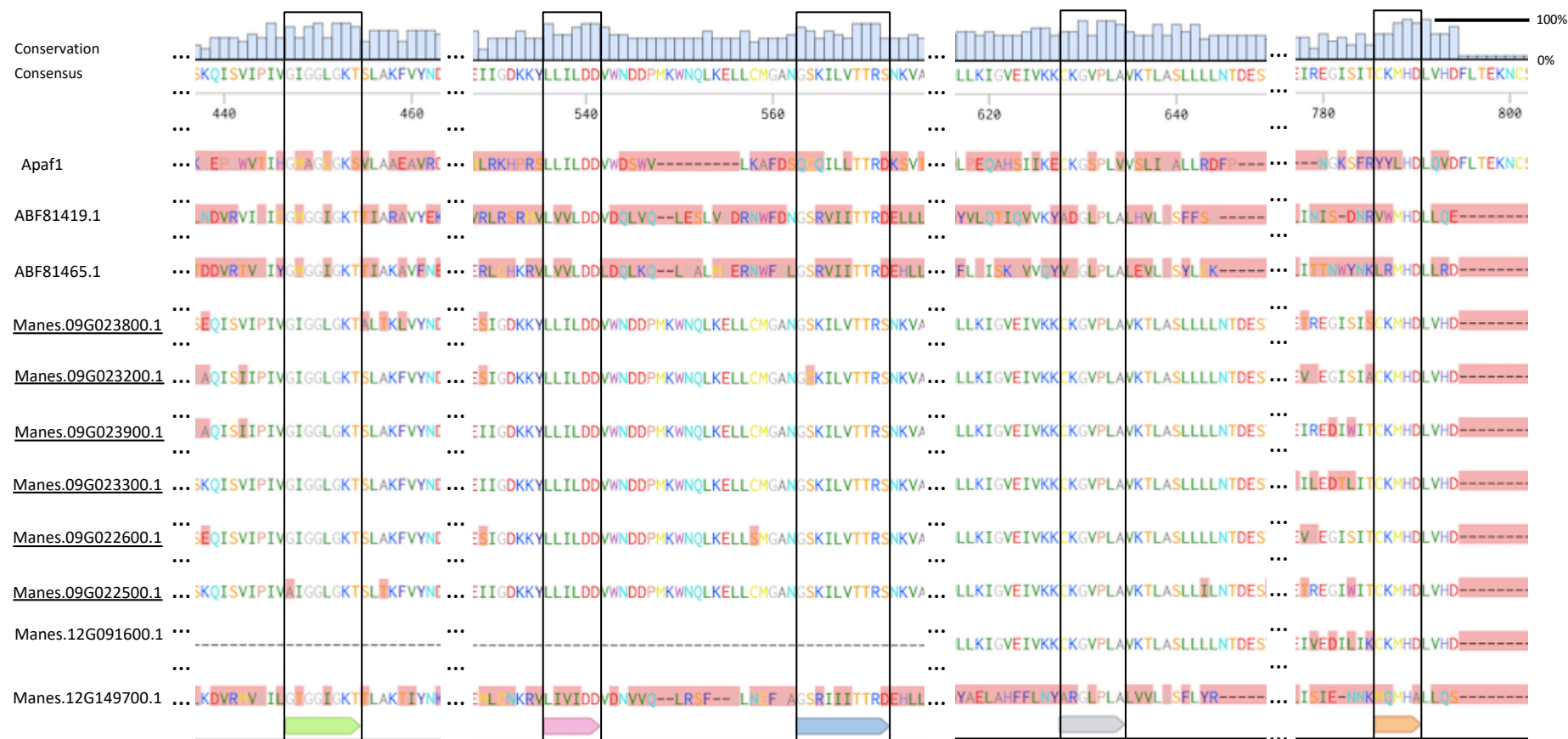


Figure 2-1. Conserved motifs of the NB-ARC domain of resistance proteins. **Green arrow**=P-loop/Walker A motif, **Pink arrow**=Walker B motif, **Blue arrow**=Resistance Nucleotide Binding Site (RNBS)-B, **Grey arrow**= GxP motif, **Orange arrow**= MHD motif.

Molecular phylogenetic analysis using Maximum Likelihood method based on the JTT matrix-based model was used to gain some insight into the diversity of the two selected chromosome 12 resistance proteins and the paralogs. The tree was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value and the tree with the highest log likelihood of -3828.66 is shown. Manes.12G091600 (non-TIR) grouped with its paralogs while Manes.12G149700 (TIR) grouped with the two TIR-NBS-LRR reference proteins (Figure 2-2).

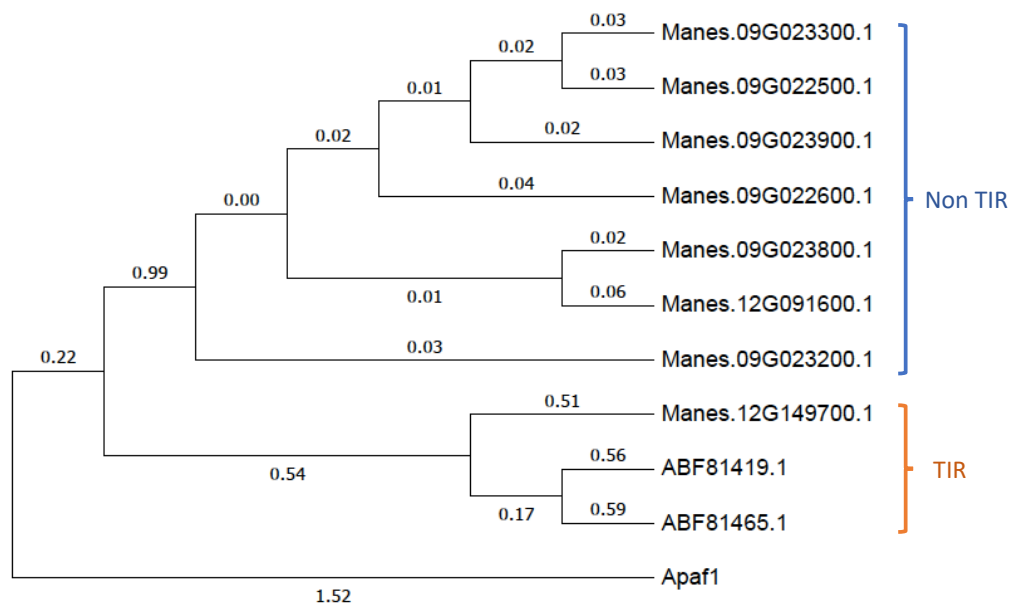


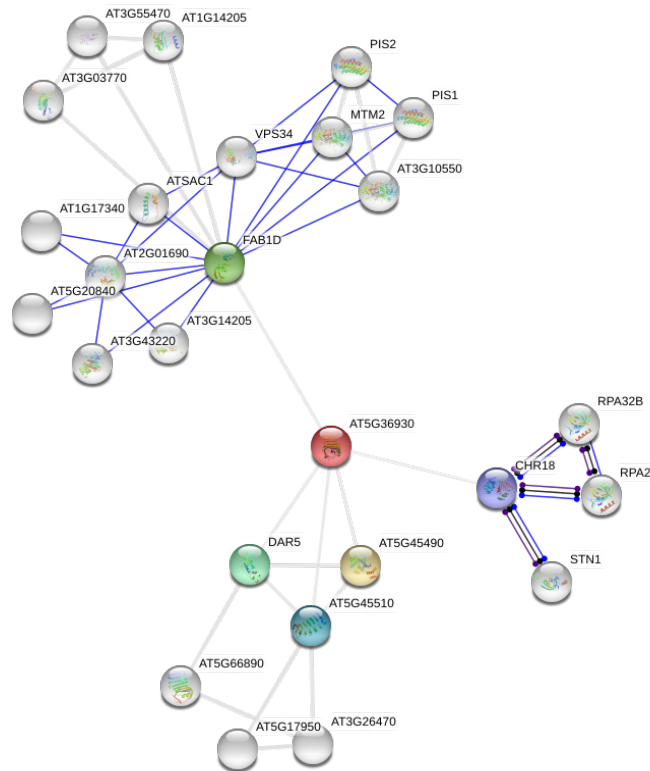
Figure 2-2. Molecular phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (-3828.66) is shown. Branch lengths are measured in the number of substitutions per site (next to the branches). The analysis involved 11 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 271 positions in the dataset. Evolutionary analysis was conducted in MEGA7.

MeTNL (AT5G36930) STRING network shows five direct interactions with DA1-related protein 5 (DAR5), chromatin remodelling factor18 (CHR18), forms aploid and binucleate cells 1A (FAB1D), and two probable disease resistance proteins AT5G45490 and AT5G45510 (Figure 2-3A). DAR5 and AT5G45490 were annotated to contain the NB-ARC domain and both function in ADP binding. However, DAR5 is also thought to be involved in ubiquitin binding in *Arabidopsis thaliana* (TAIR). AT5G45510 is a leucine-rich repeat family protein involved in ATP binding and localised in the nucleus, plasma membrane and in the plastid. FAB1D is involved in phosphatidylinositol phosphate biosynthetic process. KEGG analysis of the direct interactors and secondary interactors were annotated to eight pathways

including mismatch repair, DNA replication, phosphatidylinositol signalling system, homologous recombination, and regulation of autophagy (Appendix 2-Table 2).

MeRPPL1 (AT3G14470) network showed ten direct interactions with DA1-related protein 5 (DAR5), Anthranilate synthase alpha subunit 2 (ASA2), Cation/calcium exchanger 7 (CAX7), and the last seven proteins being from the disease resistance protein family; AT2G34930, AT5G46520, ATG72950, AT3G04210, AT1G72890, AT5G45510 and receptor-like protein 36 (RLP36) (Figure 2-3B). ASA2, together with its other interactors, play roles in biosynthesis of amino acids and secondary metabolites. CAX7 is involved in potassium and sodium ion transport and transmembrane transport, and has antiporter activity. The other proteins from the disease resistance family are involved in signal transduction and defense responses. ATG72950, AT1G72890 and AT3G04210 are also involved in innate immune response, namely apoptosis. KEGG analysis revealed that these MeRPPL1 interactors are involved in six different metabolic pathways such as amino acid metabolism and biosynthesis and biosynthesis of plant hormones (Appendix 2-Table 2).

A



B

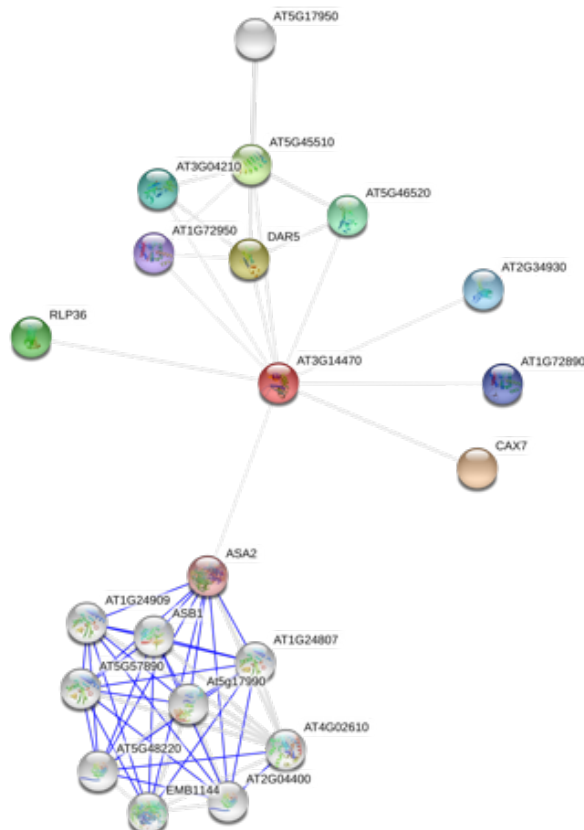


Figure 2-3. STRING protein-protein interaction analysis of (A) Manes.12G149700 *A. thaliana* homolog AT5G36930 and Manes.12G091600 (B) homolog AT3G14470.

2.3.2. Silencing constructs confirmation

All silencing constructs were successfully cloned, three into pC8A VIGS vector and two into pCambia 1380 CRISPR-Cas vector. Confirmation was done using restriction enzyme digestion and sequencing. When pC8A-MeRPPL1 vector was digested with XhoI and XbaI, it resulted in a 761 bp band pointed at by the blue arrow, corresponding to MeRPPL1 construct (Figure 2-4A). pC8A-MeTNL digestion with XhoI and SacI, resulted in a band of 803 bp (Figure 2-4B). pC8A-GFP digestion with HindIII and SacI resulted in a band of 1071 bp, which corresponded to GFP construct (Figure 2-4C). For CRISPR-Cas, pC1380-MeRPPL1 digested with HindIII and BglII, and pC1380-MeTNL digested with HindIII and Eco72I, resulted in bands of approximately 760 bp (Figures 2-4D, 2-4E), respectively. The differences between the two constructs are the gRNA target sites which are specific to each gene.

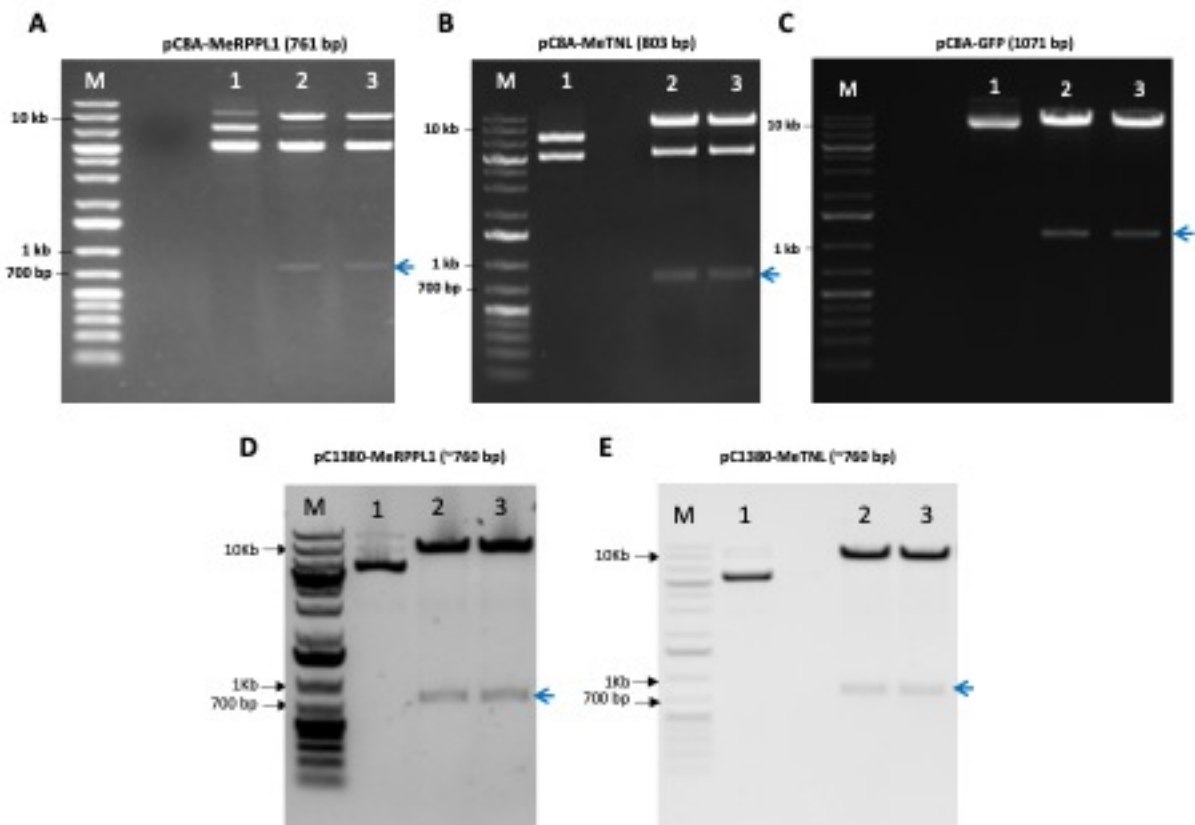


Figure 2-4. Silencing constructs confirmation using restriction enzyme digestion and agarose gel analysis. **(A)**, VIGS vector pC8A-MeRPPL1 digested with XhoI + XbaI. B resulting in a construct of 761 bp. **(B)**, VIGS vector pC8A-MeTNL digested with XhoI + SacI resulting in a construct of 803 bp. **(C)** VIGS vector pC8A-GFP digested with HindIII + SacI resulting in a construct of 1071 bp. **(D)** CRISPR vector pC1380-MeRPPL1 digested with HindIII + BglII resulting in a construct of approximately 760 bp. **(E)** CRISPR vector pC1380-MeTNL digested with HindIII + Eco72I resulting in a construct of approximately 760 bp. **M** = 1 Kb Plus molecular marker, **Lane 1** = Undigested vector, **Lanes 2 & 3** = Digested vectors. **Blue** arrows indicate silencing constructs. Samples were analysed on 1% agarose gel, with 1 µg/ml ethidium bromide.

2.3.3. Protoplast isolation and viability

Protoplasts were successfully isolated from 4-week-old leaf mesophyll cells of cassava TME3 plantlets propagated and grown under sterile and controlled conditions. When analysed under a light microscope, spherical intact protoplasts were observed as well as protoplasts with irregular shapes (Figure 2-5A). Diameter was measured from at least ten spherical protoplasts and the range for these was between 16.1 – 38.9 μm . Chloroplasts were observed around the edge of protoplasts cell membrane. Before transfection, protoplasts were assessed for viability using Evans Blue dye and as seen in Figure 2-5B, at least 90% were viable. In addition, flow cytometry was used to measure the integrity of protoplasts depending on their forward scatter (FSC) and side scatter (SSC) gating [350] and $3.8 \times 10^5/\mu\text{l}$ cell count was recorded (Figure 2-5C).

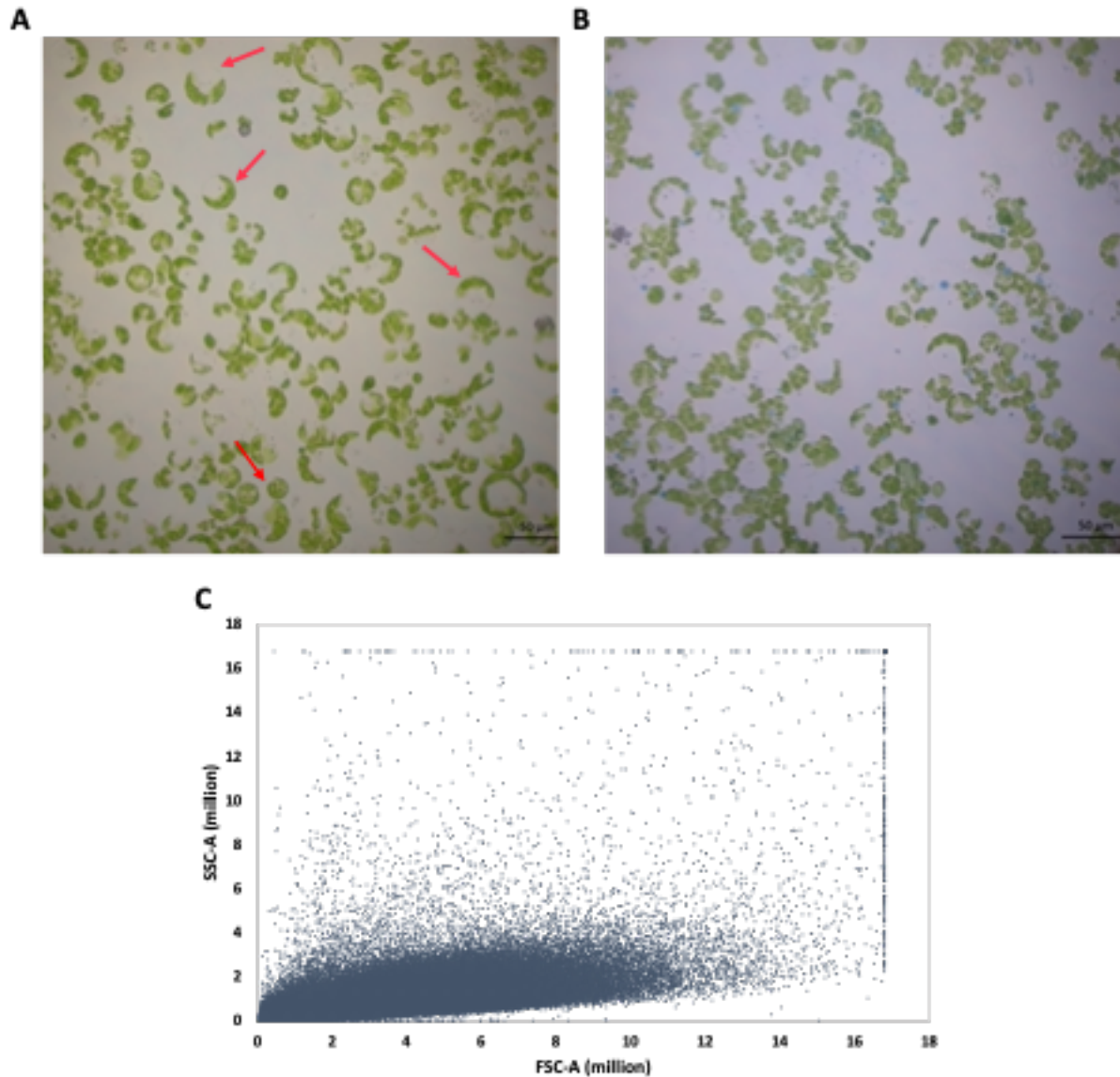


Figure 2-5. Analyses of the quality and quantity of protoplasts isolated from cassava TME3 landrace. **(A)**, Cassava protoplasts isolated from leaf mesophyll cells. **Red** arrows show spherical protoplasts with chloroplasts around the edge of the cell membrane. **(B)**, Evans Blue dye cell viability assessment on isolated protoplasts visualized under a light microscope. Viable cells do not take up the blue dye, while non-viable cells take up the blue dye. **(C)**, Protoplasts' cell count and quality measured using flow cytometry density measurement based on side scatter (SSC-A) and forward scatter (FSC-A).

2.3.4. Assessing CMD symptoms following VIGS silencing

Disease symptom evaluations of TME3 plants following VIGS silencing vector treatment and controls are depicted in Figure 2-6. *Agrobacterium Agl1*-mocked plants did not show any disease symptoms at any of the three time points (16, 28, and 75 dpi). At an early time point (16 dpi), treatment plants inoculated with pC8A-MeRPPL1 + SACMV (RS) and pC8A-MeTNL + SACMV (TS) vectors started showing

minor leaf distortion, while control plants inoculated with pC8A-GFP vector and SACMV-only remained non-symptomatic (Figure 2-6).

At the middle time point (28 dpi), severe distortion and yellow/pale green mosaic mostly around the leaf midrib connecting to the petiole was observed on leaves from plants inoculated with SACMV-only and pC8A-GFP + SACMV (controls), and pC8A-MeRPPL1 + SACMV (treatment). Plants inoculated with pC8A-MeTNL + SACMV (treatment) however, only showed minimal chlorosis and leaf distortion at this time point. Treatment plants inoculated with pC8A-MeRPPL1 + DNA B and pC8A-MeTNL + DNA B, and pC8A-GFP + DNA B vector only control plants not challenged with SACMV were still non-symptomatic at middle time point, indicated by white arrows (Figure 2-6).

At the late time point (75 dpi), pC8A-MeRPPL1 + SACMV (treatment), pC8A-MeTNL + SACMV (treatment), pC8A-GFP + SACMV (control), and SACMV-only (control) inoculated plants only showed minor leaf chlorosis and distortion CMD symptoms, representing some level of recovery on newly developed leaves compared to severe symptoms seen at 28 dpi. For the first time, leaves below the newly developed apex leaf of plants inoculated with pC8A-MeRPPL1 + DNA B, pC8A-MeTNL + DNA B, and pC8A-GFP + DNA B (control) had developed minor distortion with no mosaic (Figure 2-6).

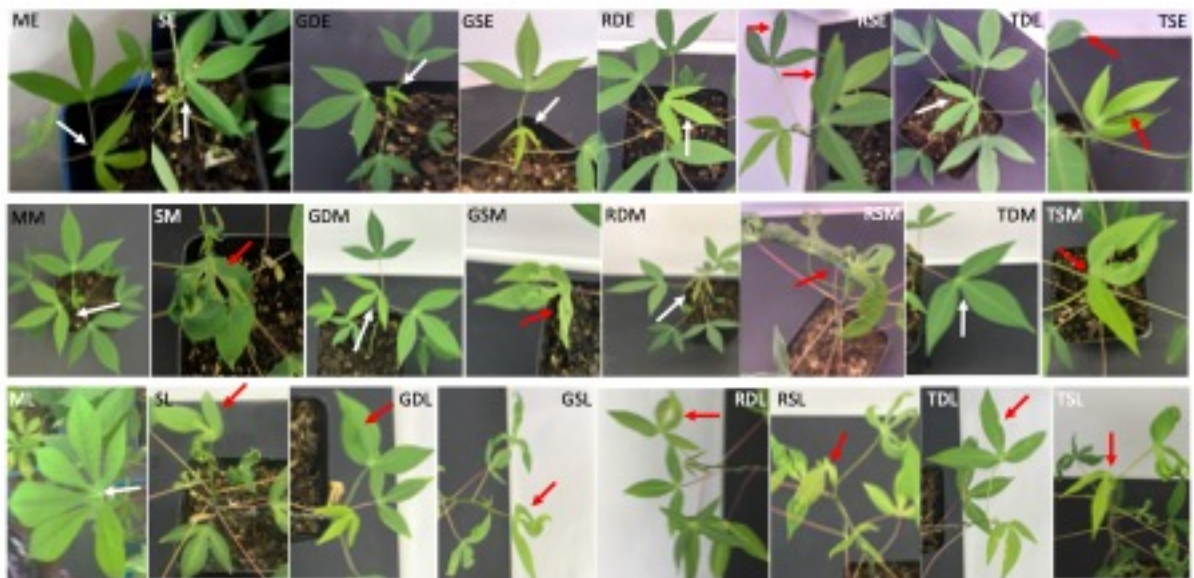


Figure 2-6. Symptoms evaluation on TME3 landrace inoculated with VIGS vectors, SACMV and Agrobacterium Agl1. **M** = Mock, **S** = SACMV infected, **GD** = pC8A-GFP + DNA B, **GS** = pC8A-GFP + DNA B + SACMV challenge, **RD** = pC8A-MeRPPL1 + DNA B, **RS** = pC8A-MeRPPL1 + DNA B + SACMV challenge, **TD** = pC8A-MeTNL + DNA B, **TS** = pC8A-MeTNL + DNA B + SACMV challenge, **E** = Early time point (16 dpi), **M** = Middle time point (28 dpi), **L** = Late time point (75 dpi). **White** arrows indicate non-symptomatic leaves, while **red** arrows indicate symptomatic leaves.

2.3.5. SACMV infection quantification

The accumulation of SACMV was assessed on plants inoculated with VIGS silencing vectors and SACMV, by quantifying the coat protein (*CP*) gene found on DNA A component of SACMV reference to cassava *UBQ10* gene using qPCR technique (Figure 2-7A). Relative viral load was performed and measured on leaf samples from plants that were inoculated with pC8A-GFP + SACMV (vector-only control), SACMV only (control), pC8A-MeRPPL1 + SACMV (treatment), and pC8A-MeTNL + SACMV (treatment). Relative qPCR was not done on samples from plants inoculated with VIGS silencing vectors + DNA B since the pC8A VIGS vector lacks *CP* gene [345]. Under-expression of *CP* was observed in all treatments and control samples at an early time point (16 dpi), indicating that there was low SACMV DNA A accumulation relative to *UBQ10* (Figure 2-7A). At the middle time point (28 dpi), the introduction of the silencing VIGS vectors significantly induced higher accumulation of SACMV DNA A in those plants when compared to control plants infected with SACMV only. pC8A-MeRPPL1 + SACMV plants had a 12.6-fold accumulation of SACMV DNA A, highest at 28 dpi, followed by pC8A-GFP + SACMV (10.1-fold), pC8A-MeTNL + SACMV (8-fold), and SACMV-only (6.7-fold) plants, at this time point. An insignificant increase in SACMV DNA A accumulation was observed in all leaf samples from TME3 at symptom recovery (75 dpi), except for pC8A-GFP + SACMV control plants which had a statistically significant higher DNA A accumulation of 11.4-fold compared to 7.7-fold in SACMV-only infected plants (Figure 2-7A). pC8A-MeRPPL1 + SACMV inoculated plants retained the highest SACMV virus accumulation with a fold change of about 12.4, however it was not significant when compared to that of plants infected with SACMV only. SACMV DNA A accumulation increase by 0.9-fold in pC8A-MeTNL + SACMV plants from 28 dpi to 75 dpi, but it was not significant.

The effects of SACMV on VIGS-silenced TME3 plants were further quantified by looking at the severity of CMD symptoms development in these plants using a symptom scoring scale (Figure 2-7B). pC8A-MeTNL + SACMV and pC8A-MeRPPL1 + SACMV plants scored 0.5 at an early time point (16 dpi). Plants inoculated with pC8A-MeRPPL1 + SACMV (score 5) and SACMV-only (score 4.7) both exhibited intense widespread leaf mosaic with severe leaf deformation and reduction in leaf size, achieving the highest scores at 28 dpi. However, comparing SACMV DNA A accumulation at 28 dpi, pC8A-MeRPPL1 + SACMV plants had significantly higher accumulation than SACMV-only infected plants (Figure 2-7A). Similarly, pC8A-MeTNL + SACMV (TS) and pC8A-GFP + SACMV (GS) control plants had the same symptoms score of 4.3, however, SACMV DNA A accumulation differed significantly between the two, GS with 10.1-fold and TS with 8.0-fold. At 75 dpi, pC8A-MeRPPL1 + SACMV and SACMV-only plants both had clear mosaic with slight leaf deformation (score 2), while pC8A-GFP + SACMV plants had a symptom score of 1.7 and pC8A-MeTNL + SACMV had the lowest score of 1.3. As observed in Figure 2-6, plants that

were infected with VIGS vectors + DNA B only (no SACMV challenge) had mild leaf deformation and no chlorosis, 0.5 score for pC8A-GFP +DNA B (GD) and pC8A-MeTNL + DNA B (TD), and score 1.0 for pC8A-MeRPPL1 + DNA B (RD) at 75 dpi.

SACMV DNA A viral accumulation in protoplasts at 24 h post transfections showed that protoplasts that were only transfected with pBIN19-SACMV-DNA A and pBIN19-SACMV-DNA B infectious clones (S) had the highest accumulation of 2.5-fold with reference to *18S rRNA* (Figure 2-7C). Protoplasts transfected with pC1380-MeRPPL1 and pC1380-MeTNL vectors + Cas9 + SACMV both had an elevated expression of SACMV *CP* gene DNA with 1.6 and 1.5-fold, respectively.

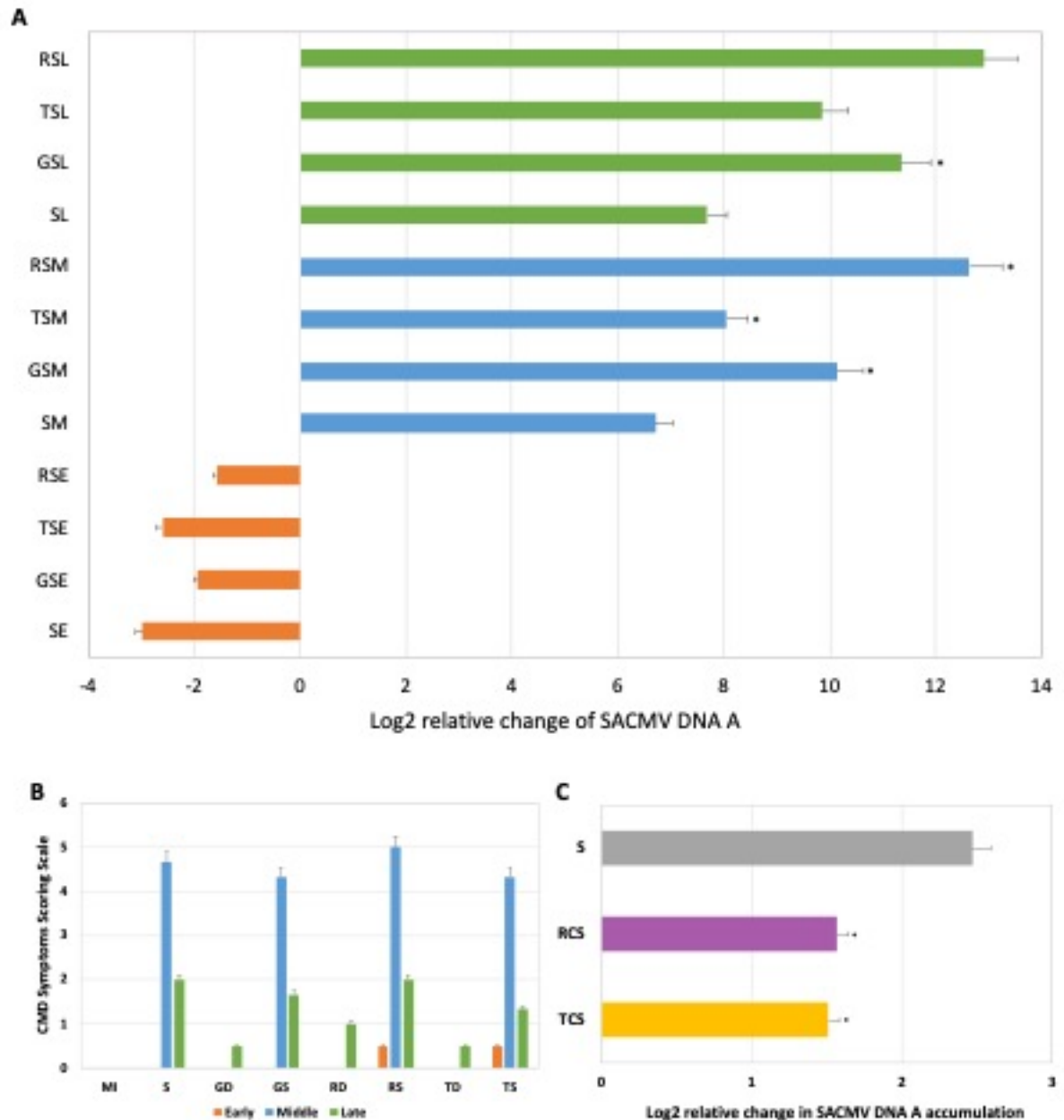


Figure 2-7. (A), Relative accumulation of SACMV DNA A relative to *UBQ10* gene following TME3 VIGS silencing. **S** = TME3 infected with SACMV, **GS** = pC8A-GFP + DNA B + SACMV, **RS** = pC8A-MeRPPL1 + DNA B + SACMV, **TS** = pC8A-MeTNL + DNA B + SACMV. **E** = Early time point (16 dpi), **M** = Middle time point (28 dpi), **L** = Late time point (75 dpi). **(B)**, Cassava mosaic disease symptoms scoring following VIGS silencing. **MI** = Agl1 mock--inoculated, **GD** = pC8A-GFP + DNA B, **RD** = pC8A-MeRPPL1 + DNA B, **TD** = pC8A-MeTNL + DNA B, **S** = TME3 infected with SACMV. **(C)**, Relative accumulation of SACMV DNA A relative to *18S rRNA* gene in protoplasts following CRISPR knockdown, **RC** = pC1380-MeRPPL1 + Cas9, **TC** = pC1380-MeTNL + Cas9, **S** = TME3 infected with SACMV. Error bars represent means $\pm$ S.D. ($n = 3$). Statistical differences were measured at $p \leq 0.05$ using T-test. Asterisks indicate statistically significance compared to SACMV-infected sample at $p \leq 0.05$. **Scale:** **0** = No symptoms, **0.5** = No chlorosis, slight leaf deformation, **1** = Mild chlorosis, slight leaf deformation, **2** = Clear mosaic with/without slight leaf deformation, **3** = Severe mosaic, distortion reduced size, **4** = Strong mosaic all over the leaf with severe leaf deformation, **5** = Severe mosaic and severe reduction of leaf size.

2.3.6. VIGS silencing of *MeRPPL1* and *MeTNL* in TME3 plants

To silence Manes.12G091600 (*MeRPPL1*) and Manes.12G149700 (*MeTNL*), cassava TME3 plants at a 4-6 leaf stage were agro-injected with pC8A-*MeRPPL1* + DNA B and pC8A-*MeTNL* + DNA B, respectively, and challenged with SACMV DNA A and B seven days later. Real-time qPCR was performed on RNA extracted from treated plants using *MeRPPL1* and *MeTNL* primers relative to *UBQ10* in order to measure the effect and extent of VIGS silencing. The same primers for the target genes were used to amplify mRNA from plants that were inoculated with the pC8A-GFP vector (vector-only control) which does not have a target gene sequence within the cassava genome.

Comparing to pC8A-GFP + DNA B vector only control, *MeRPPL1* was under-expressed in plants inoculated with pC8A-GFP + SACMV (GS), pC8A-*MeRPPL1* + DNA B (RD), and pC8A-*MeRPPL1* + SACMV (RS) with fold change of -1.3, -0.5, and -0.7, respectively at an early time point (16 dpi). At the middle time point (28 dpi), SACMV resulted in a significant over-expression of *MeRPPL1* in pC8A-GFP + SACMV plants (0.7-fold), while plants inoculated with pC8A-*MeRPPL1* + DNA B (-2.6-fold change) and pC8A-*MeRPPL1* + SACMV (-1.8-fold) both had under-expression of *MeRPPL1*. Late time point saw a significant increase in the expression of *MeRPPL1* in all treated plants. pC8A-GFP + SACMV plants had the highest expression with a fold change of 1.3, pC8A-*MeRPPL1* + SACMV with a fold change of 1.0, and pC8A-*MeRPPL1* + DNA B with a fold change of 0.5 (Figure 2-8A).

There was a significant under-expression of *MeTNL* in all treated samples at an early time point, all with a fold change of less than 1.0 compared to the expression in pC8A-GFP + DNA B vector only control plants. *MeTNL* was still under-expressed at 28 dpi (middle time point). pC8A-*MeTNL* + SACMV had a fold change of -3.6, pC8A-GFP + SACMV with a fold change of <-0.5, and pC8A-*MeTNL* + DNA B with a fold change of -1.5. At a late time point (75 dpi), the expression of *MeTNL* exponentially increased in plants inoculated with pC8A-GFP vector only control and challenged with SACMV, resulting in over-expression with fold change of 6.2. pC8A-*MeTNL* + SACMV (-2.2-fold) and pC8A-*MeTNL* + DNA B (-1.4-fold) plants persisted with the under-expression of *MeTNL* at 75 dpi (Figure 2-8B).

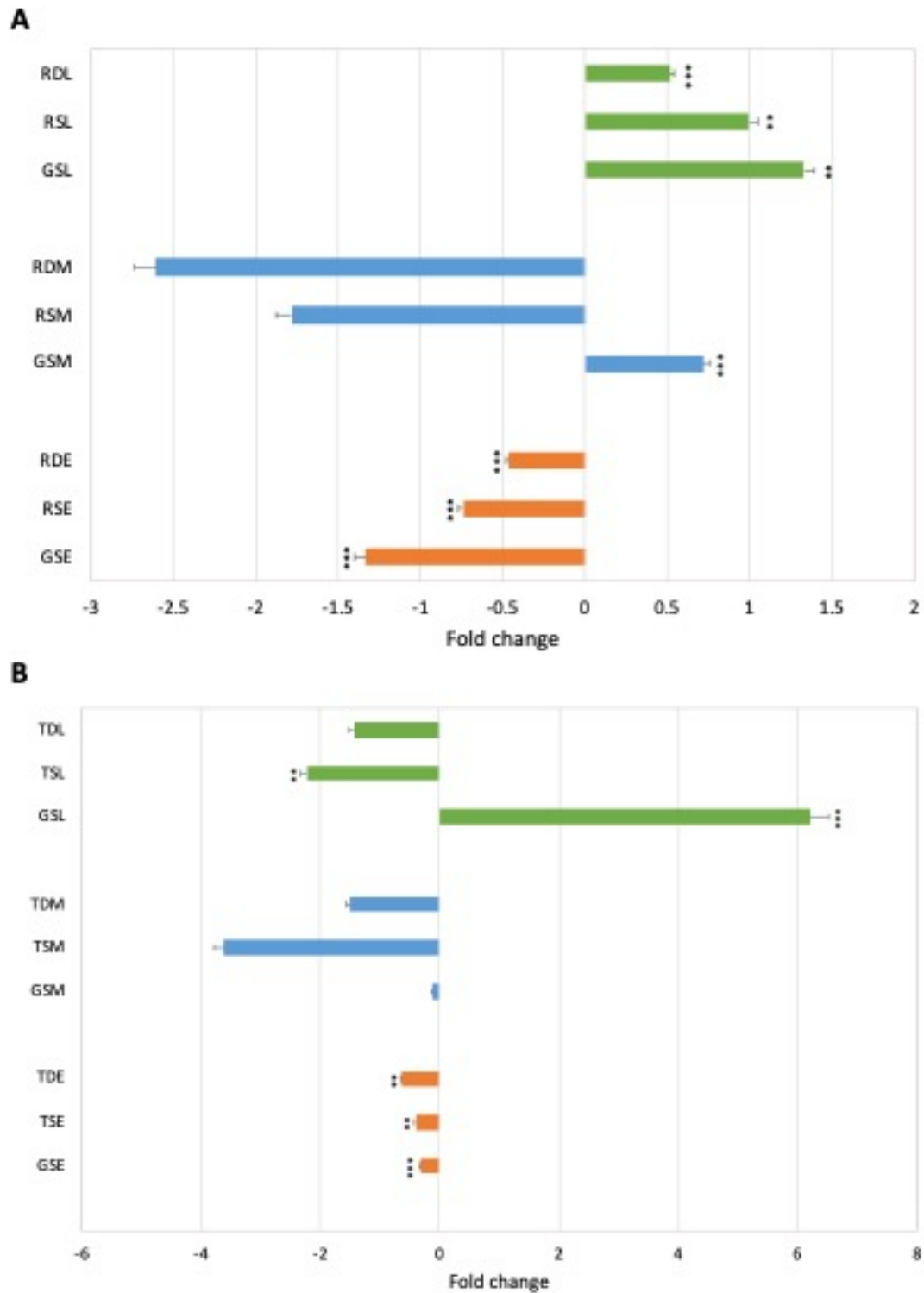


Figure 2-8. Relative expression of (A) MeRPPL1 and (B) MeTNL relative to *UBQ10* gene in cassava TME3 following inoculation with VIGS silencing vectors. **GS** = pC8A-GFP + DNA B + SACMV challenge, **RS** = pC8A-MeRPPL1 + DNA B + SACMV challenge, **RD** = pC8A-MeRPPL1 + DNA B, **TS** = pC8A-MeTNL + DNA B + SACMV challenge, **TD** = pC8A-MeTNL + DNA B, **E** = Early time point (16 dpi), **M** = Middle time point (28 dpi), **L** = Late time point (75 dpi). Error bars represent means $\pm$ S.D. ($n = 3$). Two and three asterisks indicate statistically significant differences of treated samples compared to GFP vector only control, $p \leq 0.01$, and 0.001 , respectively, using t-test.

2.3.7. CRISPR knockdown of *MeRPPL1* and *MeTNL* in TME3 protoplasts

To assess the relative expression of CRISPR-targeted genes in TME3 protoplasts, real time qPCR was performed on RNA extracted from protoplasts using the same *MeRPPL1* and *MeTNL* primers used for VIGS qPCR, with *18S rRNA* as a reference gene. The expression of *MeRPPL1* and *MeTNL* genes in pC1380-*MeRPPL1* + Cas9 and pC1380-*MeTNL* + Cas9 (treatments), respectively was compared to the expression in SACMV only and pC1380 - Cas9 control CRISPR vector protoplasts. Following transfection, protoplasts were given 24 h to allow the expression of genes delivered by CRISPR vectors and silencing target genes. There was a significant over-expression of *MeRPPL1* in treated samples and SACMV-only control when compared to the pC1380 – Cas9 control vector. *MeRPPL1* expression was highest in protoplasts transfected with SACMV only with a fold change of 3.2, followed by 1.6-fold change in pC1380-*MeRPPL1* + Cas9 + SACMV protoplasts, and 0.9-fold change in pC1380-*MeRPPL1* + Cas9 protoplasts (Figure 2-9). *MeTNL* was significantly over-expressed in treated and control protoplasts compared to pC1380 – Cas9 control vector, with the highest expression observed from protoplasts transfected with pC1380-*MeTNL* + Cas9 with fold change of 2.6. SACMV-only transfected protoplasts had *MeTNL* fold change of 0.8, while pC1380-*MeTNL* + Cas9 + SACMV protoplasts had the lowest fold change of 0.4.

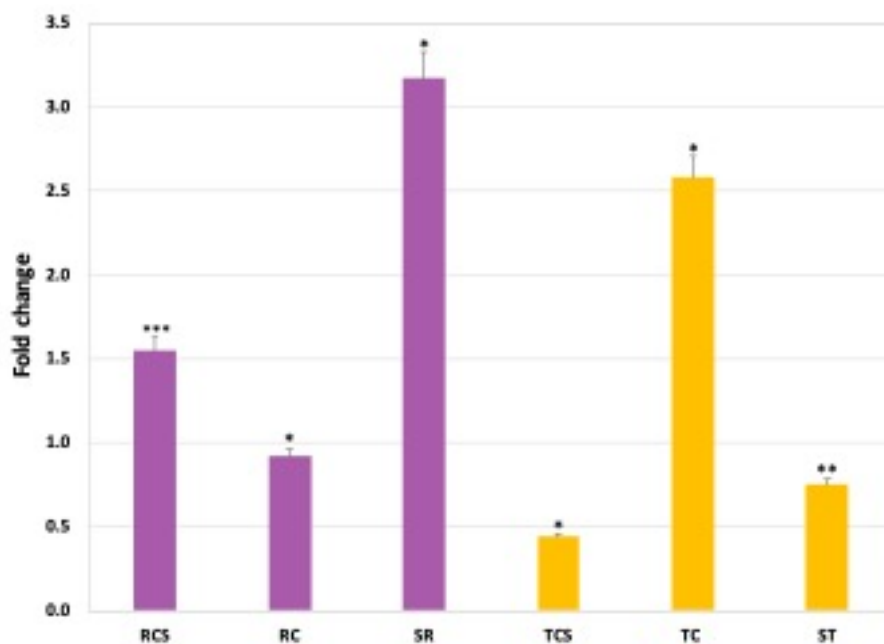


Figure 2-9. Relative expression of *MeRPPL1* and *MeTNL* relative to *18S rRNA* gene in cassava TME3 protoplasts following transfection with CRISPR vectors. **S** = TME3 protoplasts transfected with pBIN19-SACMV-DNA A and pBIN19-SACMV-DNA B infectious clones, **R** = *MeRPPL1*, **T** = *MeTNL*, **RC** = pC1380-*MeRPPL1* + Cas9, **TC** = pC1380-*MeTNL* + Cas9. Error bars represent means $\pm$ S.D. (n = 3). One, two and three asterisks indicate statistically significant differences between treated samples and pC1380 CRISPR vector control, $p \leq 0.05$, 0.01, and 0.001, respectively using t-test.

DpnI-treated DNA extracted from CRISPR-edited and SACMV-infected TME3 protoplasts was sent to Inqaba Biotech (Pretoria, South Africa) for the sequencing of the two CRISPR-targeted genes, *MeRPPL1* and *MeTNL*. Only *MeRPPL1* was able to be successfully sequenced and the results are shown herein. Different primers and conditions were tried for *MeTNL* but there was no amplification. *MeRPPL1* sequencing results of ten amplicons from a polyclonal mix revealed the occurrence of different types of mutations, where substitution mutations occurred randomly at a 90 – 100% frequency, while addition mutation upstream of *MeRPPL1* gRNA2 in CRISPR-silenced + SACMV samples occurred at a 10% frequency (Figure 2-10A). These mutations resulted to at least 40 – 100% frequency in the alteration of amino acid sequences. Interestingly, pC1380-MeRPPL1 + Cas9 + SACMV (TME3 RCS) and SACMV only (TME3 S) samples led to the alteration in the MHD domain (position 177) of the MeRPPL1 peptide, whereas pC1380-MeRPPL1 + Cas9 (RC) samples still had the domain conserved (Figure 2-10B). Looking at the entire *MeRPPL1* sequenced region, it is clear that substitution mutations occurred randomly throughout the gene region leading to changes in amino acid sequences of the peptide, compared to the peptide sequence of wild-type AM560-2 and TME3 MeRPPL1 homologs (Figure 2-10C). SACMV only without CRISPR-silencing vector also resulted in random substitution mutations that resulted in altered gene and translated peptide sequences.

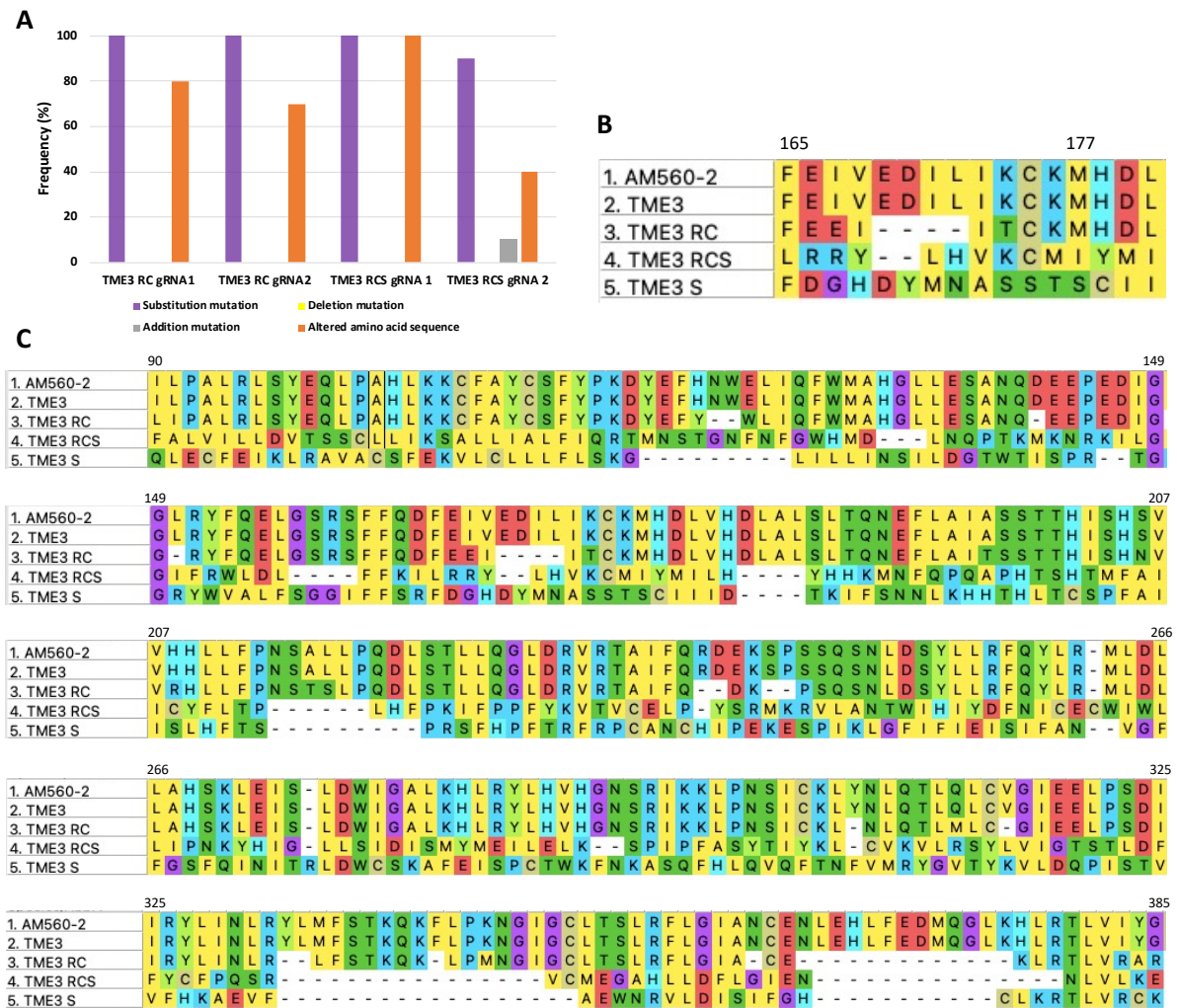


Figure 2-10. Amino acid sequence alignment of MerPPPL1 in TME3 protoplasts subjected to different treatments. **(A)** A summary of types of mutations observed and their frequency ($n = 10$ amplicons per genotype). **(B)** SACMV-infected CRISPR-edited MerPPPL1 (TME3 RCS) and SACMV-infected only (TME3 S) causes mutation in the conserved MHD domain. **(C)** An overview of all mutations induced in TME3 RCS, TME3 S, and CRISPR-edited MerPPPL1 – SACMV (TME3 RC). Mutations in MerPPPL1 were determined by aligning the sequences of treated samples to MerPPPL1 homologs from wild type reference AM560-2 and TME3 (GenBank ID GCA_003957995.1) using ClustalW algorithm in MEGA X software.

2.4. Discussion

To date, the molecular mechanisms which bring about cassava mosaic disease (CMD) resistance or tolerance are not fully comprehended. However, progress has been made through functional characterisation of genes that are suspected to play important roles in bringing about host resistance. Disease resistance genes encoding NBS-LRR proteins are known natural resistance genes and their proteins are known for their role as receptors that recognise pathogen effectors and trigger plant effector triggered immunity (ETI) responses, including reactive oxygen species, accumulation of

salicylic acid, and transcriptional activation of genes involved in defense response [351]. While many studies have been undertaken in experimental hosts such as *Arabidopsis* and *Nicotiana benthamiana*, and a range of crops, there are few studies on R proteins in cassava. It was for this reason, together with the fact that *MeRPPL1* and *MeTNL* map to chromosome 12 associated with the CMD2 resistance-associated QTL, that these R proteins were selected for this study. Specifically, *MeRPPL1* and *MeTNL* were targeted for silencing in order to determine putative roles in resistance/tolerance to CMD. Furthermore, a previous report postulated that R proteins play a role in tolerance to SACMV in cassava landrace TME3 [85].

2.4.1. *In planta* VIGS silencing

2.4.1.1. SACMV accumulation following VIGS silencing

VIGS silencing vectors pC8A-MeRPPL1 + DNA B and pC8A-MeTNL + DNA B, and pC8A-GFP (vector only control) + DNA B only induced minimal symptoms such as leaf distortion at a later time point (75 dpi) in TME3, but not at earlier time points (16 and 32 dpi) (Figure 2-6). This demonstrated the important role played by coat protein (CP) in SACMV infection cycle, and in inducing symptoms in infected plants. pC8A VIGS vector was constructed from SACMV-DNA A component, but with the CP reading frame removed [345], since cassava is restricted to infection by few viruses, and cannot be infected with any of the known plant virus silencing vectors, such as tobacco rattle virus (TRV) and potato virus X (PVX) [352]. In addition to genome packaging, the CP has been associated with several other functions, including shuttling of geminiviral DNA into and out of the nucleus in monopartite and bipartite geminiviruses [353]. Although bipartite begomovirus CPs may not be required for infection, they have been shown to enhance cell-to-cell and/or systemic spread of several geminiviruses, including African cassava mosaic virus [354]. It has also been shown that the absence of CP results in reduced levels of viral ssDNA in several begomoviruses. Amongst other studies, the coat protein (CP) of non-geminiviruses such as *Tomato mosaic virus*, *Potato virus X* and *Cucumber mosaic virus* accumulate in the chloroplast and induce viral replication and severe mosaic symptoms formation [355]–[357]. Collectively, this suggests that the absence of CP compromises SACMV replication and movement, which explains why only minor leaf distortion CMD symptoms were observed in plants that were only infected with VIGS silencing vectors + DNA B. This is important as it also reveals that VIGS pC8A-silencing vectors do not cause highly detrimental symptoms.

At an early infection stage (16 dpi), also referred to as non-symptomatic stage, SACMV replication is usually minimal to non-detectable in SACMV infection trials, as the virus is still establishing itself inside the host TME3 plants and as such there are no CMD symptoms at this time point [78]. Accordingly,

control plants infected with SACMV only and pC8A-GFP vector only + SACMV did not show CMD symptoms at 16 dpi. However, treatment plants infected with pC8A-MeRPPL1 + SACMV and pC8A-MeTNL + SACMV displayed minor leaf distortion, symptoms associated with SACMV infection (Figure 2-6). Because of this observed difference between control and treatment plants, we could conclude that pC8A-MeRPPL1 and pC8A-MeTNL silencing vectors also played a role in inducing the observed early symptoms in addition to SACMV. This is in agreement with what was observed with other geminivirus VIGS vectors from cabbage leaf curl virus (CaLCuV), SACMV, and African cassava mosaic virus (ACMV) which induced symptoms in *Arabidopsis*, *Nicotiana benthamiana* and cassava [345,358–360], respectively.

At the systemic infection stage (28 dpi), the expression of *MeRPPL1* was up-regulated in pC8A-GFP vector only control plants challenged with SACMV, compared to the unchallenged pC8A-GFP vector only control. This is in agreement with what was observed in TME3 plants, where a number of R genes were up-regulated as a result of SACMV infection [78,85], however, it was noteworthy that the silencing vector induced expression of *MeRPPL1*. *MeRPPL1* expression was down-regulated in MeRPPL1-silenced plants with/without SACMV challenge (Figure 2-8A), confirming the silencing of this gene by the VIGS silencing vector. Similarly, MeTNL-VIGS silencing vectors with/without SACMV successfully silenced *MeTNL* gene at this time point (Figure 2-8B). SACMV, however, did not induce an up-regulation of *MeTNL* in pC8A-GFP vector only control plants challenged with SAMCV. It was suggested that this may be a result of counteraction of cassava defence responses by SACMV down-regulating this gene via accumulation of their corresponding miRNA levels. SACMV-induced miRNA-mediated regulation of target genes has been shown in a previous study [361]. This has also been previously observed following *Soybean mosaic virus* infection [362].

TME3 landrace is tolerant to SACMV and showed a recovery phenotype [85] at 75 dpi. At this time point, newly developed leaves in all SACMV-infected TME3 plants (controls and treatments) showed a recovery phenotype in this study. This observation indicated that inoculation with the VIGS pC8A-silencing vectors compared to SACMV-only infected plants did not negatively influence TME3 tolerance or recovery to SACMV. This suggested that although *MeTNL* expression was down-regulated at this time point (Figure 2-8B), other R genes, including *MeRPPL1* (up-regulated), may have contributed to tolerance. Several other R proteins were shown to be differentially regulated at 67 dpi in TME3 [85]. It is also known that R genes are not immune to resistance-breaking caused by geminiviruses. For example, NBS-LRR R genes *Ty-1/3* and *Ty2* were introduced in tomato cultivars and conferred resistance to tomato yellow leaf curl virus (TYLCV) by increasing cytosine DNA methylation

of the viral genome, however, it was later found that the resistance was not durable against TYLCV [363–366]. TME3 plants where *MeRPPL1* and *MeTNL* were silenced (plants inoculated with pC8A-MeRPPL1 + SACMV and pC8A-MeTNL + SACMV) recorded an increase in SACMV DNA A accumulation at 75 dpi compared to the controls (Figure 2-7A). This indicated that SACMV is still actively replicating in TME3 without killing the host, but coexisted with the TME3, which has been suggested to be the basis of tolerance [85].

2.4.1.2. Relative expression of *MeRPPL1* and *MeTNL* following VIGS silencing

An increase in the expression of both *MeRPPL1* and *MeTNL* in pC8A-GFP vector + SACMV infected plants was observed (Figure 2-8), which suggested that SACMV induced expression of these R genes. Resistance genes are only activated when pathogen effectors are detected and interact with them, causing conformational changes and activation of defence-related genes [367]. An increase in expression of several R genes was previously observed in TME3 following infection with SACMV [78,85], further supporting the role of viral effector proteins in R genes activation. In comparison to pC8A-GFP + DNA B control, pC8A-MeRPPL1, and pC8A-MeTNL with/without SACMV evoked a decrease in expression of *MeRPPL1* at 16 and 28 dpi and *MeTNL* at 16, 28 and 75 dpi (Figure 2-8), respectively. This decrease in *MeRPPL1* and *MeTNL* could be linked to high SAMCV DNA A accumulation and symptoms observed at those time points (16 and 28 dpi for *MeRPPL1*, and 16, 28, and 75 dpi for *MeTNL*; Section 4.1.1.). This provides some evidence that silencing of these two R genes led to impairment of ETI, resulting in an increase in SACMV accumulation. Small RNAs silencing of R genes from *RPP5* locus in *Arabidopsis* was reported to negatively affect the host response to infection fungal pathogen *Hyaloperonospora parasitica* [368]. In another study, barley stripe mosaic virus (BSMV) VIGS was used to down-regulate the expression of several R genes including receptor-like kinase (RLK) brassinosteroid insensitive 1 (BRI1) in barley seedling resistance to *Fusarium* infection [369], and thus favouring pathogen infection. Another RLK gene, *Xa21* was silenced in rice using rice tungro bacilliform virus (RTBV)-derived VIGS and similar response was observed [370].

At the recovery stage (75 dpi), an increase in *MeRPPL1* expression was observed in pC8A-MeRPPL1 + SACMV and pC8A-MeRPPL1 + DNA B inoculated plants when compared to the expression levels in GFP-vector + DNA B only control plants (Figure 2-8A). It was surprising to see an increase in expression of the VIGS-silenced *MeRPPL1* gene due to its intended targeting, however such cases have previously been observed in cassava when the expression of *M8.B* and *M11.F* myosin genes increased following SACMV pC8A VIGS silencing [371], and also in *N. benthamiana* where the expression of a RING-type E3 ubiquitin ligase *RFH2A* silenced with TRV VIGS vector increased [372]. This increase in expression

of VIGS-silencing targeted genes may be due to the variation in efficiency of silencing induced by VIGS vectors on a plant-to-plant base, as a result of varying uptake of the VIGS vector by cassava TME3 plants [373]. *MeRPPL1* has paralogs (Figure 2-1) and it is possible that these could have been expressed when *MeRPPL1* was expressed in *MeRPPL1*-silenced samples (RSL and RDL) at the recovery stage (75 dpi). However, this was not the case as melt curve analysis showed that there was only amplification of one gene product (Appendix 3-Figure 1).

2.4.2. Transient silencing of *MeRPPL1* and *MeTNL* in protoplasts

2.4.2.1. CRISPR-Cas vectors increased SACMV and R genes expression

A rapid increase in SACMV DNA A accumulation was observed after 24 hours transient exposure to SACMV in TME3 protoplasts transfected with pC1380-*MeRPPL1* + Cas9 + SACMV and pC1380-*MeTNL* + Cas9 + SACMV (Figure 2-7C). This increase however, was lower compared to SACMV DNA A accumulation in SACMV only-infected control protoplasts. An increase in geminivirus DNA accumulation in cassava leaf mesophyll protoplasts and tobacco BY-2 protoplasts have previously been observed from 6 - 24 h [110,374] and 36 – 48 h [375] post transfection, respectively. Protoplasts lack the cell wall, making it easy for the virus to enter the cell by transfection with the cell membrane and establishing itself inside the host cells [376]. This increase however, was lower compared to SACMV DNA A accumulation in SACMV only-infected control protoplasts, suggesting that silencing of these two R genes play a role in reduction of SACMV replication levels. Notably, CRISPR-Cas9 silencing relative expression results demonstrated that CRISPR-silencing vectors, CRISPR + SACMV, and SACMV alone triggered an up-regulation of *MeRPPL1* and *MeTNL* in TME3 leaf mesophyll protoplasts after 24 hours compared to pC1380 CRISPR vector only control (Figure 9).

Resistance gene-encoded proteins are key players in host ETI, and as such it is commonly reported that up-regulation of R genes and over-expression of R proteins are induced upon external pathogens such as SACMV. Herein, the CRISPR vector was also perceived by the host and appeared to induce up-regulation of *MeRPPL1* and *MeTNL*. The results are in agreement with the previously reported up-regulation of genes [78,85] and proteins [340] involved in disease response in TME3 landrace infected with SACMV. Recently, a *MeE3L* gene was significantly up-regulated in TME3 protoplasts following transfection with CRISPR-silencing vector + SACMV and SACMV alone [110], also supporting the increase of disease response-related genes. Other studies that observed up-regulation of a number of R gene transcripts was in TME3 plants upon infection with SACMV [78], in mulberry, *N. benthamiana*, and tomato [377–379], for protection against bacterial infection and viruses such as *Tomato mosaic virus*. From the increase in expression of *MeRPPL1* and *MeTNL* results (Figure 2-9), we propose that

the expression of *MeRPPL1* and *MeTNL* was induced as a response by TME3 against SACMV in protoplasts and thus may be associated with tolerance.

2.4.2.2. Mutations induced by CRISPR-silencing vector and SACMV

Both CRISPR-Cas vectors and SACMV alone (and together) induced random mutations that resulted in about 40 – 100% alterations in the translated protein of the *MeRPPL1* gene in TME3 protoplasts (Figure 2-10), which may affect the functionality of MeRPPL1 protein to detect SACMV as part of the ETI defense response by this cassava landrace. Similar results have been reported in a number of studies where viruses, including geminiviruses such as TYLCV, SACMV, cotton leaf curl Kokhran virus (CLCuKoV) and tobacco rattle virus (TRV) co-infected with CRISPR vectors, induced random mutations in targeted genes in different hosts [78,110,253,254,380]. Resistance genes have also been found to be susceptible to these mutations caused by both viral and bacterial pathogens [83–86], as observed when *Potato virus X* induced random mutations in twenty-five CC-NB-LRR R genes in potato and in addition, resulted in the disruption of the NBS domain's highly conserved and strictly ordered MHD motif in *Rx* R gene [381].

Mutations caused by SACMV resulted in amino acid sequence disruptions that led to the replacement of the MHD motif in pC1380-MeRPPL1 + Cas9 + SACMV transfected and SACMV only infected protoplasts (Figure 2-10B). The MHD motif is found near the C-terminal end of the NBS domain and functions in nucleotide-dependent conformational changes [367] and mutations in this motif has been linked to the auto-activation of many NBS-LRR proteins [378,381,382,385,386], but the histidine residue was noted to be key to the functioning of this motif in transducing pathogen perception by LRR [84,387]. This is not the first report of the disruption of the MHD motif in cassava TME3 landrace, as it was previously reported that SACMV induced amino acid substitutions *in planta* that resulted in disruption and the total removal of this motif in some R gene accessions [85]. Therefore, we propose that the absence or disruption of the histidine residue of the MHD motif leads to the auto-activation (self-hypersensitive response) of R genes which in turn results in loss of gene function [146,378,388]. We can conclude that the increase in SACMV DNA A accumulation in protoplasts transfected with pC1380-MeRPPL1 + Cas9 + SACMV and SACMV only (Figure 2-7C) resulted from the observed lack of the MHD motif or mutations in the MHD motif induced by silencing vector and SACMV in *MeRPPL1* gene and possibly other R genes as previously noted [85].

2.4.3. Resistance proteins function in a network

Resistance proteins function in a network and in direct interactions with various interconnected protein groups including other resistance proteins as was observed with MeTNL and MeRPPL1 interactions with other proteins including resistance proteins (Figure 2-3). Although cassava gene functions are not always characterised, we can simulate based on TAIR *Arabidopsis* homologs annotations that these proteins identified in the networks are involved directly or indirectly in disease response mechanisms in TME3. However, functional characterisation of these proteins will need to be done in future to confirm their functions. DAR protein, which directly interacts with MeRPPL1 and MeTNL resistance proteins has previously been reported to be involved in virus DNA replication [389], while ASA protein interacting with MeRPPL1 is involved in the production of ascorbic acid and has also been linked to begomovirus resistance [390]. DAR has not been studied much in plant viruses but in viruses from the *Flaviviridae* RNA viruses, it is known to modulate replication by forming an interaction between 5' DAR and 3' DAR to activate viral RNA-dependent RNA polymerase required for replication [391]. But herein, we propose that binding of a SACMV protein, such as the CP or other encoded protein effector, to MeRPPL1 elicits expression (up or down) of its interacting partner DAR which in turn influence the replication of SACMV, however, we cannot conclude whether this interaction is contributing positively or negatively to TME3 tolerance. As such, understanding how DAR influence geminivirus replication is worth investigating.

2.5. Conclusions

Viral induced gene silencing (VIGS) and CRISPR-Cas9 system were used to induce gene silencing in order to determine loss of function effects of two R genes on SACMV infection. VIGS induced knockout of genes while CRISPR-Cas9 system introduced random point mutations which would result in polypeptide amino acid alterations. Comparing the two techniques, VIGS-silencing vectors induced under-expression of both *MeRPPL1* and *MeTNL* genes in TME3 plants, which led to increased SACMV DNA A accumulation, while CRISPR-Cas9 silencing vectors with/without SACMV resulted in increased expression of the two target genes in TME3 protoplasts. Because of the roles R genes play in ETI response mechanisms, increased expression is expected following the introduction of external effectors such as the coat protein of SACMV and silencing vectors. This however, was only observed in CRISPR-silencing in protoplasts and not in VIGS-silenced TME3 plants due to the fact that VIGS was used to silence genes. Further studies would be needed to perform complete knockouts of *MeRPPL1* and *MeTNL* to assess how they will influence SACMV infection in TME3 protoplasts and how the influence will be correlated to VIGS silencing results *in planta*.

CRISPR silencing in protoplasts further revealed that CRISPR-Cas silencing vector + SACMV and SACMV-only induced random amino acid mutations, including mutations in the highly conserved MHD motif of MeRPPL1 which is found in NBS-LRR proteins. The consequence of these mutations is the total replacement of M, H, and D amino acids with I, Y, M in MeRPPL1-CRISPR silenced protoplasts and C, I, I in SACMV-only transfected protoplasts, which may have affected the activation of MeRPPL1 and further, its interaction with SACMV effector. It is also possible that MeRPPL1 could be autoactivated together with some R proteins in TME3 and associated with tolerance as it was previously suggested by Louis and Rey, 2015 [85]. Thus, activation of downstream signalling and defence pathways would have not occurred, leading to high virus replication in TME3 protoplasts. Therefore, we can conclude that *MeRPPL1* and *MeTNL* play a role in TME3 tolerance to SACMV *in planta* and/or in protoplasts, but further investigations such as modelling the three-dimensional structure of MeRPPL1 peptide to see the effects of MHD motif disruption on the binding of this protein need to be conducted.

Chapter 3: Proteome mapping of tolerant TME3 and susceptible T200 cassava landraces infected with *South African cassava mosaic virus*

3.1. Introduction

Cassava (*Manihot esculenta* Crantz), an exotic perennial shrub and root crop belonging to the *Euphorbiaceae* family, is the third most important food crop in tropical regions after rice and maize. It is grown in over 90 countries as it is a good source of carbohydrates and produces higher yields in adverse conditions than most other crops. According to Faostat (<http://www.fao.org/faostat/>), over 303 million tonnes of cassava were produced in the year 2019, with Africa being the major contributor (63.3% production share). Cassava does not only provide food but also brings employment to the impoverished regions of Africa. Cassava has many other uses such as industries producing food, ethanol, paper and cardboard, textiles, pharmaceuticals, glues and adhesives. Cassava roots are high in carbohydrates, whereas its leaves are an excellent source of both proteins and vitamins [392]. There is no doubt that the cassava crop is of importance, however its production is threatened by pests and pathogens resulting in yield losses.

South African cassava mosaic virus (SACMV) [42] is amongst nine cassava mosaic geminivirus species [15], which cause the devastating cassava mosaic disease (CMD) [23,24]. SACMV is a bipartite single-stranded DNA genome virus consisting of DNA-A and DNA-B components, both crucial for systemic infection [393]. DNA-A is necessary for transcription and replication of the virus, while DNA-B is crucial for cell-to-cell and long-distance movement [32]. DNA-A has six open reading frames (ORFs) namely AC1/Rep, AC2/TrAP, AC3/REn, AC4, AV1/CP and AV2. DNA-B has two ORFs; BV1/NSP and BC1/MP [394]. These encoded proteins influence defence responses, hormonal responses and cell cycle regulation, ubiquitin-proteasomal pathways, and protein-signalling cascades [395]. CMD is dated back to the late 19th century, and has since resulted in major cassava production and economic losses to the value of US\$ 1300-2300 million in Africa [396]. In susceptible cultivars, CMD has caused yield losses up to 82% [336] by significantly reducing the number and size of tubers. However, the severity of the damage is determined by the strain of the virus, cassava variety, age and sensitivity of the host, and environmental factors such as soil fertility [397]. CMD presents itself with symptoms of yellow and/or pale green chlorotic mosaic of leaves accompanied by distortion and an overall reduction in leaf size [398].

Cassava landraces TME3 and T200 have shown different responses to SACMV infection at different infection stages. TME3 is tolerant to SACMV as shown by a recovery phenotype 67 days post-infection (dpi), whereas T200 is susceptible to the virus throughout the infection period. Findings from a transcriptome profiling study showed many down-regulated and up-regulated genes in the two landraces, including several resistance (R) gene transcripts over-expressed in TME3 and under-expressed in T200 [78]. Although this study revealed some interesting findings which could potentially give some clues to the molecular mechanisms that underly the tolerance phenotype of TME3, levels of mRNA in the cell are not directly proportional to the expression level of the proteins they code for. Proteomics studies the complete set of proteins expressed in a specific cell, tissue, or organism and thus bridges the gap between the other omics [399]. As such, it is important to look at the proteome as it offers a more robust approach to discover protein networks and pathways that are crucial for stress responsiveness and tolerance, and that will enable further dissecting of the biochemical and molecular responses of cassava against viruses.

The number of proteome mapping studies of crops have increased, and so has the pool of proteomic databases that are now available in the research community. In addition, proteomics has other potentials in the biotechnology field including the identification of molecular markers to assist in plant breeding, keeping track of changes in protein profiles under different abiotic and biotic treatments at different timelines, understanding pathogen resistance or tolerance mechanisms, and the possibility of developing proteomics-based fungicides [400,401]. Together with the availability of fully sequenced genomes, researchers are now able to integrate different omics data into systems biology in order to seek an understanding into the physiological systems of a cell or tissue in response to different stimuli. Gel-based proteomics techniques such as two-dimensional electrophoresis (2-DE), and gel-free techniques such as mass spectrometry (MS)- based and Isobaric tag for relative and absolute quantitation (iTRAQ), have been used to identify proteomes in different crops [402]. Recently, a review examined tomato plant proteomes performed on different tissues, and using different proteomic techniques under different stress/physiological conditions [403]. Two of these studies investigated virus interactions with tomato. Casado-Vela et al. 2006 [404] studied the differences in protein profile in tomato fruit infected with tobacco mosaic virus (TMV). Pathogenesis-related (PR) proteins such as CHI and GLU and antioxidant enzymes such as SOD, DHAR, GST, PHGPX, APX and TPX were found to protect the plant against TMV infection [404]. PR proteins were further confirmed to be involved in defence against TMV infection in model plant *Nicotiana tabacum* [405].

Global plant proteome changes during viral infection have been reported in several other studies, showing a range of functions impacted such as translation, protein processing and degradation, intracellular trafficking, primary metabolism, amino acid metabolism, stress responsive proteins and cell wall biogenesis [406]. In tomato plants infected with cucumber mosaic virus (CMV), the responsive leaf proteome revealed proteins involved in photosynthesis, primary metabolism and defence activity to be under-expressed following infection [314]. Furthermore, they found that CMV did not systematically spread due to single-chain variable fragment (scFv)-G4 antibody which are specific for CMV CP. Several proteome studies have been performed on geminivirus-tomato interactions comparing resistant and susceptible germplasm, revealing hypothetical models for host cell responses to infection [331,407]. A cassava proteome has been investigated in storage roots and leaves under abiotic stress and physiological conditions [305,408–412] however, there has only been one study that investigated the leaf proteome in response to viral infection. In this study, cassava was infected with both Indian cassava mosaic virus (ICMV) and Sri Lankan cassava mosaic virus (SLCMV) which causes CMD [413]. Proteins such as DnaJ-like proteins involved in molecular chaperone functions, signalling and disease resistance (ascorbate peroxidase and protein kinase-coding resistance proteins) were over-expressed, implying their involvement in viral infection resistance. Proteomes have also been established from other crops such as sorghum, rice, soybean, wheat, maize and mungbean [304,414–418] in response to different stimuli.

This study is the first to profile a cassava proteome in response to infection by a geminivirus, SACMV, on the sub-Saharan African continent. Furthermore, the study compares changes in cellular proteins between a susceptible T200 and tolerant TME3 landrace infected with SACMV at the systemic infection stage (32 dpi) and recovery time point (67 dpi). Overall, the results showed differences in protein expression profiles between the two landraces and also revealed some proteins that are proposed key players in reduced symptoms and virus in tolerant TME3. The results will go towards understanding biochemical mechanisms involved in both susceptibility and symptom/virus recovery phenotypes in cassava infected with CMD.

3.2. Materials and Methods

3.2.1. Cassava Infection with SACMV

Nodal cutting of cassava T200 and TME3 landraces were micro-propagated on full Murashige and Skoog (MS) medium [346] supplemented with 2% sucrose, 0.78% plant tissue culture agar, 0.002 mM CuSO₄ and a pH of 5.8. To induce root growth, the nodal cultures were incubated in a temperature-controlled growth room at 28°C under a 12 h photoperiod at a light intensity of 150 $\mu\text{Em}^{-2}/\text{s}$. Once the

roots appeared (approximately 3 weeks), the plantlets were transplanted into 40 mm x 45 mm Jiffy® pellets (Jiffy International AS), placed in a plastic container and covered with a plastic film for acclimatization. Two slits were made to the plastic film to gradually acclimatize plantlets until they could be infected with SACMV at the 4-6 leaf stage. Agro-inoculation of the plantlets with SACMV infectious dimer clones, DNA A and DNA B, in *Agrobacterium Agl1* was performed as outlined by Allie et al. 2014 [78], with minor modifications. *Agrobacterium* harbouring the infectious clones were grown in yeast extract peptone medium (1% tryptone, 1% Bacto peptone, 0.5% sodium chloride, pH 7) supplemented with 50 mg/ml carbenicillin and 100 mg/ml kanamycin, while *Agrobacterium Agl1* medium was only supplemented with 50 mg/ml carbenicillin. The cultures were grown at 30°C until an OD600 between 1.8-2.0 was reached. SACMV DNA A and DNA B cultures were mixed in equal volumes before plantlets were agro-inoculated along the stem with 100 µl of the culture using a hypodermic needle. Control plants were mock-inoculated with *Agrobacterium Agl1* only and incubated in a temperature-controlled growth room and monitored over a period of 67 days.

3.2.2. Protein extraction and isolation by TCA/Acetone precipitation

Leaves below the apex from SACMV-infected and mock-inoculated plants were sampled at time points 32 and 67 dpi. Three infection trials (biological replicates) were done independently for both T200 and TME3 landraces, with two leaves from a single plant and nine plants sampled per treatment per trial. Leaves from 3 plants were pooled into one tube/sample and each treatment had 3 samples. Sampled leaves were immediately flash frozen in liquid nitrogen to halt any metabolic activity. A total of 2 g of crushed leaf samples were used for protein extraction using a HEPES-based buffer [50 mM HEPES-KOH, pH 7.4; 100 mM KOAc; 2 mM MgCl₂; 0.5 mM PMSF and 1% proteinase inhibitor (ThermoFisher 78438), 0.1% Triton x-100]. Samples containing the extraction buffer were gently mixed on a low-speed vortex and placed on ice for 30 min before centrifuging at 4°C for 10 min at 3000 x g. A total of 400 µl lysate was transferred to a 2 ml tube. Proteins were precipitated by the addition of 1.6 ml of ice-cold TCA extraction buffer [10% v/v TCA (trichloroacetic acid; Sigma T9159) in acetone (Sigma 34850)] with 2% 2-mercaptoethanol (Honeywell 63700) and incubated at -20°C for a minimum of 1 h. Protein was pelleted by centrifugation at 21 000 x g for 15 min at 4°C. The supernatant was then removed and the pellet washed with 500 µl of ice-cold acetone. This was repeated a further two times for a sum of three washes in total. The pellets were then dried in a fume hood. Protein pellets were solubilised in 20 µl of 4% sodium dodecyl sulphate (SDS; Sigma 71736), 100 mM triethylammonium bicarbonate (TEAB, Sigma T7408), briefly vortexed and then heated at 95°C for 5 min. Insoluble debris was removed by centrifugation at 10 000 x g for 5 min. The supernatant was then transferred to a new

tube and quantified using the QuantiPro BCA assay kit (Sigma QBCA) according to the manufacturer's instructions.

3.2.3. On-bead hydrophilic interaction liquid chromatography (HILIC) digest

In preparation for the HILIC magnetic bead workflow, the HILIC beads (ReSyn Biosciences, HLC010) were aliquoted into a new tube and the shipping solution removed. Beads were then washed with 250 µl wash buffer [15% acetonitrile (ACN), 100 mM ammonium acetate (Sigma 14267) pH 4.5] for 60 s. This was repeated for a total of two washes. The beads were then resuspended in loading buffer (30% ACN, 200mM ammonium acetate pH 4.5) to a concentration of 5 mg/ml. A total of 50 µg of protein from each sample was transferred to a 96-well protein LoBind plate (Merck, 0030504.100). Protein was reduced with tris (2-carboxyethyl) phosphine (TCEP; Sigma 646547) which was added to a final concentration of 10 mM TCEP and incubated at 60°C for 1 h. Samples were cooled to room temperature and then alkylated with methyl-methane-thiosulphonate (MMTS; Sigma 208795) which was added to a final concentration of 10 mM MMTS and incubated at room temperature for 15 min. HILIC magnetic beads were added at an equal volume to that of the sample and a ratio of 5:1 total protein. The plate was then incubated at room temperature on the shaker at 900 rpm for 30 min for binding of protein to beads. After binding, the beads were washed four times with 500 µl of 95% ACN for 60 s. For digestion, trypsin (Promega PRV5111), made up in 50 mM TEAB was added at a ratio of 1:12.5 total protein and the plate was incubated at 37°C on the shaker for 4 h. After digestion, the supernatant containing peptides was removed and dried down. Samples were then resuspended in LC loading buffer (0.1% FA, 2% ACN) prior to clean up by Zip-Tip (Sigma Z720070). Samples were then dried down once more and resuspended in a final volume of 12 µl LC loading buffer.

3.2.4. Liquid chromatography-mass spectrometry (LC-MS)

LC-MS analysis was conducted with a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) coupled with a Dionex Ultimate 3000 nano-UPLC system. Data was acquired using Xcalibur v4.1.31.9, Chromeleon v6.8 (SR13), Orbitrap MS v2.9 (Build 2926) and Thermo Foundations 3.1 (SP4). Peptides were dissolved in 0.1% Formic Acid (FA, Sigma 56302), 2% acetonitrile (ACN, Burdick & Jackson BJLC015CS) and loaded on a C18 trap column (PepMap100, 9027905000, 300 µm × 5 mm × 5 µm). Approximately 400 ng of peptide was injected. Samples were trapped onto the column and washed for 3 min before the valve was switched and peptides eluted onto the analytical column as described hereafter. Chromatographic separation was performed with a Waters nanoEase (Zenfit) M/Z Peptide CSH C18 column (186008810, 75 µm × 25 cm × 1.7 µm) as described below. The solvent system employed was solvent A: LC water (Burdick and Jackson BJLC365), 0.1% FA and solvent

B: ACN, 0.1% FA. The multi-step gradient for peptide separation was generated at 300 nL/min as follows: time change 100 min, gradient change: 5 - 30% Solvent B, time change 10 min, gradient change 30 – 80% Solvent B. The gradient was then held at 80% Solvent B for 10 min before returning it to 5% Solvent B and equilibrating the column for 15 min. All data acquisition was obtained using Proxeon stainless steel emitters (Thermo Fisher TFES523). The mass spectrometer was operated in positive ion mode with a capillary temperature of 320°C. The applied electrospray voltage was 1.95 kV.

3.2.5. Viral load

Genomic DNA was extracted from SACMV-infected leaf tissue using a CTAB-based method [347] with modifications. CTAB extraction buffer was supplemented with 40 mg/ml polyvinylpolypyrrolidone (PVPP) and 0.2% 2-mercaptoethanol before the buffer was preheated at 65°C for 10 min. A total of 500 µl of CTAB buffer was added to 0.5 g ground leaf tissue and incubated at 65°C for 45 min. Chloroform-isoamyl alcohol (24:1 ratio) was added to the samples, followed by gentle mixing and centrifuging at full speed for 10 min. Top aqueous phase was transferred into a new tube and the latter step repeated. Ice cold isopropanol (1:1 ratio) was added, samples thoroughly mixed and incubated in the freezer for 45 min. Samples were then centrifuged at full speed for 10 min and the DNA pellet was washed with ice cold 70% and 95% ethanol, respectively and air-dried. The DNA was resuspended in nuclease free water with 1% RNase and incubated at 37°C for 1 h to allow digestion. Thereafter, DNA was stored at 4°C overnight, then quantified and stored at -20°C. The DNA (25 ng) was used as template for SACMV relative viral load quantification using qPCR. Quantification was done in triplicates using Maxima SYBR Green/ROX qPCR Master Mix (2X) (ThermoFisher Scientific) with SACMV CP primers (Fwd: 5' ACGTCCGTCGCAAGTAC 3', Rev: 5' ATTGTCATGTCGAATAGTACG 3'). SACMV viral load were calculated using the $2^{(\Delta\Delta CT)}$ method [348]. The student's t-test was used to access statistical differences between samples.

3.2.6. Gene expression validation

RNA was extracted from SACMV-infected and *Ag/1*-mock inoculated leaf tissue using QIAzol Lysis Reagent (QIAGEN, Maryland, USA) according to manufacturer's protocol. RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific) was used to synthesise first strand cDNA using Oligo(dT)18 primer according to manufacturer's protocol. To validate genes using relative expression, RT-qPCR was done in triplicate with cDNA as template using Maxima SYBR Green/ROX qPCR Master Mix (2X) with primers listed in Appendix 4-Table 1. *UBQ10* was used as a reference gene for qPCR

quantifications. Gene expression fold change was calculated using the $\log 2^{(\Delta\Delta CT)}$ method [348]. The student's t-test was used to access statistical differences genes.

3.2.7. Bioinformatics

Relative quantification of identified proteins was conducted using Progenesis Q1 for Proteomics v2.0.5556.29015 (Nonlinear Dynamics, UK). Data processing included peak picking, run alignment and normalisation (singly charged spectra were removed from the processing pipeline). Only proteins that contained two or more unique peptides were considered for further analysis. Database interrogation was performed with Byonic Software v3.8.13 (Protein Metrics, USA) using a cassava reference proteome (Cassava_RefProt_UP000091857_25032020.fasta) sourced from UniProt (www.uniprot.org) dated 25/03/2020. Phytozome v13 (<https://phytozome-next.jgi.doe.gov/>) was used to assess *M. esculenta* v8.1, v7.1 and v6.1 genome and retrieve protein family data. UniProt and TAIR were used to retrieve theoretical molecular weight and *Arabidopsis* homologs of identified proteins, respectively. STRING version 11.0 (<https://string-db.org/>) was used to study protein-protein interaction networks, gene ontology (GO) annotation and KEGG analysis of DE proteins.

3.3. Results

3.3.1. SACMV infection development and viral load

CMD characteristic symptoms were observed in both susceptible T200 and tolerant TME3 landraces at 32 and 67 dpi with SACMV (Figure 3-1a). These included leaf deformation, yellow/pale-green mosaic and reduced leaf size (red arrows). Agl1 mocked plants did not show any disease symptoms (white arrows). It is important to note that minor symptoms were observed on newly formed leaves of TME3 at recovery (67 dpi), whereas, severe symptoms were still observed in T200 plants. T200 infected plants generally showed severe symptoms, with significantly high CMD symptoms score of 5 at 67 dpi as compared to TME3 infected which had a lower symptom score of 2.7 (Figure 3-1b). There was significant difference in the accumulation of SACMV DNA A in T200 and TME3 at 32 and 67 dpi (Figure 3-1c). T200 had viral load of 7.7 and 10.4 folds higher relative to *UBQ10* at 32 and 67 dpi, respectively. TME3 had fold change of 6.8 and 8.0 relative to *UBQ10* at 32 and 67 dpi respectively, which was generally lower compared to T200 fold change.

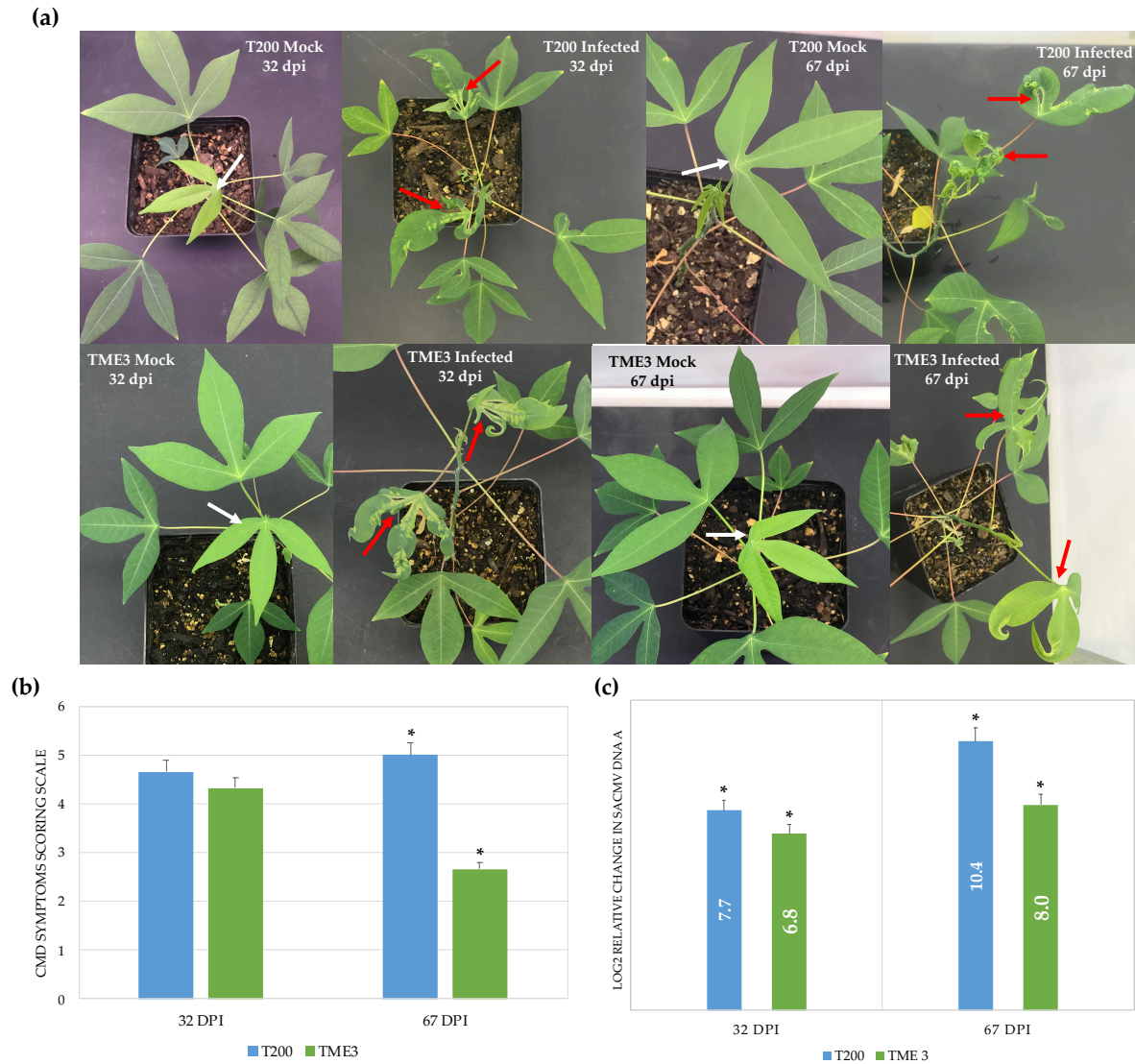


Figure 3-1. SACMV symptoms scores and viral load quantification in T200 and TME3 landraces at 32 and 67 dpi. **(a)** Characteristic CMD symptoms on T200 and TME3 compared with mock-inoculated plants. White arrow points at CMD-free leaves, while red arrow points at CMD-infected leaves. **(b)** CMD symptoms scoring on infected plants. **(c)** Relative change of SACMV DNA A to internal reference gene *UBQ10* in infected plants. Error bars represent means $\pm$ S.D. ($n = 3$). Asterisks indicate statistical significance at $p \leq 0.05$ using t-test.

Symptoms Scoring Scale: 0 = No symptoms, 0.5 = No chlorosis, slight leaf deformation, 1 = Mild chlorosis, slight leaf deformation, 2 = Clear mosaic with/without slight leaf deformation, 3 = Severe mosaic, distortion reduced size, 4 = Strong mosaic all over the leaf with severe leaf deformation, 5 = Severe mosaic and severe reduction of leaf size.

3.3.2. Protein identification and quantification

Leaf tissue proteins were extracted and isolated from T200 and TME3 landraces inoculated with SACMV infectious dimers, DNA-A and DNA-B, using HEPES-based buffer and 10% TCA/acetone, respectively. Leaves were sampled at 32 and 67 dpi from three independent biological trials. Proteins

were identified using LC-MS analysis and relative quantification was conducted using Progenesis Q1 for Proteomics v2.0.5556.29015. Only proteins that contained two or more unique peptides were considered for further analysis. Database interrogation was performed with Byonic Software v3.8.13 using a cassava reference proteome (Cassava_RefProt_UP000091857_25032020.fasta) sourced from UniProt dated 25/03/2020. Overall, 2682 proteins were identified in both T200 and TME3 at 32 dpi and 49 of the proteins were differentially expressed with fold change >1.0 ($q < 0.05$) (Table 3-1, Appendix 5-Table 1). At 67 dpi, a total of 2817 proteins were identified in both T200 and TME3, with 206 of the proteins differentially expressed with fold change >1.0 ($q < 0.05$) (Table 3-1, Appendix 5-Table 1). Ten proteins were similar to both TME3 and T200 at both time points. A large number of proteins were not characterised based on the status of the current cassava genome and protein annotations (Manihot esculenta v6.1; phytozome v12.1; <https://phytozome.jgi.doe.gov>).

Table 3-1. Summary of differentially expressed proteins in SACMV infected T200 and TME3 plants compared with mock-inoculated plants at 32 and 67 dpi.

Time point	Regulation	Protein count	Protein characterisation	GO categories ^a	Functional groups
32 dpi	Under-expressed	30 T200	14 characterised	49 CC	19
		27 TME3	35 uncharacterised	42 BP	35
	Over-expressed	19 T200		36 MF	13
		22 TME3		49 KEGG	11
		Total = 49			
67 dpi	Under-expressed	63 T200	128 characterised	198 CC	21
		81 TME3	78 uncharacterised	178 BP	44
	Over-expressed	143 T200		198 MF	23
		125 TME3		199 KEGG	22
		Total = 206			

^a Categories based on cellular component, CC; biological process, BP; molecular function, MF and KEGG analysis of the total number of differentially expressed proteins.

Figure 3-2 shows a heatmap of all DEPs in T200 and TME3 at 32 and 67 dpi. The dendrogram showed differences between the leave proteome at 32 dpi and 67 dpi, with the proteome of T200 at 67 dpi clustering separately from T200 at 32 dpi and TME3 at 32 and 67 dpi. At 67 dpi, proteins that were relatively over-expressed grouped together (red band), while under-expressed proteins grouped together (dark green band). Most over-expressed proteins branched out from the same node at 67 dpi and the same was observed for under-expressed proteins. Interestingly, at both 32 and 67 dpi, an opposite pattern between susceptible T200 and tolerant TME3 was observed, where over-expression of a group of proteins in one landrace was under-expressed in the other landrace (Figure 3-2).

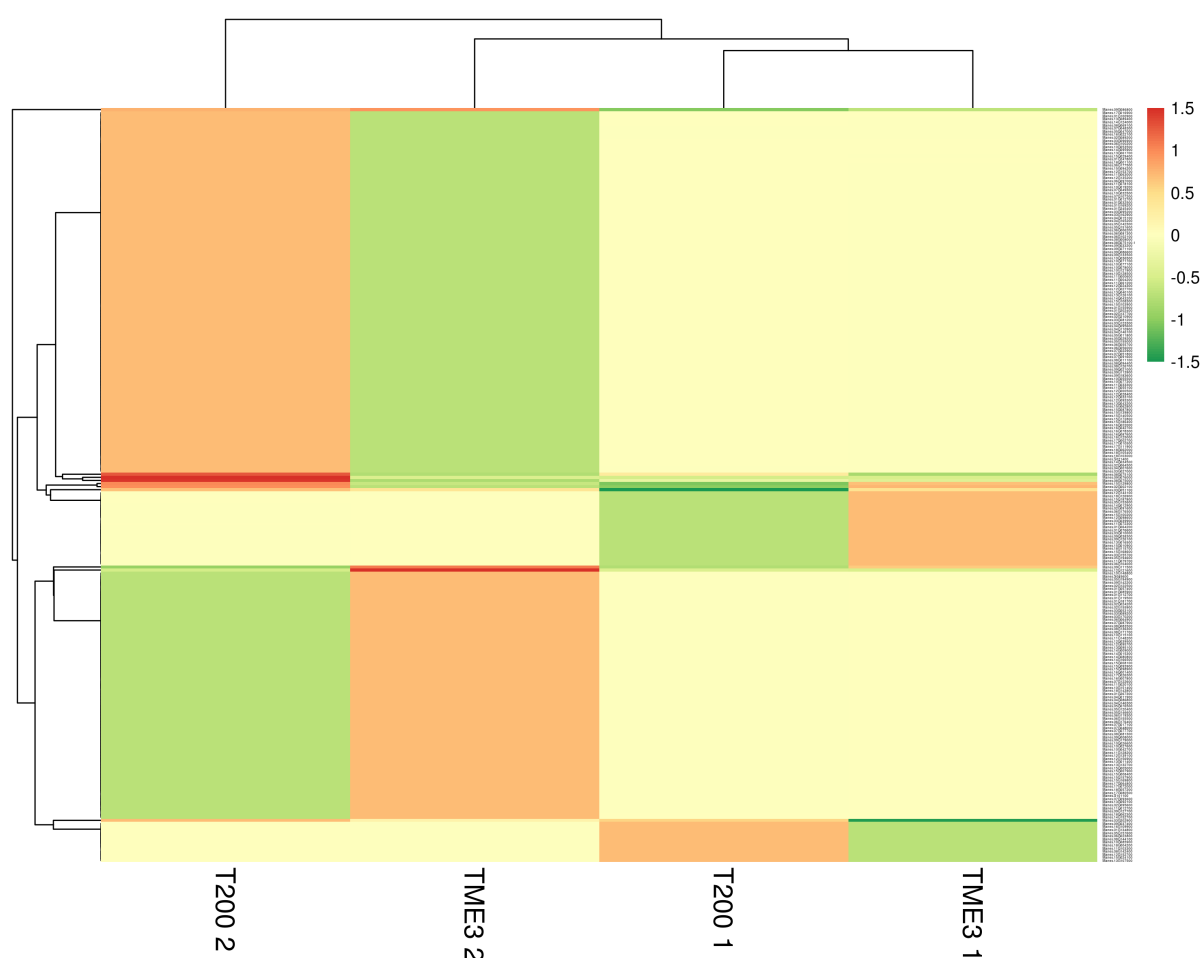


Figure 3-2. Heatmap of TME3 and T200 differentially expressed proteins at 32 and 67 dpi. **1** represents 32 dpi and **2** represents 67 dpi. ClustVis [419] was used to generate the heatmap. Rows are centred; unit variance scaling is applied to rows. Imputation is used for missing value estimation. Both rows and columns are clustered using Euclidean distance and average linkage.

3.3.3. Gene ontology analysis

Differential expressed proteins were analysed for molecular function, biological process and cellular component. In T200 at 32 dpi 30 proteins (61% of the total DEPs) were under-expressed and 19 (39%) were over-expressed (Table 3-1). Biological processes of 27 under-expressed proteins (90%, 33 functional groups) were mostly involved in response to stress (30%), response to abiotic stress (26%), biosynthetic process (26%), catabolic process (22%), multicellular organism development (19%), cellular component organisation (15%), lipid metabolic process (15%), nucleobase-containing compound metabolic process (15%), reproduction (15%), other cellular processes (59%) and other metabolic processes (55%) (Appendix 6-Figure 1a). For over-expressed proteins, 15 proteins (79%, 20 functional groups) were mostly involved in response to stress (33%), catabolic process (20%), protein metabolic process (20%), response to chemical (20%), response to external stimulus (20%), response

to biotic stress (13%), secondary metabolic process (13%), other metabolic processes (60%) and other cellular processes (67%) (Appendix 6-Figure 1b). The molecular function of under-expressed proteins (83%, 14 functional groups) was mainly catalytic activity (32%), hydrolase activity (24%), protein binding (24%), RNA binding (16%), transferase activity (16%), and other binding (36%). Other molecular functions include nucleotide binding, nucleic acid binding, lipid binding, DNA binding, kinase activity, transporter activity and enzyme regulator activity with less than 10% of the protein each (Appendix 6-Figure 1a). Over-expressed proteins (74%, seven functional group) were predicted to function in catalytic activity (57%), hydrolase activity (36%), transferase activity (29%), nucleotide binding (21%), protein binding (7%), transporter activity (7%) and other binding (43%) (Appendix 6-Figure 1b). Cellular components that were under-expressed included 30 proteins (100%, 20 component groups) located in the chloroplast (50%), cytoplasm (40%), cytosol (40%), plastid (40%), nucleus (33%), plasma membrane (27%), extracellular region (23%), vacuole (13%), other membranes (17%) and other intracellular components (23%) (Appendix 6-Figure 1a). Over-expressed proteins (100%, 12 components groups) were located in the cytoplasm (58%), mitochondrion (37%), plastid (26%), cell wall (16%), chloroplast (16%), cytosol (16%), plasma membrane (16%) and other intracellular components (32%) (Appendix 6-Figure 1b). Some under-expressed proteins included unknown biological process (8%), molecular function (8%) and cellular component (3%) predicted.

In TME3 at 32 dpi, 27 proteins (55% of the total DEPs) were under-expressed and 22 proteins (45%) over-expressed (Table 3-1). Twenty three under-expressed proteins (85%, 31 functional groups) were mostly involved in biosynthetic process (39%), response to stress (26%), multicellular organism development (22%), response to abiotic stress (22%), carbohydrate metabolic process (17%), catabolic process (17%), lipid metabolic process (17%), protein metabolic process (17%), response to chemical (17%), response to external stimulus (17%), response to light (17%), other cellular processes (65%) and other cellular processes (70%) (Appendix 6-Figure 2a). Four percent of under-expressed proteins did not have any known biological process. In comparison, over-expressed proteins (86%) were divided into 22 functional groups with most of them involved in response to stress (37%), catabolic process (26%), cellular component organisation (11%), protein metabolic process (11%), response to abiotic stress (11%), response to external stimulus (11%) and transport (11%) (Appendix 6-Figure 2b). Molecular function of under-expressed proteins (89%, 14 functional groups) included catalytic activity (38%), hydrolase activity (25%), protein binding (25%), transferase activity (25%), RNA binding (13%), nucleotide binding (13%) and other binding (38%) (Appendix 6-Figure 2a). In over-expressed proteins (68%, 8 functional groups) had functions mostly in catalytic activity (47%), hydrolase activity (33%), nucleotide binding (13%), transferase activity (13%) and other binding (40%). A total of 13% of proteins

did not have any known molecular function (Appendix 6-Figure 2b). Under-expressed proteins (100%, 17 cellular component groups) were mostly located in the cytoplasm (56%), chloroplast (44%), plastid (41%), cytosol (30%), extracellular region (30%), nucleus (30%), plasma membrane (22%), mitochondrion (22%) and other intracellular components (22%) (Appendix 6-Figure 2a). On the other hand, over-expressed proteins (100%, 16 cellular component groups) were mostly located in cytoplasm (36%), cytosol (32%), chloroplast (27%), plastid (27%), plasma membrane (23%), mitochondrion (23%) and other intracellular components (32%) (Appendix 6-Figure 2b).

At 67 dpi in T200, 143 proteins (69%) were over-expressed and 63 (31%) were under-expressed (Table 3-1). In over-expressed proteins, 127 proteins (89%, 42 functional groups) were mostly involved in biosynthetic process (36%), response to chemical (35%), response to stress (31%), response to abiotic stress (22%), anatomical structure development (17%), catabolic process (17%), multicellular organism development (17%), transport (14%), other metabolic processes (63%) and other cellular processes (77%) (Appendix 6-Figure 3). Biological processes that had at least 14% of under-expressed proteins (50, 37 functional groups) were cellular protein modification process (24%), response to stress (24%), biosynthetic process (22%), nucleobase-containing compound metabolic process (20%), protein metabolic process (20%), response to chemical (20%), anatomical structure development (16%), cellular component organisation (16%), multicellular organism development (16%), response to abiotic stress (16%), transport (14%), other metabolic processes (54%) and other cellular processes (62%) (Appendix 6-Figure 4). A total of 8% under-expressed and 4% over-expressed proteins had unknown biological processes. A total of 129 over-expressed proteins (90%, 21 functional groups) had functions in catalytic activity (38%), other binding (36%), transferase activity (24%), protein binding (23%), hydrolase activity (22%) and RNA binding (12%) (Appendix 6-Figure 3). Molecular functions that were more enriched in under-expressed proteins (78%, 16 functional groups) included catalytic activity (41%), protein binding (27%), RNA binding (24%), hydrolase activity (22%), other binding (20%) and transferase activity (14%) (Appendix 6-Figure 4). Over-expressed had 5% of proteins with unknown molecular function, while under-expressed had 6%. Cellular components, where most over-expressed proteins (98%, 22 cellular component groups) were located, included cytosol (50%), cytoplasm (47%), chloroplast (45%), nucleus (29%), plastid (25%), other intracellular components (23%), mitochondrion (20%) and plasma membrane (20%). Down-regulate protein (92%, 20 cellular component groups) were located in the chloroplast (48%), cytosol (38%), cytoplasm (34%), extracellular region (33%), nucleus (31%), plastid (29%), other intracellular components (29%), plasma membrane (28%), mitochondrion (24%), other membranes (22%), cell wall (21%) and thylakoid (16%) (Appendix 6-Figure 4).

At 67 dpi in TME3 125 proteins (61%) were over-expressed and 81 proteins (39%) under-expressed (Table 3-1). Out of 125 over-expressed proteins, 108 proteins (86%, 39 functional groups) were mostly involved in other cellular processes (71%), other metabolic processes (67%), biosynthetic process (35%), response to chemical (32%), response to stress (31%), response to abiotic stimulus (22%), catabolic process (19%) and cellular protein modification process (14%) (Figure 3-3). In under-expressed proteins, proteins (84%, 40 functional groups) were mostly involved in other cellular processes (75%), other metabolic processes (51%), biosynthetic process (28%), response to chemical (28%), response to stress (28%), anatomical structure development (24%), multicellular organism development (22%), response to abiotic stimulus (19%), transport (19%) and nucleobase-containing compound metabolic process (18%) (Figure 3-4). Molecular functions that over-expressed proteins (85%, 19 functional groups) are involved in include catalytic activity (46%), other binding (31%), hydrolase activity (27%), transferase activity (25%), protein binding (23%) and RNA binding (13%) (Figure 3a). Under-expressed proteins (88%, 17 functional groups) also had high proteins numbers in similar molecular functions as over-expressed proteins but with slightly different proteins percentage. These include other binding (32%), catalytic activity (28%), protein binding (25%), RNA binding (20%), hydrolase activity (15%) and transferase activity (15%) (Figure 3-4). Over-expressed proteins (94%) were located across 20 cellular components and most proteins were found in the chloroplast (56%), cytosol (53%), cytoplasm (44%), plastid (32%), nucleus (26%), other intracellular components (25%), mitochondrion (24%), plasma membrane (22%), other membranes (18%), extracellular region (17%) and vacuole (16%). Cellular components with the least proteins included external encapsulating structure (1%), nucleolus (3%) and peroxisome (Figure 3-3). All under-expressed proteins were located across 24 cellular components and the cytoplasm (43%), cytosol (40%), nucleus (35%), chloroplast (31%), other intracellular components (25%), plasma membrane (22%), other membranes (21%), extracellular region (20%), mitochondrion (17%) and plastid (17%) had the most percentage of proteins. External encapsulating structure, cytoskeleton, endosome, nuclear envelope and peroxisome were amongst cellular components with the least number of proteins (Figure 3-3).

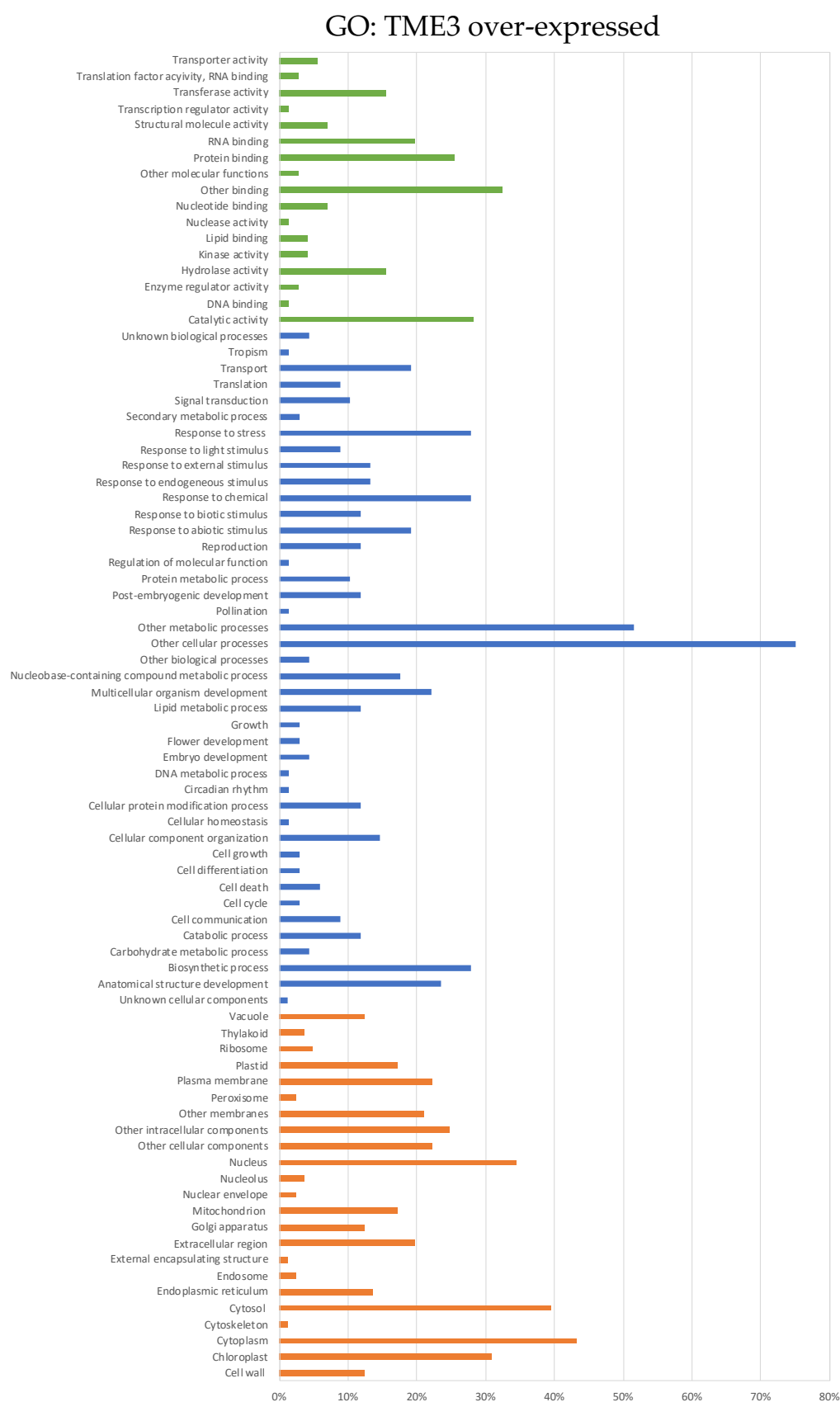


Figure 3-3. Gene ontology enrichment analysis of over-expressed proteins from TME3 at 67 dpi. **Green** colour represents molecular function; **Blue**, biological process; **Orange**, cellular component.

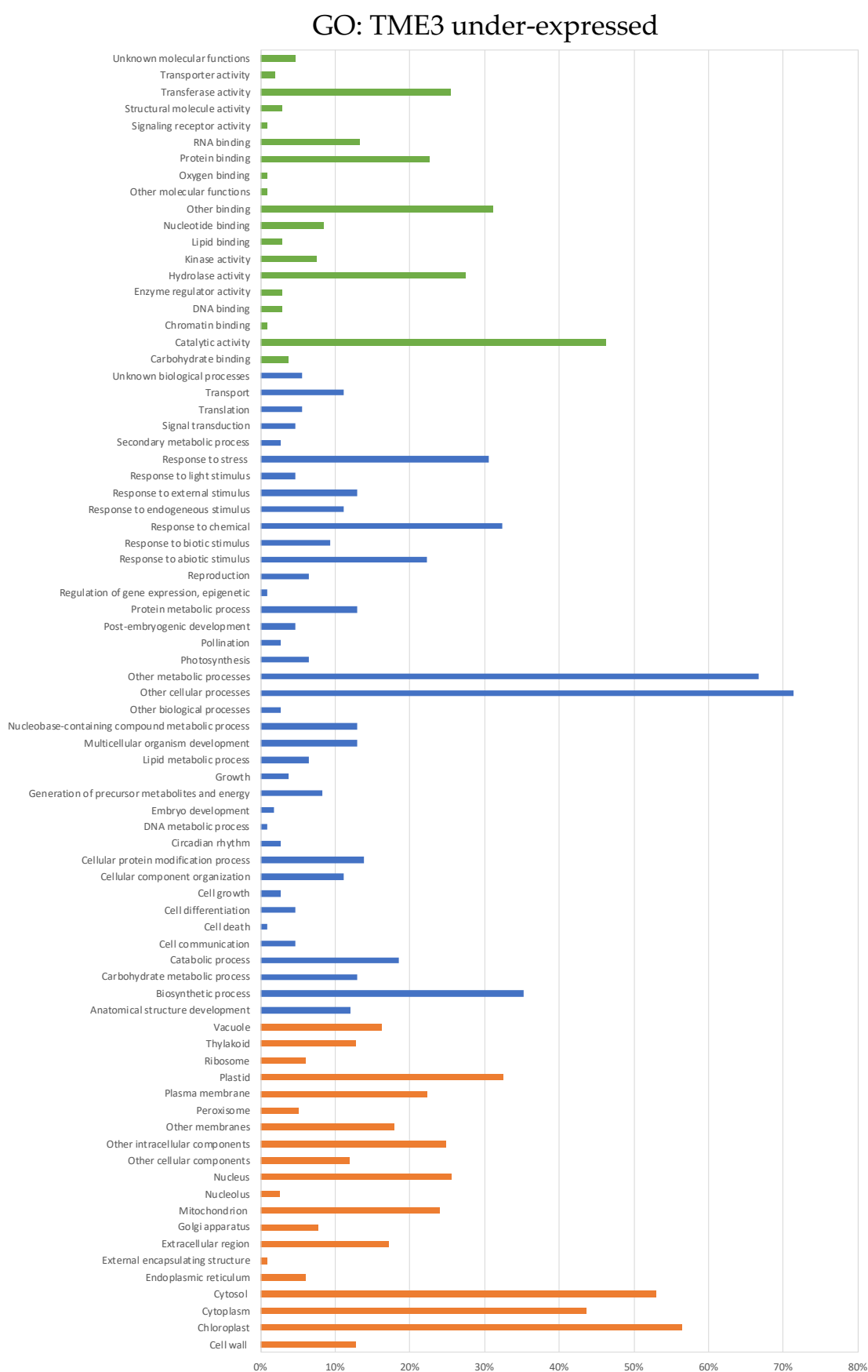


Figure 3-4. Gene ontology enrichment analysis of under-expressed proteins from TME3 at 67 dpi. **Green** colour represents molecular function; **Blue**, biological process; **Orange**, cellular component.

3.3.4. KEGG, Protein-protein interaction network and reactome pathway analysis

KEGG analysis of DEPs in T200 and TME3 at 32 dpi resulted in 6 significantly enriched pathways whereas 22 pathways were significantly enriched at 67 dpi (Table 3-2). KEGG pathways that were unique to 67 dpi included nitrogen metabolism, pyruvate metabolism, glycolysis/gluconeogenesis, ribosome and proteasome. STRING applies known and predicted protein-protein interactions extracted from genomic context predictions, high-throughput lab experiments, co-expression, automated text mining and previous knowledge in databases to create interaction networks. At 32 dpi, differentially expressed protein-protein network (p -value $<1.0e-16$) revealed four major functional groups: DNA repair and replication, secondary metabolite biosynthesis, glycolysis and plant innate immunity (Figure 3-5a).

Proteins such as suppressor of the G2 allele of *skp1* (SGT1) subunit A and SGT1B were some of the interactors with functions in plant innate immunity. SGT1 directly interacts with heat shock protein 90 (HSP90) and *Arabidopsis* homolog of Manes.09G037400 (AT3G12050; activator of Hsp90 ATPase family) which was under-expressed in both TME3 and T200 with ratio of 1.61 and 1.48, respectively (Figure 3-5a; Table 3-3). SGT1 is also an essential S-phase kinase-associated protein (SKP1)-interacting eukaryotic protein which is part of the Cullin1-based RING ligases SCF complexes that function in plant-virus interactions. At 67 dpi, proteins mainly formed two groups: proteasome and translation and translation regulation (Figure 3-5b). Regulatory particle triple-ATPase (RPT) subunit 2a and RPT2b were directly interacting with Manes.01G032500 (AT1G29150, Proteasome component family) and Manes.05G155000 (AT1G45000, Holliday junction DNA helicase RuvB P-loop family) both over-expressed in T200 and under-expressed in TME3 (Figure 3-5b, Table 3-3). Furthermore, STRING analysis gave predictions of reactome pathways that DEPs were involved in. At 32 dpi metabolism (6 proteins) was the only pathway that was significantly enriched (FDR < 0.05). Three pathways were significantly enriched at 67 dpi: metabolism (28 proteins), L13a-mediated translational silencing of Ceruloplasmin expression (8 proteins) and antigen processing-Cross presentation (3 proteins).

Table 3-2. KEGG analysis of differentially expressed proteins in SACMV-infected T200 and TME3 plants at 32 and 67 dpi.

Time point	Pathway	Protein count	FDR ^a
32 dpi	Metabolic pathways	13	2.30E-04
	Biosynthesis of secondary metabolites	8	2.30E-03
	Carbon metabolism	5	7.30E-04
	Arginine biosynthesis	3	5.40E-04
	Biosynthesis of amino acids	3	3.86E-02
	Carbon fixation in photosynthetic organisms	2	3.64E-02
67 dpi	Metabolic pathways	56	1.46E-18
	Biosynthesis of secondary metabolites	36	2.88E-13
	Biosynthesis of amino acids	14	1.04E-07
	Glycine, serine and threonine metabolism	9	1.04E-07
	Carbon metabolism	9	1.30E-03
	Arginine biosynthesis	7	2.83E-07
	Ribosome	7	3.26E-02
	Amino sugar and nucleotide sugar metabolism	6	2.80E-03
	Alanine, aspartate and glutamate metabolism	5	4.80E-04
	Carbon fixation in photosynthetic organisms	5	1.60E-03
	2-Oxocarboxylic acid metabolism	5	1.80E-03
	Glyoxylate and dicarboxylate metabolism	5	1.90E-03
	Glycolysis / Gluconeogenesis	5	9.10E-03
	Arginine and proline metabolism	4	4.60E-03
	Pyruvate metabolism	4	1.92E-02
	Nitrogen metabolism	3	2.07E-02
	Ascorbate and aldarate metabolism	3	2.32E-02
	Valine, leucine and isoleucine degradation	3	2.32E-02
	Pentose phosphate pathway	3	3.41E-02
	Proteasome	3	3.41E-02
	Histidine metabolism	2	3.26E-02
	Other glycan degradation	2	3.41E-02

^a false discovery rate.

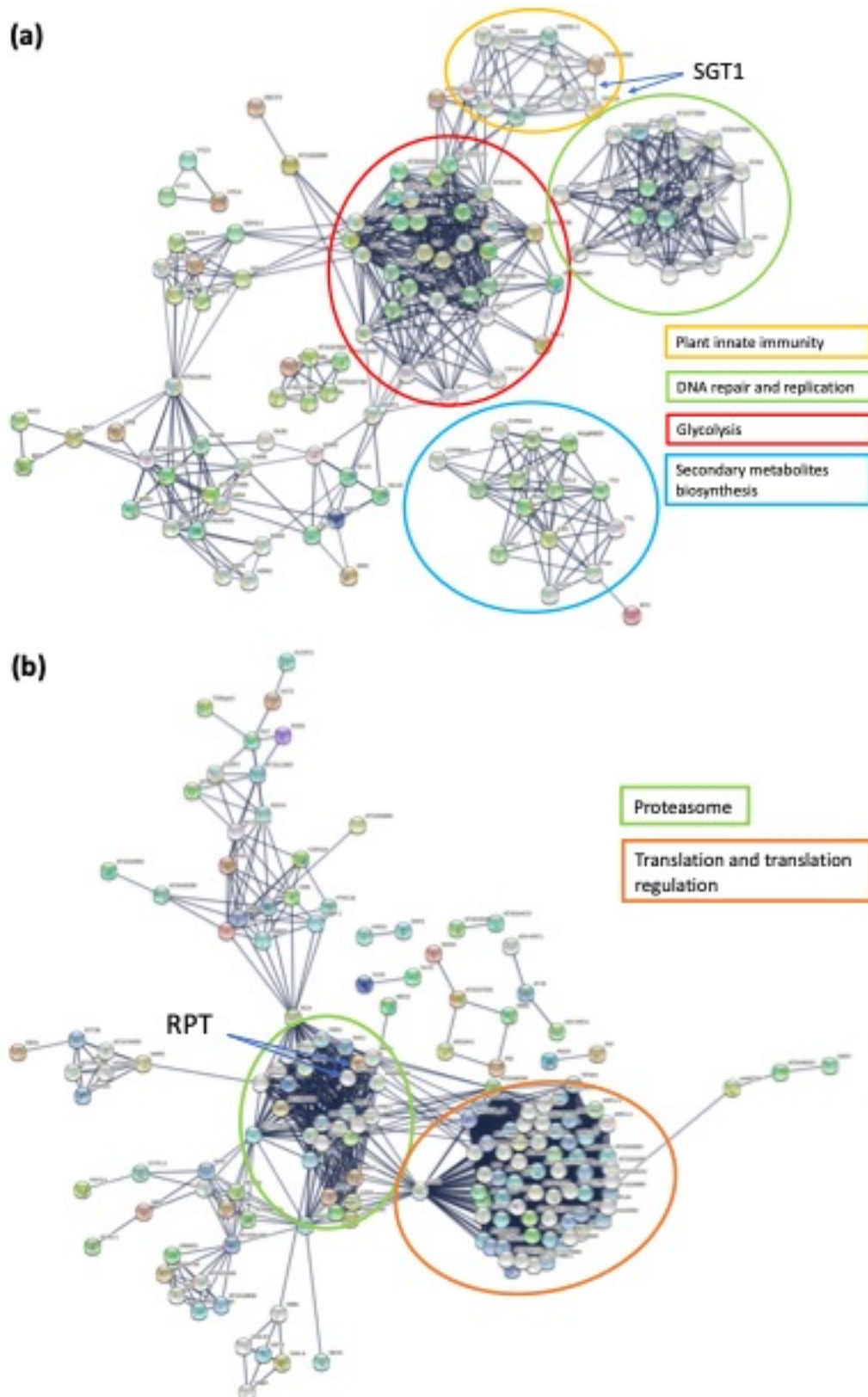


Figure 3-5. STRING protein-protein interaction network of differentially expressed proteins with p -value $< 1.0 \times 10^{-16}$ in T200 and TME3 at 32 dpi **(a)** and 67 dpi **(b)**. Network Settings: Full network; Confidence edge; Textmining, Experiments, Databases, Co-expression Interaction sources; Confidence of 0.8 and 0.9, respectively; More than 50 interactors 1st and 2nd shell; Hide disconnected nodes in the network.

3.3.5. Differentially expressed protein groups in response to SACMV infection at 67 dpi

3.3.5.1. Metabolic pathways

A total of 56 proteins were involved in primary metabolic pathways in TME3 infected with SACMV at 67 dpi (Table 3-2). Functions of up- and under-expressed DEPs in this group included amino acid metabolism, secondary metabolism, photorespiration and carbohydrate metabolism. Primary metabolites are crucial for the normal development of the plant. In T200, glycine cleavage system H proteins (Manes.07G087900, Manes.15G169800) and aminotran_5 domain-containing protein (Manes.01G181700), also functioning in the biosynthesis of secondary metabolites were under-expressed. In addition, phosphoglycerate kinase (PGK, Manes.14G009000), a major role player in energy generation was also under-expressed (Table 3-3). In *Nicotiana benthamiana*, chloroplast PGK was found to be one of the proteins that binds to bamboo mosaic virus (BaMV) RNA [420]. Trehalose-6-phosphate synthase 7 (Manes.16G042700) is another protein that has been shown to have play a role in plant growth, developments and also regulation of defence response against pathogens. This protein was over-expressed T200 and under-expressed in TME3. Other proteins involved in the biosynthesis of metabolites include Inositol-1-monophosphatase, Phosphoacetylglucosamine mutase, AA-kinase domain-containing protein and Arginine biosynthesis bifunctional protein, to mention a few (Table 3-3). One protein of note was uncharacterised Manes.09G142200 belonging to the family enoyl-(Acyl carrier protein) reductase which was highly over-expressed by 67-fold in TME3 and under-expressed (1.63-fold) in T200.

3.3.5.2. Biosynthesis of secondary metabolites

A total of 36 proteins were involved in the biosynthesis of secondary metabolites (Table 3-2). Plant hosts require secondary metabolites for several roles including pigmentation, growth, reproduction and resistance to pathogens, amongst many others. Peroxidase protein, Manes.15G103900 was over-expressed in T200 and under-expressed in TME3 (Table 3-3). Peroxidases are known to play important roles in oxidative stress, auxin catabolism and cross-linking of structural cell wall proteins amongst others. In this study, a methyltransferase protein (Manes.05G142300) was under-expressed almost seven-fold in TME3 and over-expressed three-fold in T200 (Table 3-3). Manes.16G087600 (3-ketoacyl-CoA synthase) and AB hydrolase-1 domain containing proteins (Manes.12G095700, Manes.04G110900, Manes.05G029200 and Manes.13G092100) are some of the structural proteins that play a role in lipid homeostasis (Table 3-3) [421,422]. Other characterised proteins in this group include PALP domain-containing protein, Phospholipase D, Serine hydroxymethyltransferase, FAD-binding PCMH-type domain-containing protein, GPAT_N domain-containing protein, SAICAR_synt domain-containing protein, inositol-1-monophosphatase and F420_oxidored domain-containing

protein (Table 3-3). Some of the proteins however were not characterised but these belonged to protein families including KR domain (Manes.10G026600), p450 (Manes.06G006200 and Manes.09G183600) and aminotransferase_3 (Manes.13G089400), just to mention a few.

3.3.5.3. Glycolysis/gluconeogenesis

This pathway is mainly for the generation of energy. When a plant is attacked by an external stimulus, it directs most of its energy to defence response mechanisms and less to cellular metabolic pathways. In this group, five proteins are involved. Three proteins were over-expressed in both T200 and TME3 and these are also involved in metabolic pathways: FBPase domain-containing protein (Manes.08G083500), pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit alpha (Manes.18G001100) and Manes.04G146300 (uncharacterised). FBPase (fructose 1, 6-bisphosphatase) catalyses the hydrolysis of fructose 1, 6-phosphate to fructose 6-phosphate in gluconeogenesis and the Calvin cycle. The reverse is true for glycolysis with phosphofructokinase (PFK) and pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit alpha enzymes catalysing the reaction. With FBPase over-expressed, it means that the plant had enough cellular ATP concentration and there was no need to produce more in both T200 and TME3 at 67 dpi. Uncharacterised Manes.04G146300 protein is from the family glycoside hydrolase family 19 which consists of chitinase class I. Phosphoglycerate kinase (Manes.14G009000) was over-expressed in TME3 with ratio of 1.51 and under-expressed in T200 with ratio of 1.12 (Table 3-3). This enzyme catalyses the high-energy phosphoryl transfer of the acyl phosphate of 1,3-bisphosphoglycerate to ADP to produce ATP [423]. The last protein in this group is uncharacterised (Manes.08G094400), belongs to the thiamine pyrophosphate enzyme (TTP) family and it was only over-expressed in T200 (ratio 1.91; Table 3-3).

3.3.5.4. Ribosome

Ribosomal proteins are known to be components of the protein synthesis machinery. However, with recent studies it would seem that these proteins have extra functions such as stress signalling [424]. Seven DEPs were grouped to ribosome pathway (Table 3-3), and three of these are uncharacterised. Three proteins were under-expressed in T200 and over-expressed in TME3, three over-expressed in T200 and under-expressed in TME3, and one was under-expressed in both landraces. For example, ribosomal_L14e domain-containing protein (Manes.03G162900) was over-expressed with ratio of 4.36 in T200 and under-expressed with ratio of 1.86 in TME3 (Table 3-3). This protein binds to the 60S ribosomal subunit and plays a role in translation [425]. The 40S ribosomal protein S4 (Manes.03G095200) had ratios of 1.59 in T200 and -1.06 in TME3. An increase in transcript levels of this protein was observed in *Vanilla planifolia* Jacks infected with fungi [426], whereas there was a

decrease in transcript in *Arabidopsis* 48 h after inoculation with *A. tumefaciens* [427]. The 60S ribosomal protein L13 (Manes.07G017100) was under-expressed in T200 (3.86) and over-expressed in TME3 (2.11). This protein has been shown to interact with CMV P6 (translation reinitiator) in *A. thaliana* [428]. Uncharacterised proteins, Manes.10G151400 (-3.90 ratio in T200 and -1.14 in TME3), Manes.17G064800 (-4.61 in T200 and 1.83 in TME3) and Manes.10G019200 (3.30 in T200 and -1.16 in TME3) are members of the ribosomal protein family L3, L32 and L35Ae. The last protein of interest in this group was ribosomal_L7Ae domain-containing protein (Manes.12G139100), which was under-expressed in T200 with ratio of 5.24 and over-expressed in TME3 with the ratio of 2.18 (Table 3-3). Like the other proteins in this group not much has been reported on this protein, however, its molecular function includes RNA binding.

3.3.5.5. Proteasome

Proteasomes form a large portion of the ubiquitin proteasome system, responsible for degrading intracellular proteins involved in most cellular metabolic processes. Three proteins, also involved in metabolic pathways function as proteasomes, were only over-expressed in T200. PCI domain-containing protein (Manes.01G032500) was over-expressed by up to 7 folds ratio compared to mock sample in T200. PCI (Proteasome, COP9 signalosome, and initiation factor 3) domain mediates and stabilize protein-protein interactions within multi subunit protein complexes [429]. The other two proteins are both AAA domain-containing proteins with ratios of 4.75 in T200 and -1.32 in TME3 (Manes.13G043200) and 2.82 in T200 and -1.07 in TME3 (Manes.05G15500) (Table 3-3). ATPase associated with various cellular activities (AAA) plays a role in the cell including cell-cycle regulation, protein proteolysis and disaggregation, organelle biogenesis and intracellular transport.

Table 3-3. Differentially expressed proteins between mock-inoculated and SACMV-infected leaves at 67 dpi. Proteins with at least a ratio of 2.0 in one landrace are presented here.

Accession	Manes ID	Protein name	Unique peptides	q Value	T200 Ratio ^a	TME3 Ratio ^a
tr A0A2C9U824	Manes.16G022000	PKS_ER domain-containing protein	3.00	2.83E-04	2.38	-1.15
tr A0A2C9VEA2	Manes.08G075000	Uncharacterized protein	4.00	7.56E-04	2.37	-2.04
tr A0A2C9U4J9	Manes.17G026300	Lactoylglutathione lyase	2.00	2.59E-03	-1.35	2.82
tr A0A2C9VIL5	Manes.07G049300	Proline iminopeptidase	7.00	3.91E-03	2.26	1.05
tr A0A0A1E5H6	Manes.11G078100	Phosphate transporter	2.00	4.63E-03	5.62	1.01
tr A0A251JR98	Manes.11G020100	Germin-like protein	6.00	5.29E-03	-3.26	-1.03
tr A0A2C9VJP2	Manes.07G087900	Glycine cleavage system H protein	2.00	6.82E-03	-3.32	1.43
tr A0A2C9WL85	Manes.01G155900	Uncharacterized protein	2.00	6.82E-03	2.46	-2.07
tr A0A2C9V1F6	Manes.11G148200	Uncharacterized protein	3.00	7.50E-03	-2.79	5.63
tr A0A2C9UCJ8	Manes.15G029400	Cysteine synthase	10.00	7.85E-03	2.94	1.25
tr A0A2C9VK05	Manes.07G093600	GLOBIN domain-containing protein	2.00	1.11E-02	1.14	3.05
tr A0A2C9UGM3	Manes.15G103900	Peroxidase	9.00	1.16E-02	2.09	-1.03
tr A0A2C9UYJ6	Manes.11G012700	Aminotran_1_2 domain-containing protein	10.00	1.16E-02	1.01	2.01
tr A0A2C9VAT8	Manes.09G071100	Uncharacterized protein	2.00	1.22E-02	2.23	1.27
tr A0A2C9VSW1	Manes.05G029200	AB hydrolase-1 domain-containing protein	2.00	1.26E-02	2.09	1.25
tr A0A2C9WBE7	Manes.02G064500	PALP domain-containing protein	3.00	1.30E-02	3.27	1.14
tr A0A2C9URN3	Manes.13G132700	Uncharacterized protein	4.00	1.32E-02	-1.40	3.51
tr A0A2C9VLP8	Manes.06G006200	Uncharacterized protein	3.00	1.34E-02	3.25	-1.85
tr A0A2C9UGX0	Manes.15G172800	Phosphoacetylglucosamine mutase	9.00	1.49E-02	2.46	-1.12
tr A0A2C9VP29	Manes.06G081300	Uncharacterized protein	3.00	1.51E-02	18.46	-1.91
tr A0A2C9UBX9	Manes.15G008100	Bet_v_1 domain-containing protein	9.00	1.76E-02	-1.11	3.20
tr A0A2C9V4E1	Manes.10G042700	Uncharacterized protein	2.00	1.76E-02	-1.20	2.12
tr A0A2C9WLB8	Manes.01G085900	Uncharacterized protein	2.00	1.76E-02	1.17	2.43
tr A0A251K3C9	Manes.09G021000	HP domain-containing protein	18.00	1.81E-02	2.18	-1.71

tr A0A2C9UTJ3	Manes.12G039500	Uncharacterized protein	6.00	1.81E-02	-1.18	2.10
tr A0A2C9W314	Manes.04G086800	SUMO-activating enzyme subunit	2.00	1.81E-02	-5.15	4.65
tr A0A251K980	Manes.08G011100	Uncharacterized protein	3.00	1.81E-02	11.82	-2.66
tr A0A251K915	Manes.08G008000	Uncharacterized protein	4.00	1.81E-02	1.82	-2.13
tr A0A2C9VGL2	Manes.08G075100	VWFA domain-containing protein	11.00	2.00E-02	3.13	-1.29
tr A0A2C9WEH9	Manes.02G089200	Uncharacterized protein	6.00	2.07E-02	2.41	1.99
tr A0A2C9UID8	Manes.15G186400	Uncharacterized protein	6.00	2.07E-02	4.47	-5.30
tr A0A2C9UV72	Manes.12G093300	Glycosyltransferase	2.00	2.24E-02	9.06	-1.92
tr A0A2C9VFU9	Manes.08G126700	Protein kinase domain-containing protein	2.00	2.66E-02	23.94	-1.43
tr A0A2C9W2J9	Manes.04G146100	Uncharacterized protein	7.00	2.66E-02	2.21	-1.44
tr A0A2C9UPJ8	Manes.13G061700	FAS1 domain-containing protein	10.00	2.66E-02	-1.45	-2.01
tr A0A2C9U6Y4	Manes.17G080500	Uncharacterized protein	5.00	2.67E-02	-2.30	1.59
tr A0A2C9U1Y7	Manes.18G103000	Uncharacterized protein	3.00	2.68E-02	3.18	-1.22
tr A0A2C9UQH1	Manes.13G092100	AB hydrolase-1 domain-containing protein	18.00	2.70E-02	1.30	2.76
tr A0A2C9UYF6	Manes.11G055100	Phospholipase D	34.00	2.73E-02	2.05	-1.17
tr A0A251K1M8	Manes.10G151400	Uncharacterized protein	2.00	2.73E-02	-3.90	-1.14
tr A0A2C9WAR9	Manes.03G202900	Uncharacterized protein	9.00	2.73E-02	2.28	1.45
tr A0A2C9UXW4	Manes.12G139100	Ribosomal_L7Ae domain-containing protein	3.00	2.73E-02	-5.24	2.18
tr A0A2C9UYT0	Manes.12G153700	Uncharacterized protein	9.00	2.79E-02	2.40	1.20
tr A0A2C9UNI9	Manes.14G166500	Non-specific lipid-transfer protein	6.00	2.81E-02	-5.88	10.33
tr A0A2C9V3Z4	Manes.10G027600	ATPase_AAA_core domain-containing protein	3.00	2.89E-02	-52.29	6.89
tr A0A251JSV5	Manes.11G063000	Uncharacterized protein	3.00	2.94E-02	2.16	1.11
tr A0A2C9WF62	Manes.02G137700	Alpha-mannosidase	13.00	3.05E-02	2.32	1.63
tr A0A2C9W858	Manes.03G096900	M20_dimer domain-containing protein	3.00	3.05E-02	2.47	1.62
tr A0A2C9VPI9	Manes.06G102100	Uncharacterized protein	2.00	3.05E-02	2.09	-2.96
tr A0A2C9VT96	Manes.06G155500	Glyco_hydro_18 domain-containing protein	4.00	3.05E-02	2.67	2.71
tr A0A2C9VIN8	Manes.07G051800	Uncharacterized protein	3.00	3.07E-02	2.44	1.35
tr A0A2C9UXQ1	Manes.11G033300	TauD domain-containing protein	4.00	3.14E-02	2.87	1.67

tr A0A0C4ZQZ2	Manes.12G135200	Annexin	13.00	3.14E-02	2.78	1.41
tr A0A2C9UCR5	Manes.15G007900	Bet_v_1 domain-containing protein	2.00	3.14E-02	-1.08	3.69
tr A0A2C9U3Q4	Manes.17G010500	RRM domain-containing protein	4.00	3.14E-02	2.41	-1.45
tr A0A2C9WMF8	Manes.01G202400	Uncharacterized protein	3.00	3.21E-02	6.53	-1.13
tr A0A2C9VRV6	Manes.06G177500	Uncharacterized protein	2.00	3.26E-02	3.26	1.27
tr A0A2C9UUG2	Manes.12G027700	Uncharacterized protein	14.00	3.32E-02	2.28	1.56
tr A0A2C9U185	Manes.18G062000	NAD(P)H-hydrate epimerase	7.00	3.42E-02	4.79	-2.12
tr A0A2C9V3P1	Manes.10G055500	WD_REPEATS_REGION domain-containing protein	2.00	3.43E-02	2.24	-1.84
tr A0A2C9VBZ8	Manes.09G142200	Uncharacterized protein	2.00	3.59E-02	-1.63	67.74
tr A0A2C9UV10	Manes.12G095700	AB hydrolase-1 domain-containing protein	3.00	3.59E-02	1.42	2.21
tr A0A2C9UJE4	Manes.14G034500	UMP-CMP kinase	2.00	3.59E-02	3.10	1.14
tr A0A2C9WRD2	Manes.01G245400	Importin N-terminal domain-containing protein	9.00	3.59E-02	3.16	-1.16
tr A0A251KS16	Manes.05G011800	Uncharacterized protein	12.00	3.61E-02	2.05	-1.21
tr A0A2C9UGV6	Manes.15G140500	Eukaryotic translation initiation factor 3 subunit I	10.00	3.61E-02	2.10	-1.40
tr A0A2C9UA86	Manes.16G087600	3-ketoacyl-CoA synthase	2.00	3.61E-02	8.18	-6.12
tr A0A2C9UX42	Manes.12G156900	Hist_deacetyl domain-containing protein	5.00	3.74E-02	-4.08	1.33
tr A0A2C9W735	Manes.03G081200	FAD-binding PCMH-type domain-containing protein	8.00	3.77E-02	5.66	-1.54
tr A0A2C9VMD1	Manes.07G122600	AAI domain-containing protein	4.00	3.77E-02	-10.20	-1.40
tr A0A2C9VAA7	Manes.09G127700	Bet_v_1 domain-containing protein	7.00	3.77E-02	1.32	2.22
tr A0A2C9URF0	Manes.13G126100	Uncharacterized protein	2.00	3.77E-02	2.16	-1.04
tr A0A2C9U7U7	Manes.16G007800	Uncharacterized protein	7.00	3.77E-02	-2.85	1.88
tr A0A2C9W3J5	Manes.04G095600	eRF1_1 domain-containing protein	4.00	3.77E-02	2.26	-1.02
tr A0A199UC10	Manes.s021400	ZnMc domain-containing protein	2.00	3.77E-02	3.57	1.09
tr A0A2C9WH55	Manes.01G012700	Vac14_Fab1_bd domain-containing protein	3.00	3.80E-02	2.00	1.10
tr A0A2C9U3Q3	Manes.18G143800	Uncharacterized protein	3.00	3.80E-02	-5.26	1.18
tr A0A2C9V4F7	Manes.10G077300	Uncharacterized protein	2.00	3.80E-02	2.46	1.12
tr A0A251LVH3	Manes.01G267300	Uncharacterized protein	3.00	3.87E-02	1.54	2.11
tr A0A2C9UJT8	Manes.14G080800	Peptidyl-prolyl cis-trans isomerase	2.00	3.90E-02	-3.45	4.03

tr A0A2C9V4B1	Manes.10G077100	Lipase_3 domain-containing protein	5.00	3.92E-02	2.03	-1.24
tr A0A2C9VG07	Manes.08G081300	Uncharacterized protein	2.00	3.93E-02	-5.02	6.17
tr A0A2C9WEB2	Manes.02G155900	Uncharacterized protein	4.00	3.93E-02	1.17	4.80
tr A0A2C9V5Y8	Manes.10G128500	Uncharacterized protein	5.00	3.93E-02	4.19	-1.96
tr A0A2C9WJN9	Manes.01G112700	AAI domain-containing protein	3.00	4.06E-02	-2.73	6.45
tr A0A2C9VN74	Manes.06G056000	Uncharacterized protein	2.00	4.06E-02	3.26	-1.66
tr A0A2C9WNU1	Manes.01G247800	Glycosyltransferase	2.00	4.06E-02	2.17	1.39
tr A0A2C9VYT5	Manes.05G155000	AAA domain-containing protein	9.00	4.06E-02	2.82	-1.07
tr A0A2C9W393	Manes.04G165200	Uncharacterized protein	4.00	4.06E-02	4.42	-1.42
tr A0A2C9VKB0	Manes.07G107200	Chalcone-flavonone isomerase family protein	10.00	4.06E-02	2.89	1.20
tr A0A2C9UBT3	Manes.15G008400	Bet_v_1 domain-containing protein	7.00	4.06E-02	2.85	13.10
tr A0A2C9V9W8	Manes.09G113900	Uncharacterized protein	4.00	4.06E-02	2.31	1.48
tr A0A2C9WD78	Manes.02G122500	Alpha-1,4 glucan phosphorylase	5.00	4.06E-02	2.32	4.83
tr A0A2C9WCC9	Manes.02G095600	CTP_transf_like domain-containing protein	3.00	4.18E-02	1.16	2.35
tr A0A2C9UNS6	Manes.13G043200	AAA domain-containing protein	9.00	4.22E-02	4.75	-1.32
tr A0A2C9U6K5	Manes.17G064800	Uncharacterized protein	2.00	4.23E-02	-4.61	1.83
tr A0A2C9VE34	Manes.08G069100	Uncharacterized protein	15.00	4.30E-02	2.06	1.34
tr A0A2C9V5M6	Manes.10G121900	Uncharacterized protein	13.00	4.30E-02	2.52	-1.27
tr A0A2C9U8P4	Manes.17G111900	Uncharacterized protein	3.00	4.30E-02	2.33	1.50
tr A0A2C9USP9	Manes.12G024300	Uncharacterized protein	2.00	4.30E-02	2.64	-1.54
tr A0A2C9V2I1	Manes.10G019200	Uncharacterized protein	2.00	4.34E-02	3.30	-1.16
tr A0A2C9VWB9	Manes.05G146600	Peptidyl-prolyl cis-trans isomerase	3.00	4.34E-02	-1.52	2.06
tr A0A2C9UGS7	Manes.15G108300	Uncharacterized protein	6.00	4.45E-02	3.10	-1.11
tr A0A2C9VJ85	Manes.07G017100	60S ribosomal protein L13	2.00	4.45E-02	-3.86	2.11
tr A0A2C9UTD1	Manes.12G033700	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	4.00	4.49E-02	5.39	-1.86
tr A0A2C9WHE0	Manes.01G032500	PCI domain-containing protein	2.00	4.52E-02	7.14	-1.40
tr A0A2C9UFY6	Manes.15G139800	Transmembrane 9 superfamily member	4.00	4.55E-02	4.51	-2.11

tr A0A2C9VXS5	Manes.05G194900	Protein kinase domain-containing protein	2.00	4.55E-02	-8.12	-2.18
tr A0A2C9VQH2	Manes.06G055700	Uncharacterized protein	7.00	4.57E-02	2.10	-2.39
tr A0A2C9VYM5	Manes.05G142300	Methyltransferase	2.00	4.62E-02	3.32	-6.67
tr A0A2C9TZI5	Manes.18G001100	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha	3.00	4.62E-02	2.13	1.34
tr A0A2C9V233	Manes.11G128200	Uncharacterized protein	3.00	4.65E-02	-6.76	9.16
tr A0A2C9V6N1	Manes.09G008000	F420_oxidored domain-containing protein	4.00	4.68E-02	1.08	2.91
tr A0A251JLF7	Manes.12G028400	Carbonic anhydrase	2.00	4.68E-02	2.95	-1.11
tr A0A2C9W4R1	Manes.03G053100	Uncharacterized protein	5.00	4.68E-02	-5.50	1.68
tr A0A2C9V313	Manes.10G032500	NAD(P)-bd_dom domain-containing protein	3.00	4.68E-02	3.11	1.28
tr A0A2C9VBY7	Manes.09G183600	Uncharacterized protein	3.00	4.68E-02	3.66	-1.04
tr A0A2C9UMZ5	Manes.13G011400	AAI domain-containing protein	5.00	4.68E-02	-9.13	6.96
tr A0A2C9VRT4	Manes.06G097000	Plasma membrane ATPase	6.00	4.69E-02	5.75	-1.45
tr A0A2C9WA59	Manes.03G162900	Ribosomal_L14e domain-containing protein	2.00	4.69E-02	4.36	-1.86
tr A0A2C9VYM8	Manes.04G011900	Carboxypeptidase	3.00	4.69E-02	-1.36	3.72
tr A0A251K5T0	Manes.09G086600	Uncharacterized protein	6.00	4.69E-02	3.00	-1.22
tr A0A2C9V462	Manes.10G078000	Glycosyltransferase	5.00	4.71E-02	3.12	-1.63
tr A0A2C9VLD9	Manes.07G091600	Uncharacterized protein	4.00	4.81E-02	4.30	-2.64
tr A0A2C9W8C0	Manes.03G123300	Trafficking protein particle complex subunit	3.00	4.81E-02	2.54	-1.12
tr A0A2C9W8D3	Manes.03G089200	Uncharacterized protein	2.00	4.87E-02	-14.91	2.30
tr A0A199U962	Manes.S101100	Lipase_GDSL domain-containing protein	3.00	4.87E-02	-2.51	3.28
tr A0A2C9UL26	Manes.14G124000	Protein kinase domain-containing protein	3.00	4.89E-02	-1.70	-2.19
tr A0A2C9U8J5	Manes.16G001400	Abhydrolase_2 domain-containing protein	2.00	4.89E-02	-1.41	8.09
tr A0A2C9VH42	Manes.08G171700	Non-specific lipid-transfer protein	3.00	4.97E-02	-13.04	6.81
tr A0A2C9VWH1	Manes.05G151600	Uncharacterized protein	10.00	4.97E-02	3.19	-3.49

^aNegative ratio = under-expression, positive ratio = over-expression.

3.3.6. Validation of changes in RNA level

Based on proteomics analysis, ten proteins that were differentially expressed in response to SACMV were selected for transcription analyses using RT-qPCR (Figure 3-6). These selected proteins have functions in disease response and cell morphogenesis and were differentially expressed in both landraces; two at 32 dpi (C3HC4-type RING finger and LRR) and eight at 67 dpi (kinase, *SnRK1.1*, two receptor-like kinases, *LRX2*, receptor-like protein, two pathogenesis-related proteins). At 32 dpi, RT-qPCR results demonstrated that C3HC4-type RING finger (Manes.08G075100) was expressed with fold change of 0.5, whereas LRR had a fold change of -1.7 compared to mock-inoculated control (Figure 3-6). Transcription levels of the latter genes did not reflect protein translation expression regulation (Table 3-3). At 67 dpi, all eight genes were demonstrated to be over-expressed with fold change ranging from 1.7 to 10.8 higher than the mock-inoculated control, with pathogenesis-related protein_2 (Manes.15G007900) showing the highest expression levels (Figure 3-6). Transcripts of receptor-like kinases (Manes.05G194900, Manes.14G124000), Kinase (Manes.08G126700) and receptor-like protein (Manes.06G055700) were over-expressed with fold change of 7.9, 3.8, 1.7 and 2.5, respectively (Figure 3-6), whereas, the expression levels of their proteins were under-expressed based on the proteomics results (Table 3-3). The results herein suggest differences between transcript and protein levels may be due to post-transcriptional regulatory processes.

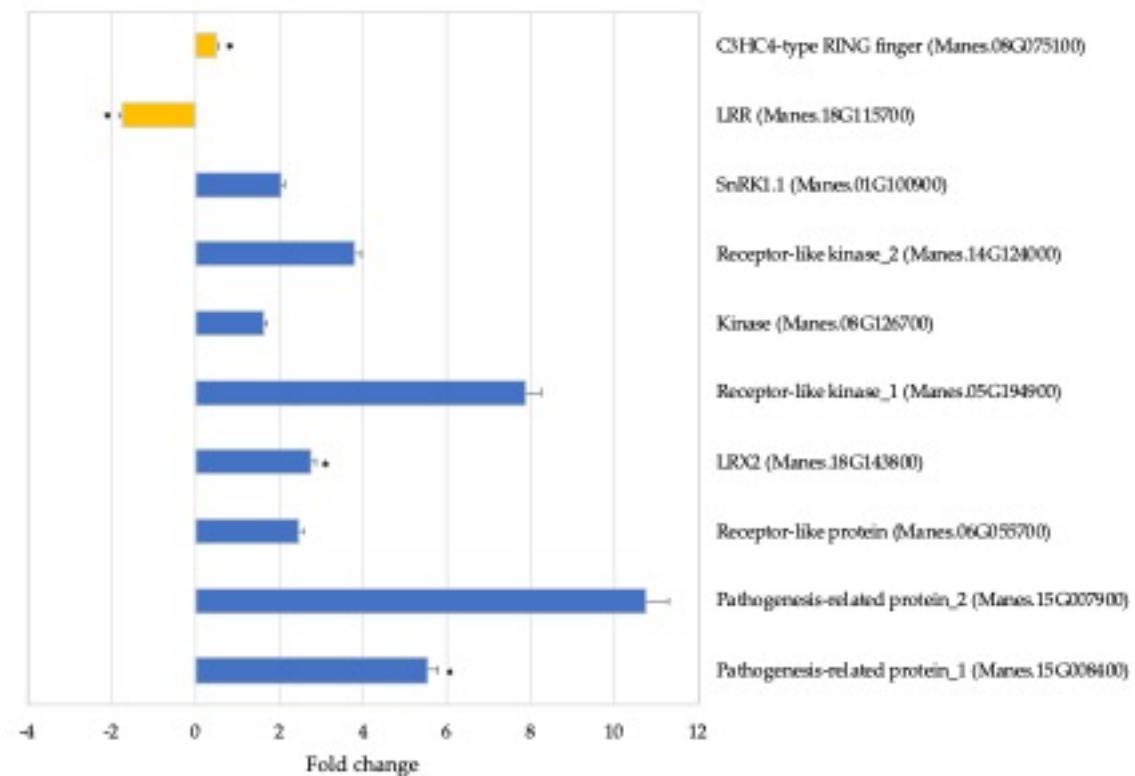


Figure 3-6. Validation of cassava gene expression levels by real time RT-qPCR in TME3. **Gold** represents 32 dpi; **Blue** represents 67 dpi. Readings are averaged from three biological replicates normalized against *UBQ10*. Error bars represent means $\pm$ S.D. (n = 3). Asterisks indicate statistical significance at $p \leq 0.05$ using t-test.

3.4. Discussion

Due to its ability to withstand drought prone regions, cassava has become an economically important crop and a major source of nutrition in the tropical and sub-tropical regions of Africa and Asia. However, cassava production is threatened by viral diseases such as CMD caused by a number of viruses including geminiviruses. As such, it is important to identify proteins that are differentially expressed in response to SACMV as these will help bridge the gap that exist in understanding plant-virus interactions in this and other orphan crops, and furthermore inform engineering strategies for crop varieties that will better tolerate and/or resist virus diseases. This study reports the first SACMV-cassava proteome map comparing susceptible and tolerant landraces and further contributes new information to the proteomic data pool. Herein we used LC-MS to identify proteins that were differentially expressed between SACMV-infected cassava landraces T200 (susceptible) and TME3 (tolerant), and to determine which proteins may be involved in the recovery tolerant phenotype at 67 dpi in TME3. During SACMV infection, 49 proteins were differentially expressed in both landraces at 32 dpi and 206 at 67 dpi (Tables 3-1, Appendix 5- Table 1).

While it is well documented that protein modifications are a consequence of pathogen infection of plants, fewer studies have been done on virus infections, in particular geminiviruses. It is noteworthy that eight out of 18 genes involved in processes related to protein modifications or protein metabolism, such as ubiquitination, rubylation, phosphorylation, acetylation or protein folding in *N. benthamiana* infected with the geminivirus tomato yellow leaf curl Sardinia virus (TYLCSV) [372]. It is therefore, not surprising that in SACMV-infected T200 and TME3, seven of the DEPs were related to the ribosome pathway. Ribosomal proteins are not only known to be components of the protein synthesis machinery, but also are involved in stress signalling [424]. In cassava infected with Indian cassava mosaic virus (ICMV) and Sri Lankan cassava mosaic virus (SLCMV), upregulation of components of the 80S ribosome and 40S small subunit was upregulated in susceptible cassava cultivar H226 [297]. In a proteome study of the begomovirus, Tomato chlorotic mottle virus (ToCMoV), in resistant tomato, two ribosomal proteins (40S ribosomal protein S7 and ribosomal protein L11/12) were also differentially expressed [407]. These results suggest that translational control via ribosome activity may be a general response to geminivirus-induced stress. Since T200 and TME3 showed contrary expression (up or down) in six ribosomal proteins, we conclude that these play a role in susceptibility and tolerance in T200 and TME3, respectively. Perturbations in protein translation also reflect dual expression of virus and plant host proteins induced during the interaction.

Carbon metabolism associated DEPs were also highly represented in T200 and TME3 at 32 and 67 dpi (Table 3-2). Manipulation of photosynthesis and primary metabolism by plant viruses is widely recorded. Photosynthetic and carbon fixation proteins were also differentially regulated in another geminivirus proteome study of

mungbean yellow mosaic India virus (MYMIV) infected *Vigna mungo* [418]. In a proteome study by Huang et al. 2016 [331] in Tomato yellow leaf curl virus (TYLCV) resistant and susceptible tomato cultivars, eighty-six DEPs were identified after infection. Many of these were also associated with photosynthesis and primary metabolism, such as chlorophylla-b binding proteins, ribulose biphosphate carboxylase and ferredoxin-NADP reductase. While viral targets in primary metabolic pathways vary between virus and host species, and also between infection time and plant age, proteome data provides compelling evidence that carbon metabolism is a key target for virus manipulation. Interestingly, most proteins in the resistant cultivar Zheza-301 showed downregulation profiles [331]. Of the 71 DEPs in Zheza-301, 28 and 43 proteins were over-expressed and under-expressed, respectively. In cassava, at 32 dpi, a similar trend was observed in both landraces where under-expressed DEPs were under-represented. However, at 67 dpi in cassava, while there was no significant difference (8%) between the two landraces between over-expressed and under-expressed DEPs, notably the percentages of over-expressed DEPs were higher than under-expressed DEPs in both T200 (38%) and TME3 (22%). This suggests that the higher percentage of over-expressed proteins at 67 dpi in both landraces may reflect partial recovery of protein-related functions compared to 32 dpi when virus replication is high and symptoms are severe.

Notably, most of the DEPs were annotated by GO to be located in different cellular components, but mostly in the cytoplasm and plastid, including the chloroplast and thylakoid (Figure 3-3, Appendix 6-Figures 1, 2, 3). From this observation, we conclude that the chloroplast may be involved in response to SACMV. It is expected to see large fractions of proteins in the cytoplasm since this is where most cellular activities such as metabolic pathways take place. On the other hand, plastids have versatile roles including providing essential metabolic and signalling functions as such photosynthetic process, affect plant growth and development, and play a role in plant-microbe interactions [430]. For the longest time, chloroplasts were known to be a factory for photosynthesis, however this fact is changing with more of its functions being reported. Functions include chloroplast involvement in plant defense responses through the production of reactive oxygen species, defense-related hormones, and plastid-to-nucleus retrograde signalling pathways [431]. Together with the fact that chloroplasts are central hubs for facilitating communication with different plant cellular compartments, chloroplasts functions have become targets of plant pathogens. Pathogens deliver micro molecules and effector proteins that disrupts the normal functioning of the chloroplast and this results in suppressed defense response and induced infection [432]. We propose that identified chloroplast proteins in our study play a role in virus tolerance and susceptibility in cassava. The majority of over-expressed proteins (56%) in TME3 at 67 dpi were localized in the chloroplast (Figure 3-3), whereas 31% of these chloroplast proteins were under-expressed in T200 at 67 dpi (Appendix 6-Figure 3). Manes.15G029400 *Arabidopsis* homolog AT4G14880, a chloroplast protein whose gene expression is mediated by abscisic acid (ABA) was over-expressed in TME3 (1.25 ratio) at a symptom recovery stage (67 dpi).

ABA is an essential signal for plant resistance to pathogens and it is synthesized in the chloroplast. The over-expression of Manes.15G029400 indicates that ABA production in the chloroplast was not affected by SACMV infection. We conclude that ABA-mediated defence mechanism was active in TME3, and plays a role in the reduction of SACMV replication and symptom reduction at 67 dpi. Results from other studies have shown the importance of ABA in biotic stress and host defence against viral pathogens. For example, in *N. benthamiana*, it was proved that *Chinese wheat mosaic virus* suppressed the ABA pathway which was important in inducing plant host defence against this virus [306]. Although the contribution of these chloroplast-associated proteins to tolerance is not currently known, we suggest that the over-expression of chloroplast proteins in tolerant TME3 at 67 dpi plays a role in virus and symptom recovery phenotype (Figure 3-1).

TME3 landrace shows a recovery phenotype and significant reduction of SACMV DNA accumulation at 67 dpi (Figure 3-1) as compared with susceptible T200 landrace. A decrease in DNA also implies that virions are not formed and coat protein synthesis is decreased. Differences in GO analysis and KEGG pathways will provide evidence for differences in SACMV responses in the T200 and TME3 landraces at 67 dpi. Coat protein (CP) of a number of viruses including TMV, *Potato virus X* and CMV has been shown to accumulate in the chloroplast of their host and cause chloroplast ultrastructure disruption as well as the development and severity of mosaic or chlorosis symptoms caused by viral infections [355–357]. TME3 landrace shows a recovery phenotype and significant reduction of SACMV DNA accumulation at 67 dpi (Figure 3-1) as compared with susceptible T200 landrace. A decrease in DNA also implies that virus coat protein synthesis is decreased as these are co-regulated, which suggests a decrease in CP in chloroplasts in TME3 at 67 dpi could additionally contribute to symptom attenuation at the recovery stage. It was also reported that some viral CPs are associated with the thylakoid [433,434]. In this study, a relatively high number of over-expressed proteins (15; 13%) were localised in the thylakoid at 67 dpi in TME3 (Figure 3-3). Proteins such as Manes.10G027600 (Rubisco activase; 6.89 ratio) and Manes.14G009000 (Phosphoglycerate kinase 1; 1.15 ratio) were amongst over-expressed proteins localised in the thylakoid and known to interact with virus nucleic acids or proteins [432] (Table 3-2). Another difference that was seen between T200 and TME3 at 67 dpi is the presence of Manes.08G136300 (linker histone; 1.08 ratio) protein involved in epigenetic regulation of gene expression in TME3 (Table 1, Figure 3-3). Linker histones are major negative regulators by limiting the accessibility of DNA to various trans-acting factors which in turn enables epigenetic suppression of genes [435]. It is not clear how the mechanism of this protein would contribute to SACMV tolerance phenotype in TME3, however, further studies on this protein are warranted. Of particular interest with regard to SACMV DNA accumulation, was the upregulation and downregulation of SUMO-activating enzyme subunit in T200 and TME3 at 67 dpi, respectively (Table 3-1). SUMOylation is an essential post-translational modification in plants and is also affected by oxidative stresses. Geminiviruses replicate in nuclei of infected plant cells using the plant DNA replication machinery, including proliferating cellular nuclear antigen

(PCNA), a cofactor that orchestrates genome duplication and maintenance by recruiting crucial players to replication forks. Posttranslational modification of PCNA by SUMO plays an essential role in the switching of PCNA between interacting partners during host replication, recombination, and repair mechanisms. The geminivirus Rep hijacks and induces the accumulation of the host replication machinery by interfering with the cell cycle, and impairs SUMO conjugation of PCNA (proliferating cellular nuclear antigen), and the SUMO E2 conjugation enzyme (SCE1) [436]. The significant down-regulation (-5.5 ratio) of SUMO-activating enzyme subunit in T200 would lead to impairment of SUMO conjugation and switch on the host cell machinery for its own replication. This would contribute to the persistent high SACMV replication levels in T200 at 67 dpi (Figure 3-1). In contrast, the up-regulation (4.65 ratio) of SUMO-activating enzyme in TME3 would contribute to reduction in SACMV replication leading to recovery at 67 dpi.

For successful long-term infection, begomoviruses need to restrict their detrimental effects upon infecting their hosts in order to prevent drastic defence responses from the host but at the same time maintain virus replication. At 32 dpi (systemic infection stage) virus replication and symptoms are high in both landraces. At this stage STG1A and the molecular chaperone HSP90.1 were over-expressed. These proteins directly interact with AT3G12050, a homolog of Manes.09G037400 from the Activator of Hsp90 ATPase family (Figure 3-5a). SGT1 binds to HSP90, resulting in the positive regulation of disease resistance conferred by many Resistance (NB-LRR) proteins in plants [143]. In *N. benthamiana*, a study found that SGT1a controls the abundance of an R protein Rx after silencing of SGT1a resulted in the reduction in steady-state levels of Rx. It is understood that any compromise in the activity of HSP90 and amount of SGT1a leads to reduced accumulation of R proteins [143,437]. This is in agreement with what was observed in this study; Hsp90 activator was under-expressed in both T200 (-1.48 ratio) and TME3 (-1.61 ratio) which may have compromised HSP90 and the implications of this would be the observed severe CMD symptoms (Figures 3-1a, 3-1b) as well the accumulation of SACMV DNA (Figure 3-1c) at 32 dpi. Moshe et al. 2016 [438] also proved the role that SGT1a and HSP90 play in susceptibility to TYLCV in infected tomatoes. Interestingly, in ICMV and SLCMV infected susceptible cassava, a member of the HSP70 chaperones, namely chaperone DNAj, was upregulated. It has been suggested that chaperones may be involved in cell-to-cell movement via modified plasmodesmata (PD), and molecular chaperones are known to be involved in modification of the size exclusion limit of PD [409]. This makes a case for a role for HSP90.1 in virus movement in T200 and TME3 at 32 dpi, corresponding to high virus loads observed in both landraces.

At a SACMV recovery time point (67 dpi) in TME3 but not in T200, we discuss two unique protein interactors selected from 1) all DEPs in the two landraces and 2) over-expressed DEPs in TME3, from the STRING network (Figure 3-4b, Appendix 6-Figure 5a). The first one, RPT2 is a member of proteasome regulatory subunits, known to be of importance in defence response [439]. *Arabidopsis* homologs of Manes.01G032500 (AT1G29150; 7.14

ratio in T200 and -1.40 ratio in TME3) and Manes.05G155000 (AT1G45000; 2.82 ratio in T200 and -1.07 ratio in TME3) (Table 3-3) interact directly with RPT2a and RPT2b proteins (Figure 3-5b). RPT2a has been linked with PAMP triggered immunity (PTI) response and the establishment of the long lasting, broad-spectrum pathogen resistance; systemic acquired response in *Arabidopsis* in response to bacterial infections. This was corroborated by an increased infection on RPT2a mutant lines due to the significant attenuation of ROS production upon induction of the oxidative burst [440], one of the first measurable responses of plants to PAMP perception [441]. In addition, there was no induction of SAR essential components FMO1 and ALD1 in RPT2a mutant systemic tissue upon priming infection. Other studies have also reported that RPT2a interacts with R proteins to activate defence signal [442]. With the up-regulation of proteins directly interacting with RPT2a, one would propose that there would be the reduction in systemic infection due to the responses that RPT2a protein has been proven to induce. However, this is not the case for susceptible landrace T200 which shows no recovery phenotype at 67 dpi as disease symptoms are still severe and SACMV accumulation is significantly high (Figure 3-1). Interestingly, a study showed that *Cauliflower mosaic virus* relies on the proteasome subunits RPT2a and RPT2b for robust infection [443], indicating that these proteasome subunits could have different functions in different host-virus interactions. This supports the results seen on our study, where higher expression of the proteasome interactors was linked to T200 with a greater virus load and down-regulation linked to TME3 (Appendix 6-Figure 5b) with low virus accumulation and a recovery phenotype at 67 dpi (Figure 3-1, Table 3-3).

The second protein of interest is Sac52, also known as the ribosomal protein L10 (RPL10), which interacts with Ribosomal_L7Ae domain-containing protein, over-expressed uniquely in TME3 only at symptom recovery (67 dpi) (Appendix 6-Figure 5a). This protein is a known key player in the NIK1-mediated antiviral immunity defence response to geminivirus infection as it plays the role of viral effector detection, triggering effector triggered immunity (ETI) mechanisms in plants [444]. This defence mechanism is established with interaction between membrane bound LRR receptor-like serine/threonine kinase and its virulence target the begomovirus nuclear shuttle protein (NSP) found on DNA B component of the bipartite geminiviruses. During geminivirus infection, NIK1 autophosphorylates the kinase domain on threonine-469 and threonine-474, resulting in NIK1 kinase activation [395,445]. Once activated, NIK1 indirectly phosphorylates the cytoplasmic RPL10, which is then subsequently translocated to the nucleus where it interacts with the L10-interacting MYB domain-containing protein (LIMYB). This interaction mounts a defence response in the form of translational inhibition of several host genes and decreased geminiviral transcript association with polysomes, consequently negatively impacting virus infection. The binding of NSP to NIK1 kinase domain counteracts this defensive signalling by preventing NIK1 phosphorylation, and thus interrupt downstream signalling by trapping RPL10 within the cytoplasm and ultimately enabling virus proliferation and spread [218,446]. In this study, RPL10 directly interacts with Ribosomal_L7Ae domain-containing protein homolog (Manes.12G139100, AT1G77940) which is under-

expressed (5 ratio) in T200 while over-expressed (2 ratio) in TME3 (Table 3-3). This interaction occurs in the cytoplasm; however, it is not known whether or not both these proteins are translocated into the nucleus after RPL10 is phosphorylated. Both proteins are involved in translation. With regard to the role that RPL10 may play in TME3 tolerance to SACMV, we propose that this protein may be expressed in abundance and then phosphorylated by membrane-bound NIK1, which would then lead to downstream transitional suppression of viral proteins and thus virus movement which could contribute to the symptom and virus recovery phenotype observed in TME3 (Figure 3-1a). This is in agreement with what was observed in other different plant hosts [65,218,445,447–449]. It is also possible that there could have been some NSP-NIK1 interactions as well, although this might have been limited in the tolerant landrace. It is also important to note that the RPL10 interactor (Manes.12G139100) is located on chromosome 12, known to be the location of the QTL for dominant CMD2 resistance [18,207,339], thus supporting the likelihood of this protein being a key player in TME3 tolerance to SACMV.

Pathogenesis-related (PR), RING finger and R proteins were also induced by SACMV infection in T200 and TME3 at 32 and 67 dpi. PR proteins are an imperative component of innate immune responses in plants and they are also associated with SAR of plants against diverse pathogens [450]. In SACMV-infected leaves, five PR proteins belonging to the Bet v1 family were differentially expressed, with three over-expressed in T200 (Manes.09G127700, Manes.15G008400, Manes.15G129800) and four in TME3 (Manes.15G007900, Manes.09G127700, Manes.15G008400, Manes.15G008100) (Table 3-3, Figure 3-6). Bet v1 allergen is one of the ubiquitous PR plant proteins that acts as a plant steroid carrier and has ribonuclease activity, suggesting its possible involvement in the plant defence response against pathogens [450–454]. Up-regulation of Bet v1 PR proteins were also observed in rice leaves following infection with *Rice stripe virus* [312], suggesting that an increase in expression of these PR proteins, observed in SACMV-infected cassava, would improve the transport of steroids such as brassinosteroid which is involved in signalling pathways, and also known to boost ribonuclease activities and overall antiviral defence mechanisms in plants.

Receptor-like kinases (RLKs) and receptor-like proteins (RLPs) are well-documented to be involved in pathogen defence in plants [455,456]. At 67 dpi, RLKs Manes.05G194900 and Manes.14G124000 were under-expressed in both landraces, while RLPs Manes.06G055700 and Manes.18G115700 were over-expressed in susceptible T200 and under-expressed in tolerant TME3, and Manes.18G143800 under-expressed in T200 and over-expressed in TME3 (Table 3-3). RLK proteins that play crucial roles in the recognition of pathogen-associated molecular patterns and the activation of downstream PTI [455,457]. RLPs on the other hand differ from RLKs by their lack of a cytoplasmic kinase domain [456]. A RLK protein was also highly expressed in cassava following infection with ICMV and SLCMV in leaf tissue [297], suggesting this may be a general response to cassava geminiviruses.

Ubiquitination of geminiviral proteins can contribute to plant defence and geminivirus often subvert or hijack ubiquitin-proteasomal pathways [34]. At 67 dpi, one of the represented functional group proteins was the proteasome (Figure 3-5b). PCI domain-containing protein (Manes.01G032500), involved in the COP9 signalosome (CSN), was over-expressed (7.14 ratio) compared to mock sample in T200, suggesting a major role in susceptibility via interference of this complex. Plant geminiviruses have been shown to redirect ubiquitination by interfering with the activity of the CSN complex [458]. The geminivirus C2 protein has been demonstrated to compromise CSN activity on CUL1. Among several responses regulated by the CUL1-based SCF ubiquitin E3 ligase, is response to jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA). Exogenous jasmonate treatment of *Arabidopsis thaliana* plants disrupts geminivirus infection [458], suggesting that the suppression of the jasmonate response might be crucial for infection. Notably, a C3HC4-type RING finger protein, Manes.08G075100 was found to be over-expressed at 67 dpi in T200, the opposite being true for TME3 (Table 3-3). At 32 dpi this C3HC4-type RING finger protein was under-expressed in T200 and over-expressed in TME3, supporting a strong role for ubiquitination in TME3 tolerance. This RING finger protein is an E3 ubiquitin ligase (E3L) that is a component of the ubiquitin proteasome system (UPS), with many of their functions involved in plant stress resistance [110,459–461]. Recently, it was shown that CRISPR knockdown of a E3L in cassava TME3 protoplasts resulted in an increase in SACMV replication, suggesting that E3L target proteins may play a role in SACMV tolerance [110]. Ubiquitin activating enzyme *UBA1* and *RING-type E3 ubiquitin ligase* are also reported by Lozano-Duran et al. 2011 [372] to be transactivated by the geminivirus C2 protein. A proteome study on ToCMoV resistant tomato showed a decrease in levels of proteins involved in ubiquitination pathway, including ubiquitin and threonine endopeptidase [365]. These proteins are positively modulated by viruses in host plants, providing additional evidence for the role of the ubiquitin-proteasome in response to geminivirus infection. Further gene editing *in planta* studies on the role of the UPS in cassava in response to SACMV would prove invaluable.

3.5. Conclusions

SACMV is a highly pathogenic virus in susceptible hosts, leading to major yield losses. In perennial hosts like cassava, tolerance may represent accommodation of the persistent virus infection throughout its life span [85]. Our proteome results identified proteins involved in different metabolic pathways, some with functions in defense responses to SACMV. We identified proteins in the ribosome, chloroplasts and the cytoplasm and we proposed that these proteins and their connected networks are contributors to SACMV virus and symptom recovery in TME3. One of these proteins is Sac52 which interacts with NIK1 and may contribute to an anti-virus response. This is highly significant since this finding provides more evidence that PAMP triggered immunity (PTI) may be involved in resistance to plant geminiviruses. While ETI has long been recognized as an efficient specific defence against viruses, PTI characterized in non-viral pathogen-plant interactions has not been found to

operate against plant viruses until recently [462]. Geminivirus nucleic acids can act as viral pattern associated molecular patterns (PAMPs) and activate transmembrane receptor NIK1, which shares PTI co-receptors regulatory mechanisms for activation [462]. However, geminiviral PAMPs and their cognate PRRs have yet to be identified, and investigation of antiviral PTI mechanisms in cassava is highly warranted. In cassava TME3 landrace, we conclude that tolerance allows the virus to accumulate to some degree without causing significant loss of fitness, implying that the virus and host co-exist without one killing each other. This could be attributed to the fact that the host demonstrates metabolic homeostasis and can perform its physiological and molecular processes. The results herein support the proposal that symptom recovery in TME3 may be associated with stress tolerance and maintenance of cellular homeostasis. In perennial crops this would be advantageous as long-term defence responses are energy-intensive. SACMV-tolerance in TME3 would mitigate energy costs leading to growth, recovery and a reduction in symptoms and virus load. The results from this study forms the foundation for further *in planta* functional studies to understand cassava tolerance to geminiviruses.

Chapter 4: General conclusions and recommendations

Cassava is an economically important staple crop responsible for feeding more than 800 million people globally and the 3rd largest source of carbohydrates in Africa. Although cassava can thrive in drought prone regions, no cassava genotype has overcome the threat by begomoviruses, which causes cassava mosaic disease and can lead to up to 82% cassava production losses. When plants are faced with abiotic and biotic stress factors, molecular networks that involves genes, proteins, and metabolites; which aid in the perception of stress and the activation of cascades of stress defense responses are triggered, and subsequently determines the survival of the plant. Considering the importance of this process, it was timely for this study to aim to identify proteins stimulated by the *South African cassava mosaic virus* (SACMV) infection in cassava tolerant TME3 and susceptible T200 landraces, and to further characterise the function of *MeRPPL1* and *MeTNL* resistance genes analogs in tolerant TME3 landrace. The proteomic study identified proteins involved in metabolic pathways, biosynthesis of secondary metabolites, glycolysis, ribosome, and proteasome to be differentially expressed, and to contribute to TME3 tolerance to SACMV infection at 67 dpi. One protein that spiked our interest is the ribosomal_L7Ae domain-containing protein (Manes.12G139100) that is located within chromosome 12 of *Manihot esculenta*, known to be the host of cassava mosaic disease (CMD) resistance QTL. This protein was 5-fold under-expressed in T200 and 2-fold over-expressed in TME3 and plays a key role in TME3 tolerance and recovery at 67 dpi by directly interacting with ribosomal protein Sac52 to activate NIK-mediated antiviral immunity. Results from the proteomic study serves as framework for studies aimed at characterising and understanding plant defence mechanisms and as such, we recommend that differentially expressed proteins to be further characterised using molecular biology tools such as VIGS and CRISPR-Cas9 to characterise their functions in cassava and other plants. *MeRPPL1* and *MeTNL* have been reported to be amongst resistance genes (R genes) that had increased expression levels in TME3 following infection with SACMV [78], [85], and proposed to be associated with recovery of TME3 landrace at 67 dpi. VIGS silencing results showed that reduced expression levels of *MeRPPL1* and *MeTNL*, promoted SACMV replication and CMD infection in TME3 plants, which supports the notion of their involvement in SACMV tolerance and recovery. CRISPR-Cas9 vectors with/without SACMV provided partial silencing of the two genes and as a result, there was an up-regulation in the expression of these two R genes in TME3 leaf mesophyll protoplasts, as it would be expected due to these genes involvement in disease response. Furthermore, CRISPR silencing vector + SACMV and SACMV alone induced mutations in *MeRPPL1* polypeptide which led to the disruption of the MHD motif, crucial for the interaction of the NLR in NB-LRR proteins with effectors. It is possible that this disruption could have resulted in a loss of ATP binding and a change in the activation status of *MeRPPL1*, thus promoting the SACMV infection as it was observed. However, Further 3D modelling of *MeRPPL1* polypeptide would need to be done to ascertain the effects of non-functional MHD motif.

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Appendices

Appendix 1: Silencing constructs design.

MeTNL CRISPR-Cas Construct (~760 bp)

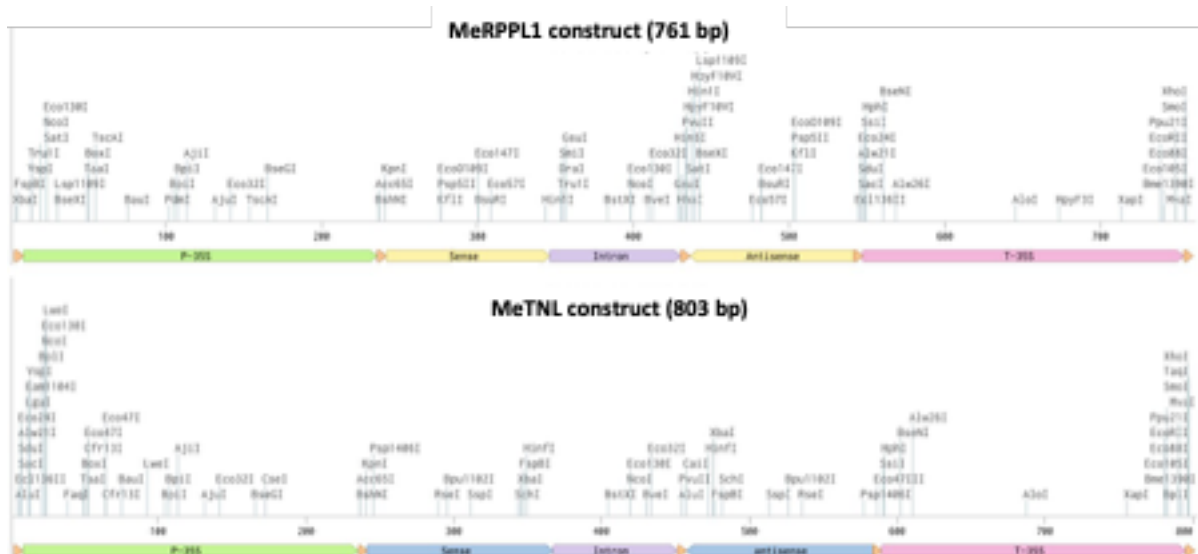
Aag ctt CTT TTT TTC **TTC TTC TTC** GTT CAT ACA GTT TTT **TTT** TGT TTA TCA GCT TAC ATT TTC TTG AAC
 CGT AGC TTT CGT TTT CTT **CTT** TTT AAC TTT CCA TTC GGA GTT TTT GTA TGT TGT TTC ATA GTT TGT CCC
 AGG ATT AGA ATG ATT AGG CAT CGA ACC TTC AAG AAT TTG ATT GAA TAA AAC ATC TTC ATT CTT AAG ATA
 TGA AGA TAA TCT TCA AAA GGC CCC TGG GAA TCT GAA AGA **AGA** GAA GCA GGC CCA TTT ATA TGG GAA AGA
 ACA ATA GTA TTT CTT ATA TAG GGC CAT TTA AGT TGA AAA CAA TCT TCA AAA GTC CCA CAT CGC TTA GAT
 AAG AAA ACG AAG CTG AGT TTA TAT ACA GCT AGA GTC GAA GTA GTG **ATT** **cc atg ga** ACA GAG CAC
CAG TGG TCT AGT GGT AGA ATA GTA CCC TGC CAC GGT ACA GAC CCG GGT TCG
ATT CCC GGC TGG TGC A **GG TAA GCT ACG ACG GCT TGG gtt tta gag cta gaa aat agc aag tta**
aaa taa ggc tag tcc gtt atc aac tlg aaa aag tgg cac cga gtc ggt gc g ga ACA GAG CAC CAG
TGG TCT AGT GGT AGA ATA GTA CCC TGC CAC GGT ACA GAC CCG GGT TCG ATT CCC
GGC TGG TGC A **TT CGG GAA TCA ATT TCG TAG gtt tta gag cta gaa aat agc aag tta aaa taa ggc**
tag tcc gtt atc aac tlg aaa aag tgg cac cga gtc ggt gct ttt tt c aag tg

MeRPPL1 CRISPR-Cas Construct (~760 bp)

Aag ctt CTT TTT TTC **TTC TTC TTC** GTT CAT ACA GTT TTT **TTT** TGT TTA TCA GCT TAC ATT TTC TTG AAC
 CGT AGC TTT CGT TTT CTT **CTT** TTT AAC TTT CCA TTC GGA GTT TTT GTA TCT TGT TTC ATA GTT TGT CCC
 AGG ATT AGA ATG ATT AGG CAT CGA ACC TTC AAG AAT TTG ATT GAA TAA AAC ATC TTC ATT CTT AAG ATA
 TGA AGA TAA TCT TCA AAA GGC CCC TGG GAA TCT GAA AGA **AGA** GAA GCA GGC CCA TTT ATA TGG GAA AGA
 ACA ATA GTA TTT CTT ATA TAG GGC CAT TTA AGT TGA AAA CAA TCT TCA AAA GTC CCA CAT CGC TTA GAT
 AAG AAA ACG AAG CTG AGT TTA TAT ACA GCT AGA GTC GAA GTA GTG **ATT** **cc atg ga** ACA GAG CAC
CAG TGG TCT AGT GGT AGA ATA GTA CCC TGC CAC GGT ACA GAC CCG GGT TCG ATT CCC GGC TGG
TGC A **GG ACT ATG AAT TCC ATA ACT gtt tta gag cta gaa aat agc aag tta aaa taa ggc tag tcc gtt atc aac**
ttg aaa aag tgg cac cga gtc ggt gc g ga ACA GAG CAC CAG TGG TCT AGT GGT AGA ATA GTA CCC TGC
CAC GGT ACA GAC CCG GGT TCG ATT CCC GGC TGG TGC A **CA AAC TTT ACA ATT ATG TGT gtt tta gag**
cta gaa aat agc aag tta aaa taa ggc tag tcc gtt atc aac tlg aaa aag tgg cac cga gtc ggt gct ttt tt a gat ct

KEY:
 Arabidopsis U6-26 Promoter
 Pre-trRNA
 gRNA
 SpCas9 tracrRNA (scaffold)
 terminator

Appendix 1-Figure 1. CRISPR-Cas9 silencing constructs.



Appendix 1-Figure 2. SACMV VIGS silencing constructs.

Appendix 2: Datamining of selected target genes.

Appendix 2-Table 1. Amino acid sequence percent identity matrix for Manes.12G091600 and its cassava paralogs[#].

Protein ID	1	2	3	4	5	6	7
1:Manes.09G022600.1	100	91	89	91	91	91	90
2:Manes.09G023300.1		100	94	90	93	92	92
3:Manes.09G022500.1			100	90	92	91	91
4:Manes.12G091600.1				100	90	92	91
5:Manes.09G023900.1					100	94	93
6:Manes.09G023800.1						100	94
7:Manes.09G023200.1							100

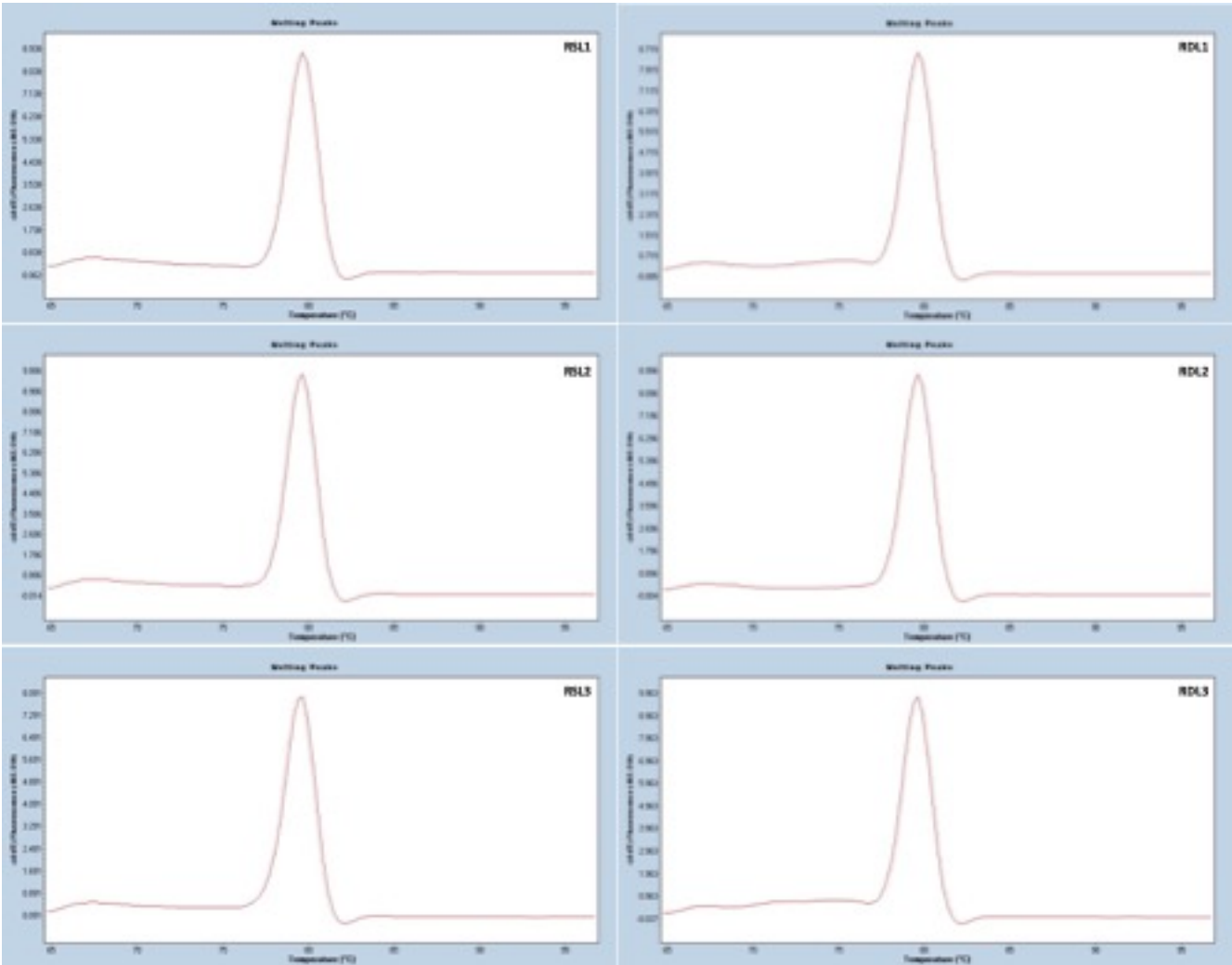
[#]Percent Identity Matrix was created by Clustal2.1. Percentage identity was determined from Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) multiple amino acids sequence alignment of the individual paralogs and the reference Manes.12G091600.

Appendix 2-Table 2. KEGG pathway analysis of protein interacting partners of MeTNL (AT5G36930) and MeRPPL1 (AT3G14470).

AT5G36930 INTERACTING PARTNERS	KEGG Pathway*	Genes	p-value	FDR	AT3G14470 INTERACTING PARTNERS	KEGG Pathway*	Genes	p-value	FDR
	Inositol phosphate metabolism	AT1G60490 AT1G68000 AT4G38570	6.31e-06	2.84e-05		Phenylalanine, tyrosine and tryptophan biosynthesis	AT1G24807 AT2G04400 AT5G57890 AT4G02610 AT5G48220 AT1G25220 AT2G29690 AT5G17990 AT1G48850	1.59e-20	9.53e-20
	Phosphatidylinositol signalling system	AT1G68000 AT1G60490 AT4G38570	4.97e-06	2.84e-05		Biosynthesis of phenylpropanoids	AT1G24807 AT2G04400 AT5G57890 AT4G02610 AT5G48220 AT1G25220 AT2G29690 AT5G17990 AT1G48850	8.07e-14	1.61e-13
	Homologous recombination	AT2G24490 AT3G02920	2.92e-04	7.05e-04		Biosynthesis of alkaloids derived from shikimate pathway	AT1G24807 AT2G04400 AT5G57890 AT4G02610 AT5G48220 AT1G25220 AT2G29690 AT5G17990 AT1G48850	3.82e-15	1.14e-14
	Mismatch repair	AT2G24490 AT3G02920	3.13e-04	7.05e-04		Biosynthesis of plant hormones	AT1G24807 AT2G04400 AT5G57890 AT4G02610 AT5G48220 AT1G25220 AT2G29690 AT5G17990 AT1G48850	5.09e-13	7.64e-13
	DNA replication	AT2G24490 AT3G02920	5.98e-04	1.08e-03		Metabolic pathways	AT1G24807 AT2G04400 AT5G57890 AT4G02610 AT5G48220 AT1G25220 AT2G29690 AT5G17990 AT1G48850	9.29e-08	1.11e-07
	Glycerophospholipid metabolism	AT1G68000 AT4G38570	9.72e-04	1.35e-03		Glycine, serine and threonine metabolism	AT4G02610	0.0323	0.0323
	Nucleotide excision repair	AT2G24490 AT3G02920	1.05e-03	1.35e-03					
	Regulation of autophagy	AT1G60490	9.89e-03	0.0111					

*KEGG pathway analysis were done on an online webserver Plant GeneSet Enrichment Analysis (PlantGSEA; <http://structuralbiology.cau.edu.cn/PlantGSEA>). The search query was again Arabidopsis thaliana whole genome with the following parameters chosen; Fisher statistical test method and Hochberg (FDR) multi-test adjustment method both at 0.05 significance level.

Appendix 3: Gene expression melt curves.



Appendix 3-Figure 1. MerPPL1 melt curves in MerPPL1 VIGS-silenced samples at a late time point (75 dpi). **RSL** = pC8A-MerPPL1 + DNA B + SACMV challenge sample at a late time point, **RD** = pC8A-MerPPL1 + DNA B sample at a late time point.

Appendix 4: Quantitative PCR primers used in gene validation.

Appendix 4-Table 1. Primers used for RT-qPCR.

Gene Name	Primer Sequences
<i>UBQ10</i>	Fwd: 5' TGCATCTCGTTCTCCGATTG 3' Rev: 5' GCGAAGATCAGTCGTTGTTGG 3'
Manes.05G194900 (Receptor like kinase_1)	Fwd: 5' AGTCAGACCTCATCGACCCA 3' Rev: 5' AACCTGAGCAACTCGACCAG 3'
Manes.06G055700 (Receptor like protein)	Fwd: 5' GCCCCTACCTTCACAGCATT 3' Rev: 5' AACAGCAGCAGTCTCAGCAT 3'
Manes.08G126700 (Kinase)	Fwd: 5' GTGGGCCTGTAAAGGATGCT 3' Rev: 5' TCGATGCCGAGACAAACCA 3'
Manes.14G124000 (Receptor like kinase_2)	Fwd: 5' TCCACCATTAACTGGGCAC 3' Rev: 5' CCCATGAGGAACAGCACGAT 3'
Manes.18G115700 (LRR)	Fwd: 5' GCCTCAAACCTCACCTCAGCT 3' Rev: 5' GCTGAGCATAGCAGATGGGT 3'
Manes.08G075100 (C3HC4-type RING finger)	Fwd: 5' CCGTTTTATGCCCTCTCTGT 3' Rev: 5' TCTCACCTCTGGCCTCTCTC 3'
Manes.15G008400 (Pathogenesis-related protein_1)	Fwd: 5' TGAAGGTGATCCGTGGTTGG 3' Rev: 5' TGGTGCACTTGCTGATGGAT 3'
Manes.18G143800 (<i>LRX2</i>)	Fwd: 5' ATTTCCGGGATCACCACCAC 3' Rev: 5' CTGTTGCTGAGGAGGTGGAG 3'
Manes.01G100900 (<i>SnRK1.1</i>)	Fwd: 5' AGAAGTCCTCAAAGCCCTGC 3' Rev: 5' CCTGGAATCCACCTGCACTT 3'
Manes.15G007900 (Pathogenesis-related protein_2)	Fwd: 5' TCCGGCAAAGCTGTACAGAG 3' Rev: 5' TTCTTGATGGTCCAGGCC 3'

Appendix 5: Differentially expressed proteins from SACMV-infected T200 and TME3 at 32 and 67 dpi.

Appendix 5-Table 1. Differentially expressed proteins between T200 and TME3 mock-inoculated and SACMV-infected leaves at 32 and 67 dpi.

Protein Number	Accession	Description	Manes ID	Unique peptides	q Value	T200 Ratio ^a	TME3 Ratio
32 dpi							
1	tr A0A251JUP0	Uncharacterized protein	Manes.12G152700	17	7.6E-05	1.35	1.02
2	tr A0A2C9V9U7	Uncharacterized protein	Manes.09G038300	4	3.4E-04	-1.46	1.77
3	tr A0A2C9W4F1	Germin-like protein	Manes.03G039900	2	2.7E-03	-2.91	-1.26
4	tr A0A2C9UP99	Urease	Manes.13G016500	3	2.7E-03	1.09	1.16
5	tr A0A2C9W667	Aminopeptidase	Manes.03G051100	2	2.7E-03	-2.08	1.25
6	tr A0A2C9UVT3	Uncharacterized protein	Manes.12G121600	3	2.7E-03	-1.59	-1.61
7	tr A0A2C9UFR6	Uncharacterized protein	Manes.15G129800	5	2.7E-03	-1.79	1.06
8	tr A0A2C9W7V0	Uncharacterized protein	Manes.03G155700	2	3.8E-03	-2.52	1.53
9	tr A0A2C9UHT2	Uncharacterized protein	Manes.14G012900	3	3.8E-03	-1.72	-1.13
10	tr A0A2C9W9Q3	Uncharacterized protein	Manes.02G002100	5	3.8E-03	-1.77	1.11
11	tr A0A2C9VGL2	Uncharacterized protein	Manes.08G075100	11	3.9E-03	1.02	-1.48
12	tr A0A2C9WJ97	Uncharacterized protein	Manes.01G018800	9	3.9E-03	1.46	1.46
13	tr A0A2C9U0A3	Uncharacterized protein	Manes.18G008600	6	4.2E-03	1.06	1.06
14	tr A0A2C9VPS7	Uncharacterized protein	Manes.06G104000	2	4.3E-03	-3.38	-1.46
15	tr A0A2C9V1A4	Molybdopterin molybdenumtransferase	Manes.11G103300	13	6.7E-03	1.37	-1.16
16	tr A0A2C9V934	Uncharacterized protein	Manes.09G037400	3	6.7E-03	-1.48	-1.61
17	tr A0A2C9WAR9	Uncharacterized protein	Manes.03G202900	14	6.7E-03	2.01	-1.26
18	tr A0A2C9V631	Uncharacterized protein	Manes.10G085900	3	1.1E-02	1.28	-1.12
19	tr A0A2C9V927	Arginine biosynthesis bifunctional protein ArgJ, chloroplastic	Manes.09G086800	6	1.3E-02	-1.90	-1.27
20	tr A0A2C9VA66	Inositol-1-monophosphatase	Manes.09G076000	4	1.3E-02	-1.56	-1.49
21	tr A0A2C9UFN9	Chalcone-flavonone isomerase family protein	Manes.15G100200	4	1.4E-02	-1.80	-1.25
22	tr A0A2C9WDT7	Uncharacterized protein	Manes.02G091600	10	1.4E-02	-1.85	-1.11
23	tr A0A2C9VA92	Uncharacterized protein	Manes.09G126100	9	1.5E-02	-1.34	1.06
24	tr A0A2C9VG94	Glutamate dehydrogenase	Manes.08G144100	4	1.7E-02	1.87	-1.22
25	tr A0A2C9UYG9	Uncharacterized protein	Manes.12G143100	4	1.7E-02	-1.40	-1.28
26	tr A0A251J7N7	Pectin acetyltransferase	Manes.15G187800	14	1.7E-02	-1.87	-1.03
27	tr A0A2C9U2L8	Uncharacterized protein	Manes.18G115700	2	2.1E-02	-1.11	1.20
28	tr A0A2C9UYW9	Uncharacterized protein	Manes.11G072300	3	2.1E-02	-2.29	-1.11
29	tr A0A2C9VBJ0	Uncharacterized protein	Manes.09G111500	6	2.5E-02	-1.37	1.06
30	tr A0A2C9VI86	Uncharacterized protein	Manes.08G142400	7	2.5E-02	-1.47	-1.99
31	tr A0A251JTC4	Uncharacterized protein	Manes.11G079700	9	3.0E-02	-1.46	1.12
32	tr A0A2C9WKE4	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	Manes.01G134800	14	3.1E-02	1.41	1.24
33	tr A0A2C9W3I4	Uncharacterized protein	Manes.03G010000	3	3.1E-02	-1.04	1.59
34	tr A0A2C9VWI2	Purple acid phosphatase	Manes.05G153600	3	3.1E-02	1.85	2.91
35	tr Q52QX9	Aldo/keto reductase AKR	Manes.05G194600	23	3.2E-02	-1.33	-1.08
36	tr A0A2C9UAX3	Malic enzyme	Manes.16G109900	21	3.2E-02	2.03	1.28
37	tr A0A2C9UWL8	Uncharacterized protein	Manes.12G098600	12	3.3E-02	-2.06	-1.45
38	tr A0A2C9VEA2	Uncharacterized protein	Manes.08G075000	3	3.3E-02	-1.05	-1.30
39	tr A0A2C9UQV9	Uncharacterized protein	Manes.13G107500	8	3.3E-02	1.66	1.18
40	tr A0A2C9U378	Uncharacterized protein	Manes.18G126900	4	3.4E-02	-2.09	-1.70

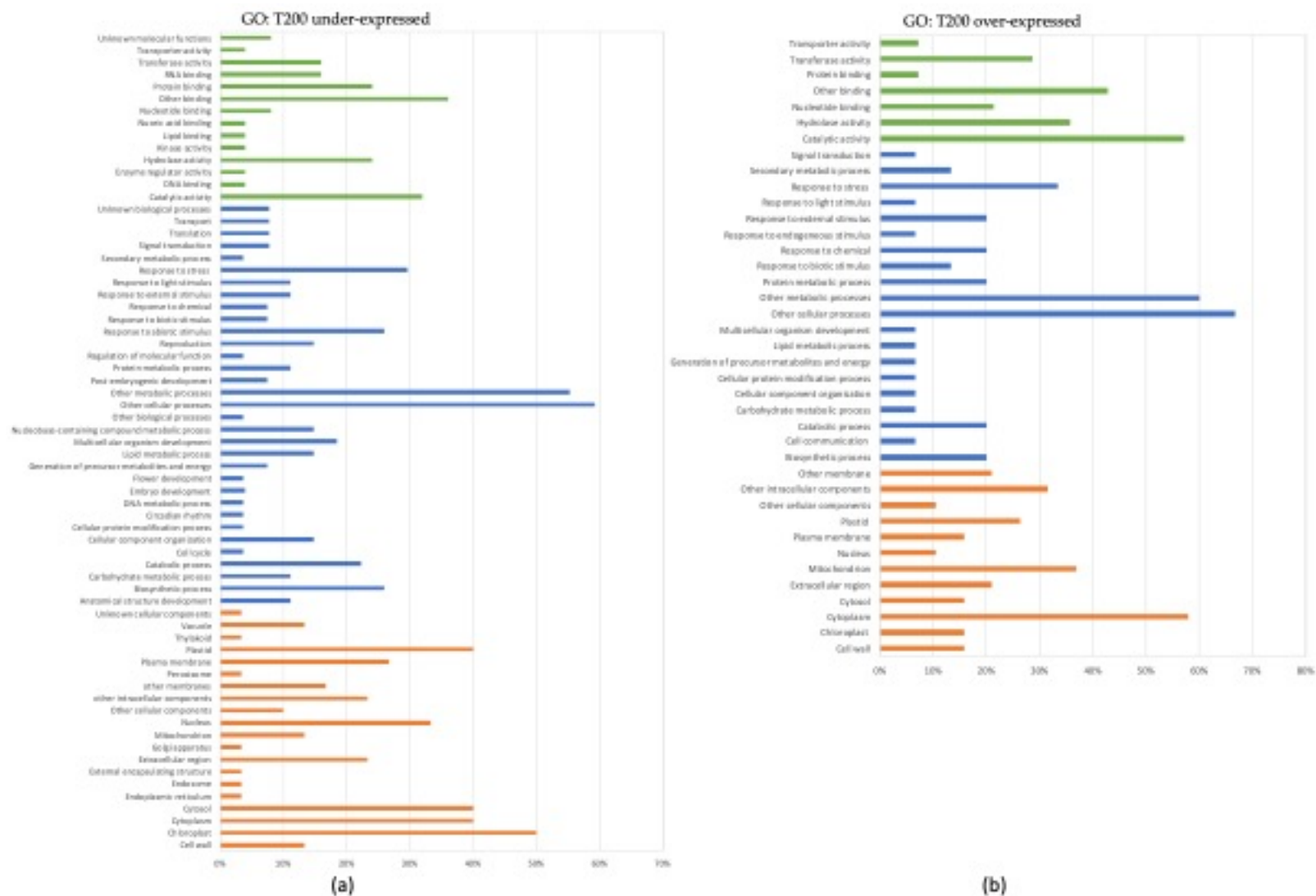
41	tr A0A2C9VNR2	Uncharacterized protein	Manes.06G023800	6	3.4E-02	1.17	1.13
42	tr A0A2C9VVR2	Uncharacterized protein	Manes.05G157600	3	3.7E-02	1.57	-1.12
43	tr A0A2C9WIE5	Uncharacterized protein	Manes.01G064200	5	3.8E-02	1.28	1.89
44	tr A0A2C9UGQ4	Uncharacterized protein	Manes.15G168600	4	4.6E-02	-1.49	1.53
45	tr A0A2C9WIT7	Uncharacterized protein	Manes.01G076600	10	4.6E-02	-2.17	-1.08
46	tr A0A2C9UC96	Uncharacterized protein	Manes.15G024100	3	4.7E-02	1.31	-1.20
47	tr A0A2C9VRW1	Phosphoglycerate kinase	Manes.06G176500	19	4.7E-02	-1.85	-1.20
48	tr A0A2C9UCU5	Uncharacterized protein	Manes.15G010900	8	4.7E-02	1.00	1.44
49	tr A0A2C9U034	Uncharacterized protein	Manes.18G004200	2	4.9E-02	3.29	-1.40
67 dpi#							
50	tr A0A2C9UDC1	Uncharacterized protein	Manes.15g062800	4	4.77E-04	1.57	-1.49
51	tr A0A2C9W667	Aminopeptidase	Manes.03g051100	4	1.26E-03	1.68	1.19
52	tr A0A251JUP0	PKS_ER domain-containing protein	Manes.14g152700	18	1.26E-03	1.01	1.42
53	tr A0A2C9W9Q3	DUF4033 domain-containing protein	Manes.02g002100	5	2.40E-03	1.53	-1.23
54	tr A0A2C9UEI7	Uncharacterized protein	Manes.15g098900	3	5.99E-03	-1.19	1.03
55	tr A0A2C9UVT3	Peptidase_M3 domain-containing protein	Manes.12g121600	9	6.82E-03	-1.67	1.00
56	tr V9NC17	Purple acid phosphatase	Manes.06g119300	5	6.82E-03	-1.37	1.12
57	tr A0A2C9V927	Arginine biosynthesis bifunctional protein ArgJ, chloroplastic	Manes.09g086800	3	7.18E-03	1.21	1.48
58	tr A0A2C9U9T9	PKS_ER domain-containing protein	Manes.16g022100	9	1.11E-02	1.82	1.42
59	tr A0A2C9W111	AB hydrolase-1 domain-containing protein	Manes.04g110900	9	1.16E-02	1.29	-1.10
60	tr A0A2C9VGR9	Uncharacterized protein	Manes.08g094400	6	1.16E-02	1.91	-1.41
61	tr A0A2C9UHP4	Phosphoglycerate kinase	Manes.14g009000	18	1.22E-02	-1.12	1.15
62	tr A0A2C9V475	CPSF_A domain-containing protein	Manes.10g036500	2	1.26E-02	-1.02	-1.27
63	tr A0A2C9V2K1	Uncharacterized protein	Manes.10g026600	3	1.31E-02	-1.32	1.22
64	tr A0A2C9UX46	OMPdecase domain-containing protein	Manes.11g004200	5	1.32E-02	1.31	-1.06
67	tr A0A2C9VLW2	AA_kinase domain-containing protein	Manes.07g077700	2	1.34E-02	1.06	1.19
68	tr A0A2C9UQN2	Uncharacterized protein	Manes.13g040100	2	1.34E-02	1.24	-1.28
69	tr A0A2C9WLL0	NAD(P)-bd_dom domain-containing protein	Manes.01g169200	7	1.81E-02	1.37	-1.36
70	tr A0A251LE04	rRNA N-glycosidase	Manes.04g007600	13	2.07E-02	1.26	1.06
71	tr A0A2C9W7J2	40S ribosomal protein S4	Manes.03g095200	2	2.33E-02	1.59	-1.06
72	tr A0A2C9W0W5	Uncharacterized protein	Manes.04g015100	11	2.55E-02	1.41	-1.24
73	tr A0A2C9V5M3	Uncharacterized protein	Manes.10g071700	30	2.63E-02	1.03	-1.13
74	tr A0A2C9UE23	Probable bifunctional methylthioribulose-1-phosphate dehydratase/enolase-phosphatase	Manes.15g087800	7	2.66E-02	1.78	1.18
75	tr A0A2C9UQE1	Uncharacterized protein	Manes.13g089400	12	2.66E-02	1.84	1.10
76	tr A0A2C9WA83	S1 motif domain-containing protein	Manes.02g024200	5	2.66E-02	-1.03	1.02
77	tr A0A2C9UWY1	Uncharacterized protein	Manes.11g000600	7	2.66E-02	1.60	1.08
78	tr A0A2C9UQK9	Uncharacterized protein	Manes.13g095100	8	2.70E-02	-1.07	1.65
79	tr A0A2C9WI78	Pyr_redox_2 domain-containing protein	Manes.01g057400	18	2.71E-02	1.11	1.36
80	tr A0A2C9VU66	Epimerase domain-containing protein	Manes.05g076500	28	2.73E-02	-1.08	1.45
81	tr A0A2C9VRX3	Phosphoglycerate kinase	Manes.06g176400	6	2.81E-02	-1.09	1.24
82	tr A0A2C9VVJ7	Glycosyltransferase	Manes.05g120400	5	2.81E-02	1.23	1.54
83	tr A0A2C9UBD7	Uncharacterized protein	Manes.16g125000	3	2.81E-02	1.69	1.06
84	tr A0A2C9U6L0	Uncharacterized protein	Manes.17g072000	4	2.94E-02	-1.83	1.46
85	tr A0A2C9WMB0	Glutamine synthetase	Manes.01g119500	5	2.94E-02	-1.95	1.85
86	tr A0A2C9VBj0	Uncharacterized protein	Manes.09g111500	5	3.05E-02	-1.68	1.92
87	tr A0A2C9UHV3	Glycine cleavage system H protein	Manes.15g169800	7	3.05E-02	-1.30	1.78

88	tr A0A2C9UDJ0	Usp domain-containing protein	Manes.15g005000	2	3.21E-02	-1.35	1.45
89	tr A0A2C9VIN2	Peptide-methionine (R)-S-oxide reductase	Manes.07g048000	4	3.22E-02	-1.75	1.91
90	tr A0A2C9VG29	FBPase domain-containing protein	Manes.08g083500	17	3.35E-02	1.04	1.31
91	tr A0A2C9VAJ1	Uncharacterized protein	Manes.09g133500	3	3.46E-02	1.63	-1.07
92	tr A0A2C9UU70	Uncharacterized protein	Manes.12g000500	12	3.46E-02	1.72	1.03
93	tr A0A2C9WI85	AA_TRNA_LIGASE_II domain-containing protein	Manes.02g210900	8	3.59E-02	1.50	1.27
94	tr A0A2C9V7L1	Lipoxygenase	Manes.10g148800	32	3.61E-02	1.31	1.39
95	tr A0A2C9VPJ9	Aldedh domain-containing protein	Manes.06g100200	11	3.74E-02	1.93	1.37
96	tr A0A2C9U9Y1	Serine hydroxymethyltransferase	Manes.16g078300	14	3.74E-02	1.66	1.24
97	tr A0A251K8T5	Uncharacterized protein	Manes.11g061200	3	3.77E-02	-1.17	-1.65
98	tr A0A2C9V5I8	TYR_PHOSPHATASE_2 domain-containing protein	Manes.10g053500	7	3.77E-02	1.83	1.18
99	tr A0A2C9V5D4	Cytochrome c domain-containing protein	Manes.10g115100	5	3.77E-02	1.19	1.29
100	tr A0A2C9U4A0	GPAT_N domain-containing protein	Manes.17g016900	4	3.90E-02	1.35	1.28
101	tr A0A2C9W416	Uncharacterized protein	Manes.03g027000	7	3.90E-02	1.49	1.38
102	tr A0A2C9UIA3	Uncharacterized protein	Manes.15g157900	2	3.90E-02	1.13	1.58
103	tr A0A2C9VA66	Inositol-1-monophosphatase	Manes.09g076000	3	3.93E-02	1.63	-1.46
104	tr A0A2C9UIE7	Ribonuclease	Manes.14g043200	18	3.99E-02	1.55	-1.37
105	tr A0A2C9UL99	Uncharacterized protein	Manes.14g095900	16	4.06E-02	1.61	1.10
106	tr A0A2C9VIK5	Uncharacterized protein	Manes.07g048300	12	4.06E-02	1.68	1.35
107	tr A0A2C9VHU1	Uncharacterized protein	Manes.07g022900	11	4.06E-02	1.38	1.29
108	tr A0A2C9VG21	H15 domain-containing protein	Manes.08g136300	3	4.06E-02	-1.85	1.08
109	tr A0A2C9U8F0	Trehalose-6-phosphate synthase 7	Manes.16g042700	8	4.18E-02	1.39	-1.82
110	tr A0A2C9VND6	CTP_transf_like domain-containing protein	Manes.06g064900	3	4.18E-02	-1.04	1.38
111	tr A0A2C9UFH3	Bulb-type lectin domain-containing protein	Manes.15g094200	3	4.18E-02	-1.15	-1.97
112	tr A0A2C9W9S7	Peptidase_S9 domain-containing protein	Manes.03g170200	20	4.26E-02	1.25	1.44
113	tr A0A2C9U4P5	Cupin_3 domain-containing protein	Manes.17g002700	3	4.30E-02	1.76	-1.13
114	tr A0A2C9U121	SAICAR_synt domain-containing protein	Manes.18g062300	7	4.30E-02	1.66	1.90
115	tr A0A251LSN4	Aminotran_5 domain-containing protein	Manes.01g181700	4	4.30E-02	-1.23	1.96
116	tr A0A2C9UFH5	Bulb-type lectin domain-containing protein	Manes.15g093900	8	4.48E-02	-1.28	-1.09
117	tr A0A2C9UFR6	Bet_v_1 domain-containing protein	Manes.15g129800	2	4.57E-02	1.57	-1.08
118	tr A0A2C9V7I3	Uncharacterized protein	Manes.09g033200	3	4.68E-02	1.17	-1.11
119	tr A0A2C9VVI6	TRANSKETOLASE_1 domain-containing protein	Manes.05g047000	55	4.68E-02	1.49	1.08
120	tr A0A2C9U368	Uncharacterized protein	Manes.18g105400	5	4.69E-02	-1.04	-1.17
121	tr A0A2C9VDK9	Epimerase domain-containing protein	Manes.09g179000	25	4.71E-02	-1.21	1.18
122	tr A0A2C9U1L7	CBM-like domain-containing protein	Manes.18g057200	3	4.71E-02	-1.95	1.15
123	tr A0A2C9WLR0	Non-specific serine/threonine protein kinase	Manes.01g100900	3	4.75E-02	1.42	1.30
124	tr A0A199UAF9	Amidase domain-containing protein	Manes.s085600	7	4.81E-02	-1.19	1.21
125	tr A0A251L4D3	Uncharacterized protein	Manes.04g146300	5	4.87E-02	1.41	1.78
126	tr A0A2C9UIV7	Uncharacterized protein	Manes.14g015300	7	4.87E-02	1.51	1.89

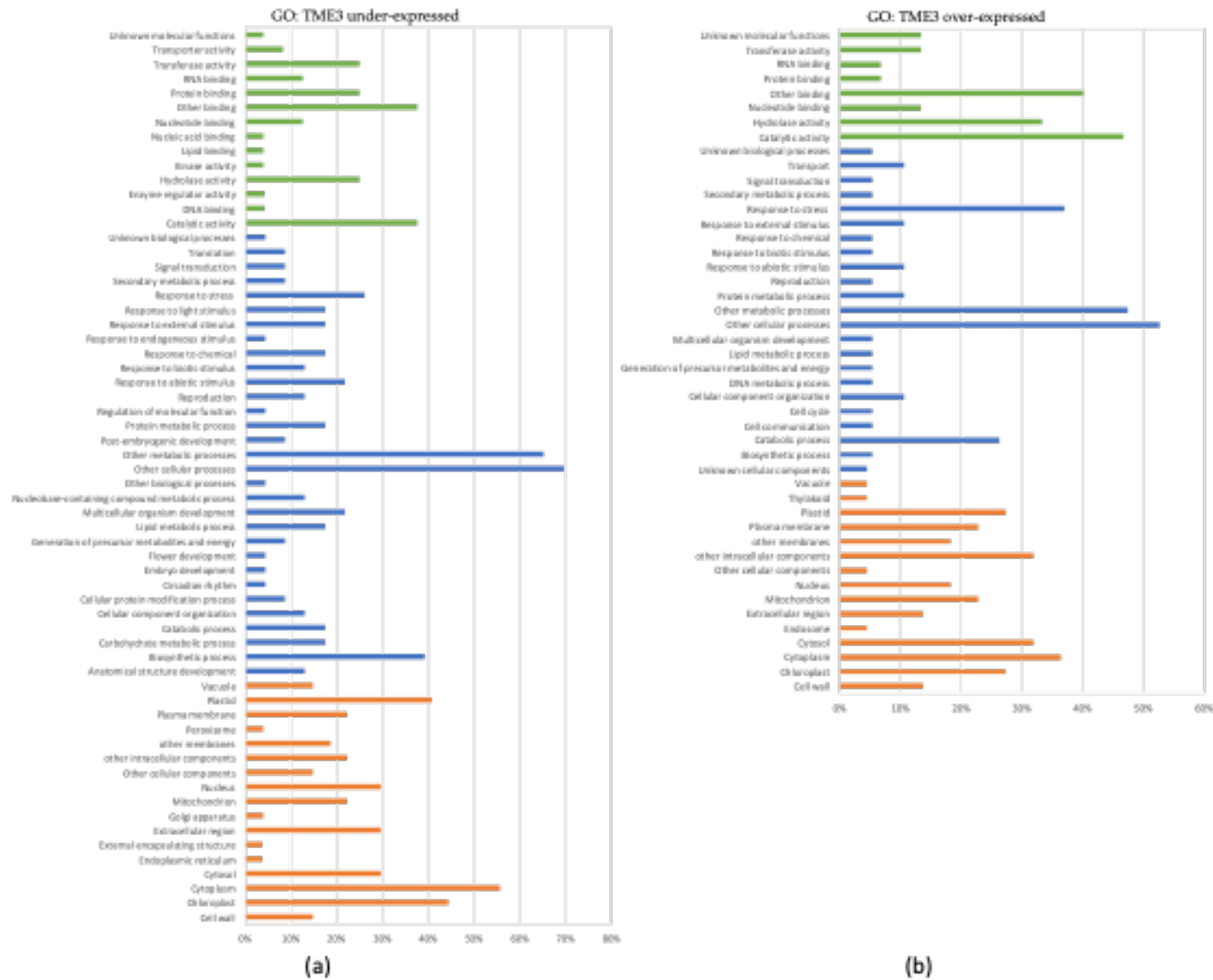
^aNegative ratio = under-expression, positive ratio = over-expression.

[#]Differentially expressed proteins with a ratio of < 2.0.

Appendix 6: Bioinformatics analysis of differentially expressed proteins in T200 and TME3 at 32 and 67 dpi.

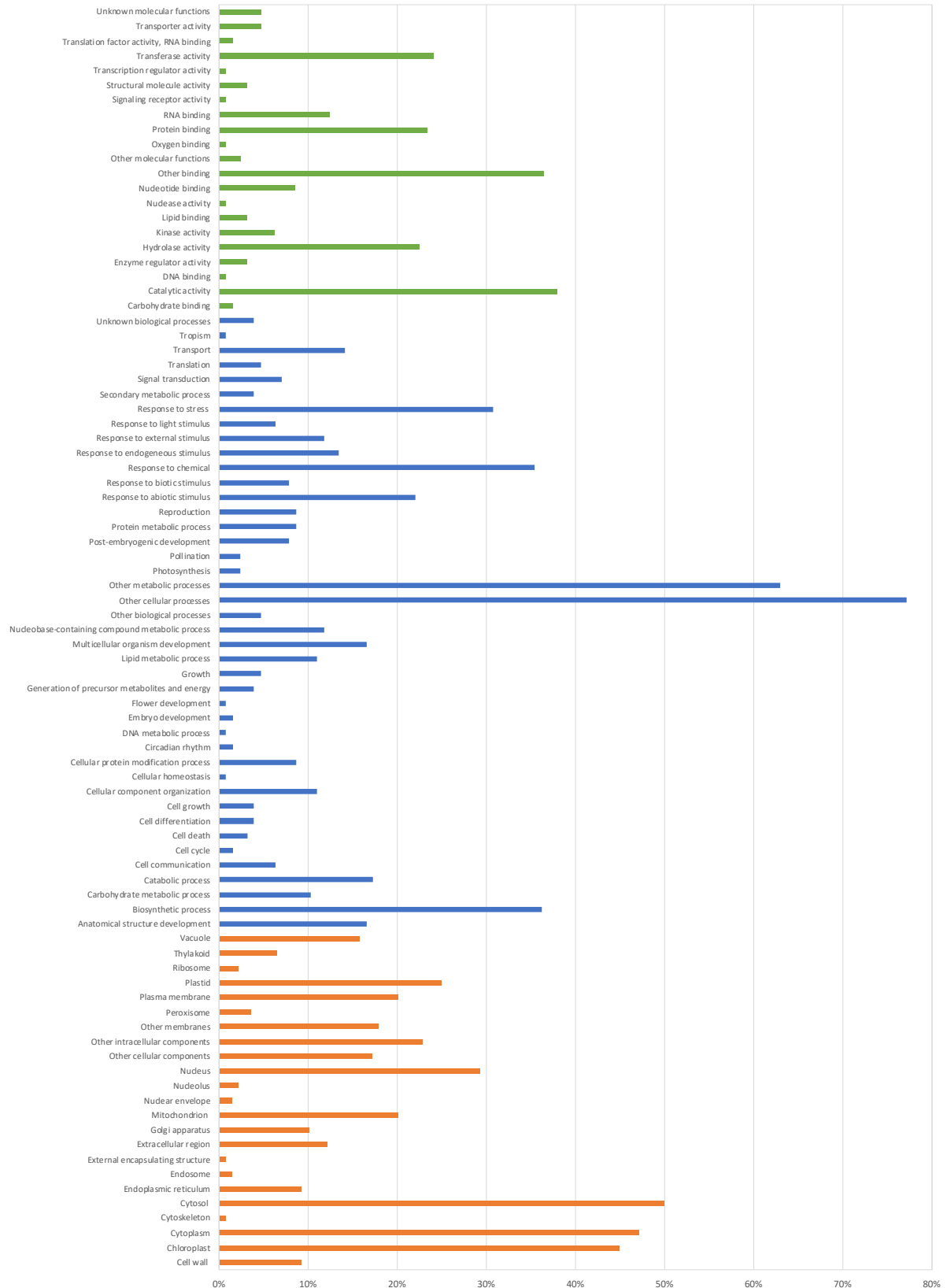


Appendix 6-Figure 1. Gene ontology enrichment analysis of differentially expressed proteins from T200 at 32 dpi. **(a)** Functional grouping of under-expressed proteins. **(b)** Functional grouping of over-expressed proteins. Green colour represents molecular function; blue, biological process; orange, cellular component.

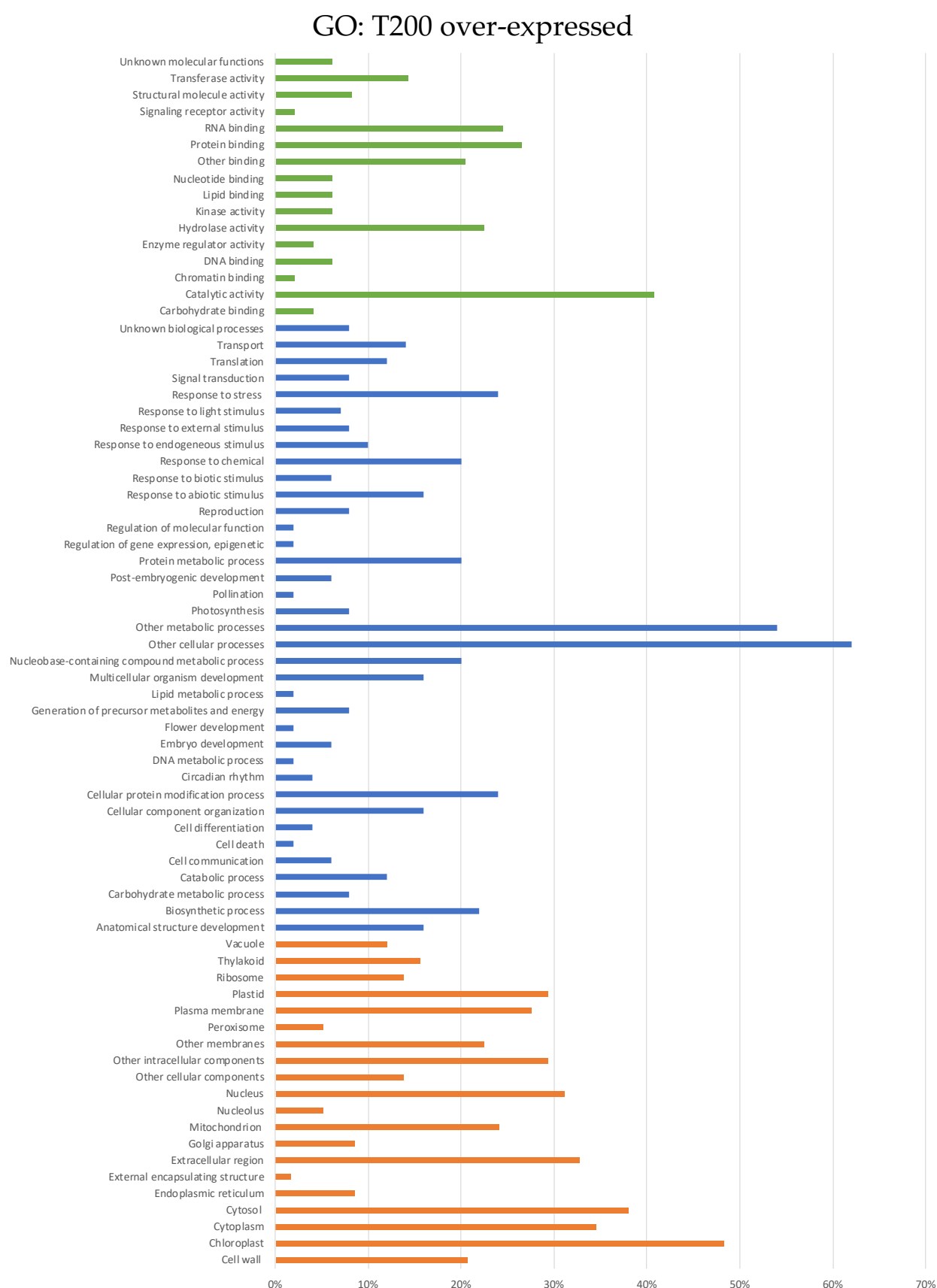


Appendix 6-Figure 2. Gene ontology enrichment analysis of differentially expressed proteins from TME3 at 32 dpi. **(a)** Functional grouping of under-expressed proteins. **(b)** functional grouping of over-expressed proteins. Green colour represents molecular function; blue, biological process; orange, cellular component.

GO: T200 under-expressed

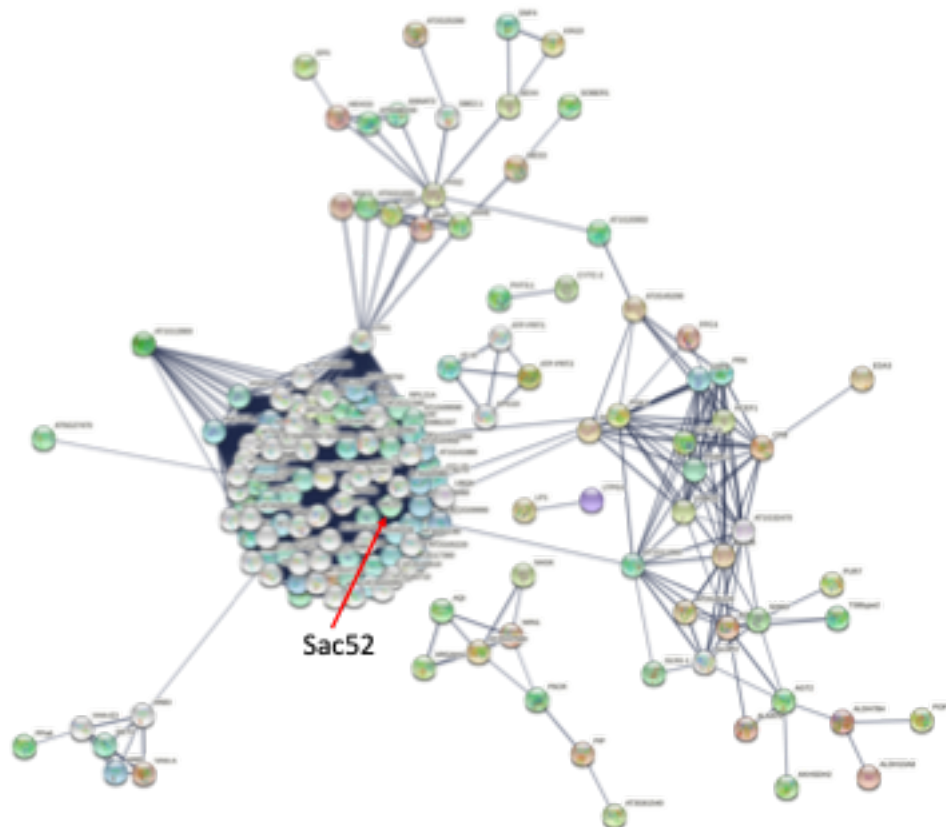


Appendix 6-Figure 3. Gene ontology enrichment analysis of under-expressed proteins from T200 at 67 dpi. **Green** colour represents molecular function; **Blue**, biological process; **Orange**, cellular component.

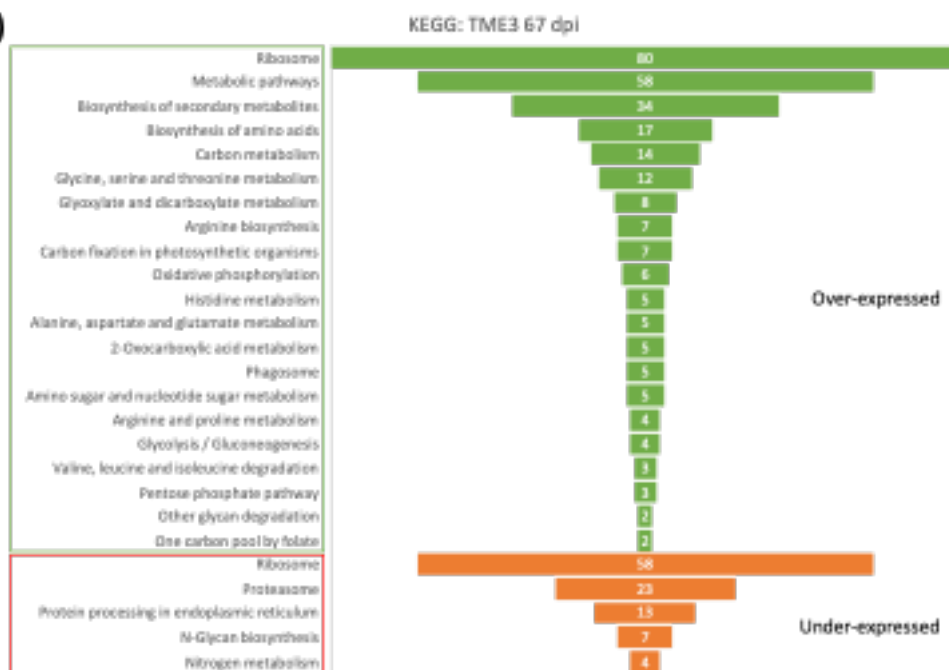


Appendix 6-Figure 4. Gene ontology enrichment analysis of over-expressed proteins from T200 at 67 dpi. **Green** colour represents molecular function; **Blue**, biological process; **Orange**, cellular component.

(a)



(b)



Appendix 6-Figure 5. TME3 differential expressed protein interactions at 67 dpi. (a) STRING protein-protein interaction network functional enrichment analysis of over-expressed proteins with p -value $< 1.0e-16$. Network Settings: Full network; Confidence edge; Textmining, Experiments, Databases, Co-expression Interaction sources; Confidence of 0.7; More than 50 interactors 1st and 2nd shell; Hide disconnected nodes in the network. (b) KEGG pathways analysis of over-expressed and under-expressed proteins with false discovery rate (FDR) < 0.05 .

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