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Cassava brown streak viruses: interactions in cassava and transgenic control

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Cassava Brown Streak Viruses:

Interactions in Cassava and Transgenic Control

A thesis presented by

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to

The Faculty of Science, University of the Witwatersrand, Johannesburg

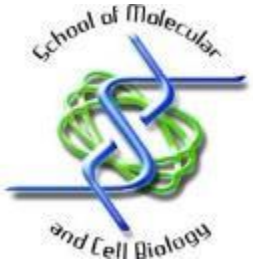
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Project Supervisor: Prof. M.E.C Rey

Co-supervisor: Nigel J. Taylor (PhD)

Abstract

Cassava brown streak disease (CBSD) ranks among the top seven biological threats to global food security and is considered to be a major risk to food security in tropical Africa. In Uganda, overall CBSD incidence has increased by c. 20% since 2004, and persistently reduces cassava yields and storage root quality. Presently the disease negatively impacts the livelihoods of over 80% of the farming families who rely on cassava as a staple food and source of income. Two distinct ipomoviruses, *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) cause CBSD. The viruses systemically infect primary host plants and accumulate, and cause severe disease symptoms as the plant matures, reducing yields through the induction of necrotic lesions in the storage roots and suppressing utility of cassava stems for subsequent vegetative propagation. Effective control strategies require screening of available germplasm for sources of natural resistance in combination with improved understanding of host-virus interaction to facilitate targeted breeding. Due to a lack of known sources of resistance to CBSD in the cassava germplasm, incorporating new virus resistance into existing cassava genotypes through transgenic RNA interference (RNAi) approaches offers an additional, relevant avenue to reduce the increasing impact of CBSD. The research presented in this thesis provides insights into the complex mechanisms of virus-host interactions linking genotype to phenotype in CBSV- and UCBSV-cassava pathosystems and provides proof of principle for CBSD control by RNAi-mediated technology. Both are contributions to progress towards potential control of the CBSD epidemic in East Africa.

To correlate CBSD symptoms with virus titer, within-host CBSV and UCBSV accumulation was studied in leaf, stem and storage root samples collected from 10 genotypes of field-grown cassava with varied levels of resistance to CBSD. CBSV was found to be present in 100% of CBSD samples collected from symptomatic plants. Presence of both CBSV and UCBSV was seen in 45.3% of the samples. Quantitative PCR (RT-qPCR) analysis showed that tolerant genotypes were infected with CBSV alone

and accumulated lower virus titer compared to susceptible genotypes, which were co-infected with CBSV and UCBSV. To further comprehend the molecular interaction between CBSD viruses and cassava, deep sequencing was performed to compare profiles of virus-derived small RNAs (vsRNAs) in CBSV- and UCBSV-infected cassava genotypes of NASE 3 (CBSD tolerant), TME 204 and 60444 (CBSD susceptible). The results showed an abundance of 21-24 nt sized vsRNAs which when mapped were shown to cover the entire CBSV and UCBSV genomes. The 21- and 22-nt sizes were predominant compared to the 23- and 24-nt size classes. CBSV-infected plants accumulated higher populations of vsRNAs across the genotypes compared to UCBSV-infected plants, which accumulated moderate amounts of UCBSV-derived sRNAs in TME 204 and 60444, and insignificant amounts in UCBSV-challenged NASE 3, respectively.

Quantitative RT-PCR analysis was performed to determine transcript levels of cassava homologues of Dicer (DCL) proteins, particularly DCL4 and DCL2, which are involved in the biogenesis of 21- and 22-nt small RNAs, and to correlate to the abundance of 21- and 22-nt vsRNAs in CBSV- and UCBSV-infected cassava. Similarly, RT-qPCR was performed to determine the expression of Argonaute (AGO) proteins, specifically AGO2 which preferentially sort and bind sRNAs with 5' adenine (A) or uracil (U) to effector complexes to target mRNAs repression or cleavage, since in this study a major proportion of the vsRNAs were found to have A or U at the first 5'-end. Expression levels of cassava homologues of AGO2, DCL2 and DCL4, which are core components of the gene-silencing pathway, were found to be affected in virus-infected plants across all three genotypes. The levels of viral RNA and vsRNAs correlated with disease phenotype in infected plants. CBSV-infected plants showed more severe CBSD symptoms compared with UCBSV-infected plants of the same genetic background. These results showed that CBSV is more aggressive compared to UCBSV and supports the hypothesis of occurrence of genotype-specific resistance to CBSD viruses. The abundance of 21- and 22-nt vsRNAs in CBSV- and UCBSV-infected plants signifies the viruses activated the RNA-silencing mechanism, referred to as transcriptional or post-transcriptional gene silencing (TGS or PTGS).

To test efficacy of RNAi-mediated resistance to control CBSD under field conditions, 14 lines of cassava plants transgenically modified to express, as inverted repeats, two RNAi constructs p718 and p719 targeting near full-length (894 bp) and N-terminal (402 bp) portions of UCBSV coat protein sequence were tested under confined field trial conditions at Namulonge, Uganda. Transgenic plants expressing p718 showed a 3-month delay in CBSD symptom development, while 100% of non-transgenic plants (n = 60) developed CBSD shoot symptoms. Over the 11-month trial duration, 98% of clonal replicates within line 718-001 were found to remain free of CBSD symptoms. RT-PCR analysis detected UCBSV within leaves of 57% of non-transgenic plants compared to only 0.5% across the 14 transgenic lines. Presence of the non-homologous CBSV was detected in all transgenic plants that developed CBSD symptoms. However, 93% of plants of line 718-001 were free of CBSV and UCBSV. At harvest, 90% of storage roots of non-transgenic plants showed severe necrosis, whereas plants of lines 718-001 and 718-005 showed significant suppression of CBSD. Line 718-001 had 95% of roots free from necrosis and was RT-PCR negative for presence of both viral pathogens.

To determine durability of RNAi-mediated resistance to CBSD, stem cuttings were obtained from mature plants of lines p718-001, p718-002 and p718-005, replanted and monitored for 11 more months. CBSV but not UCBSV was detected in tissues of plants of lines p718-002 and p718-005, whereas all leaves and roots of p718-001 plants were free of CBSV and UCBSV. Thus, RNAi constructs conferred durable CBSD resistance across the vegetative cropping cycle, providing proof of concept for application of RNAi technology to control CBSD in farmers' fields. The findings presented in this thesis contribute to understanding the complex interconnected mechanisms involved in CBSV- and UCBSV-host interactions and will contribute to the long-term goals of devising new methods of CBSD control.

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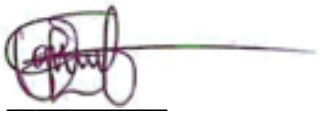
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Declaration

I, **Emmanuel Ogwok (701419)**, am a student registered for the degree of Doctor of Philosophy in Molecular and Cell Biology in the academic year 2012. I hereby declare the following:

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Signature  Date: 31st day of July 2015

Research Outputs

Original Publications

1. **Emmanuel Ogwok**, Muhammad Ilyas, Titus Alicai, Chrissie M. Rey, and Nigel J. Taylor (2015). Comparative analysis of vsRNAs within cassava (*Manihot esculenta* Crantz) infected with cassava brown streak viruses. Virus Research (*Submitted*).

2. **Emmanuel Ogwok**, Titus Alicai, Chrissie M. Rey, Getu Beyene, and Nigel J. Taylor (2014). Distribution and accumulation of cassava brown streak viruses within infected cassava (*Manihot esculenta* Crantz) plants. Plant Pathology. Doi: 10.1111/ppa.12343.

3. John Odipio, **Emmanuel Ogwok**, Nigel J. Taylor, Mark Halsey, Anton Bua, Claude M. Fauquet and Titus Alicai (2014): RNAi-derived field resistance to Cassava brown streak disease persists across the vegetative cropping cycle. GM Crops and Food: Biotechnology in Agriculture and the Food Chain, **Vol. 5 (1):** 16-19.

4. **Emmanuel Ogwok**, John Odipio, Mark Halsey, Eliana Gaitán-Solís, Anton Bua, Nigel J. Taylor, Claude M. Fauquet and Titus Alicai (2012): Transgenic RNAi-derived Field Resistance to Cassava Brown Streak Disease. Molecular Plant Pathology. **Vol. 13 (9):** 1019-1031.

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1. **Emmanuel Ogwok**, Muhammad Ilyas, Titus Alicai, Chrissie M. Rey, and Nigel J. Taylor (September 2014). Comparative analysis of virus-derived small RNA profiles of cassava brown streak viruses in infected cassava (*Manihot esculenta* Crantz) plants. Donald Danforth Plant Science Center, 16th Annual Fall Symposium, St. Louis, MO, USA (Poster presentation).

2. **Emmanuel Ogwok,** Nigel Taylor, Chrissie M. Rey, Claude M. Fauquet, and Titus Alicai (January 2013): Distribution of CBSD Causing Viruses in Different Cassava Tissues. 12th International Plant Virus Epidemiology Symposium, Arusha, Tanzania (Poster presentation).

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List of Abbreviations

aa	amino acid
ACMV	African cassava mosaic virus
AGO	Argonaute protein
BaMMV	*Barley mild mottle virus*
BaYMV	*Barley Yellow mosaic virus*
BlVY	*Blackberry virus Y*
bp	Base pairs
BtMV	*Beet mosaic virus*
CBSD	Cassava brown streak disease
CBSV	*Cassava brown streak virus*
cDNA	Complementary DNA
CFT	Confined field trial
CGM	Cassava green mite
ClYVV	*Clover yellow vein virus*
CM	Cassava mealy bug
CMD	Cassava mosaic disease
CMBs	Cassava mosaic begomoviruses
CMV	*Cucumber mosaic virus*
COX	Cytochrome c oxidase
CP	Coat protein
Cq	Quantification cycle
CVYV	*Cucumber vein yellowing virus*
CymRSV	*Cymbidium ring spot virus*
DCL	Dicer-like proteins
DNA	Deoxyribonucleic acid
DNA-B	DNA component B
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
EACMV	*East African cassava mosaic virus*

EACMV-UG	*East African cassava mosaic virus-Uganda*
ER	Extreme resistance
GFP/RFP	Green fluorescent protein/Red fluorescent protein
GTPb	GTP binding protein
Ha	Hectares
hpRNA	Hairpin RNA
HR	Hypersensitive response
IR/CR	Intergenic region/common region
MAP	Months after planting
miRNA	microRNA
mL	Milliliter
MT	Million metric tons
NARO	National Agricultural Research Organization
nM	Nano meter
nt	Nucleotide
OMV	*Oat mosaic virus*
ORF	Open reading frame
PCR	Polymerase chain reaction
PDR	Pathogen-derived resistance
PMMoV	*Paper mild mottle virus*
PP2A	Serine threonine phosphatase 2
PPV	*Plum pox virus*
PR	Pathogenesis-related protein
PRSV	Papaya ring spot virus
PTGS	Post-transcriptional gene silencing
PVA	*Potato virus A*
PVX	*Potato virus X*
PVY	*Potato virus Y*
RDR	RNA-dependent RNA polymerase
RGMV	*Rye grass mosaic virus*
RISC	RNA-induced silencing complex

RNA	Ribonucleic acid
RNAi	RNA interference
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
ScSMV	*Sugarcane streak mosaic virus*
siRNA	Small interfering RNA
SPMMV	*Sweet potato mild mottle virus*
SqVYV	*Squash vein yellowing virus*
sRNA	Small RNA
ssDNA	Single-stranded DNA
ssDNA	Single-stranded DNA
DNA-A	DNA component A
SSR	Simple sequence repeat
TCV	*Turnip crinkle virus*
TGS	Transcriptional gene silencing
TomMMV	*Tomato mild mottle virus*
TriMV	*Triticum mosaic virus*
TRV	*Tobacco rattle virus*
TuMV	*Turnip mosaic virus*
TVMV	*Tobacco vein mosaic virus*
TYLCV	*Tomato yellow leaf curl virus*
UBQ10	Ubiquitin 10
UCBSV	*Ugandan cassava brown streak virus*
UTR	Untranslatable region
VIRCA	Virus Resistant Cassava for Africa project
vsRNA	Virus-derived small RNA
WMV	*Watermelon mosaic virus*
WSMV	*Wheat streak mosaic virus*
ZYMV	*Zucchini yellow mosaic virus*
μg	Microgram
μM	Micro molar

Chapter 1

Introduction

1.1 Cassava

Cassava (*Manihot esculenta* Crantz, Family: *Euphorbiaceae*) is a perennial crop that originated from the southwestern rim of the Amazon basin of South America (94). Portuguese traders introduced cassava into Africa around the 16th century, and into Asia in the 18th and 19th centuries. By this time cassava cultivation was widespread in West Africa, from where it was later introduced into Central and East Africa through the Cape of Hope via Madagascar and Zanzibar (73). Cassava cultivation has since become integral to the farming systems of tropical Africa (64). In Uganda, cassava cultivation was established in the 19th century, developing to become a valuable food security crop during the 20th century.

1.1.1 Production and utilization

Cassava is now the third most important tropical food crop and central to the livelihoods of over 800 million people in African, Asian, Latin American and Oceania (131). Global cassava production presently stands at c. 277 million metric tons (MT), on an area of *c.* 20 million hectares. Although more than half (53.9%) of total production is in sub-Saharan Africa, this continent has the lowest average cassava yields of tuberous roots (11.5 t/ha), compared to Latin America (13.5 t/ha) and Asia (21.1 t/ha) (47, 91). The eight largest cassava producing African countries include Nigeria (33.6%), Democratic Republic of Congo (10.5%), Angola (10.4%), Ghana (10.1%), Mozambique (6.3%), Uganda (3.3%) Malawi (3.1%) and Tanzania (3.0%) (47).

Cassava is cultivated both for subsistence and commercial purposes with international trade mostly confined to East and Southeast Asia, especially in the form of chips, starch, and flour. Thailand, in particular, is the world's leading international supplier of cassava products with Europe and China the

major importers of cassava products (47). In sub-Saharan Africa, cassava is mostly used as a food security crop, accounting for a third of the total food produced (45), while in South American and some parts of Asia, the starchy roots are processed into diverse commercial and industrial products. The starchy cassava storage roots, with a dry matter content of 30-40%, are a highly effective source of energy for humans and livestock (53). Cassava leaves are a good source of proteins, vitamins and minerals, and are therefore used as food and fodder, in addition to genotypes with variegated leaves serving ornamental purposes (25). Cassava is also increasingly being developed for industrial applications in the form of ethanol, starch, and bioenergy (45).

In Uganda, cassava ranks second after banana in terms of production and consumption, and is a priority staple crop for household food and income security (142). The widespread adoption of cassava in Uganda and elsewhere has resulted from its broad adaptability including its ability to produce acceptable yields during unreliable rain and drought conditions, on low fertility soils, and under a number of low input production systems.

1.1.2 Production constraints

In Africa, diverse biotic and abiotic constraints impede cassava production. The economically most important biological constraints are the viral diseases: cassava mosaic disease (CMD) and cassava brown streak disease (CBSD). Both diseases were recognized and have been known to restrict cassava production since the 1930s (92). A severe new outbreak of CMD in Uganda was reported in the 1980s (90) and by early 1990s had become an epidemic that nearly wiped out cassava as a crop in the country (142). While CBSD was endemic in the coastal lowlands of East Africa for over 70 years, the disease has recently attained an epidemic status (10, 90). The crop is also attacked by bacterial and fungal pathogens, many of which were inadvertently introduced into Africa. Examples are cassava

bacterial blight (CBB), cassava anthracnose (CA) and cassava root rot, which significantly contribute to reduction in cassava production and productivity (91, 142). Major pests limiting cassava production include cassava green mites (CGM), *Mononychellus tanajoa;* cassava mealy bug, *Phenacoccus manihoti* Matt.-Ferr; leafhoppers; rodents; and the whitefly, *Bemisia tabaci*, which in addition to being a pest, also acts as the vector for CMD- and CBSD-causing viruses (107). The long cropping cycle of cassava (8 – 24 months) exacerbates many of the disease and pest issues, while its clonal propagation via stem cuttings means that infection persists across cropping cycles. Lack of a formal seed system for ensuring access to clean planting materials leads to increased pest and disease loads within farmer's fields. Physiological deterioration of cassava storage roots within 24 to 72 hours after harvest (154) remains a major constraint for economic development of the crop. Farmers relying on fresh roots as a source of food and income must consume, sell or process fresh cassava roots almost immediately after harvesting. This presently limits the marketing potential of cassava and cassava products.

1.2 Viruses infecting cassava in Africa

Globally, cassava is infected by at least 20 different viruses. In Africa, 15 virus species have been identified infecting the crop (91). In East Africa, the most economically important viruses are cassava mosaic viruses, which cause cassava mosaic disease (CMD) and cassava brown streak viruses, which cause cassava brown streak disease (CBSD) (90, 145).

1.2.1 Cassava mosaic disease

Cassava mosaic disease is caused by cassava mosaic begomoviruses (CMBs) (family *Geminiviridae*, genus *Begomovirus*). CMD occurs in all major cassava-growing areas of sub-Saharan Africa (145). The disease is known to be caused by at least nine species of CMBs, eight of which have been

reported in Africa (180). The two most important in East Africa are *Africa cassava mosaic virus* (ACMV) and *East Africa cassava mosaic virus* (EACMV). The CMBs are comprised of two circular single stranded DNA (ssDNA) components (DNA-A and DNA-B) (156), and are efficiently transmitted by the whitefly vector, *Bemisia tabaci* (61). Both DNA components (A and B) are *c.* 2,800 bp in size, encased within a twinned protein coat of 30 x 20 nM and replicate in infected cells by rolling cycle amplification through a double-stranded DNA (dsDNA) intermediate (51). DNA-A and DNA-B differ in their sequences except for the intergenic region (IR), also called the common region (CR), which contains the origin of replication. The CR is a stem-loop structure containing the TAATATTAC sequence required for cleavage and joining of the viral DNA during replication (51). DNA-A contains six open reading frames (ORF), which encode six proteins, namely *AV1* and *AV2* translated in the virion-sense and *AC1*, *AC2*, *AC3* and *AC4* translated in the virion-antisense. *AC1* encodes the replication associated protein (Rep); *AC2* the translational activator (TrAP); *AC3* the replication enhancer (REn); *AC4* the RNA-silencing suppressor; *AV1* the coat protein (CP) and *AV2* the pre-coat protein. Whereas, DNA-B has two ORFs, the sense BV1, which encodes the nuclear-shuttle protein and the antisense BC1, which encodes the movement protein (MP) (51, 91).

The CMBs can cause disease in host plants singly or in combination. Symptoms of CMD include distinct leaf chlorosis, leaf deformation resulting in significantly suppressed storage root yields. Prior to the 1980s, CMD caused mostly mild symptoms in all cassava growing areas in Africa. However, a severe CMD epidemic occurred in the 1980s and 1990s in north-central Uganda (33, 90, 143), which was later found to be associated with emergence of a recombinant CMB viral variant, *East Africa cassava mosaic virus*-Uganda (EACMV-UG). Mixed virus infections of EACMV-UG and the ACMV caused very severe symptoms and crop losses in the susceptible farmer landraces, due to synergistic interactions between the two virus species (52, 61, 149). Although the CMBs have been well studied

since the early 1990s and management strategies developed through conventional breeding (90), new spread of the disease has recently been reported in West Africa (9, 84)

Storey and co-workers carried out breeding programs for CMD resistance between 1937 to 1958 at Amani in Tanzania (133) by introgression of resistance genes from *Manihot glaziovii, a* wild relative of cassava, and backcrossing into cultivated cassava. This generated moderately CMD resistant clones (such as 5318/34 and 58308), which were introduced into Nigeria and subsequently used as breeding stock. In 1971 the Roots and Tubers Improvement Program of International Institute for Tropical Agriculture (IITA) was established with the priority of incorporating disease resistance into local cassava cultivars (137). Within 10 years, the team generated the Tropical Manihot Selection (TMS) series of highly CMD resistant cassava varieties (TMS 30395, TMS 30555, TMS 30572, TMS 30001 etc.), which increased cassava yields in farmers' fields by *c.* 40% (91, 137). In the 1990s breeders in the major African cassava-producing countries used these varieties as parents in their breeding programs to generate new germplasm with increased CMD resistance. Pyramiding of the polygenic recessive resistant genes (CMD1) obtained from the Amani clones with a dominant CMD resistance gene (CMD2) obtained from Nigerian landraces were used to develop cassava varieties, with not only durable CMD resistance, but agronomic and culinary attributes desired by farmers and consumers (153).

1.2.2 Cassava brown streak disease

Cassava brown streak disease is considered to be one of the world's most serious threats to food security (147, 148). The disease is caused by two viral pathogens, *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV). Both are species of the genus *Ipomovirus*, family *Potyviridae* (112, 199) transmitted by the whitefly vector *Bemisia tabaci*, in a non-circulative, semi-

persistent manner (38, 107). CBSD can result from single or dual infections by these two positive single stranded RNA (+ssRNA) viruses.

1.2.2.1 History of CBSD

The first report of CBSD was in 1930s from East Africa during the early days of the Amani (northeastern Tanzania) research program (166). At that time the disease was confined to low altitudes (below 1000 m above sea level) along the coastal regions of Kenya and Tanzania, around Lake Malawi and in Zanzibar (166). Relative incidence and severity was low. In the early 2000s, however, CBSD was reported to be present at mid-altitude (above 1000 m above sea level) in areas of northwestern Tanzania (71) and Western Kenya (136). In Uganda, the disease was first reported in 1934 but was effectively eradicated by destroying all plants showing CBSD symptoms (25). The disease re-emerged in Uganda in 2004 and has continued to increase in importance (10).

Recent nationwide surveys of cassava diseases and pests in Uganda indicated that CBSD is present throughout the country with greatest incidence in the south and central regions where mainly CBSD susceptible varieties (TME 204 and TME 14) introduced to combat CMD are grown, and lowest in northeast where CBSD-susceptible varieties have been replaced with tolerant ones (NASE 3 and NASE 14) (5). The overall national CBSD prevalence increased from 3.3% in 2005, peaked at 45.2% in 2011 and declined slightly to 40.5% in 2013, whereas disease incidence increased from 1% in 2005 to 21.8% in 2013 (5). To gather farmers' perception on the impact of cassava diseases and pests on household livelihood, a total of 1,068 households were interviewed in a survey in 2010 (21). The results indicated that 82% of the households dedicated c. 9.6 acres of their land for cassava production, mainly for food (60%) or food and cash income (23%). Most households cultivated improved cassava varieties (67%) though some households (23%) used local landraces. Interestingly, over 75% of the

households abandon cultivation of particular cassava varieties due to the effect of diseases (mainly CBSD) and pests (60%), and for local cassava varieties, low yield (47%). The survey data clearly demonstrated the significant and important effect of CBSD to the farming communities in Uganda. Reports of similar diseases and pests survey from other countries including Malawi (114) and Tanzania (72), and some areas within eastern and Central Africa region (90, 136) confirm the devastating effect of CBSD in such farming communities. CBSD continues to spread and has been reported in Mayotte Island (157), Democratic Republic of Congo (128), Rwanda (90), Burundi (15) and threatens to spread further towards Nigeria, currently Africa's most populous country and the world's largest cassava producer (91).

1.2.2.2 Economic impact of CBSD

Studies have shown that CBSD causes losses to cassava production in a number of ways. These include stunting of affected plants, constriction and necrotic rotting of roots, reduction in root numbers and root weight, adverse effect on starch quality of affected roots and reduced price at the marketplace. In susceptible varieties, this can result in yield losses of up to 70% (65). The severity of the disease increases with plant age, becoming more severe as the plant matures. This leads to secondary losses as farmers are inclined to harvest the plants early to avoid extensive root damage. In 2001, estimated overall production loss was US $6-7 million, whereas, for the farmers in Tanzania alone, an annual loss of about US $45 million due to CBSD has been reported (24), and losses of US $75 million was recently estimated for all the eight affected East African countries (101).

1.2.2.3 CBSD symptoms and host range

CBSD was first described by Storey (166), who recognized that the disease expresses itself in all parts of affected plants, producing diverse symptoms. The major symptom types include yellowing of older leaves along the major veins, brown coloured round, or elongated streak-like lesions on the stems and most destructively, production of brown, corky, necrotic lesions within the storage roots (63, 118, 166). CBSD affects cassava, but evidence has also been reported of CBSV infecting the cassava relative Manihot glaziovii (113). The viral pathogens causing CBSD are transmitted by whiteflies (107) between cassava plants, and mechanically by propagation of infected stem cuttings by farmers. Mechanical transmission by sap inoculation under controlled growth conditions is also possible to several herbaceous plants species of different families (96, 140), and by grafting between cassava plants. Mechanical transmissibility of the viral pathogens suggest they are not phloem limited but are also present in epidermal and mesophyll tissues (38). Infections caused by different isolates of CBSV and UCBSV might produce different CBSD symptoms in affected plants but there are large overlaps (72, 118), and environmental condition, age and genotype of the cassava plant tend to affect symptom severity (70, 140, 146). Comparison of symptom severity has revealed that CBSV-infected plants produce more severe CBSD symptoms than UCBSV-infected plants (76, 113, 118, 138, 199). Mild CBSD symptoms are sometimes problematic to distinguish, especially in plants suffering from other stresses such as insect damage and nutritional deficiencies. This can make it difficult to distinguish between uninfected and infected plants. Unfortunately, this leads to propagation of the disease through planting infected materials.

1.3 Ipomoviruses

Ipomoviruses (genus *Ipomovirus,* family *Potyviridae*) are whitefly-transmitted viruses characterized by filamentous flexible particles and a single-stranded positive-sense RNA genome of ~9 kb. The viral genome is translated into a polyprotein precursor, which is subsequently cleaved by virally encoded proteases into 9 – 10 functional proteins (7, 38). Members of the genus *Ipomovirus* at present include: *Sweet Potato mild mottle virus* (SPMMV), *Cucumber vein yellowing virus* (CVYV), *Squash vein yellowing virus* (SqVYV), CBSV and UCBSV. In addition, there is another tentative member, *Tomato mild mottle virus* (TomMMoV) and the Israeli isolate of TomMMoV (TomMMoV-IL) that have recently been characterized (38) (Fig. 1.1). These viruses are frequently present as part of the natural pathogen population causing chronic losses in yield and quality of many crop species globally. Notable among the important crop hosts are sweet potato (*Ipomea batatas*), cassava (*Manihot esculenta*), cucurbits (*Citrullus lanatus, Cucumis melo,* and *Cucumis sativus*), eggplants (*Solanum melongena*) and tomato (*Solanum tycopersicum*) (38). All ipomoviruses, apart from CBSV and UCBSV have been shown to produce characteristic pinwheel-like or cylindrical inclusion bodies in the cytoplasm of infected tissues (8, 38, 39, 86).

1.3.1 Ipomoviruses genome organization and gene functions

The complete genome of ipomoviruses exhibit considerable variation in both length, ranging from ~9069 nt (CBSV) to 10818 nt (SqVYV), and the domain encoding the three proximal genes at the 5' terminus. Nonetheless, the genomes generally encode similar proteins, namely: the first protein (P1) proteinase, helper component cysteine proteinase (HC-Pro), the third protein (P3), a short overlapping Pretty Interesting Potyviridae open reading frame (PIPO), two 6 kDa proteins (6K1 and 6K2), cylindrical inclusion protein (CI), viral genome-linked protein (VPg), viral proteinase a (NIa-Pro), replicase (NIb)

and coat protein (CP) (Fig. 1.2). Based on the variation of proteins encoded at the 5' terminus (P1 to P3), the viruses can be categorized into three groups; (i) members that encode P1 and HC-Pro (SPMMV and TomMMoV), (ii) members that encode two repeats of P1: P1a and P1b and lack HC-Pro (SqVYV and CVYV), and (iii) members that encode a single P1 and lack HC-Pro (CBSV and UCBSV). In addition, the genomes of CBSV and UCBSV encode a HAM1h protein, a putative nucleoside triphosphate pyrophosphatase, situated between the viral replicase (NIb) and coat protein (CP) in the C-proximal part of the polyprotein (111).

HAM1h shares homology with the Maf/HAM1 superfamily of proteins, which are known to intercept non-canonical nucleotide triphosphates and prevent their incorporation into nucleic acids, and thereby lowering viral mutation rates (55, 112, 199). *Euphorbia ring spot virus* (ERSV), belonging to the genus *Potyvirus* (29), is the only other virus known to encode the HAM1-like protein. The P1 proteins of SPMMV and TomMMoV and the P1b protein of CVYV and SqVYV, respectively, play the role of gene silencing suppression (38). Presumably, the P1 protein of CBSV and UCBSV also possesses the silencing suppression activity, since it contains the basic LxKA and Zn-finger motifs, which confer RNA-silencing suppression activity to the P1 of SPMMV and P1b of SqVYV and CVYV, respectively (38, 112, 146). Conversely, potyviral HC-Pro is a multifunctional protein involved in RNA-silencing suppression, utilizing the FRNK motif, to prevent small RNA loading in the RNA silencing complex (RISC), viral transmission by aphids, genome replication and symptoms development (99). The P3 of all ipomoviruses encode a short overlapping ORF, P3N-PIPO, generated by a +2 frameshift. The P3N-PIPO protein interacts with CI to form a complex that helps in intercellular virus movement across plasmodesmata-associated structures (26, 198). The remaining genes encoded by CBSV and UCBSV have similar functions to that of their homologues in SqVYV, CVYV and SPMMV (26, 38).

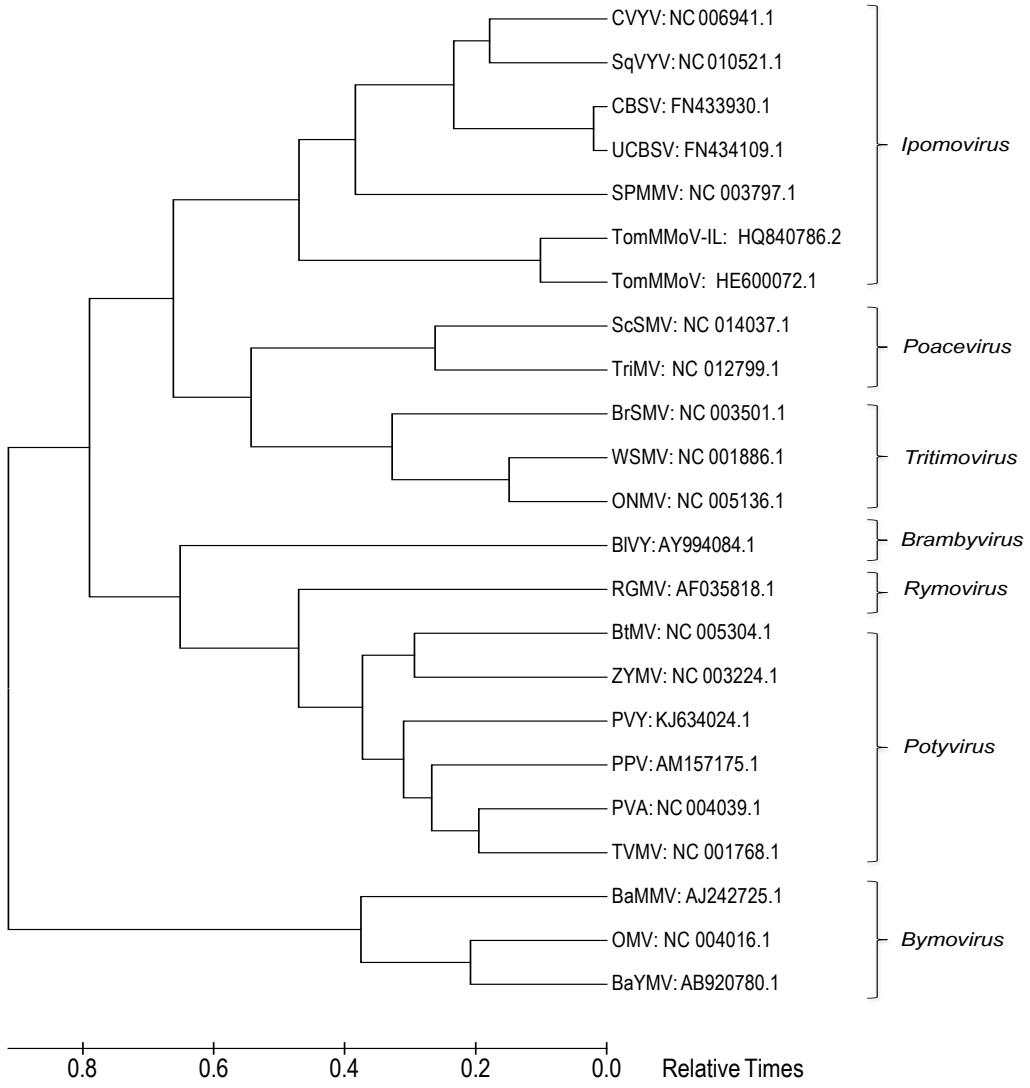


Fig. 1.1: Phylogenetic tree based on complete genome sequences corresponding to 23 viruses belonging to seven genera in the family Potyviridae. All members of the genus Ipomovirus are represented: Cucumber vein yellowing virus (CVYV), Squash vein yellowing virus (SqVYV), Cassava brown streak virus (CBSV), Uganda cassava brown streak virus (UCBSV), Sweet potato mild mottle virus (SPMMV), Tomato mild mottle virus-Israeli (TomMMoV-IL), and Tomato mild mottle virus (TomMMoV); two members of the genus Poacevirus: Sugarcane streak mosaic virus (ScSMV) and Triticum mosaic virus (TriMV); two members of Tritimovirus: Wheat streak mosaic virus (WSMV) and Oat necrotic mottle virus (ONMV); a Brambyvirus, Blackberry virus Y (BlVY); a Rymovirus, Ryegrass mosaic virus (RGMV); six Potyviruses: Beet mosaic virus (BtMV), Zucchini yellow mosaic virus (ZYMV), Potato virus Y (PVY), Plum pox virus (PPV), Potato virus A (PVA), and Tobacco vein mosaic virus (TVMV); and three Bymoviruses: Barley mild mottle virus (BaMMV), Oat mosaic virus (OMV), and Barley yellow mosaic virus (BaYMV). GenBank accession numbers are indicated against each virus.

1.3.2 Diversity of cassava brown streak viruses

Phylogenetic analysis of 12 complete genome sequences available in the public database (GenBank) indicates that CBSD is caused by two distinct virus species with several strains within each species (Fig. 1.3). The viruses share 69.0 – 70.3% nucleotide, and 73.6 – 74.4% amino acid sequence identity, respectively (110). Nucleotide identity between CBSV and UCBSV is ~70%, whereas it ranges from ~87 – 99% and ~79 – 95% among UCBSV and CBSV isolates, respectively (110). Further analysis of the sequences indicates that CBSV (~9069 nt) contains a 137 nt 5' untranslated region (UTR) followed by a single ORF, encoding a 2902 kDa polyprotein, and a 226 nt 3' UTR (111), whereas that of UCBSV (~9070 nt) has a 134 nt 5' UTR, a 2902 kDa polyprotein, and 227 nt 3' UTR .

Considerable variation between the genomes of CBSV and UCBSV, together with the presence of several isolates within each virus species (Fig. 1.3), indicates that the viruses are evolving fairly rapidly. Observed CBSD symptom differences may be a consequence of strain variations (118), suggesting that these viruses have higher recombination potentials and greater chances to generate host-range mutants with possibility of emergent epidemics (44). Recent reports from Malawi (114) and Mayotte Island (157) showed that UCBSV is more prevalent compared to CBSV, and revealed the presence of intermediate isolates in both geographical locations, which cluster between previously described isolates of UCBSV and CBSV. These reports provide evidence for the existence of extensive variability within and between the two virus species, and highlight the likelihood of the existence of yet to be detected CBSD-causing viruses with potential for causing emergent disease outbreaks.

Transport of infected planting materials within and across geographical borders may have played an important role in the occurrence of the present CBSD epidemic and a major contributor to the spread of CBSD in the East African region (72). The new occurrence of super-abundant populations of *B. tabaci*

whiteflies, which are better adapted on cassava (126), and to environmental conditions prevalent in mid-altitude areas of East and Central Africa (72), is also considered to be an important component of the CBSD resurgence and is evidence that whiteflies remain at the core of the epidemic.

1.4 Detection and diagnostics of cassava brown streak viruses

A number of tools and techniques have been developed for CBSD diagnosis. These include identification of symptoms on infected plants, electron microscopy, serology, and polymerase chain reaction (PCR)-based methods (91). The availability of CBSV and UCBSV whole genome sequences from different locations in East and Central Africa has enabled development of PCR primers that target the most conserved sequences and their use for reverse transcription PCR (RT-PCR), which is now used routinely for CBSD diagnostics (2). Quantitative RT-PCR (RT-qPCR), which is a more sensitive and reliable detection method, has been developed using both TaqMan and SYBR Green I chemistries and optimized for detection of CBSV and UCBSV in infected cassava tissues (95). TaqMan RT-qPCR was used extensively to detect CBSD viral pathogens in materials produced for distribution within countries in the Great Lakes region (6, 124, 138). Another technique, called reverse transcription loop-mediated isothermal amplification (RT-LAMP) (181), which does not require thermal cycling machines, but rather uses lateral flow devices containing specific antibodies to labeled primers, has also been developed and used for CBSV and UCBSV detection.

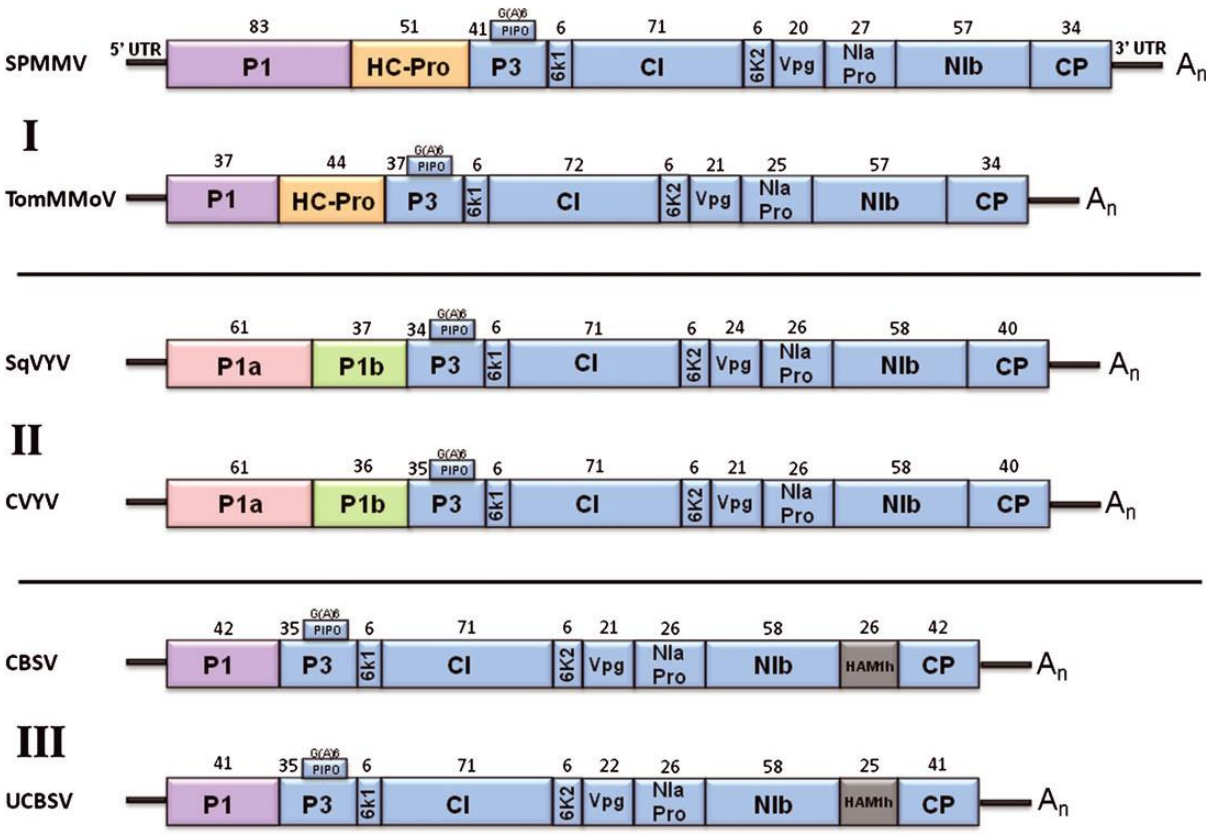


Fig. 1.2: Genome organization of Ipomoviruses (genus Ipomovirus) showing genes encoded by each virus and grouping of the viruses based on genome organization. Numbers above each genome represent the molecular weight of each of the protein (kDa) indicated. The viruses grouped into three clusters: cluster I: SPMMV (NP_620656), TomMMoV (AF359575) and TomMMoV-IL (HQ840786); cluster II: SqVYV (YP_001788991) and CVYV (NC_006941); cluster III: CBSV (ACM48176) and UCBSV (YP_004063681). Source: Dombrovsky et al., (2014). Pest Management Science 70: 1553-1567 (38)

1.5 Within-host virus distribution and accumulation

Current knowledge on host-virus interaction portrays a plant as a spatially structured environment (44). In essence, populations of viruses replicating within an infected plant are a collection of subpopulations in different parts of the plant. The virus has to move from point of infection to colonize tissues within branches, individual leaves and the root system (31). This results in significant variability of viral load between infected tissues within a given host and between different host plants within the same field

(195). In addition, numerous ecological and environmental factors including virus and host genetic diversity, infection dosage, age, size and growth rate of the host influence variation in viral load. This in turn influences virus evolution, disease spread, epidemiology and importantly the management options best suited to tackling the disease.

Identical virus populations have been observed to exhibit spatial separation or mutual exclusion in mixed infected plants (34, 168). The phenomenon of spatial separation or mutual exclusion occurs when two or more viruses, or strains of the same virus, simultaneously infect a host with antagonistic within-host interactions. Jedlinski and Brown (1965) (68) observed a classical example of mutual exclusion when they inoculated young oat plants with three strains of *Barley yellow dwarf virus* (BYDV). The plants exhibited mild symptoms soon after inoculation but completely recovered later during growth. On testing no virus could be detected. Meanwhile, spatial separation essentially reduces extensive multiple viral infection by restricting the number of viral genomes that enter and effectively replicate in a cell (60, 123). A classic example of spatial separation was demonstrated by Dietrich and Maiss (2003) (35) when they simultaneously inoculated *N. benthamiana* plants with cDNA clones of *Plum pox virus* (PPV), *Tobacco vein mottling virus* (TVMV) and *Clover yellow vein virus* (ClYVV) or identical PPV labeled with green and red fluorescent proteins (GFP and RFP). When the plants were subjected to imaging, clusters of green and red fluorescent signals were distinctly visible in neighboring cells but not in the same cell, implying that the two viral populations competed with each other during colonization of epidermal cells. Similarly, co-infection of cowpea with two strains of *Cucumber mosaic virus* (CMV) indicated that the viruses did not colonize the same cell (170). Simultaneous inoculation of *N. benthamiana* leaves with differently labeled *Bean yellow mosaic virus* (BYMV) also showed spatial separation (170). Consequently, spatial separation and mutual exclusion limit chances for recombination and pseudorecombination between viruses, and restrict generation of viral genetic

variants (44), and the likelihood of emergence of novel, highly fit virus variants (167). Little is known about within-host distribution and the nature of interaction between CBSV and UCBSV in plants carrying single or mixed infections of these pathogens.

1.6 Virus-virus and virus-host interactions

Within-host virus interactions may involve nucleic acids or proteins of one virus interacting physically with the gene or gene products of co-infecting virus(es). These can be synergistic or antagonistic in nature and are reviewed by Syller (2012) (30, 167). Both synergistic and antagonistic virus-virus interactions occur through co-infection, in which one or more viruses invade the same host simultaneously, or through super-infection where different viruses or virus strains infect the host at different times (167). Such interactions result in measurable changes in tissue tropism, viral replication, pattern of progeny production and release, latency, and pathology compared to infection of the plant with one virus only (30).

Predominantly, synergistic interactions has been observed between unrelated viruses infecting the same host cells, except for a few cases observed for related viruses such as Begomoviruses, Criniviruses, and Potyviruses (155, 172, 200). For instance, the following virus combinations were reported to exhibit synergy in the respective host plants; *Potato virus X* (PVX) and *Potato virus Y* (PVY) in *Nicotiana tabacum* and *N. benthamiana* plants (167); *Tomato infectious chlorosis virus* (TICV) and *Tomato chlorosis virus* (ToCV) in *N. benthamiana* and *Physalis wrightii* plants (200); *Sweet potato feathery mottle virus* (SFMV) and *Sweet potato chlorotic stunt virus* (SPCSV) in sweet potato (127); PVY and *Tobacco mosaic virus* (TMV) in *Solanum brevidens* (186); *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV) in wheat (172).

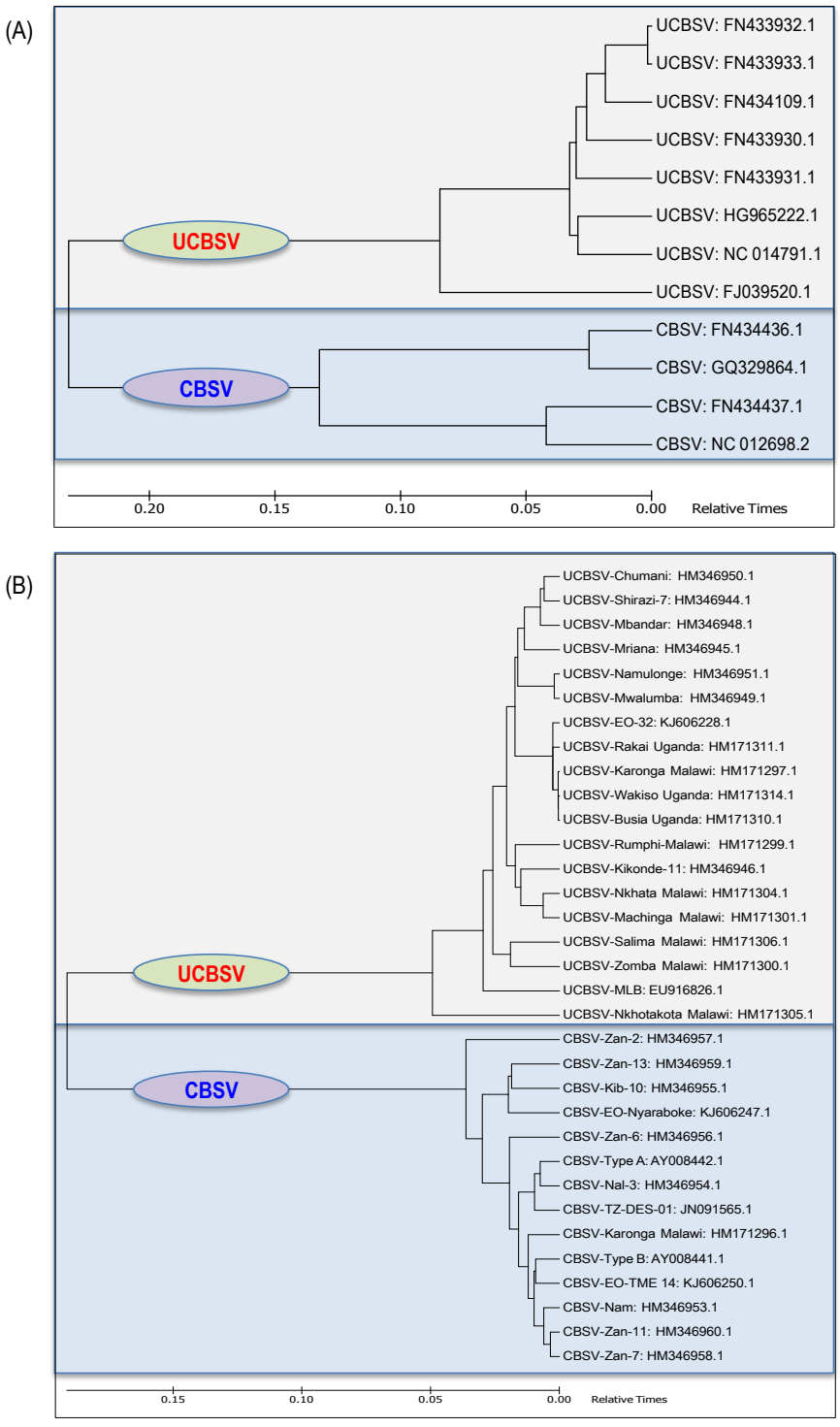


Fig. 1.3: Phylogenetic tree based on sequences of different isolates of CBSV and UCBSV. (A) Complete genome sequences showing that CBSD is caused by two distinct virus species. (B) Coat protein sequences showing diversity within isolates of CBSV and UCBSV. GenBank accession numbers are indicated against each virus isolate.

Previous studies reported that plants of *N. benthamiana* co-infected with CMBs (ACMV/EACMV) and UCBSV showed symptoms of both viruses and these were very severe, to the extent these plants eventually died (140). The authors hypothesized that UCBSV and CMBs might, therefore, interact synergistically in mixed virus infections. While this could hold true in *N. benthamiana*, observations in the field indicate that the viruses behave independently in cassava. Infection of cassava with CMBs does not seem to influence subsequent infection of the same plant with CBSD viruses and *vice versa*. Resistance to CMD does not break down in CMD resistant cassava varieties after infection with CBSD viruses, and CMD susceptible cassava varieties infected with CBSD viruses do not show CMD symptom enhancement. A recent regional CMD and CBSD epidemiology report by Legg *et al.,* (2011) (90) support these field observations. Subsequently, most synergistic virus interactions are host dependent and the patterns of infection depend not only on the host species but also on host cultivar.

Synergistic interactions involving a potyvirus (PVY, TEV, TVMV etc.) with a heterologous virus mainly result in increased concentration of the non-potyvirus and unchanged, or slightly decreased, concentration of the potyvirus (167). For example, mixed infection of *N. tabacum* with PVY and PVX resulted in disease symptoms enhancement and a 10-fold increase in PVX titer compared to single infection (90). Wintermantel and associates (2008) (200) also observed increased TICV titers and decreased ToCV titers in dual infected *N. benthamiana*, but the titers of both viruses decreased in doubly infected *Physalis wrightii* plants. Tatineni et al. (2010) (172) reported different synergy patterns for three wheat cultivars doubly infected with WSMV and TriMV. In addition, some viruses such as *Pepper huasteco virus* (PHV) and *Pepper golden mosaic virus* (PepGMV) display synergistic interactions in *N. tabacum* and *N. benthamiana* plants but antagonistic interactions in pepper. Some virus combinations can result in mutual benefit in mixed infections as reported by Mascia *et al.* (2010) (109) for CMV and PVY in doubly infected tomato. The transmission potential of a virus present in

mixed infections within the same plant depends on its concentration. Higher virus titers result in a higher likelihood of vector transmission. Conversely, environmental factors influence virus-virus interaction with consequential negative pathogenic changes in the host due to the compounded nature of mixed viral cytopathic effects (30). Together, the rate at which a virus mutates and the nature of interactions with other viruses and the environment determine viral pathogenesis and evolution.

1.7 Antiviral defense in plants

Natural disease resistance in plants includes presence of physical barriers (waxy secretion, cuticle or cell wall) to prevent pathogen entry, or constitutive and induced resistance regulated by different signaling pathways. Constitutive or induced resistance is triggered by activation of disease resistance, pathogenesis-related proteins (PRs) upon recognition of pathogen effector molecules, or induced by "challenge" inoculation with the pathogen (188). The resistance can be systemic, in which a mobile signal is generated and circulated to enhance defense mechanisms. This is termed systemic acquired resistance (SAR), or localized depending on stimuli. The most common viral recognition determinants for PR protein activation in plants include viral coat protein (CP), movement protein (MP) or the replicase (103). Upon activation, the PR proteins determine the type of resistance, whether hypersensitive resistance (HR), extreme resistance (ER) or a combination of both HR and ER. HR is associated with physiological and molecular changes in the host, which might include production of elevated levels of secondary metabolites or response elements such as ethylene, jasmonic acid, salicylic acid, or nitric oxide, and which in combination restrict virus entry into the plant system (100). Nonetheless, ER largely inhibits virus replication and accumulation. For example, in potato the PVX-CP is capable of activating both HR, controlled by *Nx* and *Nb* genes, and ER, controlled by *Rx1* and *Rx2* genes (100).

Plant-infecting viruses activate RNA interference (RNAi), an inherent antiviral defense mechanism which employs small interfering RNAs (siRNAs) to target and disarm invading nucleic acids in a sequence-dependent manner (36, 97). Plants and other eukaryotes produce endogenous siRNAs including microRNAs (miRNAs), *trans*-acting siRNAs, heterochromatin-associated siRNAs, and natural antisense transcript siRNAs in several different ways (42, 117). Endogenous siRNAs are involved in regulation of gene expression to control plant development, genome stability, stress-induced responses and defense against invading foreign molecules such as viruses and transposons (97, 117). Virus-derived siRNAs stimulate a highly effective antiviral silencing mechanisms employed by the plant which can result in complete loss of viral disease symptoms (recovery) by limiting accumulation of viral RNA in initially infected cells (37, 43), or by producing a systemic signal that is translocated by the same routes as the virus (117). In what is most likely an evolutionary response to this plant defense strategy, viruses have developed the ability to suppress siRNA-mediated resistance through suppressors. Virally encoded suppressor proteins such as potyviral HC-Pro or P1 of ipomoviruses (40) can greatly increase disease symptoms both of the primary virus and others that might simultaneously infect the host. The latter can result in synergistic interactions between the pathogens with high levels of virus accumulation for one or both viruses and severe disease impacts on the host plant (167).

During antiviral gene silencing, dsRNA molecules are produced in the cytoplasm as replication intermediates of viral RNA genome replication, or in the case of DNA viruses via bidirectional transcription in the nucleus (117). dsRNAs are also produced through formation of intramolecular hairpin structures in viral RNA genomes, by imperfect folding of self-complementary single stranded viral genomic RNA (37), or through the conversion of ssRNAs to dsRNAs by host encoded RNA-dependent RNA polymerase (RDR) (120, 161). Long virus-derived dsRNAs activate the silencing mechanisms in the plant by serving as substrates for Dicer-like (DCL) ribonucleases, which cleave the

dsRNAs to produce 21 to 24 nucleotide (nt) virus-derived small RNA duplexes (18, 119, 205). The guide strand of the virus-derived small RNAs (vsRNAs) are loaded onto Argonaute (AGO) proteins, a component of RNA-induced silencing complexes (RISC), to facilitate guidance to, and degradation of complementary viral mRNA, or repression of translation of mRNAs with imperfect sequence homology (37, 165). Virally encoded suppressor proteins inhibit one or more of these components of the gene silencing machinery, resulting in a dramatic increases in the accumulation of viral RNA of one or more co-infecting viruses (117).

The production of siRNAs requires DCLs, AGO proteins, and RDRs, which are diverse proteins encoded by multi-protein families (97, 161, 164). For instance, the *Arabidopsis* and rice genomes each possess four and eight DCLs, six and five RDRs, and ten and nineteen AGO proteins respectively. In virus-infected *Arabidopsis,* DCL4, DCL2 and DCL3 have been shown to catalyze the formation of 21, 22, and 24-nt vsRNAs, respectively to induce the antiviral response (17, 159). The DCL4-21 nt and DCL2-22 nt vsRNAs confer efficient antiviral defense in plants, but also exhibit functional redundancy (32, 41, 42, 159). Studies have shown that the 21- and 22-nt vsRNAs are the most predominant class amongst potyvirus-infected host plants (32, 36, 56), and that different host plants accumulate varying amounts of vsRNAs in response to infection by different viruses (42, 95). Similarly, multiple AGO proteins are involved in antiviral defense, including at least AGO2 and AGO5, which have been shown to bind *Cucumber mosaic virus* (CMV)-derived small RNAs (169). And, RDR1, RDR2 and RDR6 have been implicated in virus-derived small RNA biogenesis and antiviral defense in plants (36, 37, 41, 42, 56, 151).

Deep sequencing, or high-throughput sequencing techniques has been exploited to identify and sequence complete genomes of viruses, and to study the profiles of small RNAs in plants (81). Deep sequencing of vsRNAs in virus-infected plants has provided an insight into the biogenesis, composition,

and abundance of vsRNAs from many host-virus pathosystems (41, 42, 95, 116, 132, 150, 164, 192, 202). Studies in *Arabidopsis* infected with *Turnip mosaic virus* (TuMV), CMV, and *Tobacco rattle virus* (TRV) (42); *N. benthamiana* infected with *Potato virus X* (PVX), *Bamboo mosaic virus* (BMV), and *Pepper mild mottle virus* (PMMoV) (42, 95); potato infected with three different strains of *Potato virus Y* (PVY-O, PVY-N, and PVY-NTN) (132); and maize infected with *Sugarcane mosaic virus* (SCMV) (202), among others, have revealed the predominance of the 21 and 22 nt vsRNAs. This in turn, suggests activity of DCL4 and DCL2 in response to virus infection in these plant species. Particularly in *Arabidopsis*, studies revealed preferential sorting and loading of vsRNAs into multiple AGO complexes on the basis of their first 5'-end nucleotides. AGO1 has been shown to have preference for U, AGO2 and AGO4 have preference for A or U, while AGO5 prefers C at the first 5'-end of the siRNA (42, 115, 151, 169). The abundance, composition, spatial distribution and sequence diversity of the populations of vsRNAs generated by CBSV and UCBSV in infected cassava, and whether these viruses trigger RNA-silencing machinery in the cassava host-virus pathosystem remain unknown. Such information is important if researchers are to have a better understanding of the response of cassava to infection by CBSD pathogens, gain improved insight into the molecular mechanisms underlying the pathogenicity of CBSV and UCBSV, and explain the response of different cassava genotypes to CBSD.

1.8 Management of cassava viral diseases in Uganda

1.8.1 Conventional breeding approaches

Several measures have been put in place as attempts to manage CMD and CBSD in Uganda. Strategies employed include surveillance to track the presence and spread of the diseases, phytosanitation measures to ensure health of the crop plants and planting materials, quarantine systems to restrict movement of pests and pathogens along with cassava germplasm, and host plant

resistance breeding (91). Conventional host plant resistance has been the main mechanism deployed to manage the CMD pandemic. Between 1990 and 2000 at least 12 CMD resistant cassava cultivars were developed and deployed in the country (142). However, to date host plant resistance has been less successful for control of CBSD. This is problematic as the CMD resistant cultivars deployed in Uganda and elsewhere is East Africa are failing and being rejected by farmers due to poor harvests and root qualities resulting from their susceptibility to CBSD (78). The best cassava cultivars have shown at best, tolerance and not resistance to CBSD under field conditions (90, 125). Phytosanitary measures including rogueing, selection of disease-free propagation materials, planting at different times of the year and in isolation from neighbouring fields can significantly reduce the degree of CBSD spread (70). However, these measures require trained personnel and resources. This is especially true for selection of CBSD-free stem cuttings due to the difficulty in symptom recognition (90). Other approaches include the use of seed certification schemes, and eradication of disease-affected materials. This was successfully enforced by the colonial authorities in Uganda to eliminate CBSD in mid 1930s, and to replace CMD-affected local cassava varieties in northeastern Uganda by improved CMD-resistant materials in 1950s (91).

Traditional cassava breeding has been an on-going effort to address key constraints to cassava production including resistance to CMD and CBSD. The urgency of the current CBSD epidemic has made the latter a matter of high priority and urgency. Breeding for CBSD resistance started in Amani, Tanzania, where resistance genes from wild relatives of cassava were introgressed and then backcrossed into cultivated cassava (69, 133). These efforts generated cassava varieties which are still present in farmers' fields in Kenya (*Kaleso, Kibiriti Mweusi* and *Ambari*), Mozambique (*Nikwaha*, *Nachinyaya*, and *Mulaleia*), and Tanzania (*Namikonga*, *Mfaransa*, *Kalulu*, and *Kibangameno*) (64). These varieties are being used by cassava breeders in East Africa to produce the next generation of

CBSD-resistant varieties. Within a decade (2004–2014), *c*. 10,000 cassava seeds obtained from CMD-resistant materials from the IITA– East and Southern Africa Regional Center (IITA-ESARC) were screened and evaluated for CBSD resistance in Uganda (77, 135). Recently eight clones showing varied levels of resistance or tolerance to CBSD were selected and deployed to farmers (75).

Cassava possesses a biology that contributes to inefficiencies for effective breeding. It has long growth cycle and some varieties with desirable attributes do not flower or flower sparsely. Those that do flower show considerable variation in flowering time, rate and fertility, making it difficult to efficiently combine traits from different genotypes (25). Cornell University recently received $25 million in funding for the Next Generation Cassava Breeding project (27). The project aims to achieve a number of goals including improvement of flowering and seed set, development of molecular markers to ease genomic selection in breeding programs, to develop cassava databases that provide breeders and researchers with a centralized, user-friendly access to data, and the capacity building needed to elevate cassava breeding to the next level. The project is being implemented by partner institutions in Africa, Europe and USA. In addition, breeding efforts to identify molecular markers associated with CBSD resistance or tolerance are ongoing in 99 cassava genotypes for which the field reaction to CBSD is known. These are being assayed using 30 simple sequence repeat (SSR) markers (1). Similar efforts are underway using some of the Amani varieties (191). Sequencing of the transcriptomes of CBSV- and UCBSV-infected resistant and susceptible varieties to identify candidate genes involved in CBSD resistance, and validation of such genes by functional genomics approaches will facilitate improved, targeted breeding for resistance to CBSD (91).

1.8.2 Transgenic resistance strategies to CBSD

Due to lack of effective, inherent resistance to CBSD within the cassava germplasm, application of

transgenic technologies to manage this disease presents an attractive additional approach to conventional breeding. Plant genetic engineering offers a means of integrating specific virus resistance traits into plants. Resistance to RNA viruses in transgenic plants has been achieved in many species through expression of full length, translatable viral protein genes or by RNA-mediated mechanisms (28, 53). For example, expression of viral coat protein (CP) resulted in the generation of TMV resistant tobacco (4), PVY resistant potato (74), PPV resistant plum trees (160), CMV resistant Pepper (87), and *Bean common mosaic virus* (BCMV) resistant peanut (62). Similarly, expression of the replicase protein gene resulted in *Potato leaf roll virus* (PLRV) potato plants resistant to disease caused by this virus (85). This technology has resulted in successful commercialization of transgenic crop plants with resistance to RNA viruses such as squash and papaya (28). Viruses replicating within the host cell produce dsRNAs from replication intermediates. These are recognized and cleaved by Dicer proteins into siRNAs duplexes and elicit a cascade of events called posttranscriptional gene silencing (PTGS). The siRNAs are utilized as guides in the silencing pathway to target and degrade mRNAs carrying perfect sequence homology, or repress translation of mRNAs with imperfect sequence homology (14, 37, 197).

Discovered in the 1990s the RNAi mechanisms have since been exploited by researchers and exploited through several biotechnological applications. In order to achieve more reproducible siRNA-mediated gene-silencing, target sequences have been cloned to produce inverted repeat constructs. The inverted repeat construct is made by amplifying a DNA fragment from a target gene by PCR with primers containing appropriate restriction sites. The PCR product is cloned on each side of an intron in an intron-containing vector in sense and antisense orientation, resulting in an RNAi construct expressing a hairpin RNA (hpRNA) of the target gene (196). These constructs can be designed to target multiple viruses by fusing together different sequences of the target viruses. In this way, RNAi

technology has been used to achieve resistance to many virus species, mostly potyviruses, in economically important crops. For example *Papaya ring spot virus* (PRSV) resistant papaya (59), *Beet necrotic yellow vein virus* (BNYVV) resistant sugar beet (93), *Soybean dwarf virus* (SDV) resistant soybean (182), BYDV resistant barley and wheat (194, 204), *Zucchini yellow mosaic virus* (ZYMV) resistant melon (201), and *Citrus tristeza virus* (CTV) resistant grapefruit (*Citrus paradisi*) (48) etc.

Previous studies demonstrated that RNAi technology could confer resistance to CBSD in *N. benthamiana* (147) and cassava (203) under controlled growth conditions. In each of the cases, two RNAi constructs, p718 (ΔFull Length-CP) and p719 (N-terminal CP), were derived from the coat protein (CP) sequence of UCBSV (CBSUV-[Uganda:TO4-42:2004]; GenBank accession number: HM171316.1) and integrated into the plant genome of *N. benthamiana* and the susceptible cassava cultivar 60444. Transgenic lines that accumulated high levels of virus-derived siRNAs were found to be highly resistant to both CBSV and UCBSV upon inoculation with infectious sap in *N. benthamiana*, and via UCBSV-infected stem grafts on cassava. The levels of resistance correlated with the strength of the siRNA signal within individual plant lines, with some lines showing cross-protection against challenge with CBSV (147). Thus, development of resistant genotypes through introgression or engineering CBSD resistance to existing CMD resistant cultivars suitable to farmers needs is promising. However, the CBSD transgenic resistance data presented above is lab-based and need to be confirmed by field-testing selected transgenic cassava lines in a high CBSD pressure area. This fitted well within the goal of the Virus Resistant Cassava for Africa (VIRCA) project, within which multiple field trials have been undertaken in Uganda and Kenya (174).

Vanderschuren *et al.,* (2012) (189) have also demonstrated resistance to CBSD by expression of inverted repeat constructs under controlled growth conditions using a stringent top-grafting method of

virus inoculation that a sequence of the CBSV coat protein (CP) highly conserved between CBSV and UCBSV could confer very high resistance, with possible immunity against both viral species in the CBSD susceptible cassava cultivar 60444. Most transgenic lines were highly resistant to increasing viral loads, with no viral replication observed in the resistant transgenic lines, which remained free of CBSD symptoms at 7 months post-inoculation. When the same hairpin construct was transferred to a CMD-resistant farmer-preferred Nigerian landrace TME 7 (Oko-Iyawo), all transgenic TME 7 lines were immune to both CBSV and UCBSV infections, and remained so even when plants were co-inoculated with EACMV, a geminivirus causing CMD (189).

1.9 Rationale for the study

In Uganda, cassava production has fluctuated over recent years. Before the year 2000, cassava yields were low averaging 7 – 9 tons/Ha due to the impact of the severe CMD epidemic and use of inferior varieties (142). Following deployment of improved CMD-resistant varieties, cassava yields started to increase such that in 2005 Uganda produced *c.* 5.6 million MT on 350,000 hectares (Ha) of land, the highest cassava yield recorded in the history of cassava production in the country (47). Since that time the area under cassava production has increased by *c.* 44,000 Ha, but the re-emergence of CBSD in 2004 has caused a decline in cassava yields from *c.* 16 tons/Ha to *c.* 13 tons/Ha over the same period (47). Therefore, despite expansion in acreage committed to the crop, cassava production has declined by *c.* 20% within the last 10 years. This is mainly due to the impact of CBSD, although CMD and pests continue to contribute to yield losses. Importantly, the CMD resistant cassava genotypes developed and deployed to combat this threat have almost all proved to be susceptible to CBSD. Those improved and elite cassava varieties that have shown some tolerance have not necessarily proved to be popular with farmers (78, 90). Cassava is a staple and food security crop that is grown by over 80% of the farming families in Uganda (21), it is imperative therefore that control measures are sought to address

the significant impact of CBSD, and return cassava to its central role in food and economic security for small holder farmers and their communities.

CBSD viruses cause systemic infections in the primary host and reduce quality of planting materials for use in the subsequent cropping cycle through persistent infection. Development of CBSD resistant varieties is the most logical and economically feasible way to control CBSD. However, for this to be effective screening of available germplasm for sources of resistance, and an improved understanding of host-virus interaction in a number of cassava genotypes is of paramount importance to facilitate targeted breeding.

CBSD distribution, transmission and spread have been studied in affected cassava genotypes in the field situation in different agro-ecologies (90). However, within-host distribution and accumulation of the viral pathogens in naturally infected cassava is unknown. CBSV and UCBSV show indistinguishable symptoms on infected plants (118), making it difficult to determine whether the plants are infected with one or both viruses. In addition, availability of the virus to feeding vectors is determined by its concentration within different parts of the host. Information regarding CBSV and UCBSV loads within the cassava plant structure remains scant and inadequate to provide an insight into the virulence or pathogenicity and epidemiology of the viruses in nature. This makes lack of information on viral location and titer within the plant a critical gap in our knowledge and important area requiring research input.

Biotechnology strategies including cross protection and RNAi technology have been developed and used to confer durable resistance to many viruses belonging to family Potyviridae in multiple crop plant species, and the mechanisms of resistance have been characterized (28). Subsequently, similar attempts to develop transgenic resistance to CBSD are underway and preliminary lab-based tests are promising. Transgenic plants generated using RNAi technology have shown high levels of resistance

to virus challenge (147, 189, 203). However, molecular mechanisms linking host-virus interaction to phenotype in cassava is unclear. Understanding the biogenesis, nature, and extent of accumulation of sRNAs in CBSV- and UCBSV-infected cassava compared to uninfected cassava plants could relate genotype to phenotype, and help explain the occurrence of CBSD susceptible and tolerant cassava genotypes.

The present studies aim to establish within-host CBSV and UCBSV distribution and accumulation, characterize vsRNAs generated during CBSV and UCBSV infection, correlate CBSD symptoms with virus titre in organs of infected plants, and field-test to confirm lab-based CBSD transgenic resistance of selected transgenic cassava lines in a high CBSD pressure area. Information generated will contribute to leveraging sustainable management of CBSD at the field level.

1.9.1 Goal

The overall goal of this study is to develop strategies for sustainable control measures against CBSD and thereby enhanced cassava production in Uganda using knowledge gained from the research results.

1.9.2 Specific objectives

The specific objectives of the study are: -

1. To determine the distribution and accumulation of CBSD causing viruses in infected cassava plants
2. To characterize CBSV- and UCBSV-derived small RNA populations in infected cassava plants
3. To evaluate RNAi-derived transgenic cassava lines for resistance to CBSV and UCBSV under field conditions

1.9.3 Hypothesis

1. CBSD causing viruses exhibit similar distribution patterns in infected cassava plants
2. CBSD causing viruses exhibit virus-virus and virus-host interactions in mixed infections
3. RNAi-derived transgenes confer CBSD resistance in transgenic cassava under field conditions

1.9.4 Direct and longer-term impact of this study

1. Improved knowledge base regarding plant virus-host interactions
2. More effective control and disease management strategies for CBSD in place
3. Improved breeding strategies for resistance to CBSD

Chapter 2

Distribution and accumulation of cassava brown streak viruses within infected cassava (*Manihot esculenta* Crantz) plants

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2.1 Abstract

Cassava brown streak disease (CBSD), caused by *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV), ranks among the top seven biological threats to global food security. The disease poses a significant threat to cassava production in East and Central Africa (ECA). In Uganda, overall CBSD incidence increased by *c.* 20% since it re-emerged in 2004, and the disease persistently reduces cassava yields and storage root qualities. The spread of CBSD has been studied spatially in fields in different agroecologies. However, within-host distribution and accumulation of CBSV and UCBSV in naturally infected cassava plants is unknown. Therefore, within-host CBSV and UCBSV distribution was studied to correlate CBSD symptoms with virus titre in organs of infected cassava. Leaf, stem and storage root samples, with and without symptoms, were collected from 10 genotypes of field-grown cassava. Presence of CBSV and UCBSV was detected by RT-PCR and virus levels determined by RT-qPCR. CBSV was present in 100% of CBSD samples with symptoms, with 45.3% positive for presence of both CBSV and UCBSV. Tolerant cassava genotypes were infected with CBSV alone and accumulated higher titre in roots than in aerial organs. Susceptible genotypes were co-infected with CBSV and UCBSV and exhibited variation in virus titre in each organ. Across genotypes, virus titre was lowest in the youngest leaves and highest in mature non-senescing leaves. This information provides insight into the relationship between CBSV, UCBSV, and their cassava host, and is valuable for CBSD resistance breeding, epidemiology studies and CBSD control.

2.2 Introduction

Cassava brown streak disease (CBSD), caused by *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV), previously occurred only in the low altitude areas of coastal Eastern Africa, and was present at relatively low incidence and severity (90). Recent regional cassava disease and pest surveys have confirmed that CBSD is spreading rapidly and now poses a significant threat to cassava production in East and Central Africa (15, 90, 128). In Uganda, a 2013 nationwide survey conducted across 1,100 cassava fields recorded CBSD in 50 out of 55 districts assessed, with mean district incidence of up to 86% and an overall average of 24% (T. Alicai, unpublished data). This reflects an increase in overall CBSD incidence per district of *c.* 20% compared to the time when the current epidemic of the disease was first observed in Uganda in 2004 (10). As a result, the disease is impacting the food security value of cassava, reducing overall cassava yields by up to 70% (65) and quality of edible storage roots by up to 100%.

CBSD distribution and spread has been studied primarily at the field level in different agroecologies (90). Cassava affected by CBSD in the field shows diverse symptoms. Yellowing on either side of the small veins is commonly observed on older leaves, but is less obvious on younger leaves and absent on the topmost leaves (64). Brown lesions can be observed on stems, and brownish necrotic regions seen within the edible storage roots. Some cassava genotypes display very severe symptoms including complete necrosis of storage roots and stem dieback, while others show mild symptoms. In some cases, symptoms are restricted to a few branches, with the rest of the infected plant remaining symptomless.

Information describing within-host CBSV and UCBSV distribution is lab-based (124) and insufficient to explain the response of some cassava genotypes to CBSD in the field. Accumulation and location of

CBSV and UCBSV within the host, and thus their availability for transmission by feeding whitefly (*Bemisia tabaci*) vectors (107), is unknown as is the extent of mixed infections compared to single infections across different cassava genotypes. Information is also lacking to relate disease phenotype to CBSV and UCBSV accumulation and distribution patterns in host tissues/organs of naturally infected cassava genotypes. The present study was designed to establish the pattern of within-host distribution of CBSV and UCBSV in cassava genotypes considered as tolerant and susceptible to CBSD, and to assess the correlation between disease symptom expression with virus titer in different tissues and organs of CBSD affected plants. The information generated in this study provides further insight into the relationship between CBSV, UCBSV and the cassava host, and is considered important for CBSD resistance breeding, epidemiology studies and control of the disease.

2.3 Materials and methods

2.3.1 Collection of field plant samples

Plant samples were collected in October 2012 from 10 cassava genotypes growing within experimental plots at the National Crops Resources Research Institute (NaCRRI), Namulonge, Uganda, and from farmers' fields inside a 2 km radius of the research station. Cassava cultivars sampled included two Ugandan landraces, Nyaraboke and Ebwanatereka, in addition to the varieties NASE 3 (TMS 30572), NASE 14, NASE 16, TME 14, TME 204, MH97/2961, I92/00067 and 60444. Response of the cassava genotypes to CBSD in the field was categorized as CBSD susceptible (Ebwanateraka, Nyaraboke, TME 14, TME 204, MH97/2961 and 60444) and CBSD tolerant (NASE 3, NASE 14 and NASE 16) based on breeders' descriptors and/or phenotypes (1).

A total of 800 plant samples were collected from 6-month-old cassava plants. Leaf samples were collected from leaf positions 3, 6, 9, 15, 20 and 25, numbered from the first fully open top-most leaf position downwards on the major stem (Fig. 2.1A). Presence (+) or absence (-) of CBSD symptoms was noted for each leaf sampled. In addition, a stem section at mid-way of the shoot, and a sample of storage root were collected from each plant (Fig. 2.1A). Leaf and stem samples were collected using gloved hands, wrapped with aluminum foil and immediately placed in a container with liquid nitrogen. Storage root samples were obtained from whole roots that were cleaned and sliced transversely into 1–2 cm thick discs from which a representative piece was selected and preserved in liquid nitrogen. The samples were stored frozen until RNA extraction.

2.3.2 Detection and sequencing of CBSV and UCBSV from field samples

Cassava storage root discs, stem sections, and leaf samples were ground with the aid of liquid nitrogen in sterile mortar. Total RNA was extracted from the resulting powder following the CTAB protocol (98) with some modifications, as previously described (139). Extracted RNA was treated with DNase I according to manufacturer recommendations (Life Technologies, Grand Island, NY) to remove cassava genomic DNA. RNA purity, quantity and integrity of each sample were assessed using a NanoDrop (Model 2000C, Thermo Scientific, Waltham, MA), and visually by running in denaturing agarose gels. Quantitative reverse transcription PCR (RT-qPCR) was performed using RNA samples with $OD_{260/280}$ of 1.8 to 2.1. Two micrograms of total RNA was converted into cDNA using Superscript III™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA) following manufacturer recommendations.

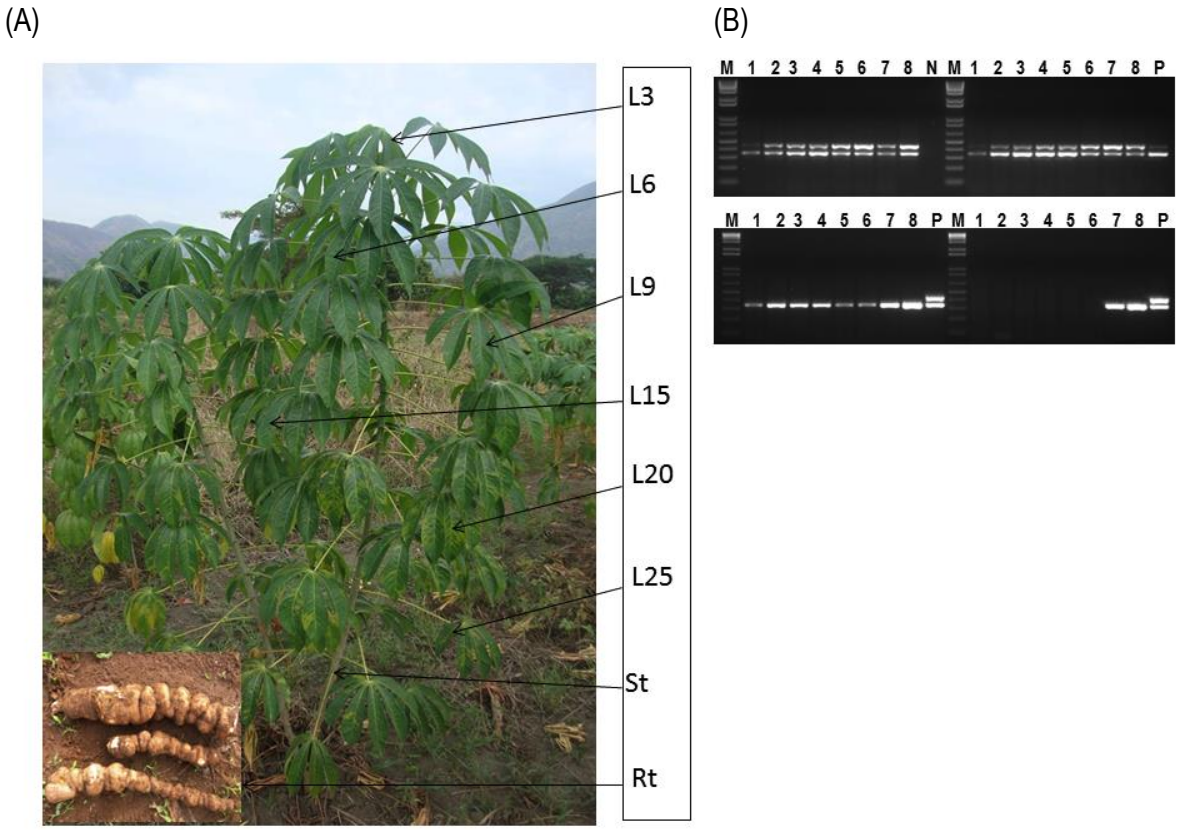


Fig. 2.1: Sampling as performed on plants with cassava brown streak disease (CBSD) symptoms and RT-PCR diagnostics for simultaneous detection of *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV). (A) Samples were collected from different leaf positions and organs of field-infected CBSD cassava plants with symptoms. Leaf numbering as assigned from the first fully expanded leaf downwards. L3 = leaf position 3, L6 = leaf position 6, L9 = leaf position 9, L15 = leaf position 15, L20 = leaf position 20, L25 = leaf position 25, St = stem, Rt = storage root. (B) RT-PCR gel electrophoresis showing presence of CBSV and UCBSV in a subset of field samples; 1–8 represent samples from different leaf positions, stem and root as described in (A), where 1 = L3 and 8 = Rt. Upper panel left and right: CBSV and UCBSV co-infection; lower panel left: presence of CBSV alone; lower panel right: presence of CBSV only in samples obtained from stem and storage root samples with symptoms and not in the samples from symptomless leaves. M = 1 kb+ DNA ladder; N = negative control (sample from known uninfected plant); P = positive control (sample from known CBSV- and UCBSV-infected plant confirmed by RT-PCR).

Presence of UCBSV and CBSV in each sample was detected by subjecting 1 μL of cDNA to conventional RT-PCR assay as described previously (139), using the primers CBSVDF2 and CBSVDR (113), which amplify *c.* 437 and 343 nucleotides of the 3'-terminal sequences of the UCBSV and CBSV genomes, respectively. DNA fragments for sequencing were amplified with Phusion High-Fidelity DNA

polymerase (New England Biolabs Inc. Ipswich, MA) using primer combination CBSVF2 (118) and CBSVDR (113), which amplify *c.* 1670 bp and 1607 bp fragments covering the entire UCBSV and CBSV coat protein (CP) and part of HAM1-like protein sequences, respectively. Reaction mixtures contained 10 μL of Phusion High-Fidelity PCR master mix, 1 μL of each primer (0.5 μM final concentration), and 1 μL of cDNA template in a total reaction volume of 20 μL. The thermal cycles used were: initial denaturation step for 1 min at 98°C, followed by 30 cycles of 10 s at 98°C, 10 s at 59°C and 40 s at 72°C, and a final extension for 5 min at 72°C. The PCR products were resolved by electrophoresis in a 1% (w/v) agarose gel containing 0.5 μg/mL ethidium bromide and fragments were purified using the QIAquick Gel Extraction kit (QIAGEN), ligated into Zero Blunt TOPO vector, and transformed into Top10 *Escherichia coli* cells (Invitrogen). Positive clones were confirmed by restriction analysis, and by sequencing using virus species-specific and M13 forward and reverse primers by GENEWIZ DNA sequencing services (GENEWIZ Inc. South Plainfield, NJ). Sequences were analyzed using BLASTN (206) and BLASTP (11) search algorithms available at the National Center for Biotechnology Information (NCBI). In order to determine phylogenetic relationships multiple nucleotide sequence alignment was made using CLUSTAL OMEGA (163) and phylogenetic tree of the aligned sequences was constructed using maximum likelihood, with 500 bootstrap repetition, using the MEGA5 software package (171).

2.3.3 Primer design and determination of virus titer with RT-qPCR

For RT-qPCR primer design, 12 complete CBSV and UCBSV sequences available in GenBank (accession numbers: FN433932.1, FN433931.1, FN434109.1, FJ039520.1, NC014791.1, HM181930.1, FJ185044.1 for UCBSV, and GQ329864.1, NC012698.2, FN434437.1, FN434436.1, GU563327.1 for CBSV) were aligned using MEGA5 (171). Conserved sequence regions unique to each virus species were selected and used as input data for the program PRIMER-BLAST of NCBI that uses the

PRIMER3 algorithm (158). Prospective primer sets targeting unique sequences of 75 – 200 bp were tested for amplification. In addition to primer sequences for amplification of UCBSV and CBSV, primer sets for amplification of the reference genes Cytochrome c oxidase (COX) (6), Ubiquitin 10 (UBQ10), serine-threonine phosphatase (PP2A), and GTP-binding (GTPb) protein (124) used for data normalization, were tested. The annealing temperatures, PCR efficiencies, and melt curve analysis of amplicons of the primer combinations UCBSVqF and UCBSVqR, CBSVqF and CBSVqR, COXF and COXR, and PP2AF and PP2AR (Table 2.1) were further optimized and used in subsequent analysis.

The RT-qPCR protocol used in this study was validated following published guidelines (20, 176). Primer pairs targeting unique sequences, 75 – 200 bp in size, were tested for amplification of CBSV and UCBSV with cDNA derived from five isolates of CBSD causal viruses maintained in *N. benthamiana* (147). For virus titer determination, cDNA was diluted ten-fold and subjected to RT-qPCR following SYBR Green I chemistry. Real-time PCR was performed using Bio-Rad CFX96 Connect RT-qPCR instrument, equipped with the CFX MANAGER software for data analysis (Bio-Rad Laboratories Inc, Hercules, CA). Reaction mixtures contained 5 μL SsoFast Advanced SYBR Green I SuperMix, 1 μL of each primer (0.5 μM final concentration), and 3 μL of diluted cDNA template in a total reaction volume of 10 μL. The RT-qPCR thermal cycles used were as follows: initial denaturation step for 3 min at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at 56°C. Data were collected at the end of each 56°C stage. The following controls were included in each plate: cDNA synthesized from RNA of tissue-cultured plants known to be free of both CBSV and UCBSV (NEG); cDNA synthesized from RNA of plants known to be infected with either or both CBSV and UCBSV (POS); RNA that went through cDNA synthesis process with no reverse transcriptase enzyme added (NRT); and water was used for the no template control (NTC). Three biological replicates for each sample and two or three technical replicates of each biological replicate were analyzed by RT-qPCR.

2.3.4 Graft challenge of NASE 3, TME 204 and 60444

Verification of infection of different cassava genotypes with CBSV and UCBSV and assessment of within-host distribution and accumulation in different plant parts was performed in a controlled environment. Tissue culture-derived plants of NASE 3, TME 204 and 60444 (*n* = 12) were used and were first determined to be free of CBSD viral pathogens by RT-PCR using primers and conditions described above. The plants were established in soilless compost (173) and bud-graft challenged at 10 weeks after planting with the CBSV Naliendele isolate (CBSV-[TZ:Nal3-1:07) and UCBSV Kabanyoro isolate (UCBSV-[UG:T04-42:04]) as described elsewhere (193). Formation of successful graft union was assessed visually 1 week after grafting (WAG) and development of CBSD symptoms monitored weekly thereafter as previously described (203). Leaf and root samples were collected 6 WAG and tested for presence or absence of CBSV and UCBSV by RT-PCR as described previously (139).

2.3.5 Data analysis

To determine relative normalized quantity for each sample, the default settings of the Bio-Rad CFX MANAGER software were used. The PCR efficiency of each primer was determined using standard curve method and input into the software. The relative virus titers were calculated using the $2^{-\Delta\Delta C_t}$ method for relative normalized expression analysis using *COX* and *PP2A* as reference genes and positive sample of predetermined virus concentration as calibrator, respectively. The data generated were exported to EXCEL (Microsoft) and AGRICULTURAL RESEARCH MANAGER (ARM; Gylling Data Management, Inc) software for analysis of variance (ANOVA). Where necessary, mean separation was done using Tukey's test.

2.4 Results

2.4.1 Detection of CBSV and UCBSV within field grown cassava

The number of plants sampled and analyzed for each genotype along with CBSD foliar and root symptom severity scores is summarized in Table 2.2. The cDNA prepared from leaf, stem and root samples was subjected to conventional RT-PCR to diagnose presence of CBSV and UCBSV. All samples obtained from plants with CBSD symptoms were infected with CBSV, 45.3% of which were positive for concurrent presence of both CBSV and UCBSV (Table 2.2). The improved cassava genotypes NASE 3, NASE 14, NASE 16, MH97/2961 and the landrace Nyaraboke were infected with CBSV only, whilst the highly CBSD susceptible genotypes TME 14, TME 204, Ebwanatereka, I92/0067 and 60444 tested positive for mixed presence of both CBSV and UCBSV (Fig. 2.1B). Across 95 plants and 760 samples tested, none were found to be positive for presence of UCBSV alone (Table 2.2).

2.4.2 Optimization of RT-qPCR

2.4.2.1 Primer optimization

The primer combinations CBSVqF and CBSVqR for CBSV and UCBSVqF and UCBSVqR for UCBSV provided the lowest quantification cycle (C_q) and no non-specific amplification; they were species-specific but sufficiently degenerate to detect diverse isolates within the two virus species (Fig. 2.2A). As the primer sets had a similar optimum annealing temperature (56ºC), both pairs could be used concurrently in the same PCR machine. Neither CBSV- nor UCBSV-specific primers provided amplification with cDNA derived from samples of healthy tissue-cultured plantlets used as negative controls, nor from the no-reverse transcription and no-template controls. Results of

agarose gel electrophoresis of CBSV and UCBSV amplicons were consistent with their expected sizes of 88 and 112 bp, respectively (Fig. 2.2B). The melting curves for CBSV, UCBSV, *COX* and *PP2A* amplicons performed at the end of the PCR cycles showed a single peak at the expected melting temperature (81.5ºC for CBSV, 82.0ºC for UCBSV, 80.5ºC for *COX* and 85.5ºC for *PP2A* amplicons, respectively; Fig. 2.2C).

Table 2.1: Reference genes, CBSD virus species and primer sequences used for RT-PCR and RT-qPCR

Gene description	Primer code	Primer sequence (5' to 3')	Size (bp)	Reference
Cytochrome c oxidase (COX)	COX F COX R	CGTCGCATTCCAGATTATCCA CAACTACGGATATATAAGRRCCRRAACTG	79	Adams *et al.* 2013
Serine-threonine phosphatase (PP2A)	PP2AF PP2AR	TGCAAGGCTCACACTTTCATC CTGAGCGTAAAGCAGGGAAG	187	Moreno *et al.* 2011
Uganda cassava brown streak virus (UCBSV)	UCBSVqF UCBSVqR	AAGGCAAGGGTGGCTCTAAC GCGTCCTTGTTGGCCCATTA	112	This study
Cassava brown streak virus (CBSV)	CBSVqF CBSVqR	GCCAACTARAACTCGAAGTCCATT TTCAGTTGTTTAAGCAGTTCGTTCA	88	Adams *et al.* 2013
3'-UTR of CBSV and UCBSV	CBSVDF2 CBSVDR	GCTMGAAATGCYGGRTAYACAA GGATATGGAGAAAGRKCTCC	437 (UCBSV) 343 (CBSV)	Mbanzibwa *et al.* 2010
CBSV and UCBSV	CBSVF2 CBSVDR	GGRCCATACATYAARTGGTT GGATATGGAGAAAGRKCTCC	1670(UCBSV) 1607 (CBSV)	Mohammed *et al.* 2012

2.4.2.2 Evaluation of RT-qPCR efficiency and choice of reference genes

Efficiency of the RT-qPCR assays was evaluated using the standard curve method as described elsewhere (176). cDNA synthesized from total RNA of CBSV and UCBSV positive controls was used to prepare sequential tenfold dilutions over eight points (10^1 to 10^8) and subjected to RT-qPCR in triplicate for each of CBSV, UCBSV, *COX* and *PP2A* primer pairs. The standard curves were made by plotting C_q values against the logarithm of the starting quantity of the template using the Bio-Rad CFX MANAGER software (Fig. 2.2D). Standard curves covered a linear range of 10^5, 10^6, 10^6, and 10^4 orders of magnitude for CBSV, UCBSV, *COX* and *PP2A,* respectively. The efficiency (E), coefficient of

determination (r^2) and slope of the curves used to evaluate RT-qPCR assay optimization for each of the primer sets were within limits desirable for RT-qPCR (Fig. 2.2D; (20, 176). The C_q values for the triplicate assays were consistent for all the optimized primers in simultaneous independent assays in the same run, or in separate runs with the same conditions. Consequently, the RT-qPCR assays were reproducible and facilitated comparison of virus titers in different samples determined among independent assays.

The four reference genes *COX*, *UBQ10*, *GTPb*, and *PP2A* were tested for use as controls to allow normalization of data by correcting differences in quantities of template cDNA (175, 190). *COX* and *PP2A* genes showed consistent expression in both CBSD-affected and healthy tissues across biological replicates for each genotype analyzed by RT-qPCR (data not shown). *COX* and *PP2A* were therefore utilized in this study as reference genes for data normalization.

Table 2.2: Foliar and root CBSD severity scores, and presence of CBSV and UCBSV in plant tissues as determined by RT-PCR

Genotype	No. plants sampled	No. plants analyzed (total no. samples)[a]	Mean severity[b]		Virus detected by RT-PCR		
			Foliar	Root	CBSV only	UCBSV only	CBSV + UCBSV
NASE 3	10	10 (80)	3.0	3.7	9	0	0
NASE 14	10	10 (80)	3.6	4.2	10	0	0
NASE 16	10	9 (72)	3.2	4.2	9	0	0
MH97/2961	10	10 (80)	3.9	4.7	10	0	0
Nyaraboke	10	10 (80)	3.4	5.0	10	0	0
cv.60444	10	10 (80)	2.9	2.8	0	0	10
TME 204	10	10 (80)	2.7	4.4	0	0	10
TME14	10	10 (80)	3.0	3.5	0	0	10
Ebwanateraka	10	10 (80)	2.1	3.3	0	0	10
I92/0067	10	6 (48)	4.0	3.1	3	0	3
Total	100	95 (760)			51	0	43

[a]Eight samples were taken per plant: six leaf samples from different positions along the main stem, one sample mid-way up the stem and one 1–2 cm storage root sample. Leaf samples were collected from leaf position 3, 6, 9, 15, 20, and 25 numbered from the first fully open top-most leaf position downwards on the major stem. [b]Severity of foliar and root damage were scored from 1 to 5 as previously described(139).

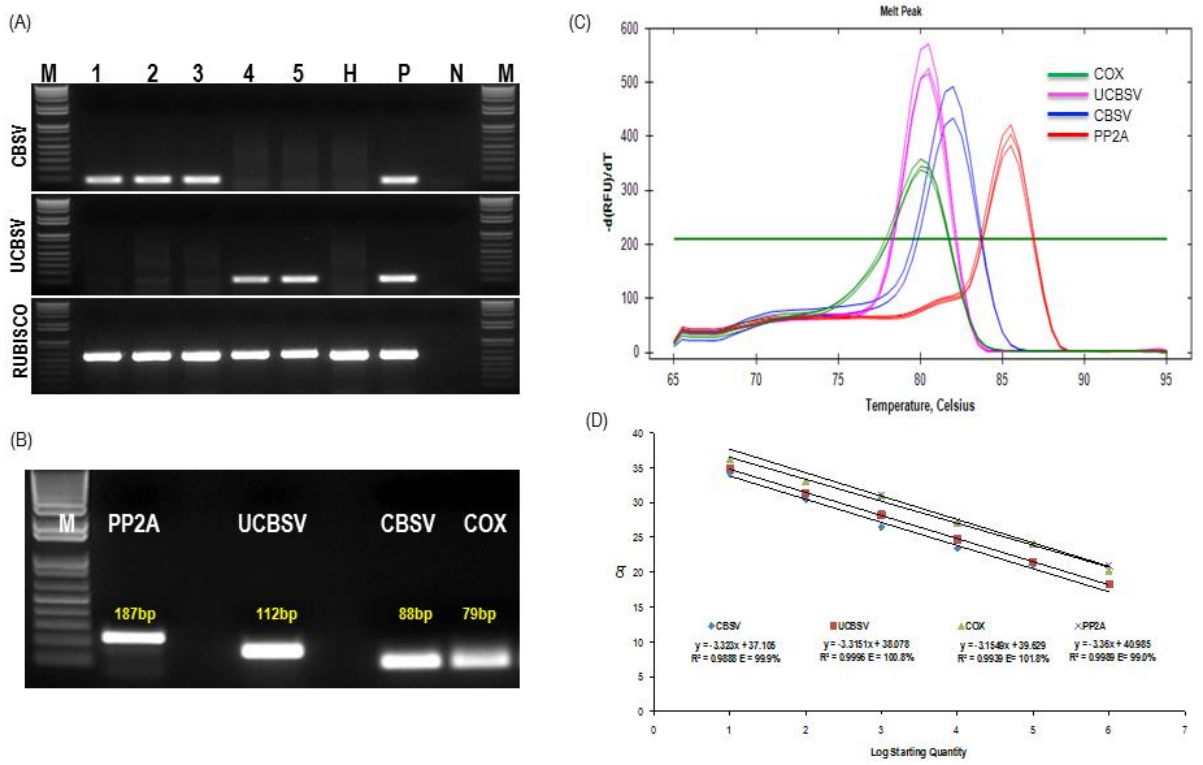


Fig. 2.2: Validation of quantitative reverse transcription-polymerase chain reaction (RT-qPCR) conditions. (A**)** Gel analysis of RT-PCR product showing primer specificity using different CBSV and UCBSV virus isolates. Lane 1 = CBSV (Nampula), lane 2 = CBSV (Naliendele), lane 3 = CBSV (Zanzibar), lane 4 = UCBSV (Kenya), lane 5 = UCBSV (Kabanyoro), lane H = healthy control, lane P = positive control (CBSV + UCBSV), lane N = no template control (NTC). (B) Gel electrophoresis analysis of RT-qPCR products showing specific amplification of serine-threonine phosphatase (PP2A, 187 bp), *Ugandan cassava brown streak virus* (UCBSV, 112 bp), *Cassava brown streak virus* (CBSV, 88 bp), and cytochrome c oxidase (COX, 79 bp) at same annealing temperature (56^{o}C), bp = base pairs. (C) Melt curve analysis of the amplicons shows a single peak indicating a single PCR product for each gene/virus species; RFU = relative fluorescent unit. (D**)** Standard curve showing efficiency (E) and coefficient of determination (r^2) for each gene/virus species.

2.4.3 Determination of viral RNA titer in field-collected samples

Quantitative PCR assays were performed using the CBSV and UCBSV species-specific primers, CBSVqF/CBSVqR and UCBSVqF/UCBSVqR (Table 2.1), to determine amount of viral RNA in at least three plants per variety. Five varieties, namely, NASE 3, NASE 14, NASE 16, MH97/2961, and the

landrace Nyaraboke, were diagnosed for presence of CBSV alone, while TME 14, TME 204, Ebwanateraka, and 60444 were diagnosed for presence of both CBSV and UCBSV (Table 2.3), confirming results of RT-PCR. Of the nine genotypes tested by RT-qPCR, 60444 accumulated the highest amount of viral (CBSV and UCBSV) RNA while NASE 16 carried the least amount of viral RNA across all three types of organs tested.

The titer of CBSV RNA present in single or mixed infection was found to vary by genotype. The highly susceptible genotypes 60444 and Ebwanateraka accumulated 17- and 8.8-fold higher total CBSV RNA than NASE 16, which accumulated the lowest total CBSV RNA. Similarly, genotypes MH97/2961 and NASE 14 accumulated 8.1- and 5.5-fold higher CBSV RNA than NASE 16, while TME 204, NASE 3, TME 14, and Nyaraboke accumulated similar amounts of CBSV RNA (1.4 to 1.8-fold) compared to NASE 16 (Table 2.3). Thus, presence of UCBSV does not seem to affect accumulation of CBSV in mixed infection.

In genotypes seen to have single CBSV infection, MH97/2961 accumulated the highest amount of viral RNA while NASE 16 accumulated the least, in all organs tested. Interestingly, the landrace Nyaraboke accumulated only 2-fold more CBSV in its organs than NASE 16. In genotypes diagnosed positive for both CBSV and UCBSV, 60444 accumulated the highest viral RNA titer in its organs, while TME 14 accumulated the least. For example, 60444 carried 12-, 11-, and 2-fold more CBSV and 1.3-, 7- and 2-fold more UCBSV viral RNA in its organs than TME 204, TME 14, and Ebwanateraka, respectively (Table 2.3b). The landrace Ebwanateraka accumulated 6- and 4-fold more CBSV and UCBSV RNA, respectively, than TME 14, while TME 204 accumulated 6-fold more UCBSV RNA than TME 14.

Table 2.3: Relative virus titer in organs of cassava genotypes with (a) single CBSV infection, (b) CBSV and UCBSV co-infection

(a)

	Cultivar									
	MH97/2961		NASE 3		NASE 14		NASE 16		Nyaraboke	
Organ	CBSV	UCBSV	CBSV	UCBSV	CBSV	UCBSV	CBSV	UCBSV	CBSV	UCBSV
Leaf 3	0.401[a]	0.0	0.0204[a]	0.0	0.6338[a]	0.0	0.0656	0.0	0.014	0.0
Leaf 6	1.141[a]	0.0	0.0541[a]	0.0	1.1560[a]	0.0	0.0277	0.0	0.026	0.0
Leaf 9	1.580[a]	0.0	0.2693[a]	0.0	1.1354[a]	0.0	0.1184	0.0	0.078	0.0
Leaf 15	0.775[a]	0.0	0.2423[a]	0.0	1.3324[a]	0.0	0.2677	0.0	0.223	0.0
Leaf 20	0.385[a]	0.0	0.0628[a]	0.0	0.2528[a]	0.0	0.3732	0.0	0.141	0.0
Leaf 25	0.651[a]	0.0	0.0720[a]	0.0	0.4627[a]	0.0	0.2105	0.0	0.113	0.0
Stem	0.988[a]	0.0	0.5764[a]	0.0	1.1489[a]	0.0	0.0154	0.0	0.033	0.0
Root	9.276[b]	0.0	1.3269[b]	0.0	4.2516[b]	0.0	0.7988	0.0	2.718	0.0
Mean	1.965		0.3414		1.3255		0.2420		0.436	
LSD	2.682		0.8112		1.953		0.8496		2.907	
P	<0.001[HS]		0.044[S]		0.013[S]		0.544[NS]		0.480[NS]	

(b)

	Cultivar							
	60444		TME 204		TME14		Ebwanateraka	
Organ	CBSV	UCBSV	CBSV	UCBSV	CBSV	UCBSV	CBSV	UCBSV
Leaf 3	0.086	0.307	0.0161	0.007	0.0607	0.0009	0.124	0.135[a]
Leaf 6	1.608	0.348	0.0345	0.082	0.0662	0.0741	3.585	1.148[a]
Leaf 9	1.337	0.437	0.2556	0.432	0.2588	0.1333	5.107	4.560[b]
Leaf 15	2.826	3.408	0.3211	2.145	0.9294	1.1050	3.257	1.023[a]
Leaf 20	14.22	6.898	0.5756	3.825	0.4072	0.3446	3.139	0.727[a]
Leaf 25	1.106	1.080	0.3545	2.159	0.3319	0.3746	1.027	0.408[a]
Stem	3.688	1.449	0.0493	0.124	0.2370	0.0712	0.068	0.013[a]
Root	7.257	2.287	1.0581	3.611	0.5403	0.0662	0.186	0.008[a]
Mean	4.188	2.102	0.3469	1.615	0.3667	0.2830	2.146	1.041
LSD	16.95	7.662	1.101	3.217	0.7940	0.8343	5.165	2.153
P	0.666[NS]	0.598[NS]	0.507[NS]	0.086[NS]	0.381[NS]	0.166[NS]	0.316[NS]	0.006[HS]

Each value is the mean from at least three plants per cultivar. Means followed by the same letters are not significantly different from each other ($P \leq 0.05$ Tukey's test). S = significant, HS = highly significant, NS = not significant. Leaf numbering as assigned from the first fully expanded leaf downwards. Leaf 3 = leaf position 3, Leaf 6 = leaf position 6, Leaf 9 = leaf position 9, Leaf 15 = leaf position 15, Leaf 20 = leaf position 20, Leaf 25 = leaf position 25, stem = a stem section collected from mid-way up the stem, root = a 1–2 cm thick storage root disc.

2.4.3.1 Relative virus titer in organs of genotypes carrying single and mixed virus infections

All genotypes displayed a similar pattern of within-host distribution of viral RNA in both single infections of CBSV and mixed infections of CBSV and UCBSV. CBSV titer was generally higher in roots than in the aerial organs (Table 2.3; Fig. 2.3). Important differences were also seen between leaves at different positions within the canopy. The titer of both viruses was found to be lowest in the youngest leaf, closer to the top of the canopy, steadily rising to a peak midway down the plants and gradually declining to a lower value in the oldest leaf furthest from the apex (Fig. 2.4).

The varieties NASE 3, NASE 14, NASE 16, MH97/2961, and the landrace Nyaraboke were diagnosed for presence of CBSV alone in all organs tested. In these cultivars, CBSV titer was significantly higher ($P \leq 0.05$) in root tissues than in aerial tissues (Fig. 2.3A), with Nyaraboke, MH97/2961, NASE 3, NASE 14, and NASE 16 shown to have accumulated 27-, 11-, 11-, 5-, and 5-fold more CBSV in storage roots than in leaves, respectively (Fig. 2.3A; Table 2.3A). Similarly, there were 2 to 8-fold and 1.2 to 3.9-fold greater viral RNA found present within mid-canopy leaves (leaf positions 9 and 15) than those at the top (leaf positions 3 and 6) and base (leaf positions 20 and 25) of the plant, respectively (Fig. 2.4A). In one NASE 3 plant, which showed only stem and root symptoms, the amount of CBSV RNA in the necrotic storage root sample was 15-fold higher than in the stem sample, while leaves had undetectable viral RNA (Fig. 2.1B; Table 2.4). Similarly, leaf samples obtained from branches with symptoms showed high virus titer while samples obtained from symptomless branches had undetectable levels of virus (Table 2.4). The viruses were undetected in samples from symptomless plants of NASE 3 and NASE 16.

The cassava genotypes TME 14, TME 204, Ebwanateraka, and 60444 were diagnosed for presence of both CBSV and UCBSV in all organs tested (Table 2.3B; Fig. 2.3B). The relative titer of CBSV RNA

was generally higher in storage roots than in leaf and stem tissues (Table 2.3B; Fig. 2.3B (i)). As seen for single infections, TME 204, cv.60444, and TME 14 accumulated 4-, 2-, and *c.* 2-fold more CBSV in storage roots than in leaf tissues, respectively. Conversely, Ebwanateraka was found to have accumulated 15-fold more CBSV in its leaves than within the storage root. Titer of UCBSV present was found to vary greatly amongst genotypes. Ebwanateraka accumulated 133-fold more UCBSV in leaf tissues than in stem or storage roots, while TME 14 had five times more UCBSV in its leaves than in stem or storage roots. TME 204 had 30- and 2.5-fold greater UCBSV titer in storage roots than in the stem and leaf samples, respectively. Only cv.60444 carried similar amounts of UCBSV in all organ types (Table 2.3B; Fig. 2.3B (ii)). Similarly, there were variations in the amount of viral RNA in the canopy. Genotypes 60444 and TME 204 accumulated more viral RNA at the base, than at the top or at mid canopy. For instance, 60444 had about 9- and 4-fold more CBSV, and 14- and 2-fold higher UCBSV, in the older leaves at the base, than at the top and mid canopy, respectively. Conversely, TME 14 and Ebwanateraka accumulated higher viral RNA at mid canopy than at the top or base of the canopy. TME 14 had 10- and 2-fold greater CBSV, and 16- and 2-fold more UCBSV, at mid canopy than at the top and base of the canopy, whereas Ebwanateraka accumulated 2- and 5-fold higher CBSV and UCBSV at mid canopy than at the top or base of the canopy (Fig. 2.4B(i) and (ii)).

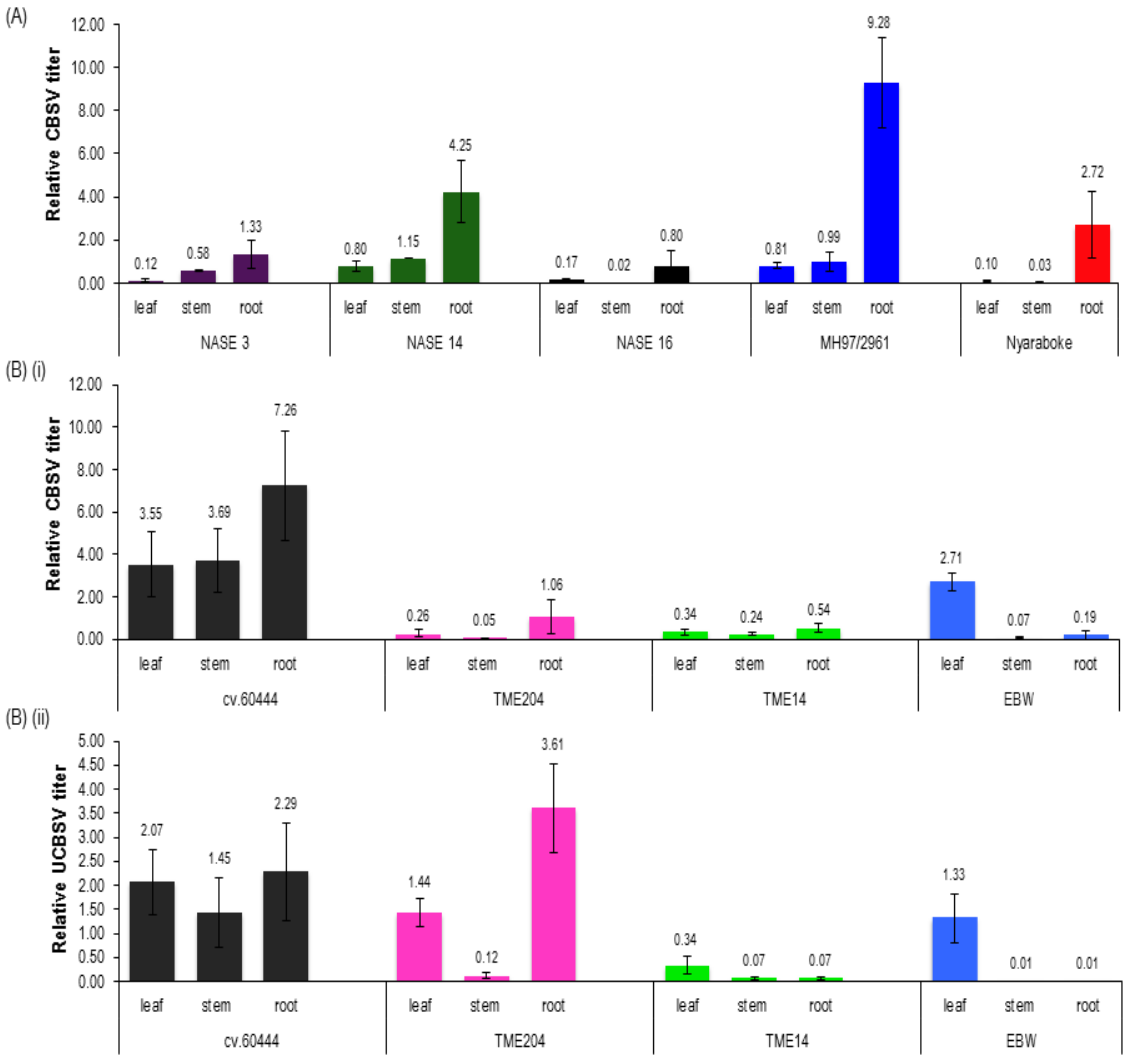


Fig. 2.3: Virus titres (relative to the titre in the positive controls) in organs of cassava genotypes infected with CBSV alone, and infected with both CBSV and UCBSV (*n* = 3). Error bars represent standard error of the mean (SEM). (A) Relative virus titres in organs of CBSV infected cassava genotypes. (B) Relative virus titres in organs of cassava genotypes infected with both CBSV and UCBSV: (i) relative CBSV titre in organs of cassava genotypes with double infection, (ii) relative UCBSV titre in organs of cassava genotypes with double infection.

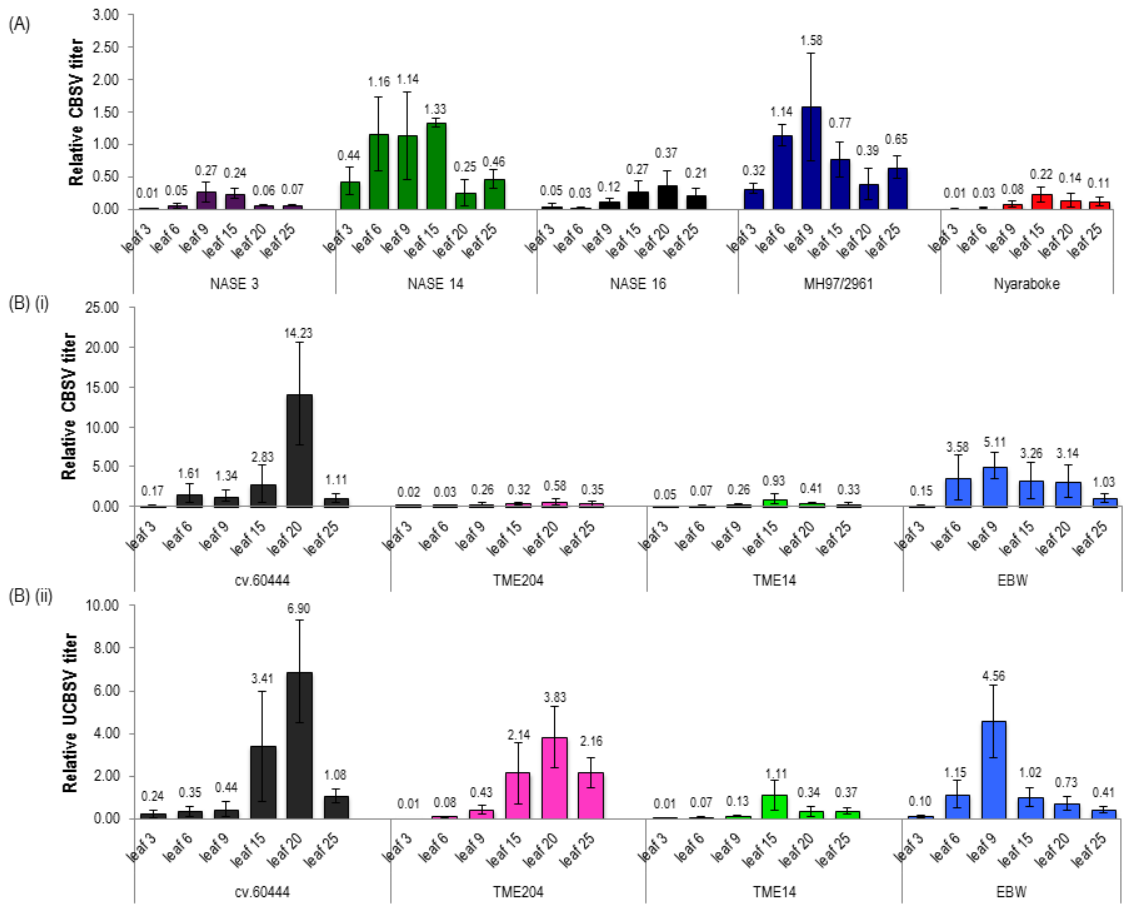


Fig. 2.4: Virus titres (relative to the titre in the positive controls) in leaf canopy of cassava genotypes infected with CBSV alone or with both CBSV and UCBSV (*n* = 3). Error bars represent standard error of the mean (SEM). (A) Relative virus titres in canopy of CBSV-infected cassava genotypes. (B) Relative virus titres in canopy of cassava genotypes with mixed infections of CBSV and UCBSV: (i), relative CBSV titre in leaf canopy of cassava genotypes with double infection; (ii), relative UCBSV titre in leaf canopy of cassava genotypes with double infection.

2.4.4 CBSV and UCBSV CP sequence analysis

Multiple alignments of the 19 partial CBSV sequences isolated from field samples revealed 91-99% nucleotide (nt) and 94-100% amino acid (aa) identity with each other. Eleven of these CBSV isolates shared 98-99% nucleotide and 99% amino acid identity with the CBSV Tanzania isolate (GenBank Accession No. GQ329864.1), while seven isolates shared 96-97% nucleotide and 97-98% amino acid identity with the CBSV PANG isolate (GenBank Accession No. GU563322.1), also from Tanzania. The

11 partial UCBSV sequences identified in this study shared 99% nucleotide and 99-100% amino acid identity with each other and showed 99% nucleotide and amino acid identity with the UCBSV Kabanyoro isolate (GenBank Accession No. HM346952.1) from Uganda. As expected, the phylogenetic analysis with sequences of the 30 isolates of CBSV and UCBSV from this study resulted in two major clades, which correspond to the two species (Fig. 2.5). The sequences identified in this study have been deposited in GenBank under the accession numbers KJ606221-KJ606250.

2.4.5 Graft challenge of NASE 3, TME 204 and 60444

In order to verify viruses infecting cassava genotypes and correlate their within-host distribution and accumulation, plants of NASE 3, TME 204 and 60444 were grafted in the greenhouse with chip buds obtained from plants infected with CBSV Naliendele isolate (CBSV-[TZ:Nal3-1:07) and UCBSV Kabanyoro isolate (UCBSV-[UG:T04-42:04]). The plants were monitored during the first week for proper graft union formation and weekly afterwards for CBSD symptom development and severity. For each variety, over 50% (*n* = 12) of plants challenged with either CBSV or UCBSV formed good graft unions (Table 2.5). Typical foliar CBSD symptoms were observed in 66.7% of TME 204 and 83.3% of 60444 6 weeks after plants were grafted with UCBSV-infected scions. No plants of NASE 3 grafted with UCBSV-infected scions developed CBSD symptoms across the 16-week observation period (Fig. 2.6A, Table 2.5). UCBSV was undetectable by RT-PCR in leaf, stem, and root tissues of these NASE 3 plants. Conversely, all leaf and root samples collected from five UCBSV graft-challenged plants of TME 204 and 60444 with symptoms tested positive for presence of this virus at 6 WAG (Fig. 2.6B).

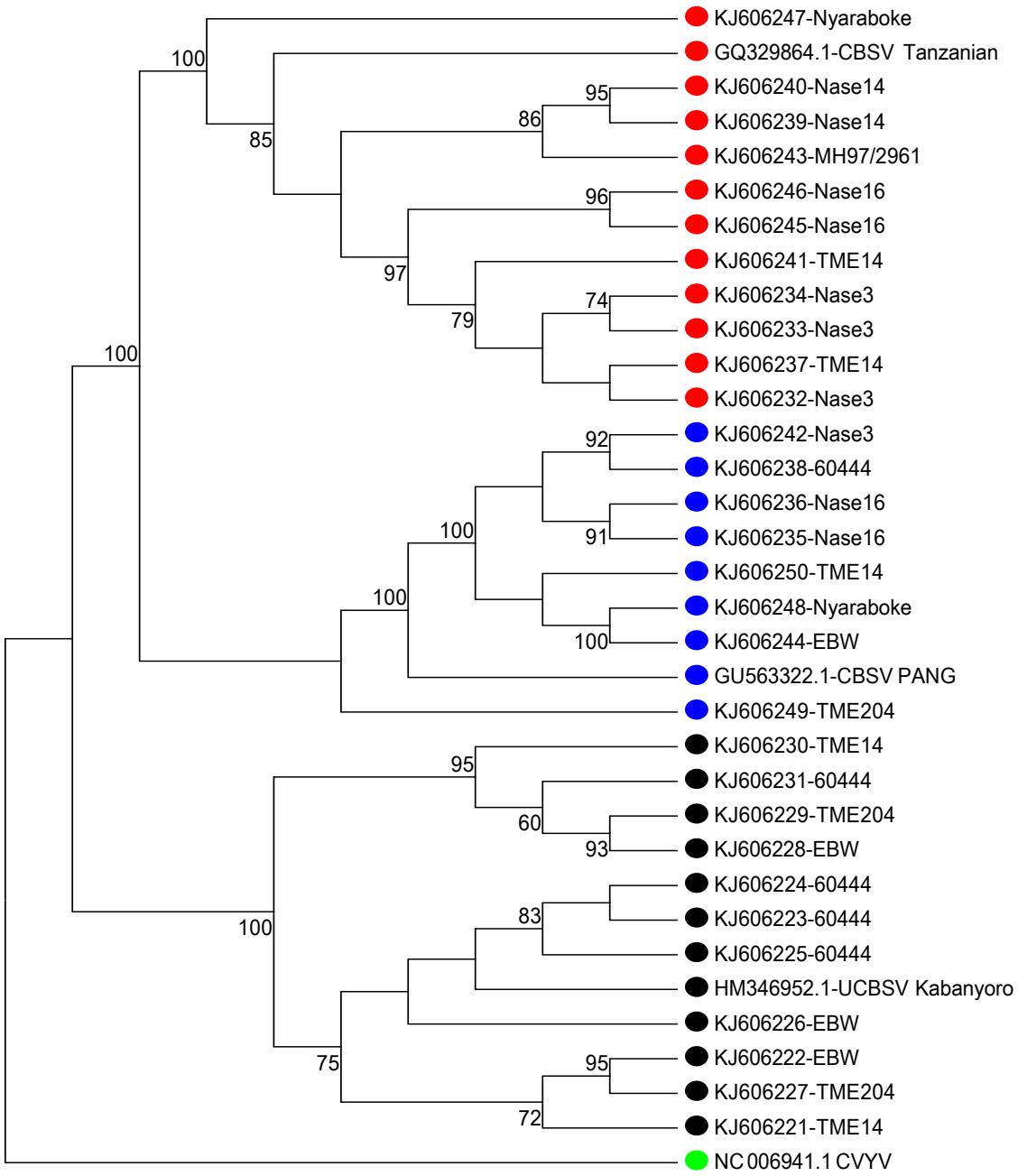


Fig. 2.5: Phylogenetic tree of partial HAM1-like and coat protein-encoding sequences of CBSV (1607 nt) and UCBSV (1670 nt) obtained from field samples. Known sequences of CBSV Tanzania and PANG isolates, and UCBSV Kabanyoro isolate were included in the phylogenetic analysis. Numbers at branches represent bootstrap values of 500 replicates. The phylogenetic tree was constructed using MEGA5 (171). Red and blue branch tips represent two clusters of CBSV sequences. Black branch tips represent UCBSV cluster, and the green branch tip represent coat protein sequence of *Cucumber vein yellowing virus* (CVYV), a distant member within the genus *Ipomovirus*.

Plants grafted with CBSV-infected bud scions showed typical foliar CBSD symptoms in 58.3, 83.3 and 92.7% of NASE 3, TME 204, and 60444, respectively at 6 WAG (Table 2.5). CBSD symptom severities were very high in plants of TME 204 (average score = 4.4 on a scale on 1 to 5) and 60444 (average score = 3.8) grafted with CBSV-infected scions (Table 2.5). All leaf samples obtained from such plants 6 WAG tested positive for CBSV by RT-PCR (Fig. 2.6B, Table 2.5). Storage roots of infected plants showed severe necrosis (Fig. 2.7B). Plants infected with CBSV developed early and more severe CBSD symptoms compared to plants infected with UCBSV, which showed milder CBSD symptoms even in susceptible varieties (Fig. 2.7C).

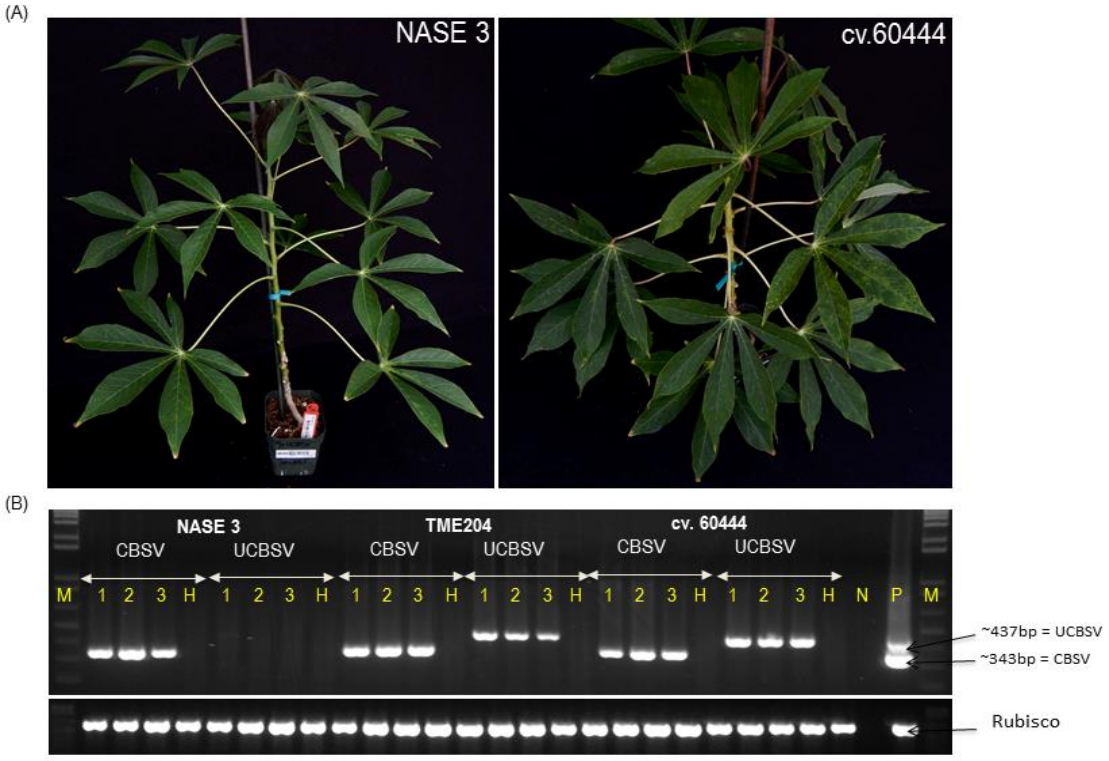


Fig. 2.6: Symptoms of CBSD and reverse transcription polymerase chain reaction diagnosis for CBSV and UCBSV in plants of NASE 3, TME 204, and 60444 grafted in the greenhouse with CBSV- and UCBSV-infected buds. (A) NASE 3 and cv.60444 grafted with UCBSV showing no CBSD leaf symptoms and mild CBSD leaf symptoms, respectively. (B) Gel electrophoresis showing presence of CBSV and UCBSV in leaf tissues of NASE 3, TME 204, and 60444 grafted with CBSV and UCBSV infected buds. 1-3 = leaf samples from plants 1-3, H = leaf samples from healthy plant, N= negative control, P = positive control, M = 1Kb+ DNA ladder.

Plants of NASE 3 behaved in a manner unlike that of other cultivars studied here. Plants showed no leaf symptoms and only localized stem lesions around the graft union area with CBSV-infected scions. CBSV was undetectable in leaf tissues of NASE 3 plants, but was detectable in stem tissues with symptoms obtained from within 2-3 cm around the graft union. Tissues in this area became increasingly necrotic and suffered die back from the shoot apical regions downwards. By 12 WAG, all plants of this genotype challenged with CBSV had died (Fig. 2.7A).

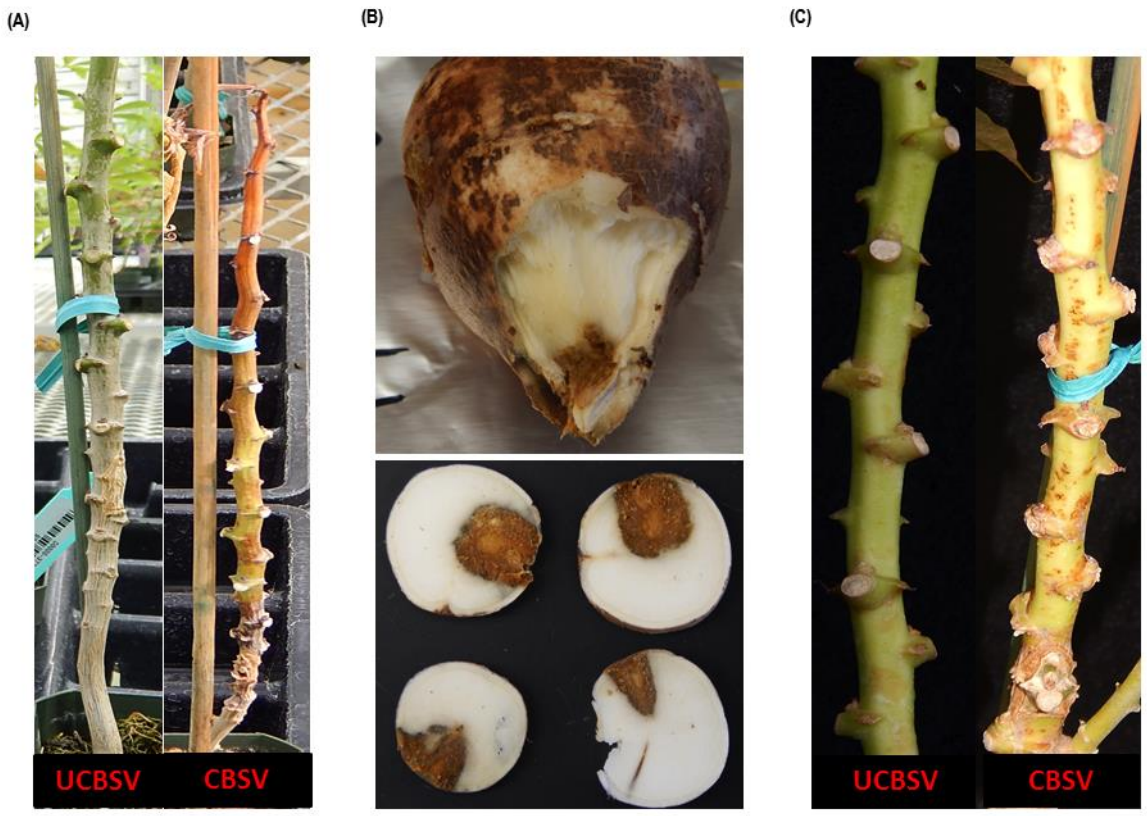


Fig. 2.7: Stem and root symptoms of CBSD on NASE 3, and 60444 after bud graft challenge. (A) No lesion and systemic necrosis and stem dieback of NASE 3 grafted with UCBSV- and CBSV-infected buds, respectively. (B) Severe necrosis in root of NASE 3 grafted with CBSV-infected bud. (C) No lesion and severe lesion on stem of 60444 grafted with UCBSV- and CBSV-infected buds respectively.

Table 2.4: Relative CBSV titers in root and leaf tissues of naturally CBSD-infected NASE 3 showing branches with and without symptoms (plants 1 and 2), and symptomless leaves but showing stem lesions and root necrosis (plant 3)

Organs	Plant 1		Plant 2		Plant 3
	Symptomless branch	Branch with symptoms	Symptomless branch	Branch with symptoms	Symptomless leaves, stems and roots with symptoms
Leaf 3	0.001	0.006	0.000	0.012	0.000
Leaf 6	0.002	0.141	0.000	0.017	0.000
Leaf 9	0.000	0.153	0.001	0.014	0.000
Leaf 15	0.000	1.189	0.001	0.227	0.000
Leaf 20	Missing	0.002	0.000	0.138	0.000
Stem	-	-	-	-	1.197
Storage root	0.543	0.543	0.162	0.162	18.636

Leaf numbering assigned from the first fully expanded leaf downwards: Leaf 3 = leaf position 3, Leaf 6 = leaf position 6, Leaf 9 = leaf position 9, Leaf 15 = leaf position 15, Leaf 20 = leaf position 20, stem = a stem section collected from mid-way up the stem, and storage root = a 1-2 cm thick storage root disc.

Table 2.5: Bud graft transmission of CBSV and UCBSV, and CBSD symptom expression under greenhouse conditions

Genotype	No. of grafted plants		No. with good graft union		No. of plants with symptoms (%)[a]		Severity 6 weeks after grafting[a]		No. analyzed by RT-PCR[b]		Virus detection by organ[b]					
											CBSV			UCBSV		
	CBSV	UCBSV	CBSV	UCBSV	CBSV	UCBSV	CBSV	UCBSV	CBSV	UCBSV	leaf	stem	root	leaf	stem	root
NASE 3	12	12	7	6	7 (58.3)	0 (0.0)	5.0	1.0	5	5	0	5	5	0	0	0
TME 204	12	12	9	8	10 (83.3)	8 (66.7)	4.4	2.0	5	5	5	5	5	5	5	5
60444	12	12	10	7	11 (92.7)	10 (83.3)	3.8	2.0	5	5	5	5	5	5	5	5

[a]Development of CBSD symptoms on grafted plants was scored (severity scale 1–5) weekly as previously described (203).
[b]Mid-canopy leaf samples, a stem section from mid-way up the stem, and a 1–2 cm thick storage root disc were collected 6 weeks after grafting and tested for presence or absence of CBSV and UCBSV by RT-PCR as described previously (139).

2.5 Discussion

In this study, the presence of CBSV and UCBSV was determined by RT-PCR and virus level quantified by RT-qPCR. CBSV and UCBSV were detected in leaf, stem and storage root tissues indicating systemic infection of field-grown plants (108). Results revealed that cassava genotypes NASE 3, NASE 14, and NASE 16, which are currently recommended to farmers in Uganda as CBSD tolerant (1), were infected with CBSV only and not UCBSV. With the exception of NASE 14, which had *c.* 4-fold higher CBSV titer than CBSD-susceptible genotypes TME 14 and TME 204, the CBSD-tolerant NASE 3 and NASE 16 registered lower average titer of CBSV compared to susceptible cultivars (Table 2.3). Highly CBSD-susceptible cultivars, 60444, Ebwanateraka and TME 204, contained a higher amount of viral RNA and were infected with both CBSV and UCBSV. However, the amount of CBSV RNA was similar in cultivars infected with both CBSV and UCBSV to that in other cultivars infected only with CBSV. This indicates that presence of UCBSV does not seem to affect accumulation of CBSV RNA and implies the existence of cassava genotype-specific resistance to infection with UCBSV. Presence of CBSV in all samples studied here shows this virus to be more present at higher levels than previously reported at Namulonge (Uganda), in contrast to UCBSV (6, 90, 112, 199); this indicates that CBSV is replacing UCBSV as the more common pathogen at this location. Additional studies would be beneficial to determine if a similar pattern is occurring elsewhere in East Africa.

Variation was seen in the amount of viral RNA present in the leaf canopy with leaf age and correlated with CBSD symptom appearance (Table 2.6); this confirms previous reports (6, 124). In aerial tissues, across genotypes, the youngest symptomless leaves accumulated the lowest amount of viral RNA, senescing leaves also had low amounts, stems moderate amounts, but mature non-senescing leaves with symptoms accumulated the highest amount of viral RNA (Table 2.6). Tolerant genotypes accumulated higher titer of CBSV in storage roots than in aerial organs, but in susceptible genotypes

the CBSV and UCBSV titer in each organ varied by genotype. This data indicates that storage roots or mature leaves are the best choice when sampling for CBSV and UCBSV diagnostics.

Table 2.6: CBSD symptom severity observed on leaf, stem and storage root samples used for virus titer determination by quantitative RT-PCR

Genotype	Biological Replicate	CBSD symptom on sampled leaf/organs (+/-)							Symptom severity[a]	
		Leaf 3	Leaf 6	Leaf 9	Leaf 15	Leaf 20	Leaf 25	stem	Foliar	root
NASE 3	1	-	+	+	+	+	+	+	3	4
	2	-	-	-	+	+	+	+	3	4
	3	-	+	+	+	+	+	+	3	3
	4*	-	-	-	-	-	-	+	3	5
NASE 14	1	-	-	-	+	+	+	+	3	4
	2	-	+	+	+	+	+	+	4	5
	3	-	+	+	+	+	+	+	4	3
NASE 16	1	-	-	+	+	+	+	+	3	5
	2	-	-	-	+	+	+	+	3	4
	3	-	-	-	+	+	+	+	3	4
	4**	-	-	-	-	-	-	-	1	1
MH97/2961	1	-	+	+	+	+	+	+	4	5
	2	-	-	+	+	+	+	+	4	5
	3	-	+	+	+	+	+	+	4	5
Nyaraboke	1	-	-	+	+	+	+	+	4	5
	2	-	-	+	+	+	+	+	4	5
	3	-	-	-	+	+	+	+	4	5
60444	1	-	-	+	+	+	+	+	3	4
	2	-	-	-	+	+	+	-	2	2
	3	-	+	+	+	+	+	-	3	3
TME 204	1	-	-	+	+	+	+	+	2	5
	2	-	-	-	+	+	+	+	2	5
	3	-	-	-	+	+	+	+	3	5
TME14	1	-	-	-	+	+	+	+	3	1
	2	-	-	-	+	+	+	+	3	3
	3	-	-	+	+	+	+	+	3	3
Ebwanateraka	1	-	-	+	+	+	+	+	3	3
	2	-	-	-	+	+	+	-	2	3
	3	-	-	-	-	+	-	-	2	1

+ or – indicate presence or absence of CBSD symptoms in each leaf and on stem. [a]Foliar severity and root damage were scored as previously described (139). *NASE 3 plant with only stem and root symptoms. **Symptomless Nase16 plant. Leaf numbering assigned from the first fully expanded leaf downwards: Leaf 3 = leaf position 3, Leaf 6 = leaf position 6, Leaf 9 = leaf position 9, Leaf 15 = leaf position 15, Leaf 20 = leaf position 20, Leaf 25 = leaf position 25.

Similar observations have been reported for potato plants infected with *Potato virus Y* (PVY) (80), where low amounts of viral RNA and no viral particles were detected in leaf lamina and veins of senescent leaves, and high amounts of virus were detected in the petiole. The low virus titer in young and senescing leaves, and high virus titer in mature non-senescing leaves could be associated with rapid cell division in young leaves, high rate of virus replication in mature leaves, and viral RNA degradation as the leaves become older (80). In storage roots, the amount of viral RNA was not clearly related to the severity of CBSD observed visually (Table 2.6). Notably, 60444 and Ebwanateraka, which showed CBSD root severity scores of 3.0 and 2.3 respectively, were found to have higher viral RNA accumulation than Nyaraboke, MH97/2961, and TME 204, which were recorded with the maximum root damage severity of 5.0.

Real-Time PCR detection of CBSV and UCBSV by TaqMan chemistry (6), and of CBSV by SYBR Green I (124) have been described recently in addition to enzyme linked immunosorbent assay (ELISA) and conventional RT-PCR (113). TaqMan RT-qPCR is costly due to the requirement for fluorogenic oligoprobes. In addition, Adams *et al.* (2013) indicated that presence of CBSV limited detection of UCBSV in multiplex TaqMan RT-qPCR reactions. SYBR Green I chemistry, based on DNA-binding fluorophores instead of fluorogenic oligoprobes, is a simpler and less expensive method. Moreno *et al.* (2011) validated SYBR Green I protocol for detection and quantification of CBSV in different cassava varieties. In the present study, the use of SYBR Green I RT-qPCR has been optimized to eliminate run-to-run variation and to enable its use for simultaneous detection and quantification of CBSV and UCBSV across leaf, stem and storage root samples.

Results from analysis of field samples were verified for selected CBSD-tolerant and susceptible varieties by grafting experiments with tissue culture-derived plants. The pathogenicity phenotypes of CBSV and UCBSV in these cassava genotypes were confirmed; the tolerant NASE 3 was resistant to

UCBSV but susceptible to CBSV, whereas the susceptible TME 204 and 60444 were susceptible to both viral pathogens. Interestingly, CBSV-infected plants of NASE 3 exhibited necrosis of tissues around the graft union (Fig. 2.6A), which culminated in the death of the plants by 12 WAG. This response could be due to relatively high amounts of viral RNA inoculum in the bud graft compared to whitefly transmission. Alternatively it could indicate presence of a genotype-specific resistance mechanism worthy of further investigation as efforts continue to understand CBSD and its impact on cassava at the pathological and economic levels.

In susceptible cultivars, UCBSV caused milder foliar symptoms than CBSV. This confirms previous reports (147, 199) indicating that CBSV is the more aggressive and virulent CBSD viral pathogen. However, molecular mechanisms behind CBSV virulence or pathogenicity are unknown. Similarly, information regarding RNA movement of CBSV and UCBSV from leaves through the stem to the storage roots, and localization of CBSV and UCBSV viral components in different parts of the tuberous root is lacking. Such studies would offer insight into the mechanism of evolution and advancement of CBSD symptoms, particularly necrosis in the cassava storage roots.

Data presented demonstrate that some cassava genotypes exhibit specific resistance to CBSD viral pathogens, notably UCBSV. Consequently, plants of cassava genotypes with mixed infections of both CBSV and UCBSV tend to accumulate more viral RNA than plants of cassava genotypes with a single infection of CBSV. Additionally, symptomless branches of cassava genotypes that displayed species-specific resistance, such as NASE 3, accumulated no or negligible CBSV viral RNA. This is important information for breeders and farmers because cassava is vegetatively propagated through stem cuttings. Farmers should therefore be advised to use stem cuttings derived from symptomless branches to establish the next crop, with the intention of establishing symptomless, CBSD-free plants.

Chapter 3

Comparative analysis of virus-derived small RNAs within cassava (*Manihot esculenta* Crantz) infected with cassava brown streak viruses

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Virus Research (2015): Submitted

3.1 Abstract

Infection of plant cells by viral pathogens triggers RNA silencing, an innate antiviral defense mechanism. In response to infection small RNAs (sRNAs) are produced that associate with Argonaute (AGO)-containing silencing complexes which act to inactivate viral genomes by posttranscriptional gene silencing (PTGS) Deep sequencing was used to compare virus-derived small RNAs (vsRNA) in cassava genotypes NASE 3, TME 204 and 60444 infected with the +ssRNA viruses, *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) the causal agents of cassava brown streak disease (CBSD). An abundance of 21-24 nt vsRNAs was detected and mapped, determining that they covered the entire CBSV and UCBSV genomes. The 21-nt vsRNAs were predominant, followed by the 22-nt class with a slight bias toward sense compared to antisense polarity, and an adenine and uracil bias present at the 5'-terminus. Distribution and frequency of vsRNAs differed between cassava genotypes and viral genomes. In susceptible genotypes TME 204 and 60444, CBSV-derived sRNAs was seen in greater abundance than UCBSV-derived sRNAs. NASE 3, which is known to be resistant to UCBSV, accumulated negligible UCBSV-derived sRNAs but high populations of CBSV-derived sRNAs. The expression levels of cassava homologues of AGO2, DCL2 and DCL4, which are central to the gene-silencing complex, were differently affected in CBSV- and UCBSV-infected plants across genotypes, suggesting that these proteins play a role in antiviral defense. The results indicate disparity between CBSV and UCBSV host-virus interaction mechanisms, and provide insight to the role of virus-induced gene silencing as a mechanism of resistance to CBSD.

3.2 Introduction

Cassava brown streak disease (CBSD) has become an economically important disease of cassava in East and Central Africa (ECA) causing significant losses in both yield and quality of cassava storage roots (88, 105). CBSD is caused by the two distinct, but closely related, positive sense single-stranded RNA (+ssRNA) virus species, *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV). Both CBSV and UCBSV belong to the family *Potyviridae*, genus *Ipomovirus* (112, 121). The viral pathogens are transmitted by whiteflies, *Bemisia tabaci*, Gennadius (106), and mechanically through grafting (193, 203) and sap inoculation of herbaceous plant species (96, 140). The viruses systemically infect cassava in the field (76, 138) causing similar CBSD symptoms on affected cassava plants. CBSV is more aggressive, inducing rapid and more severe CBSD symptoms than those seen on plants infected with UCBSV alone. Recent studies have shown some cassava varieties, for example NASE 3 (138) and Kaleso (105) to be highly resistant to infection by UCBSV (Kabanyolo isolate) but less so to CBSV. The difference in response of cassava varieties to infection with CBSV and UCBSV implies a difference in the mechanism of host-virus interaction between the two virus species.

Plant-infecting RNA viruses are known to trigger RNA interference (RNAi) (36, 97), an innate antiviral defense mechanism. This involves critical steps including production of double-stranded RNA (dsRNA), processing of the dsRNA into small interfering RNA (siRNA), followed by sequence-specific targeting and silencing of viral messenger RNA (mRNA) by siRNA-incorporated effector complexes (161). The siRNA molecules associate with distinct Argonaute-containing silencing complexes to target and mediate diverse silencing effects on both host and viral genomes (37, 97, 202). During virus-host interactions, virus-specific dsRNAs are generated in a variety of ways, including activity of virus-encoded RNA polymerase during genomic replication and transcription, formation of secondary structures as a result of intramolecular base pairing between sense and antisense viral RNA strands, or

imperfect folding of self-complementary single stranded viral genomic RNA (37). Host encoded RNA dependent RNA polymerase (RDR) plays a crucial role in the maintenance of virus-induced gene silencing by converting ssRNAs to dsRNAs for secondary siRNA synthesis (120, 161). The dsRNA serves as substrates for Dicer-like (DCL) ribonucleases which cleave the dsRNAs to produce 21 to 24 nucleotides (nt) virus-derived small RNA (vsRNA) duplexes (18, 119, 205). The vsRNAs are then loaded onto Argonaute (AGO) proteins, a component of RNA-induced silencing complexes (RISC), to facilitate cleavage of complementary viral mRNA, consequently silencing the viral genome as a self-defense response of the plant (37, 165).

Studies in *Arabidopsis thaliana* and other plants species have revealed that the core components involved in plant small RNA biogenesis and the silencing pathways are encoded by multi-protein families (161). These are diverse and exhibit functional redundancy (97, 164). For instance, the *A. thaliana* and rice genomes each have four and eight DCLs, six and five RDRs, and ten and nineteen AGO proteins respectively, while *Populous* has five DCLs. *A. thaliana* possesses six silencing pathways (18, 102). It has also been shown that in virus-infected *A. thaliana*, DCL4, DCL2 and DCL3 catalyze the formation of 21-, 22- and 24-nt vsRNAs respectively to induce antiviral response (17, 159). The DCL4-21-nt and DCL2-22-nt vsRNAs confer efficient antiviral defense in plants. Nevertheless, DCL4 and DCL2 exhibit functional redundancy or cooperative interaction, since formation of the 22-nt vsRNAs mainly happens in the absence of DCL4 (32, 41, 42, 159). The 21- and 22-nt vsRNAs are the most predominant class amongst potyvirus infected host plants, with a few exceptions (32, 36, 56). Multiple AGO proteins are also involved in antiviral defense, including at least AGO2 and AGO5, which have been shown to bind *Cucumber mosaic virus* (CMV)-derived small RNAs (169). Similarly, RDR1, RDR2 and RDR6 have been implicated in virus-derived small RNA biogenesis and antiviral defense in plants (36, 37, 41, 42, 56, 151).

The biogenesis, composition, and abundance of vsRNAs have been characterized from several host-virus pathosystems (41, 42, 95, 116, 132, 150, 164, 192, 202). Small RNAs are known to play regulatory roles in defense responses against pathogens in plants (159, 161). Different host plants have been shown to accumulate varying amounts of vsRNAs in response to infection by different viruses (42, 95). Through species diversity and epidemiology studies, CBSV and UCBSV have been shown to be closely related (110, 112). This has stimulated efforts to develop successful and durable CBSD resistance by simultaneous RNAi targeting of both viruses (139, 147). However, no information is available concerning the biogenesis and composition of vsRNAs in CBSV- and UCBSV-infected cassava, nor their important, potential contribution to host plant resistance or susceptibility. The molecular mechanisms linking vsRNAs from CBSV and UCBSV to RNAi and host phenotype is therefore unclear. Alongside the premise that CBSV and UCBSV interact differently in cassava plants of the same genetic background (138), it is reasonable that the CBSV- and UCBSV-cassava pathosystem offers a good platform to study molecular interactions between closely related RNA viruses in their natural host. The present study aimed to characterize vsRNAs generated during separate CBSV and UCBSV infection in three cassava genotypes: NASE 3 (highly resistant to UCBSV but shows systemic necrosis due to CBSV infection), TME 204 (susceptible to both CBSV and UCBSV), and cultivar 60444 (highly susceptible to both CBSV and UCBSV). NASE 3 and TME 204 are farmer-preferred cassava varieties in Uganda (174), while 60444 is a model cassava genotype frequently used to study gene expression including virus-derived transgenes tailored for resistance to CBSD viral pathogens (19, 189, 203). The information generated is an important contribution for understanding the molecular mechanisms underlying the pathogenicity of CBSV and UCBSV and the response of some cassava genotypes to CBSD.

3.3 Materials and methods

3.3.1 Plant materials and virus inoculations

Micropropagated, tissue cultured-derived cassava plants of NASE 3, TME 204 and 60444 were confirmed to be free of CBSD viral pathogens by RT-PCR (139) and established in soilless compost as previously described (173). The plants were bud-graft challenged at 10 weeks after planting (WAP) with CBSV Naliendele isolate (CBSV-[TZ:Nal3-1:07]) or UCBSV Kabanyoro isolate (UCBSV-[UG:T04-42:04]) as described by Wagaba *et al*. (2013) (193). The grafted plants were assessed a week later for graft union formation and monitored visually thereafter for CBSD symptom development.

3.3.2 Sample collection, RNA extraction, and detection of virus species

Systemically infected leaves of cultivar 60444 and TME 204 showing clear CBSD symptoms and stem sections of NASE 3 showing lesions typical of CBSD infection were collected six weeks after grafting (WAG). Similar tissues were sampled for use as controls from uninfected non-inoculated plants of each genotype. Presence of CBSV and UCBSV was detected by RT-PCR and virus levels quantified by RT-qPCR as described previously (138). The plant materials were obtained from three independent biological replicates, wrapped with aluminum foil, immediately frozen in liquid nitrogen and stored at -80°C. The samples were ground with the aid of liquid nitrogen in sterile mortar. Total RNA was extracted from the resulting powder as previously described (139). The RNA was treated with DNase I (Ambion Inc. Austin, TX) according to manufacturer recommendations to eliminate genomic DNA contamination. RNA was quantified using a Nanodrop (Model 2000C, Thermo Scientific, Waltham, MA). Integrity of RNA with $OD_{260/280}$ ratios from 1.8 to 2.1 was visually checked on 2% (w/v) agarose gel. Reverse transcription of RNA to cDNA was performed from 2 µg of total RNA using Superscript III™

First-Strand Synthesis System (Invitrogen, Carlsbad, CA) following manufacturer recommendations. The RNA aliquot was used to prepare small RNA libraries, and the remainder reserved and stored at -80^{o}C.

3.3.3 Small RNA library preparation and sequencing

Small RNA libraries were prepared using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (Sets 1 and 2) (New England Biolabs, Inc. Ipswich, MA) following manufacturer recommendations. Briefly, 3' adapters were ligated to total RNA (6 µg) followed by hybridization of the reverse transcriptase primer to prevent adapter-dimer formation. Subsequently, 5' adapters were ligated and the RNA subjected to reverse transcription and PCR amplification. The PCR products were purified using a QIAQuick PCR Purification Kit (Qiagen Inc., Valencia, CA) according to manufacturer recommendations and quality-checked by electrophoresis on a 6% polyacrylamide gel. Bands of ~140 bp corresponding to the adapter-ligated small RNA fragments were isolated, crushed in elution buffer and re-precipitated using a mixture of Acrylamide, sodium acetate, and absolute ethanol. The small RNA libraries (cDNA pellet) was re-suspended in TE buffer (10mM Tris-HCl containing 1mM EDTA, pH 8) and rechecked for size and concentration by electrophoresis in 6% polyacrylamide gel.

3.3.4 Bioinformatics analysis of small RNA sequences

The small RNA libraries were sent to Genome Technology Access Center (GTAC), Washington University in St. Louis, USA for further size, quality, and integrity checking by an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc. Santa Clara, CA). Sequencing was performed by Illumina HiSeq 2,500 using 1x50 single-end read protocol. Raw sequence data received from GTAC was demultiplexed by QIIME (23). Sequence reads with quality score below 19 were discarded. Cutadapt

(104) was used to remove adapter sequences. Small RNA sequences in the size range of 21 to 24 nt were selected for downstream analysis. The total reads as well as unique reads were mapped to the CBSV and UCBSV reference genome by Bowtie (83). Mapped reads data was converted to statistical data by BEDTools (152) and all outputs were graphically presented by Shell scripts provided by Steven Hill and Doug Bryant (Bioinformatics Core Facility at Donald Danforth Plant Science Center).

3.3.5 Quantification of Dicer-like ribonucleases III and AGO2 by RT-qPCR

Real-time quantitative PCR (RT-qPCR) was performed to measure the expression levels of DCLs and AGO2 in CBSV- and UCBSV-infected cassava using cytochrome c oxidase (COX) mRNA as the reference gene for normalization as previously described (138). The primers used to amplify the DCLs and AGO2 are listed in Table 3.1. Briefly, cDNA was diluted ten-fold and subjected to RT-qPCR using BioRad CFX96 Connect instrument (BioRad Laboratories Inc, Hercules, CA). Reaction mixtures contained 5 µl SsoFast Advanced SYBR Green I SuperMix, 1 µl of each primer (0.5 µM final concentration), and 3 µl of diluted cDNA template in a total reaction volume of 10 µl. The RT-qPCR thermal cycles used were as follows: initial denaturation step for 3 min at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at 58°C. Data were collected at the end of each 58°C stage. Each RT-qPCR run included in each plate RNA that went through cDNA synthesis process with no reverse transcriptase enzyme added (NRT) and a no-template control (NTC) containing water instead of cDNA. The RT-qPCR was performed using three biological replicates for each sample and three technical replicates of each biological replicate. The mean quantification cycle (*Cq)* value of each triplicate reaction was used for further calculations by the $2^{-\Delta\Delta CT}$ method for relative normalized expression analysis using *COX* as reference gene, and CBSV or UCBSV positive samples of predetermined virus

concentration as calibrator, respectively. The relative expression levels were obtained by comparing CBSV- and UCBSV-infected plants with uninfected plants.

Table 3.1: Primer sequences and amplicon sizes of cassava homologues of agonaute 2 (AGO2), and Dicer-like proteins 2 and 4 (DCL2 and DCL4)

Gene	Primer code	Primer sequence	Amplicon size (bp)	Efficiency (%)	R^2	Transcript ID
AGO2	AGO2-F1 AGO2-R1	GGCAATCTCCAGCTTCAGCA TCCAATGAAGCAGCCGATGA	78	104.5	0.993	cassava4.1_000920m
DCL2	DCL2-F3 DCL2-R3	ATGCACACTGACCTCGTC GTCATCACAAGCACCTCA	110	99.1	0.986	cassava4.1_000931m
DCL4	DCL4-F3 DCL4-R3	TGCTACTAAAGTGGGTGAAGAAG CGCACGTCCTCTAGATGGTATG	109	106.8	0.977	cassava4.1_001038m

3.4 Results

3.4.1 Response of cassava genotypes to infection by cassava brown streak viruses

The three cassava genotypes responded differently to graft-challenge with CBSV and UCBSV. As reported earlier, plants of genotypes TME 204 and 60444 grafted with CBSV-infected scions started showing typical CBSD symptoms two WAG, a week earlier than plants grafted with UCBSV-infected scions (193). NASE 3 plants grafted with UCBSV-infected buds developed no symptoms, while plants of this cultivar grafted with CBSV-infected buds showed stem lesions around the graft union four WAG. Across all three genotypes, plants grafted with CBSV-infected buds showed more severe foliar CBSD symptoms compared to plants grafted with UCBSV-infected buds such that CBSV-challenged 60444, TME 204 and NASE 3 showed maximum severities of 4.3, 3.3 and 5.0 respectively, while UCBSV-challenged plants showed lower CBSD severities reaching maximum values of 3.0 and 2.0, respectively for plants of 60444 and TME 204 respectively.

RT-PCR analysis revealed presence of CBSV in all CBSV-challenged plants showing CBSD symptoms across the three genotypes. RT-qPCR analysis showed that NASE 3 accumulated 2.7- and 1.4-fold higher CBSV RNA than 60444 and TME 204, respectively. Similarly, UCBSV was detected in all UCBSV-challenged plants of TME 204 and 60444, with 60444 found to have accumulated 1.4-fold higher UCBSV RNA than TME 204 (Fig. 3.4C). UCBSV was not detected in plants of UCBSV-challenged NASE 3. Together, these results indicated that CBSV was able to infect and accumulate viral RNA in all plants across genotypes, whereas UCBSV infected and accumulated vsRNAs and viral RNA only in plants of TME 204 and 60444 but not in NASE 3 plants.

Table 3.2: Summary of small RNA reads from two cassava brown streak viruses in three different cassava genotypes[a] and effect of the viruses on AGO2, DCL2 and DCL4 expression

Genotype	Virus species	Mean CBSD severity 6 WAG (1-5)[b]	Total raw reads	Total clean reads	Small RNAs mapped to virus genome	Percent vsRNAs with respect to total clean reads	Effect of virus on AGO2, DCL2 and DCL4 expression		
							AGO2	DCL2	DCL4
60444	CBSV	4.3	3,198,984	130,400	24,510	18.798	na	down	down
	UCBSV	3.0	4,020,006	612,987	63,822	10.412	up	up	up
	Healthy	1.0	3,347,340	10,238	12	0.114	na	na	na
TME 204	CBSV	3.3	3,185,175	100,750	16,703	16.579	up	down	up
	UCBSV	2.0	3,994,736	1,282,370	73,202	5.708	up	down	down
	Healthy	1.0	2,740,771	86,524	8	0.009	na	na	na
NASE3	CBSV	5.0	3,019,728	188,617	40,978	21.726	up	down	up
	UCBSV	1.0	2,511,404	410,641	39	0.009	up	down	up
	Healthy	1.0	2,154,804	213,163	19	0.009	na	na	na

[a]Data presented is an average of three biological replicates per genotype. [b]CBSD symptoms on grafted plants was scored as previously described (139). na = not applicable.

3.4.2 Small RNA populations in virus-challenged cassava plants

Sequence data showing total small RNAs obtained from leaves of uninfected and UCBSV- and CBSV-infected plants of TME 204, 60444 and NASE 3 are summarized in Table 3.2. The data presented is an average of three biological replicates per virus challenge for each genotype. The small RNA clean sequence reads (21 to 24 nucleotides adapter-trimmed sequence reads with Phred Quality score 20 and above) ranged from *c.* 10,000 to ~1.3 million across libraries. The majority of small RNA reads were host derived. CBSV-derived small RNAs from cultivars TME 204, 60444 and NASE 3 challenged with CBSV accounted for up to 17%, 19% and 22% respectively of the total clean small RNA reads. Similarly, UCBSV-derived small RNAs in TME 204 and 60444 graft-inoculated with UCBSV accounted for up to 6% and 10% respectively (Table 3.2). This contrasted with negligible UCBSV-derived small RNAs (0.009%) and UCBSV viral RNA accumulation obtained from UCBSV-challenged NASE 3 plants. The population of small RNAs in uninfected plants that mapped to viral genomes was minimal, accounting for 0.009%, 0.114% and 0.009% of total clean reads in TME 204, 60444 and NASE 3, respectively (Table 3.2; Fig. 3.1A). Total small RNA reads identified from virus-infected plants were sorted according to their numbers and length. In all three cultivars, uninfected or infected with the virus, 21-nt total small RNAs were most abundant followed by 22-nt class. The 21-nt class represented 45-60% of sRNAs in infected plants compared to 30-32% in uninfected plants (Fig. 3.1B). A similar trend was observed for 22-nt siRNAs. However, in comparison to uninfected plants, the proportion of 21-nt siRNAs was very high in virus-infected plants (Fig. 3.1B). For 24-nt siRNAs the proportion found to be present in uninfected plants was 20-30% in comparison to <10% in virus-infected plants. In UCBSV-infected NASE 3 the proportion of 21-nt siRNAs was very high (40%), whereas the proportion of the 24-nt siRNAs was more than 20%, similar to the proportion in uninfected cassava plants (Fig. 3.1B).

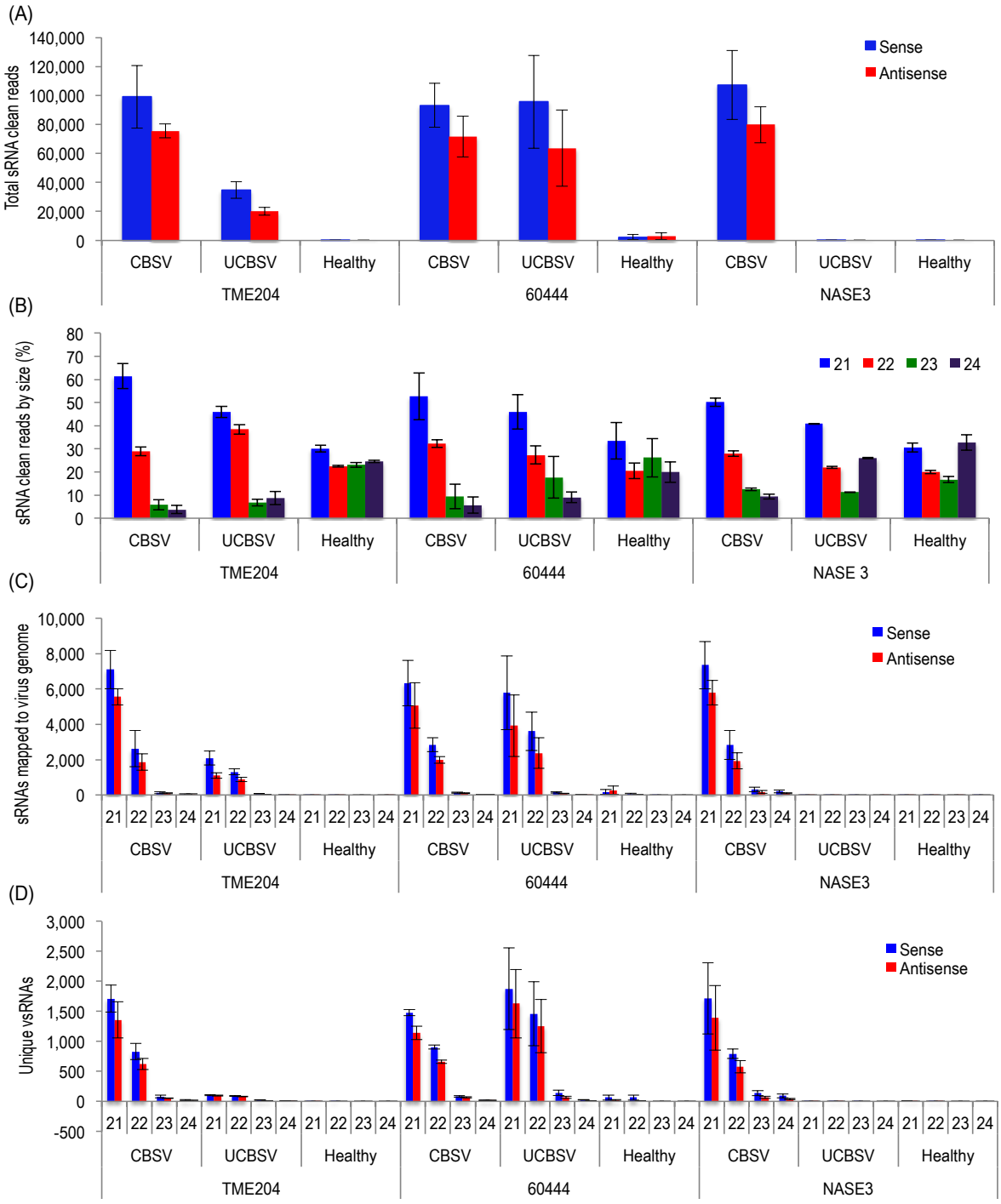


Fig. 3.1: Comparison of CBSV- and UCBSV- derived small RNA populations recovered from infected and uninfected plants of TME 204, 60444 and NASE 3 (n = 3). The histograms represent (A) total sense and antisense sRNA clean reads, (B) total sRNA clean reads by size, (C) sRNAs that mapped to virus genome by size class and orientation, and (D) unique vsRNAs in each size class and orientation. Error bars indicate standard error of the mean (SEM).

3.4.3 Characterization of CBSV- and UCBSV-derived small RNAs

To enable comparison of data across libraries, the sRNA reads were normalized as reads per million of the total clean reads of corresponding samples. Sequences showing no mismatches were regarded as CBSV- or UCBSV-derived small RNAs, and each could be unambiguously assigned to one unique genome position. Small RNAs in the range of 21-24 nucleotide size classes were included in this study. Analyses showed that the 21-nt size class was predominant (61-79%) followed by 22-nt size class (19-37%) for both viruses across all the three cassava genotypes (Fig. 3.1C). Proportions of 23- and 24-nt classes were very low (1-2%; Fig. 3.1C). The vsRNAs of all size classes were mapped throughout the CBSV and UCBSV genomes in both sense and antisense orientations. A slight bias towards the sense polarity was observed, with 56-57% of the total CBSV-derived small RNAs and 60-63% of the total UCBSV-derived small RNAs were of positive polarity. Similar results were obtained for unique vsRNAs (Fig. 3.1D), although proportion of sense unique UCBSV-derived small RNAs dropped to 53-54% (Fig. 3.1C & D).

Different proportions of CBSV- and UCBSV-derived small RNAs across cassava genotypes were also reflected in line graphs obtained from normalized genome coverage data for each virus (Fig. 3.2A-F). The major classes of vsRNAs (21- and 22-nt sizes) were produced from all parts of the viral genomes across genotypes. The distribution along the genomes were however non-homogenous. Some parts of the genomes were seen to be expressing more vsRNAs than others. Examination of the percent vsRNAs for each genomic regions showed that in CBSV-infected NASE 3 and CBSV- and UCBSV-infected 60444 and TME 204, the CI genomic region produced the highest population of vsRNAs, followed by genomic regions that encode NIb, P1, and P3 proteins, which produced moderate population of vsRNAs across viral genomes (Fig. 3.2A-E; 3.3 A-F). The 6K1 and 6K2 genomic regions produced the lowest population of vsRNAs of both orientations across viral genomes (Fig. 3.3A-F). The

profiles of CBSV- and UCBSV-derived sRNAs were different in the same cassava genotype, but similar for each virus across cassava genotypes, except for UCBSV-challenged NASE 3 (Fig. 3.2F).

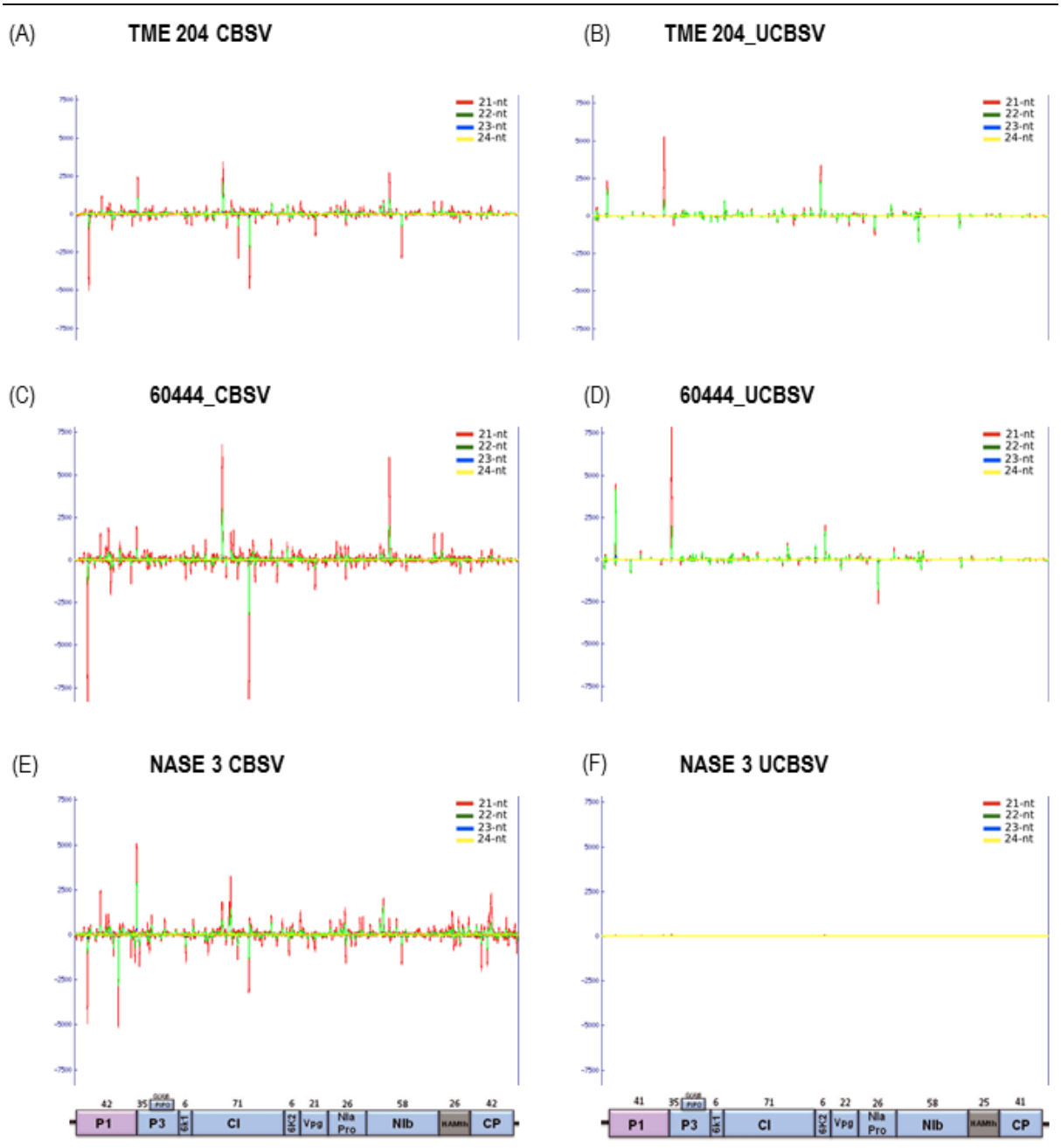


Fig. 3.2: Genomic map of CBSV- and UCBSV-inoculated libraries. The graphs plot the number of 21–24 nucleotides vsRNAs at each position along the CBSV and UCBSV genomes recovered from (A) CBSV- and (B) UCBSV-inoculated TME 204; (C) CBSV- and (D) UCBSV-inoculated 60444; and (E) CBSV-inoculated NASE 3. (F) UCBSV-inoculated NASE 3. Bars above and below the x-axis represent sense and antisense vsRNAs reads, respectively.

In *A. thaliana*, sorting of small RNAs into Argonaute complexes is directed by the first 5' nucleotide (115). To predict selective interaction of vsRNAs with specific AGOs, the relative abundance of total vsRNAs was determined based on the first 5'-terminal nucleotides. Small RNAs with G as the first nucleotide at 5' end were the least abundant (8-11%) for both viruses across all three genotypes (Fig. 3.4A). In UCBSV-infected TME 204 and 60444, the majority of UCBSV-derived small RNAs had A (40-45%) followed by U (30-34%) as the most abundant first nucleotide at 5' end. Whereas, in CBSV-challenged TME 204, 60444 and NASE 3 CBSV-derived small RNAs starting with A (32-34%), U (30-35.0%) and C (20-30%) at 5' end were all in significant proportions (Fig. 3.4A).

3.4.4 Distribution and frequency of small RNAs along CBSV and UCBSV genomes

Small RNA mapping analysis identified four major regions in each of CBSV and UCBSV genomes, which are rich in 21- and 22-nt vsRNAs (Fig. 3.2A-E). In the CBSV genome, the first hotspot was found within the cylindrical inclusion protein (CI) region from 2,216 to 4,105 nt position which correlated with 32-33% of all the CBSV-derived small RNAs produced from this region in TME 204 and 60444 (Fig. 3.3A & C). In NASE 3, only about 24% of CBSV-derived small RNAs were produced from this region (Fig. 3.3E). A hotspot was also found in the same region (from 2,209 to 4,092 nt) in the UCBSV genome, and was responsible for 17-20% of UCBSV-derived small RNAs (Fig. 3.3A & C). A second hotspot was located in the large nuclear inclusion protein (NIb) region from 5,522 to 7,026 nt in the CBSV, and 5,506 to 7,011 nt in the UCBSV genome. These hotspots produced 11-13% CBSV-derived small RNAs and 16-19% UCBSV-derived small RNAs in the CBSV and UCBSV genomes, respectively (Fig. 3.3A, C & E). The third and fourth hotspots were found located in the P1 and P3 protein regions. The P1 region in the CBSV genome (104–1,177 nt) yielded 16-20% CBSV-derived small RNAs, whereas the P3 region (1,178-2,059 nt) produced 8-12% CBSV-derived small RNAs (Fig. 3.3A, C & E). In the UCBSV genome, the P1 region (85-1,170 nt) produced 14-18% of UCBSV-derived small RNAs,

while the P3 region (1,171-2,052 nt) produced 19-20% of UCBSV-derived small RNAs (Fig. 3.3A & C). Similar trend was obtained for unique vsRNAs across genotypes (Fig. 3.3B, D & F).

3.4.5 Expression of Dicer-like proteins and AGO2 in CBSV- and UCBSV-infected cassava

Dicer-like proteins play a major role in the production of small RNAs. To understand the effect of CBSV and UCBSV infection on RNA silencing pathways, RT-qPCR analysis was performed to determine the transcript levels of cassava homologues of DCL (MeDCL) proteins and their correlation to the abundance of 21- and 22-nt vsRNAs in the infected plants. Differing patterns of expression for these genes was observed across the three cultivars when they were infected with the two viral pathogens. In CBSV-infected TME 204 there was down-regulation of *MeDCL2* mRNA and 1.2-fold up-regulation of *MeDCL4* mRNA level compared to uninfected control. In UCBSV-infected TME 204 both *MeDCL2* and *MeDCL4* transcript levels were down-regulated. In CBSV-infected 60444, the transcript levels of both *MeDCL2* and *MeDCL4* were down-regulated, whereas in UCBSV-infected 60444 plants, both *MeDCL2* and *MeDCL4* mRNAs were up-regulated (Table 3.2; Fig. 3.4B). In NASE 3, *MeDCL2* mRNA level was down-regulated in both CBSV- and UCBSV-challenged plants compared to uninfected controls. In contrast, *MeDCL4* mRNA level was up-regulated in both CBSV- and UCBSV-challenged plants compared to unchallenged controls.

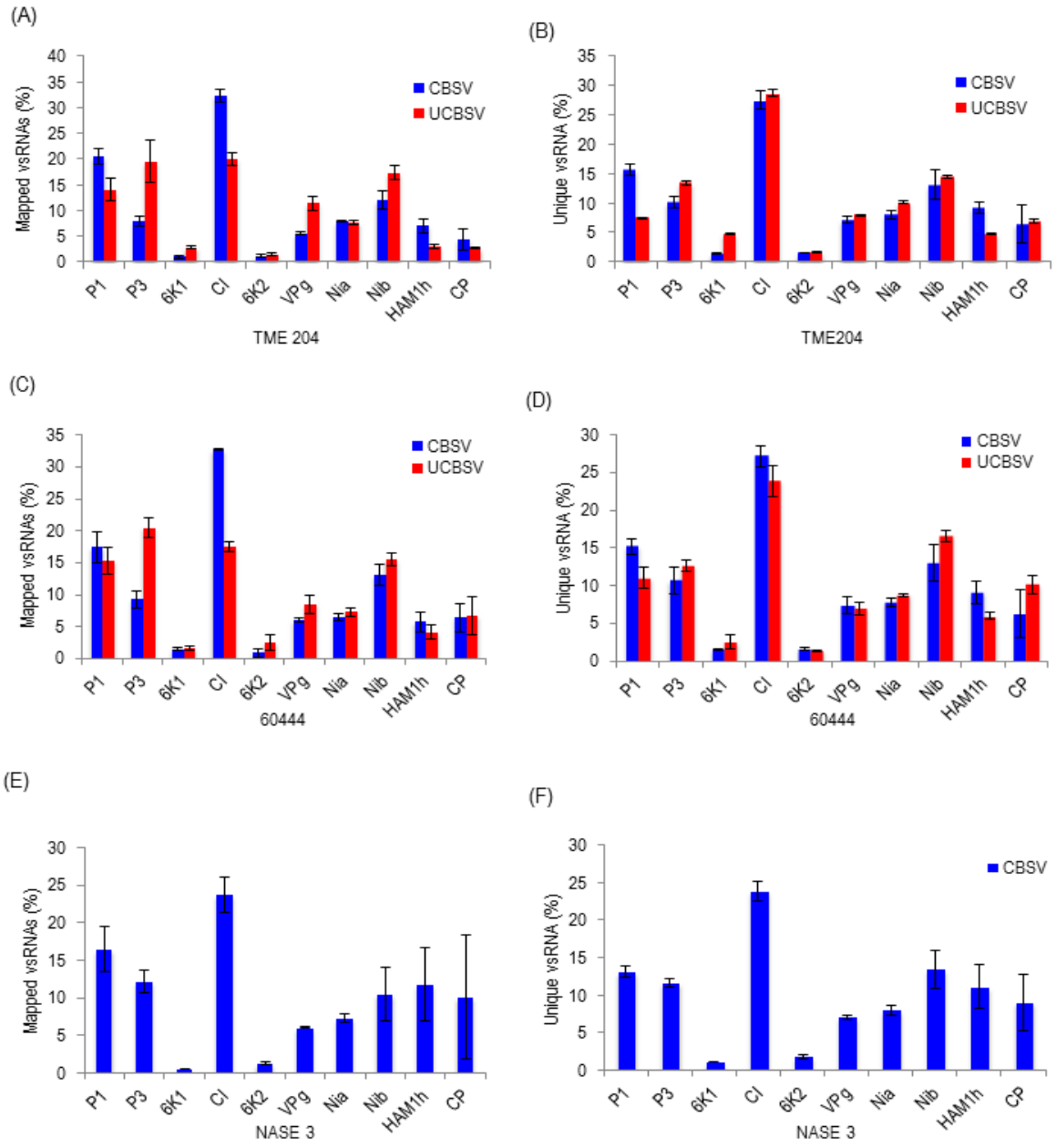


Fig. 3.3: Total versus unique virus-derived small RNA population from individual genes of CBSV and UCBSV genomes in infected plants of TME 204, 60444 and NASE 3 (*n* = 3). The histograms represent (A) total mapped and (B) unique vsRNAs CBSV and UCBSV-infected libraries of TME 204; (C) total mapped and (D) unique vsRNAs in CBSV and UCBSV-infected libraries of 60444; and (E) total mapped and (F) unique vsRNAs in CBSV-infected libraries of NASE 3. Error bars indicate standard error of the mean (SEM).

Multiple AGO-bound small RNAs guide effector complexes to the target viral mRNAs in a sequence-specific manner leading to either translational repression or mRNA cleavage (36, 37, 97). The vsRNAs, dictated by their first 5'-end nucleotides, are preferentially sorted and loaded into multiple AGO complexes (115, 169). Specifically, AGO1 has been shown to have preference for U, AGO2 and AGO4 have preference for A or U, while AGO5 prefers C at the first 5'-end of the siRNA (42, 115, 151, 169). Since in this study a major proportion of vsRNAs have A or U at the first 5' end, accumulation of cassava homologue of AGO2 (*MeAGO2*) mRNAs was determined by RT-qPCR. The results showed that *MeAGO2* mRNA was up-regulated in all three UCBSV-infected cassava genotypes and CBSV-infected TME 204 and NASE 3. However MeAGO2 expression remained unchanged in CBSV-infected 60444 (Table 3.2; Fig. 3.4B).

3.5 Discussion

To decipher the molecular mechanism and RNAi components involved in CBSV and UCBSV infection, three cassava genotypes TME 204 (CBSD susceptible), 60444 (CBSD susceptible) and NASE 3 (CBSD tolerant) (1, 76) were challenged with CBSV and UCBSV, and the population and characteristics of vsRNAs studied by next generation sequencing. Sequence analysis showed that populations of pathogen-derived siRNA varied across genotypes. Maximum populations of small RNAs that mapped to the virus genome was found in CBSV-infected plants of NASE 3 (21.7%) followed by 60444 (18.8%) and TME 204 (17.6%), respectively (Table 3.2). In UCBSV-infected plants, maximum populations of UCBSV-derived sRNAs were found in 60444 (up to 10.4%), whereas 5.7% of UCBSV-derived sRNAs accumulated in TME 204. In NASE 3, UCBSV-derived siRNA were insignificant (0.09%).

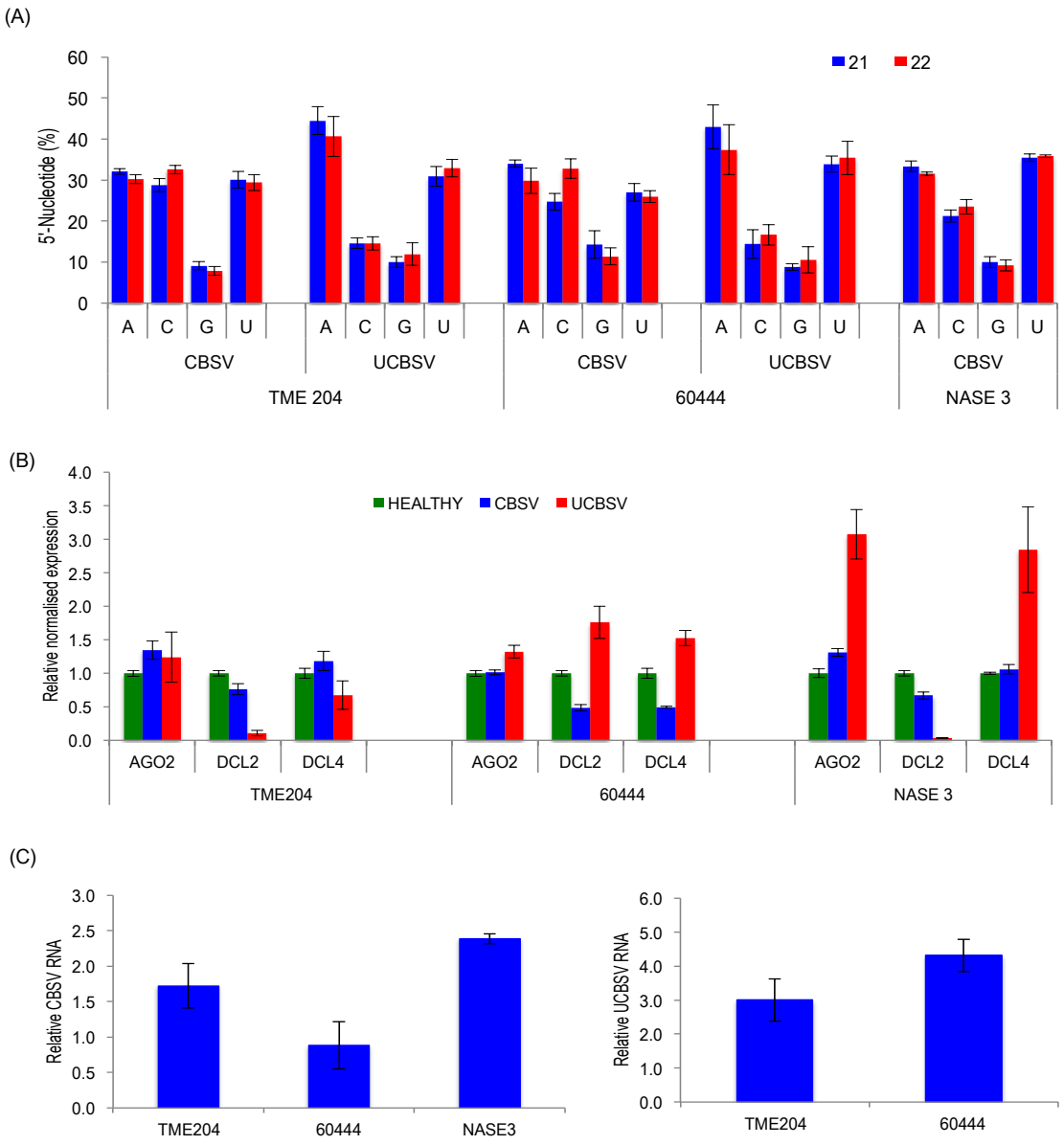


Fig. 3.4: Relative frequency of first 5'-end nucleotide and relative expression levels of Dicer and Agonaute proteins. (A) The histograms represent relative abundance of the four distinct 5'-end nucleotide in 21 and 22 nucleotide vsRNAs of CBSV- and UCBSV-infected libraries of TME 204, cv. 60444 and NASE 3 (*n* = 3). (B) Real-time PCR analysis of the expression levels of cassava homologues of AGO2, DCL2 and DCL4 in CBSV- and UCBSV-infected and uninfected plants of TME 204, cv. 60444 and NASE 3 (*n* = 3). (C) Virus titer (relative to the titer in the CBSV or UCBSV positive controls) in tissues of cassava genotypes infected with CBSV (left) and UCBSV (right). Relative gene expression and virus titer levels were normalized using cytochrome c oxidase mRNA as internal control. Error bars indicate standard error of the mean (SEM).

Quantitative RT-qPCR analysis also showed that NASE 3 accumulated 2.7- and 1.4-fold higher CBSV RNA than CBSV-infected plants of 60444 and TME 204, respectively (Fig. 3.4C). Similarly, UCBSV-infected 60444 accumulated 1.4-fold more UCBSV RNA than UCBSV-infected TME 204 (Fig. 3.4C). Similar RT-qPCR data was obtained in a previous study using field and glasshouse samples from the same cassava genotypes (138). Accordingly, the data presented indicate that UCBSV does not infect NASE 3 plants and the lack of CBSD symptom suggests this lack of infection after inoculation with UCBSV (Table 3.2). The variation in the cultivars responses could be due to the their ability to differentially recognize CBSV and UCBSV and accordingly trigger antiviral defense mechanisms, or it could be due to inherent differences in the infection cycle of the two viruses including viral replication and accumulation of vsRNAs (132).

CBSV or UCBSV infection was shown here to alter host small RNAs profiles across the three cassava genotypes studied. The level of 23- and 24-nt size classes were higher in uninfected libraries but were less abundant in virus-infected libraries across all genotypes. The 21- and 22-nt siRNAs were abundant in uninfected plants but were even higher in virus-infected plants across genotypes. Similar studies in uninfected *A. thaliana* showed that the 24-nt siRNAs were most abundant (35%) followed by 21-nt siRNAs (28%), but upon infection with *Cabbage leaf curl virus* (CaLCuV), the host siRNA profile was altered such that the 21-nt class became more abundant (32%) followed by the 24-nt class (28%) (13). Also, in *Sugarcane mosaic virus* (SCMV)-infected maize there was increased level of 21- and 22-nt size classes, whereas that of 24-nt size class decreased (202). Contrastingly, *Cauliflower mosaic virus* (CaMV)-infected *A. thaliana* resulted in overaccumulation of the 24-nt siRNAs (16).

Reports in *A. thaliana* have shown that DCL4, DCL2 and DCL3 generate the 21-, 22-, and 24-nt vsRNAs (17, 41, 159). In addition, studies in *A. thaliana* infected with *Turnip mosaic virus* (TuMV), crucifer-infecting strain of *Tobacco mosaic virus* (TMV-Cg), *Cucumber mosaic virus* (CMV), and

Tobacco rattle virus (TRV) (41, 42, 95, 151) and *Nicotiana benthamiana* infected with *Potato virus X* (PVX), *Bamboo mosaic virus* (BMV), and *Pepper mild mottle virus* (PMMoV) (42, 95); potato infected with three different strains of *Potato virus Y* (PVY-O, PVY-N, and PVY-NTN) (132); grapevines infected with *Grapevine fleck virus* (GFkV) and *Grapevine rupestris stem-pitting associated virus* (GRSPaV) (144); cotton infected with *Cotton leaf roll dwarf virus* (CLRDV) (164); and maize infected with *Sugarcane mosaic virus* (SCMV) (202), among others, revealed that majority of the vsRNAs in infected plants belonged to the 21- and 22-nt size classes, which are associated with activated PTGS (17, 32, 41). Similarly, the 21- and 22-nt CBSV- and UCBSV-derived sRNAs was shown here to be the most predominant, which suggests increased activity of homologues of DCL4 and DCL2 in response to virus infection in cassava (Fig. 3.4B). The low abundance of the 23- and 24-nt vsRNAs in both CBSV- and UCBSV-infected plants across cassava genotypes suggests marginal activity of DCL3 in cassava against these viruses (151).

Processing of dsRNAs by Dicer proteins would ideally yield an equal amount of sense and antisense vsRNAs, the life span of which depends on selective incorporation into specific AGO proteins (151). Analysis of the CBSV- and UCBSV-derived small RNA polarity showed a bias towards the sense polarity compared to antisense polarity irrespective of size class across libraries, and was consistent with previous findings in other virus-infected plants (41, 42, 132, 151). Strand biases are usually attributed to preferential processing of highly structured single-stranded genomic viral RNAs by Dicer proteins (37, 42, 164), and different viruses have been shown to produce, in the same host plant, vsRNAs with different ratios of sense to antisense polarity (42, 144). However, a correlation between vsRNAs hotspots and structured regions of genomic viral RNAs remain unclear (42).

The preferential use of vsRNAs with A or U residues as compared to C and G residues as the first 5'-end nucleotide has been reported in *A. thaliana* plants infected with TuMV and PVX, *Cucumis melo*

infected with WMV (42), and in potato plants infected with three strains of PVY (132). In contrast, a few cases of preferential use of C as the first 5'-terminal nucleotide has been reported in grapevines infected with GFkV and GRSPaV (144), and tomato plants infected with *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (116). However, most previous studies reported a tendency to avoid vsRNAs with G residues at the first 5'-end (42, 115, 144), probably due to absence of AGO proteins with known preference for G residues at the first 5'-end (115). The results presented here indicate that A and U were the most abundant nucleotides at the first 5'-end (~ 80%), while G was the least abundant at the first 5'-end (Fig. 3.4A) in UCBSV-infected plants. In addition, there was up-regulation of AGO2 mRNA in all three UCBSV-infected cassava genotypes and CBSV-infected TME 204 and NASE 3 (Fig. 3.4C). These results signify increased activity of AGO2 homologue in CBSV- and UCBSV-infected cassava. However, abundance of A, U and C at the first 5' terminus in similar proportions in CBSV-infected plants imply the contribution of multiple AGOs in sorting out the vsRNAs.

Host RDRs use viral ssRNA to synthesize dsRNAs which serve as substrates for DCL-dependent formation of secondary vsRNAs to maintain systemic silencing throughout the plant (97). In addition, DCL4 and RDR1 were reported as major contributors to the abundant pool of 21-nt TuMV-derived siRNAs in *A. thaliana* (56). However, the response of RDRs in CBSV- and UCBSV-infected cassava was not assessed in this study. Since ipomoviruses and potyviruses (TuMV) share many characteristics, such a study is worth considering.

This study provides the first high-resolution genome map of vsRNAs for an ipomovirus in the family Potyviridae. The use of deep sequencing in this study has provided an insight into the molecular interaction between CBSD causing viruses and cassava. The population of vsRNAs was abundant, diverse and revealed widespread targeting of viral genomes by machinery of the gene silencing pathway. The overall composition of vsRNAs in infected cassava unveiled the action of different Dicer

proteins in different cassava genotypes. The findings also provided an insight into the differential susceptibility of the host plants to the same virus, which is reflected in the severity of symptoms they induce. Finally the results indicate that CBSV and UCBSV interact differently in the same host genetic background.

Chapter 4

Transgenic RNA interference (RNAi)-derived field resistance to cassava brown streak disease

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4.1 Abstract

Cassava brown steak disease (CBSD), caused by the Ipomoviruses, *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV), is considered to be an imminent threat to food security in tropical Africa. Cassava plants were transgenically modified to generate small interfering RNAs (siRNAs) from truncated (894 bp) or N-terminal (402 bp) portions of the UCBSV coat protein (ΔCP) sequence. Seven siRNA-producing lines from each gene construct were tested under confined field trial at Namulonge, Uganda. All non-transgenic control plants (n = 60) developed CBSD symptoms on aerial tissues by six months after planting (MAP), while plants transgenic for the full-length ΔCP sequence showed a 3-month delay in disease development, with 98% of clonal replicates within line 718-001 remaining symptom free over the 11-month trial. Reverse transcription-polymerase chain reaction (RT-PCR) diagnostics indicated presence of UCBSV within leaves of 57% of the non-transgenic controls but in only two of 413 plants tested (0.5%) across the 14 transgenic lines. Except for line 718-001 in which 93% were confirmed to be free of both pathogens, all transgenic plants showing CBSD symptoms were PCR positive for presence of CBSV. At harvest, 90% of storage roots from non-transgenic plants were severely affected by CBSD-induced necrosis. However, transgenic lines 718-005 and 718-001 showed significant suppression of disease, with 95% of roots from the latter remaining free from necrosis, and RT-PCR negative for presence of both viral pathogens. Cross-protection against CBSV by siRNAs generated from the full-length UCBSV□ΔCP confirms a previous report in *Nicotiana benthamiana*. Information presented provides proof of principle for the control of CBSD by RNAi-mediated technology, and progress towards potential control of this damaging disease.

4.2 Introduction

Cassava brown steak disease (CBSD) is an emerging constraint to production of the tropical root crop cassava (*Manihot esculenta*), and is considered to be one of the world's most serious threats to food security (12, 148). The causal agents of CBSD are the viral pathogens, *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV), both species of the genus *Ipomovirus*, family *Potyviridae* (112, 199). The disease is transmitted by the whitefly vector *Bemisia tabaci* (107, 130) and can result from single or dual infections by these two positive single-stranded RNA viruses (110). Although known to have been present in East Africa since the 1930s (166), CBSD was mostly confined to the coastal regions and around Lake Malawi (90) until identification in Uganda in 2004 (10). Since that time, the disease has developed to epidemic proportions, representing a significant constraint to cassava production throughout the region (22, 90), with incidences reported at 93% of farmers' fields surveyed in Western Kenya (130). Unlike cassava mosaic disease (CMD), which causes distinct leaf deformation and suppressed root yields, CBSD produces yellow mottling of older leaves, stem lesions and most importantly, brown, corky, necrotic lesions within the storage roots. The necrosis of storage roots can decrease root weight in the most sensitive cultivars by up to 70% (65). In addition, necrotic roots are largely inedible and have little or no value at market (64, 65).

Cassava is central to food and economic security throughout much of East Africa (49, 141). The impact of CBSD therefore has important implications for smallholder farmers and rural communities within the region (46, 50, 185). There are also concerns that the disease is advancing south and westwards, presenting a threat to the large cassava-producing countries of Central and West Africa (3, 136). The development and deployment of CBSD-resistant germplasm suited to farmers' needs are therefore essential if the impact of the disease is to be mitigated (90).

Recently, we reported transgenically imparted resistance to CBSD via RNA interference (RNAi) technology in *N. benthamiana* (147) and cassava (203) under controlled growth conditions. In both cases, inverted repeat constructs derived from the coat protein (CP) of UCBSV were integrated into the plant genome and were shown to result in the accumulation of transgenically derived small interfering RNAs (siRNAs) when probed with CP-specific sequences. Inoculations were performed and resistance was assessed by development of CBSD leaf symptoms and reverse transcription polymerase chain reaction (RT-PCR) detection of the viral pathogens. N-terminal, C-terminal, and delta full length (ΔFL) inverted repeat CP sequences were shown to impart 100% resistance to challenge with the homologous virus (UCBSV) in tobacco, with levels of resistance correlated with the strength of the siRNA signal within individual transgenic plant lines (110). Graft inoculation with transgenic cassava also confirmed 100% resistance to, and exclusion of, the homologous virus by the transgenic scions (203). Significant cross protection was also observed when tobacco plants accumulating high levels of UCBSV ΔFL-CP-derived siRNAs were challenged with CBSV (147).

In order to assess efficacy of siRNA-imparted resistance to CBSD under conditions of naturally vectored disease pressure, a field experiment with transgenic cassava plants was established in a confined enclosure at the National Crops Resources Research Institute (NaCRRI) in Namulonge, Uganda. The data reported here demonstrate that field-grown cassava plants, transgenic for inverted repeat constructs of the UCBSV ΔCP sequence, are resistant to the homologous virus, and in some cases, capable of resisting CBSD development in the presence of whitefly-transmitted CBSV and UCBSV.

4.3 Materials and Methods

4.3.1 Plant preparation and viral diagnostics

In vitro plantlets of cassava cultivar 60444, transgenic for pILTAB718 (FL-ΔCP: (FL-ΔCP: 7947-8840 nt) and pILTAB719 (N-terminal ΔCP: 7947-8348 nt) inverted repeat constructs of the UCBSV CP gene, were generated and analyzed as described previously (173, 203). Transgenic and non-transgenic control plantlets were micropropagated at the DDPSC onto Murashige and Skoog (129) basal medium supplemented with 20 g/l sucrose (MS2) solidified with 8 g/l Noble agar. Plantlets were confirmed to be free of CMD and CBSD by diagnostic PCR. To test for presence of CMD, DNA was extracted from *in vitro* leaves using DNeasy Plant Minikit (Qiagene, Cat 69104, Germantown, MD, USA). *African cassava mosaic virus* (ACMV) was detected using primer pair JSP001 (5'-ATGTCGAAGCGACCAGGAGAT-3') and JSP002 (5'-TGTTTATTAATTGCCAATACT-3') as described by Pita *et al.* (2001) (149), and *East African cassava mosaic virus* (EACMV) was detected using primers EAB555F (5'-TACATCGGCCTTTGAGTCGCATGG-3') and EAB555R (5'-CTTATTAACGCCTATATAAACACC-3'), as reported by Fondong *et al*. (2000) (53). For the detection of CBSD causal viruses, 3-5 mg of total RNA was extracted from *in vitro* leaves using Total RNA MiniKit (IBI Scientific, Peosta, IA, USA), and reverse transcription was performed using SuperScript™ III reverse transcriptase primed with oligo$(dT)_{20}$ (Invitrogen). The resulting cDNA was used as template for PCR amplification with the universal UCBSV/CBSV primers 10F; 5'-ATCAGAATAGTGTGACTGCTGG-3' and CBSV 11R; 5'-CCACATTATTATCGTCACCAGG-3', as described by Monger *et al*. (2001) (122). The accumulation of transgenically derived siRNAs within *in vitro* leaf tissue was confirmed in transgenic cassava plant lines by Northern blot analysis, following procedures described by Yadav *et al*. (2011) (203).

Apical shoots, 2-3 cm in length, were excised from Petri dish-cultured plantlets and transferred to 50-mL sterile polystyrene Falcon tubes (BD, Franklin Lakes, NJ, USA) containing 15 mL of MS2 medium solidified with 2.2 g/L Gelzan™ (Sigma Chemical Co., St Louis, MO, USA). One cutting was placed in each tube and cultured in a growth chamber at 28°C with a 14/10 hours photoperiod and 75 μmols/m^2/s light. After 24 days, tubes were packaged and dispatched with the appropriate import and phytosanitary documentations via courier to NaCRRI, Namulonge, Uganda.

4.3.2 Hardening and soil establishment within NaCRRI screenhouses

On arrival at NaCRRI, Namulonge, Uganda, tissue culture plantlets were acclimatized in diffuse light under polythene chambers within a biosaftey level II screenhouse at 100% humidity and 24-28°C. The caps of the 50 mL tubes were loosened by one-eighth of a turn each day to allow gaseous equilibration. After 4 days, the plantlets were removed from the tubes and adhering Gelzan™ dislodged from the roots by careful agitation in warm water. Each plantlet was potted individually into a 10-cm plastic pot containing Fafard 51 soilless compost (Hummert International, Earth City, MO, USA) and the medium was saturated with water. Pots were placed within the polythene chambers and 100% humidity was maintained by flooding the floor with water twice daily. After 3 days, the plantlets were moved to open benches within the same screenhouse and grown at a relative humidity of 50-60% and temperature of 26±2°C. Plants were watered as needed, and fertilizer was applied as Phyton 27 at 2 mL/L, and as 9-45-15 NPK Jack's Professional Water Soluble Peat Lite fertilizer (J.R. Peters Inc., Allentown, PA, USA) at 0.5 g/L twice weekly.

4.3.3 Field planting, plot design, maintenance and harvesting

Plants of cassava cultivar 60444, transgenic for pILTAB718 (seven entries) and pILTAB719 (seven entries), non-transgenic control cultivar 60444 and the Ugandan farmer-preferred landrace Ebwanateraka were used to establish a field trial under confined regulated conditions at NaCRRI, Namulonge, Uganda. Hardened plants at 8 weeks of age were transferred from the screenhouse to a CFT enclosure approximately 0.5 ha in size (51 m x 95 m). The field was prepared by ploughing twice with tractor-mounted equipment. Plants were removed from their pots and planted into 20-cm diameter, 15-cm deep holes, filled with water and backfilled with soil to establish a randomized complete block experimental design with three replications. The plot configuration was 20 plants per entry (four rows wide, each with five plants) at a spacing of 1 m x 1 m (10 000 plants/ha) (Fig. 4.1). Stems cuttings of cassava cultivar TME 204, growing in the vicinity of Namulonge research station and showing foliar symptoms of CBSD, were collected, confirmed by RT-PCR to be infected with both CBSV and UCBSV (Table 4.1) and planted to form a border around and between each experimental plot (Fig. 4.1). Plants were watered daily for 1 week after planting and three times per week thereafter. Hand weeding was performed on a fortnightly basis. No agrochemicals or fertilizers were applied to the field.

4.3.4 Data collection during cultivation and at harvest

Plants were visually assessed for CBSD and CMD incidence and severity monthly until harvesting at 11 months of age. CMD symptoms were scored on all plants within the experimental plots according to the 1-5 scale reported previously (178). Symptoms of CBSD were assessed by combining leaf and stem symptoms, as described in Fig. 4.2, on all plants within the experimental plots. Plants were also assessed for average numbers of adult whiteflies (*Bemisia tabaci*) present on the underside of the uppermost five, fully expanded leaves.

(A)



(B)

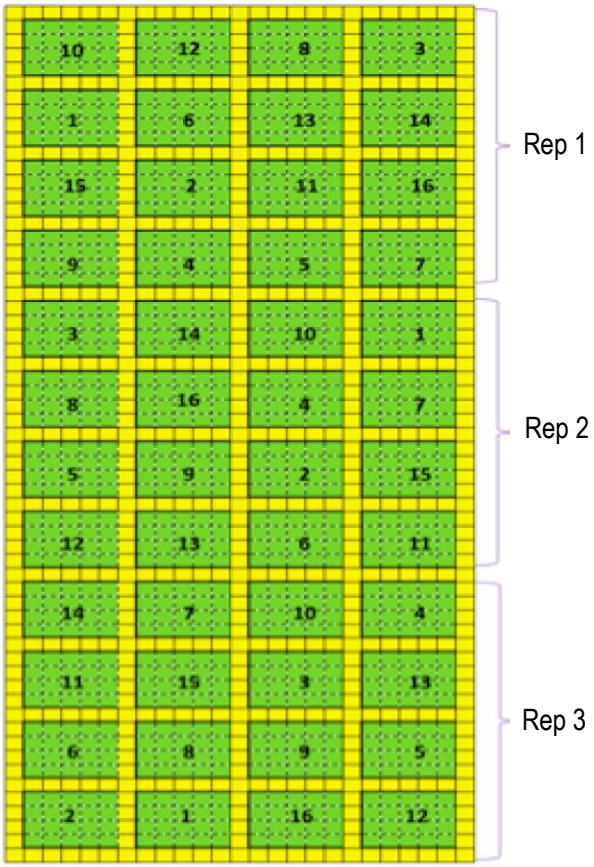


Fig. 4.1 Layout of confined field trial. (A) Plants at 3 months after planting at the research station at the National Crops Resources Research Institute (NaCRRI), Namulonge, Uganda, showing the experimental plants surrounded by a single border row of cultivar TME 204 (foreground). (B) Schematic representation of field trial plots designed to assess transgenic small interfering RNA (siRNA)-imparted resistance to cassava brown streak disease (CBSD). Each green square represents one 1-m^2 area containing one experimental plant, to generate 5 x 4 triplicated plots of the same clonal replicate. Each plot was surrounded on all sides by a single row of CBSD-infected plants of cassava cultivar TME 204 (yellow squares), collected locally and established from vegetative stem cuttings. The position of each transgenic and nontransgenic experimental plant line is shown within the plot design.

At 11 months after planting (MAP), the six innermost plants within each plot were harvested by digging with a hoe to dislodge storage roots from the soil. Stem and storage root materials were cut and weighed separately using a 200-kg scale (Hanson TM. Model No. 21, Chicago, IL, USA). "Marketable roots", defined as those cylindrical or conical-cylindrical storage roots at least 18 cm in length and 4 cm in width, of the kind usually suitable for sale in the marketplace or home consumption, were separated and weighed. Each marketable sized storage root was sliced transversely into five pieces and scored for presence and severity of CBSD using the 1-5 scale shown in Fig. 4.2. Each root was assigned the highest CBSD severity score observed within its slices. CBSD incidence was computed by expressing the number of CBSD-affected roots as a percentage of the total roots per plot. The severity of CBSD per plot was obtained by averaging the individual scores of diseased storage roots (those with scores of 2-5) within the triplicated plots. "Usable roots" were calculated from the total root weight as those with CBSD scores of 2 or less, assessed to be acceptable for sale or household consumption.

Table 4.1: Presence of UCBSV and CBSV within CBSD symptomatic cassava plants in the near vicinity of Namulonge research station, September 2010.

Sample	Sample location	Cultivar	CBSD foliar symptoms (1-5)	Virus species detected by RT-PCR
1	Nakesasa	Nase 10	3	CBSV + UCBSV
2	Nakesasa	TME 204	3	CBSV + UCBSV
3	JICA road	TME 204	2	CBSV + UCBSV
4	JICA road	Nase 3	3	CBSV
5	JICA road	TME14	3	CBSV + UCBSV
6	JICA road	Nase 3	3	CBSV + UCBSV
7	JICA road	Nase 3	3	CBSV
8	Sendusu	2961	3	CBSV
9	Sendusu	2961	3	CBSV
10	Sendusu	Akena	3	CBSV
11	Sendusu	TME 204	3	CBSV + UCBSV
12	Eastern gate*	TME 204	2	UCBSV

Leaf samples were collected from CBSV symptomatic plants at locations within 1 km of Namulonge research station in September 2010. CBSD leaf symptoms were scored on a 1-5 scale for increasing severity of yellow mottling. RT-PCR diagnostics were performed as described by Mbanzibwa et al (2011) (113).

4.3.5 Statistical analysis

Field data were analyzed using ARM8 (formerly called Agriculture Research Manager software, Gylling Data Management, Inc. Brookings, SD, USA) for analysis of variance (ANOVA), and entry means were separated using Duncan's new multiple range Test (P = 0.05).

4.3.6 Sampling and RT-PCR analysis for detection of UCBSV and CBSV in field grown plants

Unless otherwise stated, the youngest leaf showing CBSD symptoms, or equivalent non-infected tissue, was collected from field-grown plants using gloved hands, wrapped with aluminum foil and immediately placed in a container with liquid nitrogen. To collect storage root samples, whole roots sliced transversely into 1-2 cm thick discs at harvest and a representative piece was selected and preserved in liquid nitrogen. Total RNA was extracted from 0.15-0.20 g of leaf material or storage root parenchyma following the cetyltrimethylammonium bromide (CTAB) protocol (Lodhi et al., 1994), and cDNA synthesized using an Invitrogen SuperScript® III First-Strand RT-PCR Kit and oligo(dT) primers according to the manufacturer's instructions. Synthesized cDNA was subjected to UCBSV/CBSV detection by RT-PCR according to Mbanzibwa, *et al.* (2011), using primers CBSVDF2: 5'-GCTMGAAATGCYGGRTAYACAA-3'; CBSVDR: 5'-GGATATGGAGAAAGRKCTCC-3' which amplify ~437 and 343 nucleotides of the 3'-terminal sequences of the UCBSV and CBSV genomes, respectively. PCR products were resolved by electrophoresis on 1% agarose gel, fragments were visualized by UV radiation (302 nm) and gel images were recorded using a Nikon Coolpix P90 digital camera (Nikon Corporation, Tokyo, Japan).

A

Score	Leaf symptom description	Stem symptom description
1	No symptoms on leaves	No symptoms on stems
2	Mild/slight vein yellowing or chlorotic blotches on few leaves	Mild/slight brown streaks/lesions on green stem portions
3	Mild/slight vein yellowing or chlorotic blotches on many leaves	Mild/slight mild brown streaks/lesions on all stem portions
4	Severe/extensive vein yellowing or chlorotic blotches on few leaves	Severe/extensive brown streaks/lesions on all stem portions, slight defoliation with beginning of stem dieback
5	Severe/extensive vein yellowing or chlorotic blotches on many leaves	Severe/extensive brown streaks/lesions on all stem portions; severe defoliation with stem dieback

B

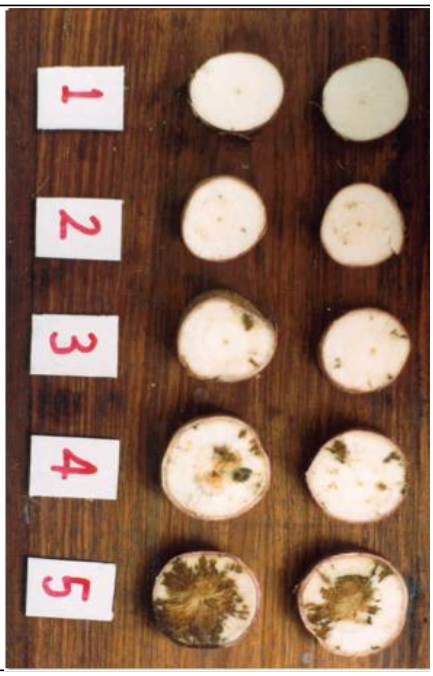
Score	Root symptom description	Pictorial
1	No symptoms on storage roots	
2	Less than 5% of storage root tissue is necrotic	
3	5-10% of storage root tissue is necrotic	
4	10-50% of storage root tissue is necrotic	
5	More than 75% of storage root tissue is necrotic	

Fig. 4.2 Scoring system utilized for the visual assessment of CBSD symptoms in plants. (A) Scoring system utilized for the visual assessment of CBSD symptoms on cassava leaves and stems. (B) Scoring criteria used for the assessment of storage root tissues and CBSD symptoms and associated severity scores on storage root slices.

4.4 Results

Prior to establishing a confined field trial (CFT) of transgenic cassava plants in November 2010, a survey was completed demonstrating that both CBSV and UCBSV were present at high incidences in the vicinity of the Namulonge research station (Table 4.1). Stem cuttings from plants of cultivar TME 204, were collected and used to establish infector row plants along all borders of each plot within the CFT (Fig. 4.1).

Seven independent lines of cassava cultivar 60444 transgenic for construct pILTAB718, and seven for pILTAB719, were selected for testing under field trial conditions. pILTAB718 carries a 894-bp inverted repeat construct for the CP of UCBSV and pILTAB719 carries a 402-bp N-terminal inverted repeat from the same CP sequence (147). The 14 transgenic events were selected from 197 originally produced on the basis of the prescence of one or two copies of the T-DNA (173), detectable expression of the expected siRNA signal and, in the case of pILTAB718, performance in previously reported glasshouse graft-inoculation studies (203). All plant lines were shown by PCR to be free of CBSD and CMD viral pathogens prior to export from St. Louis, MO, USA (results not shown). Northern blotting was also performed on these transgenic lines, confirming continued accumulation of siRNAs specific to the transgenically expressed viral sequence (Fig. 4.3). Sixty plants of each transgenic event, plus non-transgenic control plants of cultivar 60444 and the CBSD-susceptible landrace Ebwanteraka, were used to establish randomized triplicated plots under CFT conditions at the Namulonge research institute (NaCRRI). Details of field trial design are shown in Fig. 4.1.

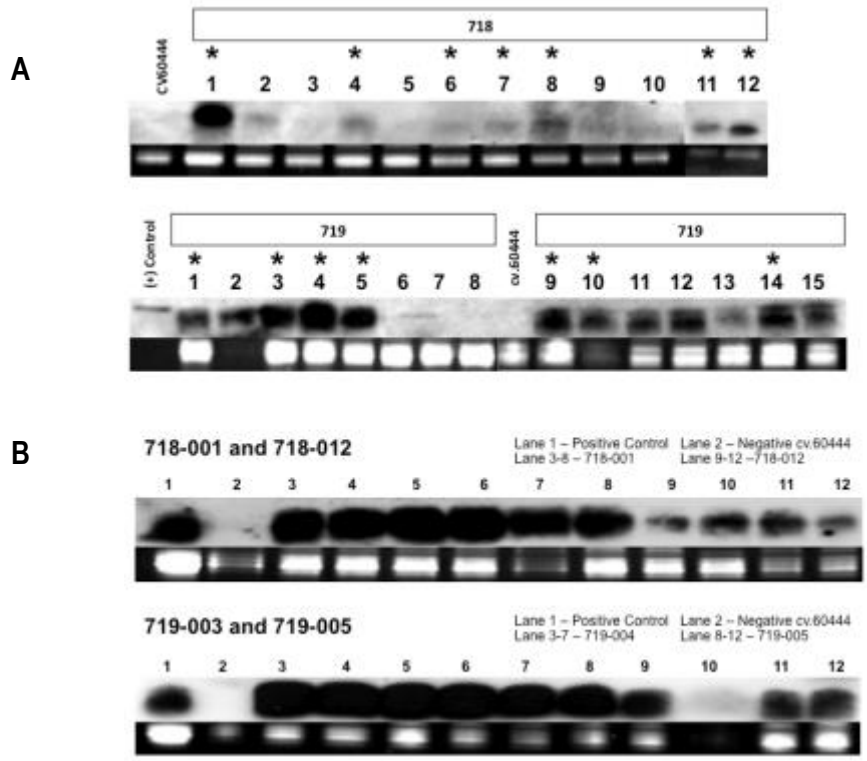


Fig. 4.3 Small interfering RNA (siRNA) accumulation from Ugandan Cassava brown streak virus (UCBSV) coat protein (CP) RNA interference (RNAi) sequences in plants transgenic for the full-length (FL)-ΔCP (pILTAB718) or the N-terminus ΔCP (pILTAB719) by Northern blotting. (A) Screening of transgenic events with a CP-specific probe for siRNA signals in leaves of *in vitro* plantlets. Asterisked plants were selected for the field trial in Uganda. (B) Example of siRNA expression from in vitro plants of field candidate transgenic lines prior to export from Donald Danforth Plant Science Center (DDPSC), St Louis, MO, USA to Uganda.

4.4.1 Development of disease symptoms on aerial tissues

Plants were assessed for the number of whitefly vectors and development of CBSD and CMD symptoms on a monthly basis for the 11-month duration of the CFT. Whitefly populations increased on all control plants of cultivar 60444 and transgenic lines of pILTAB718 and pILTAB719 from the time of planting, reaching an average of 75 to 115 per plant by 5 months after planting (MAP) (Figs. 4.5C, 4.6A, B). Elevated whitefly populations preceded the onset of CMD and CBSD (Fig. 4.6, Fig. 4.4). CMD

symptoms remained mild to moderate until 6-7 MAP (Fig. 4.4), and had little impact on the ability to observe CBSD symptoms on leaves and stems.

The development of CBSD was observed as distinct yellow-coloured mottling on mature leaves and as dark lesions on green and semi-woody portions of the stems (Fig. 4.5A, B). Symptoms appeared first on non-transgenic plants of cultivars 60444 and Ebwanateraka at 2-3 MAP, and attained 100% incidence by 6-7 MAP (Fig. 4.6C, D). Transgenic plants of pILTAB719 behaved in a similar manner, such that, by 10 MAP, all pILTAB719 transgenic plants showed typical signs of CBSD on leaves and stems (Fig. 4.6D). In contrast, transgenic lines expressing the FL-ΔCP inverted repeat construct (pILTAB718) showed a delay in the onset of CBSD compared with controls. Plants of lines 718-002, 718-003, 718-004, 718-006 and 718-007 remained asymptomatic until 5 MAP, and then started to display CBSD to reach 80-100% disease incidence by 8 MAP. In lines 718-001 and 718-005, symptom development was suppressed for the whole period for the CFT. In line 718-005, final foliar CBSD incidence was 48.4% (compared with 100% for control), whereas, in transgenic line 718-001, only one plant out of 60 (1.7%) developed visible CBSD symptoms on aerial tissues over the 11-month field trial (Fig. 4.6C). When plants were assessed for severity of CBSD symptoms (Fig. 4.6E, F; Fig. 4.2), those transgenic for pILTAB719 were no different from the controls, reaching an average severity score of 2.50-3.25 by 7 MAP before increasing to a maximum of 4.5-5.0 by the time of harvest. The severity of CBSD symptoms on diseased plants of transgenic pILTAB718 lines developed in a similar manner, except in events 718-001 and 718-005. In the latter, symptom severity did not develop above an average score of 3.0, whereas CBSD symptoms on the single symptomatic plant of line 718-001 were restricted to stem tissues only, where they reached a maximum severity of 3 by 7 MAP (Fig. 4.6E).

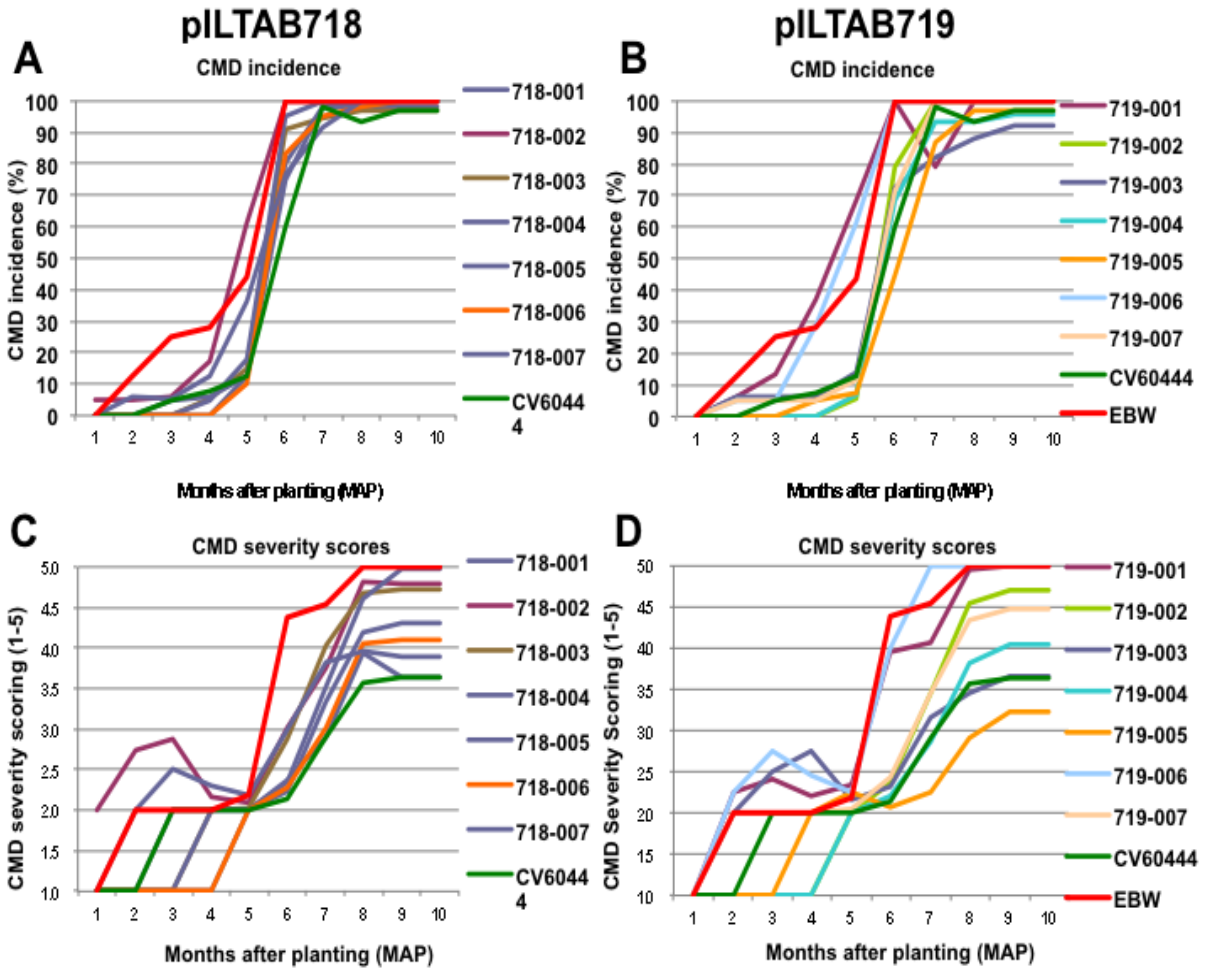


Fig. 4.4 Development of cassava mosaic disease (CMD) on plants within a confined field trial designed to assess transgenic small interfering RNA (siRNA)-imparted resistance to cassava brown streak disease (CBSD). (A, B) Incidence of CMD symptoms on experimental plants over the 11-month trial period. (C, D) Severity of CMD symptoms on experimental plant lines as determined using a visual score of 1–5. The transgenic cassava lines for pILTAB718 are shown in (C) and those for pILTAB719 are shown in (D), with the cultivars 60444 and Ebwanateraka (EBW) as nontransgenic controls.



Fig. 4.5 Cassava brown streak disease (CBSD) symptoms and whitefly vectors observed on leaves and stems of plants within the confined field trial. (A) Distinct yellow mottling caused by CBSD on mature leaves. (B) Dark brown coloured necrotic lesions seen on stems of CBSD-infected plants compared with noninfected material. (C) Significant whitefly populations present on the underside of younger leaves.

4.4.2 RT-PCR for detection of CBSV and UCBSV in plants within the CFT

Leaf samples were collected from symptomatic plants at 5.5, 7 and 11 MAP, and diagnostic RT-PCR was performed to detect the presence of UCBSV and CBSV. The primer set described previously by Mbanzibwa *et al.* (2011) (110) was employed, allowing simultaneous diagnostic amplification of both virus species (Fig. 4.7). Data are presented in Tables 4.2A-C, showing the number of plants sampled, occurrence of RT-PCR-detectable infections and number of plants found to be carrying single and dual infections with the CBSD causal pathogens. At 5.5 MAP, 248 symptomatic samples were analyzed by RT-PCR, with 232 (93.5%) testing positive for presence of one or both CBSD viruses. CBSV was found to be present at high frequency throughout the season, with non-transgenic controls showing 91% infection with this virus as early as 5.5 MAP. Levels of UCBSV were somewhat lower, with 23%, 91% and 63% of non-transgenic control plants sampled shown to be infected with this pathogen at 5.5, 7 and 11 MAP, respectively. Within the non-transgenic controls, single infections with UCBSV did not

exceed 12%, whereas single infections with CBSV reached 76%. Dual infection rates increased over the duration of the CFT, rising from 15% to 82% and 54% of PCR-positive plants at 5.5, 7 and 11 MAP, respectively (Table 4.2).

Important differences were seen for infection rates with CBSD causal viruses between transgenic plants and controls, and between plants transgenic for pILTAB718 and pILTAB719. Non-transgenic control plants of cultivars 60444 and Ebwanteraka were found to be infected with UCBSV at 23% and 42%, respectively, by 5.5 MAP, but only two plants out of 155 tested from transgenic lines pILTAB718 and pILTAB719 carried RT-PCR-detectable levels of this virus (Table 4.2A). Notably, all PCR-positive pILTAB719 transgenic plants tested at this time were found to be infected with CBSV alone. In contrast, only three samples (all from line 718-003) were confirmed to be carrying this virus species in plants engineered with the inverted repeat sequence for the FL-ΔCP of UCBSV. These data correlate with information from visual assessment of disease symptoms, which indicated that plants transgenic for pILTAB719 were diseased by 5.5 MAP, with the vast majority of pILTAB718 remaining free of CBSD at this time point (Fig. 4.6).

Frequencies of virus detection by RT-PCR from leaf samples of symptomatic plants decreased as the age of the plants increased, falling from 93% at 5.5 MAP to 66% at 11 MAP. Nevertheless, across 284 samples from symptomatic leaves tested by RT-PCR at 7 and 11 MAP, no detectable infections with UCBSV were found within transgenic plants, whereas non-transgenic controls were confirmed to be carrying this virus at frequencies of 91% at 7 MAP and 66% by 11 MAP (Table 4.2C). Conversely, incidence of CBSV increased in transgenic lines, such that, by the end of the trial, symptomatic plants of controls, pILTAB719 and pILTAB718 were confirmed to be infected with CBSV alone.

Thirty-one plants (52%) and 59 plants (98%) of transgenic lines 718-005 and 718-001, respectively, were observed to remain free of CBSD leaf and stem symptoms for the duration of the CFT. To

investigate this further, sampling was performed on a mixture of symptomatic and asymptomatic plants at 7 and 9.5 MAP. At 7 MAP, four out of nine asymptomatic 718-001 plants tested positive for the presence of CBSV, but all were negative for UCBSV (Table 4.2B). At 9.5 MAP, a more detailed study was performed in which RNA was extracted from leaves of different ages on the same plant and subjected to diagnostic RT-PCR (Table 4.3). As predicted by data from RT-PCR analysis at 7 MAP (Table 4.2B), a plant from line 719-005 was found to be infected with CBSV only, whereas, for plants of transgenic line 718-001, leaves at all positions were determined to be free of detectable CBSD viruses. The latter included the one plant of this transgenic line that showed visible CBSD-like symptoms on its stem tissues (Fig. 4.6). Further analysis was carried out on leaves from 30 plants (one symptomatic and 29 asymptomatic) of 718-001 at 11 MAP. At this time point, neither CBSV nor UCBSV could be detected in these plants (Table 4.2C).

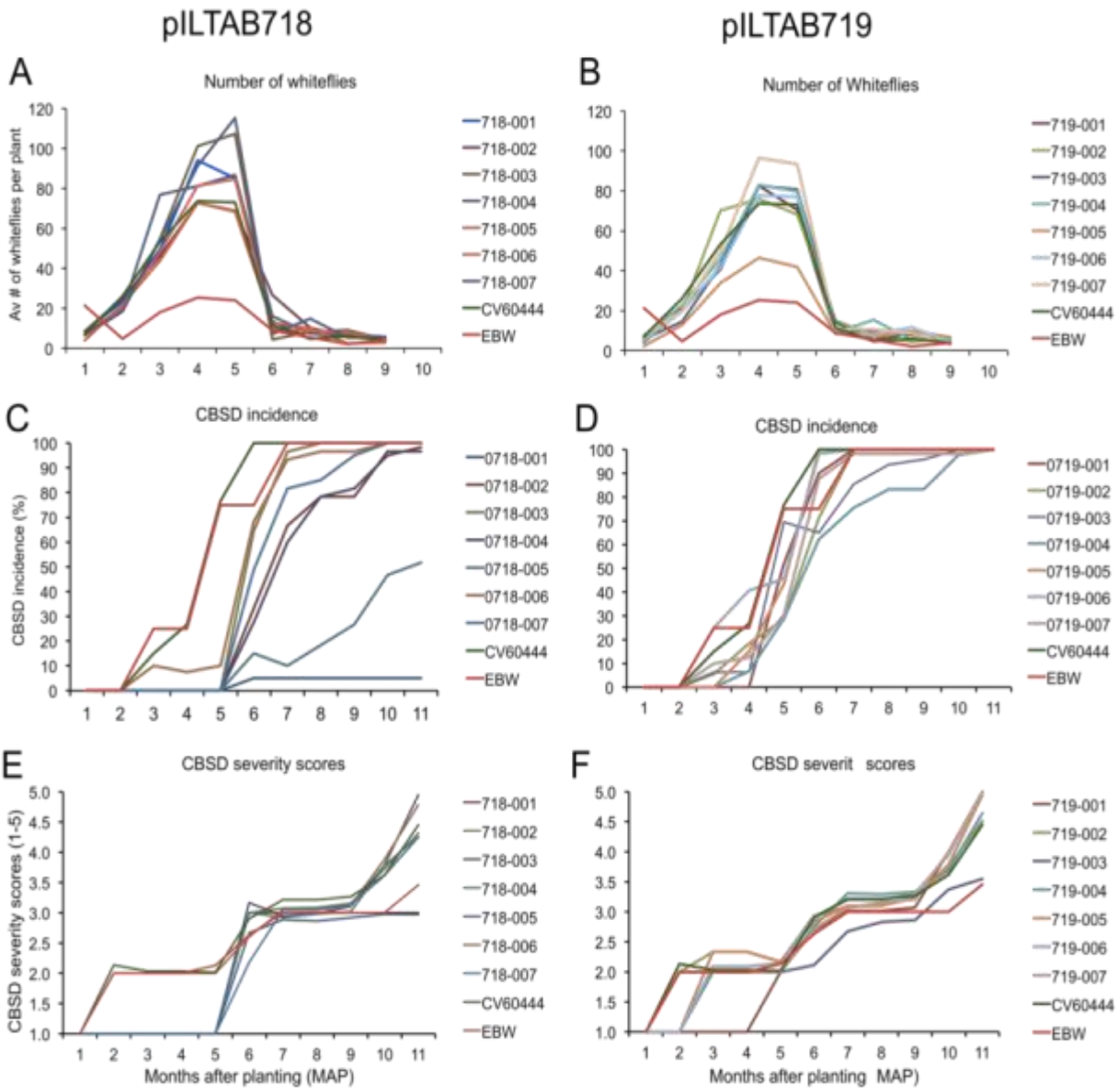


Fig. 4.6 Development of whitefly populations and visually assessed cassava brown streak disease (CBSD) symptoms on shoots of transgenic and control plants over the 11-month duration of the confined field trial. (A, B) Average number of whiteflies per plant observed on the undersides of the five uppermost leaves of transgenic pILTAB718 (A) and pILTAB719 (B) plants and nontransgenic control plants. (C, D) CBSD incidence on nontransgenic control 60444 and Ebwanateraka (EBW) cultivars and across 60 clonal replicates of seven independent transgenic lines for the inverted repeat constructs pILTAB718 (FL-ΔCP) (C) and pILTAB719 (N-terminal ΔCP) (D). (E, F) Severity of CBSD symptoms scored on a scale of 1–5 over time in nontransgenic control cultivars 60444 and EBW and across seven lines transgenic for the inverted repeat constructs pILTAB718 (FL-ΔCP) (E) and pILTAB719 (N-terminal ΔCP) (F). CP, coat protein; FL, full length.

Table 4.2: Incidence and diagnostic detection of cassava brown streak disease (CBSD) within a confined field trial of transgenic and nontransgenic cassava lines at Namulonge research station

Plant line	No. symptomatic plants (total)	No. symptomatic plants analyzed by RT-PCR	No. PCR positive samples	Virus species detected		
				Single infection UCBSV only (%)	Single infection CBSV only (%)	Dual infection UCBSV+CBSV (%)
(A) Incidence and diagnostics for presence of UCBSV and CBSV at 5.5 months after planting						
60444	44 (60)	37	34	3 (8.8)	26 (76.5)	5 (14.7)
EBW	21 (24)	45	43	18 (41.9)	19 (44.2)	6 (13.9)
718-001	0 (60)	na	na	na	na	na
718-002	1 (60)	1	0	0 (0)	0 (0)	0 (0)
718-003	4 (57)	4	4	0 (0)	3 (75.0)	1 (25.0)
718-004	0 (60)	na	na	na	na	na
718-005	0 (60)	na	na	na	na	na
718-006	8 (60)	8	6	0 (0)	6 (100)	0 (0)
719-001	31 (58)	31	26	0 (0)	26 (100)	0 (0)
719-002	16 (52)	16	16	0 (0)	16 (100)	0 (0)
719-003	23 (49)	23	23	0 (0)	23 (100)	0 (0)
719-004	14 (45)	14	13	0 (0)	13 (100)	0 (0)
719-005	23 (60)	23	22	0 (0)	21 (95.5)	1 (4.5)
719-006	22 (59)	22	22	0 (0)	22 (100)	0 (0)
719-007	21 (59)	21	21	0 (0)	21 (100)	0 (0)
Total		248	232 (93.5%)	21 (9.1)	198 (85.3)	13 (5.6)
(B) Incidence and diagnostics for presence of UCBSV and CBSV at seven months after planting						
60444	60 (60)	13	11	1 (9.1)	1 (9.1)	9 (81.8)
EBW	24 (24)	12	11	1 (9.1)	0 (0)	10 (90.9)
718-001	1 (60)	10	4	0 (0)	4 (100)	0 (0)
718-002	40 (60)	11	8	0 (0)	8 (100)	0 (0)
718-003	55 (59)	17	11	0 (0)	11(100)	0 (0)
718-004	35 (60)	16	14	0 (0)	14 (100)	0 (0)
718-005	6 (60)	12	6	0 (0)	6 (100)	0 (0)
718-006	56 (60)	17	16	0 (0)	16 (100)	0 (0)
718-007	46 (57)	15	12	0 (0)	12 (100)	0 (0)
719-001	57 (58)	13	11	0 (0)	11 (100)	0 (0)
719-002	51 (52)	11	11	0 (0)	11 (100)	0 (0)
719-003	42 (49)	12	10	0 (0)	10 (100)	0 (0)
719-004	33 (45)	10	10	0 (0)	10 (100)	0 (0)
719-005	60 (60)	10	6	0 (0)	6 (100)	0 (0)
719-006	59 (59)	14	11	0 (0)	11 (100)	0 (0)
719-007	58 (59)	14	14	0 (0)	14 (100)	0 (0)
Total		207	166 (80.2%)	2 (1.2)	145 (87.3)	19 (11.4)
(C) Incidence and diagnostics for presence of UCBSV and CBSV at eleven months after planting						
60444	60 (60)	30	26	3 (11.5)	9 (34.6)	14 (53.8)
EBW	24 (24)	12	5	0 (0)	3 (60.0)	2 (40.0)
718-001	1 (60)	1	0	0 (0)	0 (0)	0 (0)
718-002	58 (60)	12	5	0 (0)	5 (100)	0 (0)
718-003	58 (59)	30	23	0 (0)	23 (100)	0 (0)
718-004	59 (60)	12	4	0 (0)	4 (100)	0 (0)
718-005	29 (60)	30	13	0 (0)	13 (100)	0 (0)
718-006	60 (60)	12	7	0 (0)	7 (100)	0 (0)
718-007	57 (57)	12	6	0 (0)	6 (100)	0 (0)
719-001	58 (58)	12	4	0 (0)	4 (100)	0 (0)
719-002	51 (52)	12	8	0 (0)	8 (100)	0 (0)
719-003	49 (49)	12	10	0 (0)	10 (100)	0 (0)
719-004	46 (46)	12	8	0 (0)	8 (100)	0 (0)
719-005	60 (60)	12	8	0 (0)	8 (100)	0 (0)
719-006	59 (59)	12	8	0 (0)	8 (100)	0 (0)
719-007	58 (59)	12	10	0 (0)	10 (100)	0 (0)
Total		235	145 (61.2%)	3 (2.1)	126 (86.9)	16 (11.0)

na, not applicable. 60444, wild-type plants of cassava cultivar 60444; EBW, wild-type plants of Ebwanateraka. Plants were planted in a confined field trial as triplicated randomized blocks of 20 plants. 718, plant lines transgenic for the inverted repeat construct pILTAB718 consisting of the delta full length of the UCBSV coat protein (CP); 719, plant lines transgenic for the inverted repeat construct pILTAB719 consisting of the N-terminus of the UCBSV CP. Leaf samples were collected from transgenic and control cassava plant lines showing CBSD symptoms and used as a source of RNA for CBSD virus detection; 1 mL of RNA was used in reverse transcriptase-polymerase chain reactions (RT-PCR) with the primer pair CBSVDF2/CBSVDR (113) which detects and distinguishes between CBSV and UCBSV. *In this case, one symptomatic and nine asymptomatic plants were sampled. †In this case, one symptomatic and 29 asymptomatic plants were sampled. ‡Total positive samples showing infection with Ugandan Cassava brown streak virus (UCBSV) alone or UCBSV and Cassava brown streak virus (CBSV). §Total positive samples showing infection with UCBSV alone or UCBSV and CBSV.

Table 4.3: RT-PCR analysis for presence of CBSD causal viruses in leaves from various positions on transgenic and non-transgenic cassava plants 9.5 months after planting

Plant line	Plant number	CBSD severity	Leaf position	Virus present determined by RT-PCR
60444	5	3	1 9 15 27	UCBSV UCBSV + CBSV UCBSV + CBSV UCBSV + CBSV
60444	14	3	1 8 15 23	CBSV UCBSV + CBSV UCBSV + CBSV UCBSV + CBSV
60444	18	3	1 8 15 24	CBSV CBSV UCBSV + CBSV UCBSV + CBSV
719-12	17	3	1 8 15 24	CBSV CBSV CBSV CBSV
718-005	18	1	1 8 15 23	- - - -
718-001	5	3 (stem lesions only)	1 9 15 24	- - - -
718-001	6	1	1 8 15 24	- - - -
718-001	11	1	1 8 15 25	- - - -
718-001	18	1	1 8 15 23	- - - -
718-001	15	1	1 9 15 23	- - - -

Position of sampled leaf is indicted as that below from the first fully expanded leaf (position 1), downwards. CBSD severity was determined as shown in Fig. 4.1.

4.4.3 Incidence and severity of CBSD in storage roots and impact on agronomic performance

The field trial was harvested at 11 MAP. Plants were uprooted and storage roots sliced open to allow visual scoring on a 1-5 scale for presence and severity of the brown necrotic lesions typical of CBSD in these organs (Fig. 4.2). Storage roots from 16-18 plants (approximately six per triplicated plot) were assessed in this manner, and data were collected for total root production and aerial biomass. No significant differences were observed for root yields, number of roots or total biomass between 60444 control plants and the transgenic lines (Fig. 4.9).

The impact of CBSD on storage root quality was severe. Of the roots harvested from non-transgenic controls of cultivar 60444, 103 out of the 115 (90%) showed damage caused by CBSD, and disease symptoms were severe in these roots, averaging severity scores of 4.0-4.5 (Fig. 4.8A-C, Fig. 4.9E). The frequency of CBSD root symptoms was not significantly different between controls and transgenic lines of pILTAB719 and pILTAB718, except for plants of lines 718-001 and 718-005. At an average of 63%, line 718-005 differed significantly from the 60444 controls at $P>0.05$, whereas transgenic event 718-001 was significantly different from the controls and all other transgenic lines tested at $P>0.001$ (Fig. 4.9A-C). Importantly, storage roots from the latter event showed signs of brown lesions in only five of the 116 roots (4.4%) assessed, with symptom severity within these seen to be mild, at an average score of 2 (Fig. 4.8A and 5B, Fig. 4.10).

Diagnostic RT-PCR performed on total RNA isolated from peeled storage roots confirmed the presence of CBSV in secondary xylem tissues from non-transgenic cultivar 60444 and pILTAB719 transgenic lines (Fig. 4.7B). It is not known why UCBSV was not detected in storage roots of cultivar 60444, as these plants were diagnosed as infected with both viruses in their leaf tissues (Table 4.2). Interestingly, roots from four plants of the spreader row cultivar TME 204 indicated the presence of UCBSV, but not

CBSV, suggesting that the inability to detect UCBSV was not caused by technical failure, but may reflect some biological control of virus distribution within the storage roots (Fig. 4.7B). Studies are ongoing to test this hypothesis. Storage roots harvested from plants of transgenic line 718-001 were free of detectable levels of CBSV and UCBSV.

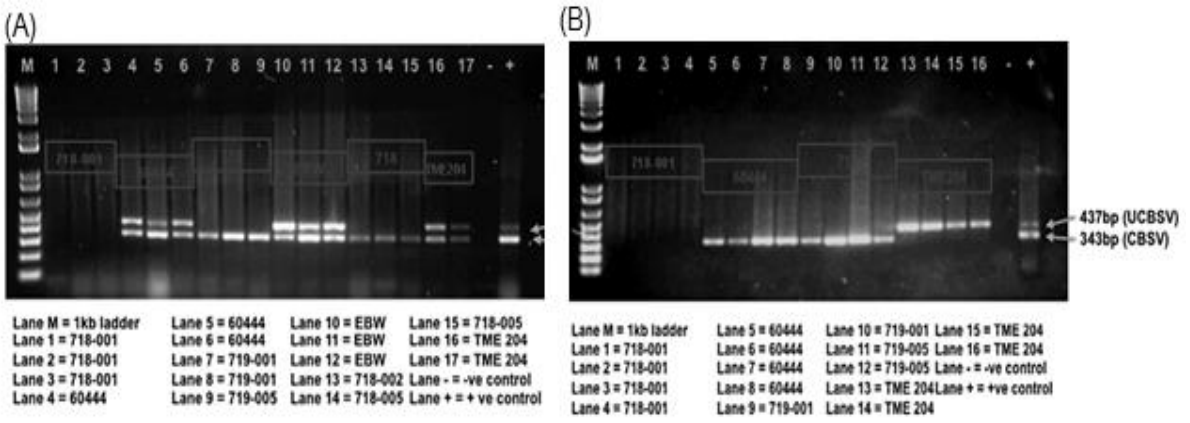


Fig. 4.7 Reverse transcriptase-polymerase chain reaction (RT-PCR) diagnostics for simultaneous detection of *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV). (A) Presence of CBSV and UCBSV in leaf tissues of a subset of field-grown transgenic and nontransgenic plants 11 months after planting. (B) Presence of CBSV and UCBSV in a subset of storage root tissues harvested from field-grown transgenic and nontransgenic plants 11 months after planting. EBW, Ebwanateraka.

The distribution of CBSD scores for storage roots across the transgenic lines and controls at harvest are shown in Fig. 4.10A. If roots scoring 1 (symptom free) and 2 (minimal disease and still usable) are combined, 100% and 50% of harvested roots from lines 718-001 and 718-005 were marketable, whereas less than 10% of the controls were fit for consumption (Fig. 4.10B). When combined with root yields from the CFT, the "usable yield" was calculated, defined as the fresh weight of storage roots with a CBSD symptom score of 2 or less. Using this assessment, the non-transgenic cultivar 60444 yielded only 2.5 t/ha, whereas the two lines of pILTAB719 and four lines transgenic for pILTAB718 achieved usable yields above 5 t/ha, with 718-001 delivering a useable yield equivalent to approximately 20 t/ha (Fig. 4.9F).

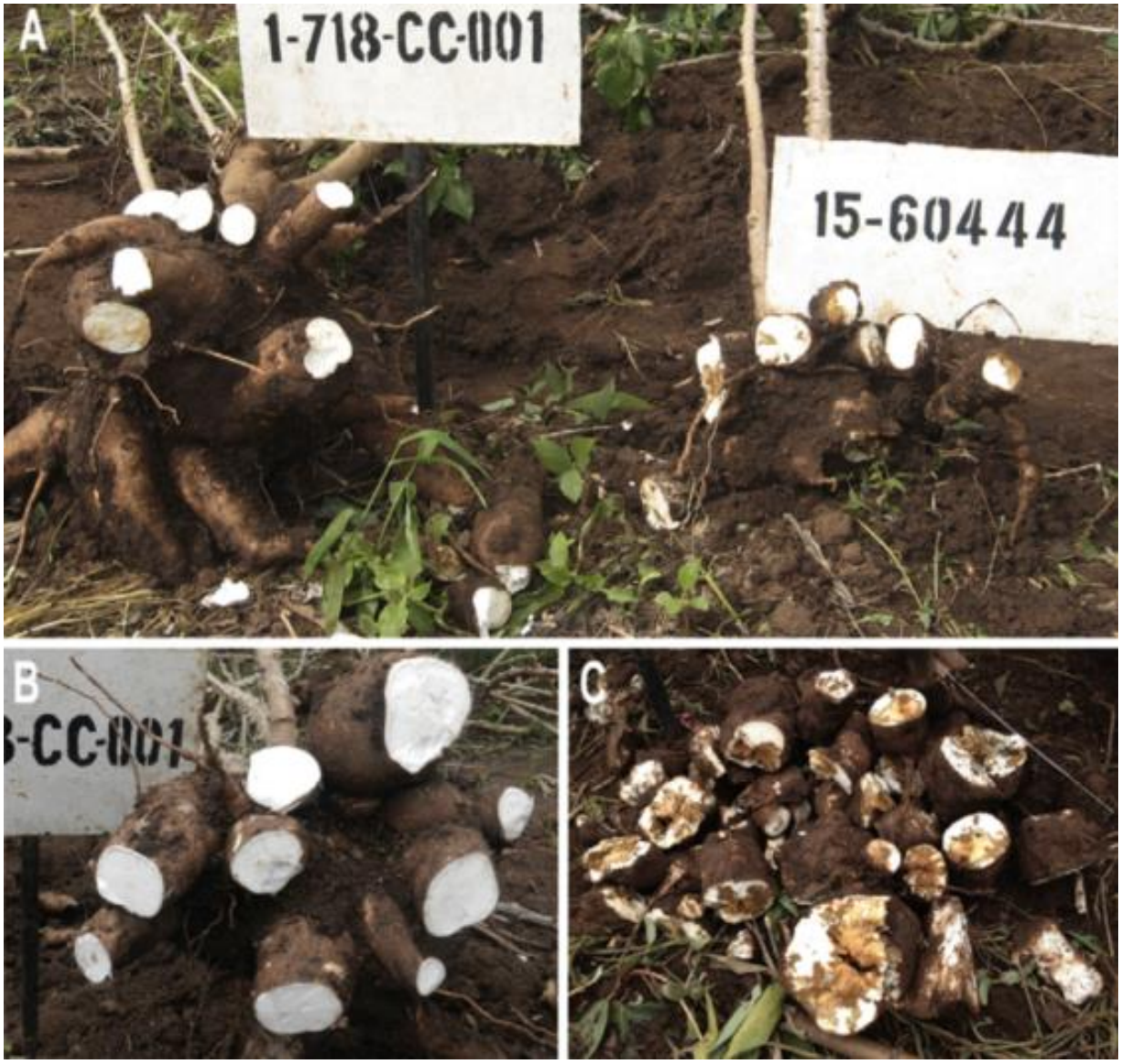


Fig. 4.8 Cassava brown streak disease (CBSD) symptoms on storage roots and stems at harvest, 11 months after planting. (A) Uprooted storage roots from a plant of line 718-001 transgenic for the inverted repeat sequence ΔFL-CP of Ugandan Cassava brown streak virus (UCBSV) beside a plant of the nontransgenic cultivar 60444. (B) Storage roots collected from plants of transgenic line 718-001 showing minimal damage caused by CBSD. (C) Storage roots of nontransgenic cultivar 60444 showing severe damage caused by CBSD.

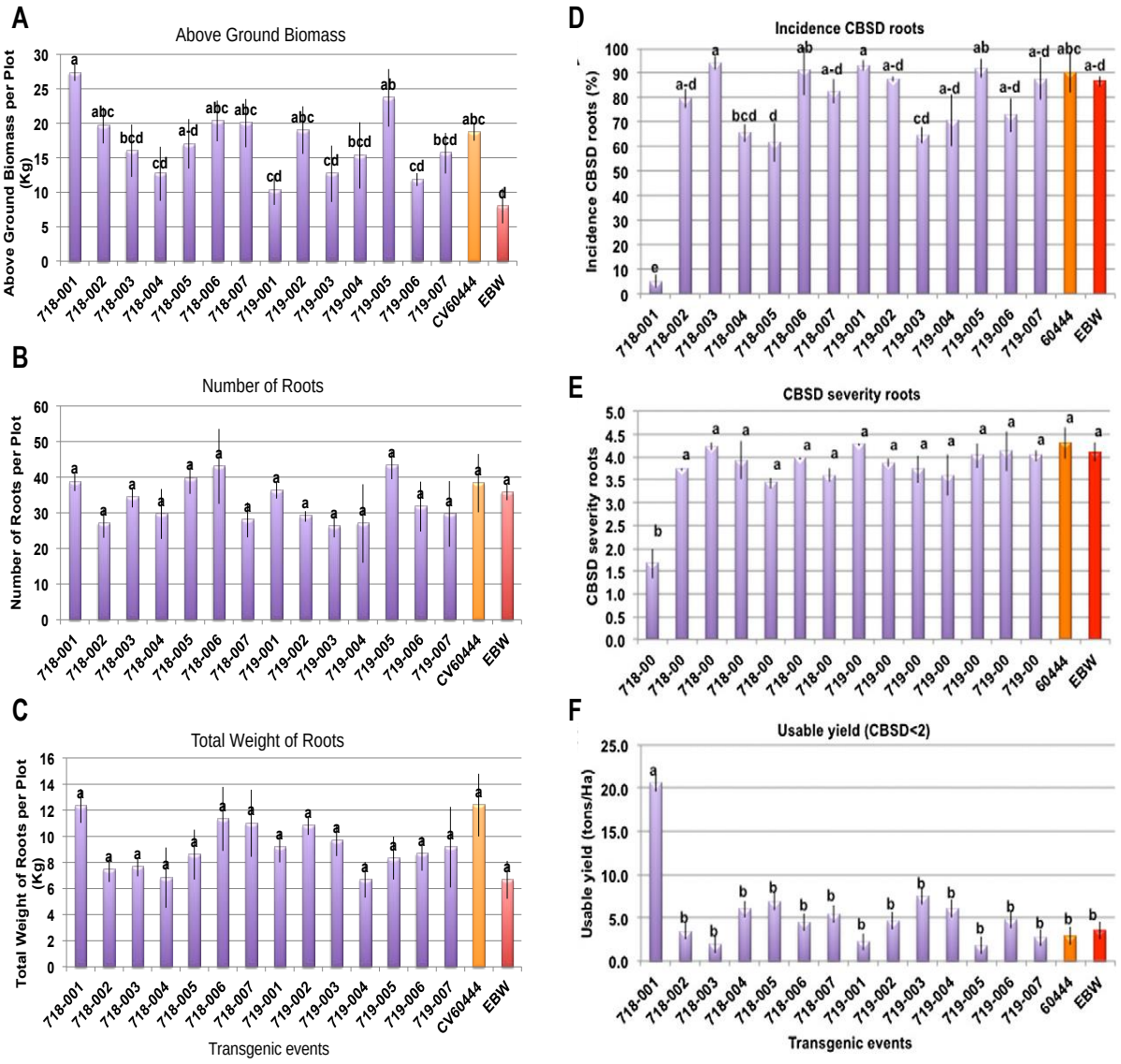


Fig. 4.9. Harvest data of plants from CBSD CFT at 11 months after planting. (A) Above ground biomass. (B) Number of roots. (C) Total root weight. (D) Incidence of CBSD in storage roots. (E) Average severity of CBSD symptoms in diseased roots. Data are shown as average values from the six innermost plants of each triplicated plot ± the standard error. (F) Yield of usable roots within transgenic and nontransgenic plots. Data are shown as average values from the six innermost plants of triplicated plots ± the standard error. Values assigned different letters are significantly different at P > 0.05 as determined by Duncan's new multiple range test. EBW, Ebwanateraka.

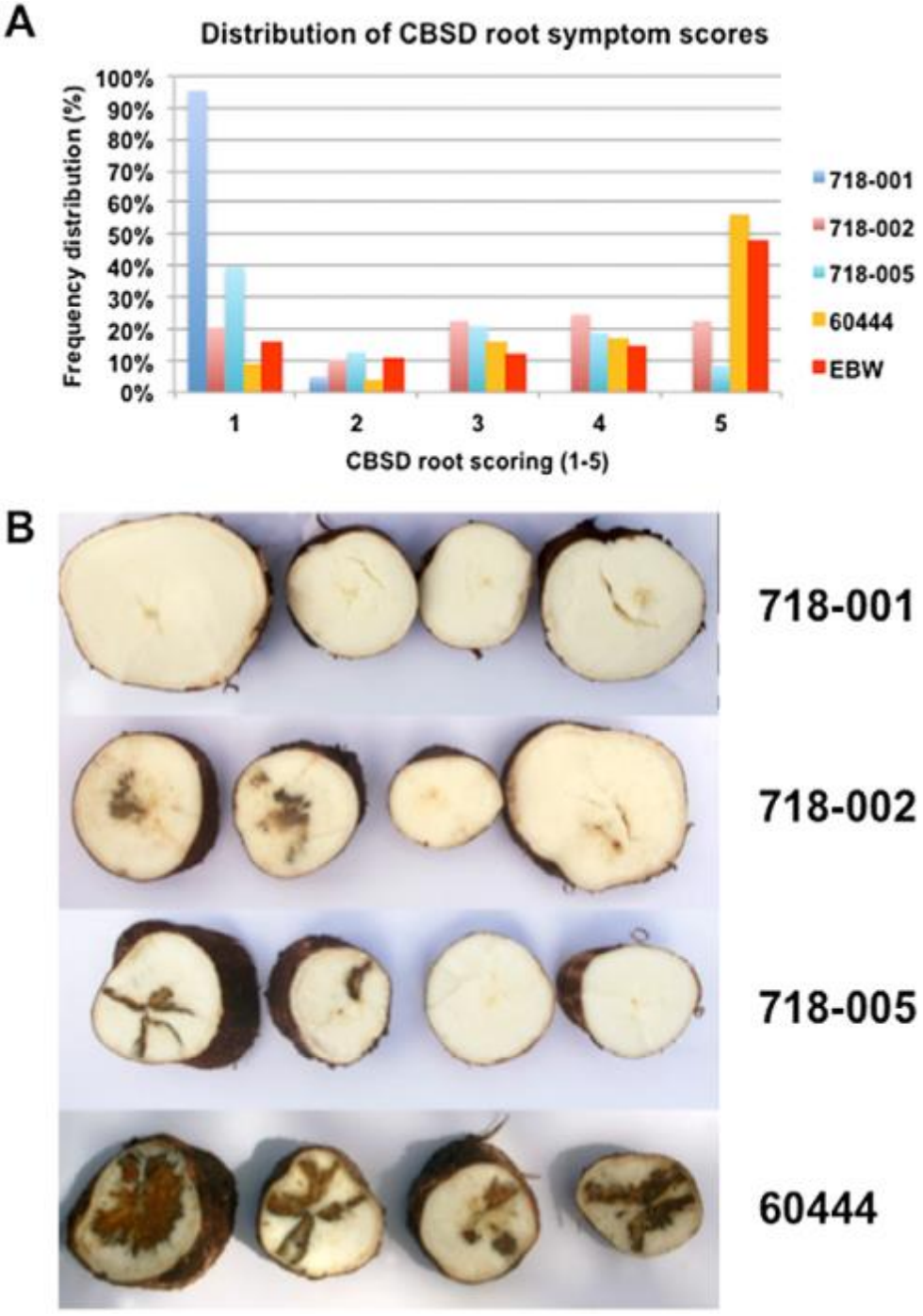


Fig. 4.10 Cassava brown streak disease (CBSD) symptom distribution in roots from harvested transgenic and nontransgenic plants. (A) Distribution of CBSD symptom values for storage roots of the three lines transgenic for pILTAB718, control cultivar 60444 and the landrace Ebwanateraka (EBW). (B) Photographs of typical CBSD severity in slices of different roots of the three lines transgenic for pILTAB718 and the control cultivar 60444.

4.5 Discussion

Plants of cassava cultivar 60444 were modified to express inverted repeat constructs of the FL-ΔCP or N-terminal ΔCP sequences from UCBSV (203), and were confirmed to be accumulating the expected siRNAs. A CFT under high disease pressure was successfully completed to assess both RNAi-mediated approaches for controlling CBSD under conditions of natural, whitefly-vectored transmission at NaCRRI, Namulonge, Uganda. Infection of non-transgenic controls reached 100% within 6 MAP; moreover, transgenic plants of pILTAB719, expressing siRNAs from the N-terminal ΔCP region of UCBSV, developed typical leaf and stem symptoms at a frequency and severity no different from the controls. In contrast, plant lines genetically modified with the ΔFL-CP displayed a 4-month delay in development of the first CBSD symptoms, with two events, 718-001 and 718-005, having 98% and 52% of their clonal replicates, respectively, remaining asymptomatic over the 11-month duration of the CFT (Fig. 4.6).

Molecular diagnostic analysis revealed almost complete exclusion of UCBSV in transgenic lines, whether as single infections with this pathogen or as dual infections in combination with CBSV (Table 4.2). Therefore, both UCBSV-ΔCP siRNA-expressing genetic constructs were capable of suppressing the replication of the homologous UCBSV pathogen to below RT-PCR detectable levels. This was evident from RT-PCR diagnostics of leaves from symptomatic and asymptomatic transgenic plants performed at four time points during the CFT, for which 393 out of 395 samples, representing both gene constructs, were negative for the presence of UCBSV (Table 4.2C). It was apparent, therefore, that CBSD symptoms in transgenic plants were caused by single infections with CBSV, and this was confirmed at the molecular level, with symptomatic plants of pILTAB719 and pILTAB718 found to be infected with this pathogen only (Table 4.2A-C). In addition, lack of CBSD symptoms on clonal plants

of transgenic line 718-001 was correlated with absence of UCBSV and CBSV, as determined by RT-PCR analysis. Apart from four plants showing a positive signal for presence of CBSV at 7 MAP, no detectable levels of UCBSV or CBSV were seen within its leaf tissues (Tables 4.2C, 4.3).

The data presented here confirm the information reported previously for RNAi-mediated resistance against CBSD by the same inverted repeat constructs under controlled growth conditions (110, 203). In glasshouse graft-inoculation studies of cassava, all lines transgenic for pILTAB718 proved to be 100% resistant to UCBSV, with plants remaining symptom free and RT-PCR negative for virus transmission when scions were grafted onto rootstocks infected with the homologous UCBSV species (203). Likewise, tobacco plants genetically modified with constructs pILTAB718 and pILTAB719 (and for an inverted repeat C-terminal version of the ΔCP) provided resistance to challenge with UCBSV, but only events expressing the FL-ΔCP (pILTAB718) provided fully effective cross-protection against infection with strains of the nonhomologous CBSV pathogen (110). Data from the field studies presented here corroborate these results, confirming that, although siRNAs from the N-terminal CP of UCBSV are sufficient to control this virus species, the FL-ΔCP sequence is required to generate robust resistance to both CBSD causal viruses.

Based on information from previously published reports describing RNAi-mediated control of RNA viruses (28, 177), inverted repeat constructs derived from the CP sequence of UCBSV were predicted to generate resistance to this virus only. Experimental evidence from the laboratory and the field studies reported here confirms, however, that a significant level of cross-protection against CBSV is also achieved. Although the CP nucleotide sequences of the two viruses vary by 30.5% (CBSV-[UG;Nam;04].CP.HM181930; CBSV-[TZ;Nal].CP.HM346954), siRNAs transgenically produced by the inverted repeat UCBSV FL-ΔCP appear to be capable of degrading the CBSV CP messenger. This is

not the case for those generated by the N-terminal RNAi construct. It is hypothesized that the siRNA population from the FL-ΔCP RNAi construct either has special sequence properties that allow at least some population to recognize the CBSV viral RNA, or that this longer fragment is differentially and better processed to siRNAs by the plant Dicers. Effective cross-protection appears to be associated with the quantity of siRNA production. In transgenic tobacco plants, cross-resistance to CBSV was correlated with plant lines possessing the highest levels of FL-ΔCP-derived siRNA accumulation (110). It should be noted that line 718-001, reported here to display greatest protection against both CBSV and UCBSV under field conditions, was seen to have a strong siRNA signal compared to other plant lines transgenic for this RNAi construct (Fig. 4.3). Further research is ongoing to better understand the mechanisms behind these results.

The suppression of aerial CBSD symptoms and RT-PCR-detectable virus in leaf tissues of plants lines 718-001 and 718-005 was correlated with reduced disease incidence in storage roots at the time of harvest. This result is critical, as it is damage to the storage roots that affects usable yields for farmers. Non-transgenic control plants carrying leaves infected with CBSV and/or UCBSV (Table 4.2) had severe damage within 90% of their storage roots (Fig. 4.8, Fig. 4.9). Tuberous roots from all plants of transgenic line pILTAB719 infected with CBSV were likewise affected. In contrast, of the 116 storage roots harvested from line 718-001, 111 were seen to be completely free of necrotic damage caused by CBSD, with the remaining five showing only mild symptoms (Fig. 4.9D). The lack of detectable virus within symptom-free storage roots of line 718-001 confirms the data from the leaf tissues (Fig. 4.7, Table 4.2) and indicates efficacy of UCBSV CP-derived siRNA populations for the suppression of the agronomic impact of CBSD in this transgenic event. As a result, the yield of usable roots, those considered fit for consumption or saleable at market, was estimated to be eight times greater for the

line 718-001 than for the non-transgenic controls, and significantly greater than that for other transgenic events that successfully excluded UCBSV but had little or no resistance to CBSV (Fig. 4.9F).

The lack of viral load in resistant plants of vegetatively propagated crops such as cassava is an essential epidemiological factor in suppressing subsequent spread and impact of disease (203) (58). It is apparent from the present studies that infection with CBSV alone is capable of causing severe storage root losses, and that both viral pathogens must be controlled to prevent root yield losses to CBSD. Simultaneous suppression of both CBSV and UCBSV to below detectable levels across all transgenic plants of 718-001 tested by RT-PCR is therefore an important outcome of the studies reported here. Stem cuttings from plants of line pILTAB718 have been replanted to assess the impact of the disease on the vigor of the infected cuttings and to examine the continued efficacy of resistance to CBSD across vegetative generations.

The data presented here provide proof of principle for the control of CBSD by RNAi-mediated technology and the first field-based evidence for transgenic control of a disease in cassava. Highly effective suppression of UCBSV has been demonstrated, in addition to partial cross-protection in one transgenic line (718-005) and significant cross-protection in a second (718-001). It is hypothesized, therefore, that co-expression of siRNAs from the CP sequences of both UCBSV and CBSV within the same plant holds promise for integration of robust field resistance to CBSD into farmer-preferred cassava cultivars. Effective RNAi-derived protection against RNA viruses has been demonstrated for papaya (54), squash (183) and plum (67) with subsequent delivery of de-regulated resistant planting materials to farmers. The Virus Resistant Cassava for Africa (VIRCA) project is exploiting the knowledge gained from the present studies, and from additional ongoing CFTs to develop CBSD-resistant cassava for deployment to farmers and breeders in Uganda and Kenya (174). The results presented here indicate that RNAi technology has the potential to be an important component in the

multifaceted approaches being brought to mitigate the impact of CBSD on farmer wellbeing in East Africa, and could be a potential strategy to combat effects of its spread to other cassava-producing regions.

Chapter 5

RNAi-derived field resistance to cassava brown streak disease persists across the vegetative cropping cycle

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5.1 Abstract

A confined field trial was established to determine durability of RNAi-mediated resistance to Cassava brown streak disease (CBSD). Stem cuttings were obtained from field-grown cassava plants of cultivar 60444 transgenic for construct p718, consisting of an 894 bp inverted repeat sequence from the *Ugandan cassava brown streak virus* (UCBSV) coat protein. Plants were established from three transgenic lines previously shown to provide complete resistance to UCBSV and differing levels of protection to the non-homologous virus species *Cassava brown streak virus* (CBSV), and grown for 11 months. CBSD symptoms were observed on shoots and storage roots of all non-transgenic 60444 control plants and transgenic lines p718-002 and p718-005, but not on p718-001. RT-PCR diagnostic showed tissues of plant lines p718-002 and p718-005 to be infected with CBSV, but free of UCBSV. All leaves and roots of p718-001 plants were confirmed to carry no detectable levels of either pathogen. Plants of 60444 in this field trial showed severe cassava mosaic disease symptoms, indicating that presence of replicating geminiviruses did not cause significant suppression of RNAi-mediated resistance to CBSD. Resistance to CBSD across a vegetative cropping cycle confirms earlier field data, and provides an important step in proof of concept for application of RNAi technology to control of CBSD under conditions encountered in farmers' fields.

5.2 Introduction

Cassava brown streak disease (CBSD) is currently considered the most important disease of cassava in East Africa and a significant threat to food security in the region (148). CBSD is caused by two distinct virus species: *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV), both of which belong to the genus *Ipomovirus*, family *Potyviridae,* and possess a ssRNA genome of messenger sense (112, 199). Both viruses are transmitted by whiteflies (*Bemisia tabaci*), resulting in CBSD-symptomatic plants carrying infections by one or both viral species. Since its re-emergence in Uganda in 2004 (10), CBSD has become prevalent in East Africa, with new outbreaks of the disease resulting from dissemination of infected cassava planting materials and high whitefly vector populations (10, 90).

Genetically engineered resistance to CBSD was reported recently in tobacco and cassava plants under greenhouse conditions (147, 203) and in cassava in a confined field trial (CFT) in Uganda (139). Two RNAi constructs targeting UCBSV were tested in the field: p718 consisting of an 894 bp inverted repeat sequence (nts 208–1101) of truncated full-length UCBSV coat protein (CP), and consisting of a 397 bp portion (nts 208–604) of the UCBSV CP N-terminal (147). Tissue culture-derived cassava plants of cultivar 60444 that were shown to be accumulating transgenically derived UCBSV CP specific siRNAs, were planted in the field and proved to be highly resistant to whitefly transmitted UCBSV. Fourteen transgenic lines were tested, seven of each of the two constructs. Across all 14 lines, greater than 98% of the plants (n = 60) remained free of UCBSV 11 months after planting (MAP), as determined by RT-PCR. Protection against the non-targeted CBSV was observed in two transgenic lines of the p718 construct (carrying the full length CP sequence). This was most striking in line p718-001 in which 54 out of 60 plants (90%) remained CBSD symptom free and RT-PCR negative for presence of UCBSV and CBSV in leaf and storage root tissues (139).

5.3 Materials and methods

Farmers propagate cassava vegetatively from field-derived stem cuttings, not from tissue culture-derived plantlets. Demonstrating proof of concept for resistance to CBSD across the vegetative cropping cycle is an essential step within the product development process to develop and deliver resistant planting materials for farmers in East Africa. A field trial was therefore established to evaluate efficacy of the RNAi-mediated resistance to CBSD through a typical vegetative propagation cycle. Woody stem cuttings measuring 25 to 30 cm in length were obtained from plants within the CFT harvested in November 2011, as previously described by Ogwok *et al.* (2012) (139). Three lines transgenic for p718 (p718-001, p718-002, and p718-005) that provided complete resistance to UCBSV and differing levels of protection to CBSV in the original field experiment were re-planted, in addition to stakes of the non-transgenic control 60444 and the CBSD tolerant cultivar TMS 30572. Stem cuttings of the highly susceptible Ugandan farmer-preferred cassava genotype TME 204 were also obtained from an experimental field at the National Crops Resources Research Institute (NaCRRI). A CFT at NaCRRI, Namulonge, Uganda was established using 36 stem cuttings per entry per plot, planted at a spacing of 1 m x 1 m in a randomized complete block design with three replications. Each plot was surrounded by a row of CBSD symptomatic plants of TME 204 to act as a supplemental source of virus inoculum. The plants were grown with no agrochemical or fertilizer applications and with hand weeding performed as necessary. Plants were visually assessed on a monthly basis for CBSD and CMD incidence and severity, and for the number of adult whiteflies (*B. tabaci*) on the underside of the uppermost five fully open leaves, as described previously by Ogwok *et al.* (2012) (139). Plants were harvested at 11 MAP.

5.4 Results

Adult whitefly populations showed a steady increase, ranging from 152 to 212 per plant by four MAP (data not shown). As expected, CMD observed on plants of 60444 within the previous CFT (139) was carried across the vegetative cropping cycle. Severe CMD symptoms were therefore seen immediately on sprouting leaves of all transgenic and non-transgenic plants of 60444, with plants reaching a maximum severity score of 5 (1–5 scale) by 7 MAP. In contrast, and as expected, plants of TME 204 and TMS 30572 exhibited very mild to moderate CMD throughout the experiment. While foliar CBSD symptoms were obscured due to CMD in some cases, typical CBSD symptoms were clearly visible on the stems (134, 139). CBSD symptoms were first seen at one MAP on plants of TME 204 and non-transgenic 60444 controls, and by three and four MAP in plants of p718-005 and p718-002, respectively. Shoot CBSD symptom severity remained mild to moderate until five MAP in the 60444 controls and all plants of p718-002 and p718-005. No CBSD was observed on leaves or stems of plants of p718-001 and TMS 30572 throughout the 11-month duration of the experiment (Table 5.1).

Table 5.1: CBSD foliar incidence and severity at 11 MAP

Plant line	Incidence (%)	Mean severity (1-5)
p718-001	0.0	1.0
p718-002	46.8	3.0
p718-005	23.7	2.6
60444	100.0	3.5
TME 204	100.0	3.4
TMS 30572	0.0	1.0

MAP: months after planting. p718: plant lines of 60444 transgenic for inverted repeat construct of 894 bp of UCBSV CP; 60444: wild type non-transgenic plants of cultivar 60444.

Damage due to CBSD-induced necrosis in storage roots was assessed at harvest 11 MAP. Storage roots from the 16 innermost plants of each plot were uprooted, sliced transversely into five pieces and sections scored visually for presence and severity of CBSD using the 1-5 scale described previously by Ogwok *et al*. (2012) (139). No CBSD necrotic symptoms were observed within 290 root slices cut from 58 storage roots obtained from 38 different transgenic plants of line p718-001 (Table 5.2, Fig. 5.1). In contrast, all (100%) roots from TME 204 and transgenic event p718-005 showed CBSD symptoms, with incidence of necrosis at 61.4%, 28.7% and 1.6% of roots harvested from plants of 60444, p718-002 and TMS 30572 respectively (Fig. 5.1). Severity of CBSD root damage remained mild to moderate in roots of p718-002, p718-005 and TMS 30572 (average score 2.5 – 3.5) (139) compared with severe to very severe root symptoms observed in roots of non-transgenic 60444 and TME 204 plants (average score 3.9 – 4.8) (Table 5.2).

Table 5.2: CBSD storage root incidence and severity at 11 MAP

Plant line	CBSD necrosis incidence (%)	Mean CBSD necrosis severity (1-5)
p718-001	0.0	1.0
p718-002	28.7	2.5
p718-005	100.0	3.5
60444	61.4	3.9
TME 204	100.0	4.8
TMS 30572	2.0	3.0

MAP: months after planting. p718: plant lines of 60444 transgenic for inverted repeat construct of 894 bp of UCBSV CP; 60444: wild type non-transgenic plants of cultivar 60444.

Molecular diagnosis was performed on total RNA extracted from the youngest leaves showing CBSD symptoms, or on equivalent non-symptomatic tissue, RNA was subjected to RT-PCR, as described previously by Ogwok *et al.,* (2012) (139), using primers CBSVDF2 (5'-GCTMGAAATGCYGGRTAYACAA-3') and CBSVDR (5'-GGATATGGAGAAAGRKCTCC-3'), which

amplify *c.* 437 and 343 nucleotides of the 3'-terminal sequences of the UCBSV and CBSV genomes, respectively. Table 5.3 summarizes diagnostic analysis of plants at 11 MAP. Virus was detected in the vast majority of plants of the susceptible cultivar TME 204 and non-transgenic 60444, with most plants found to be infected with both CBSV and UCBSV. As reported in the first CFT (139), all transgenic lines remained free of detectable UCBSV. While plants of p718-002 and p718-005 were confirmed to be infected with CBSV, all plants of p718-001 remained free of RT-PCR detectable levels of both CBSV and UCBSV (Table 5.3).

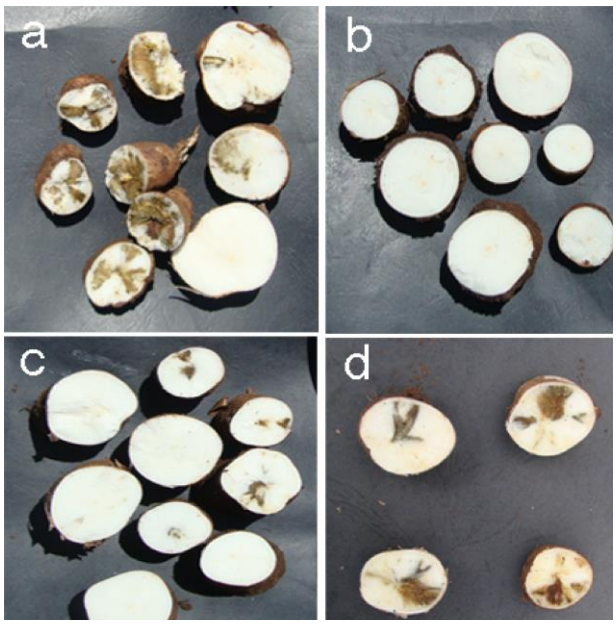


Fig. 5.1. CBSD symptoms in storage roots derived from stem cuttings at harvest 11 months after planting. (a) Non-transgenic 60444 showing severe damage due to CBSD. (b) Transgenic line p718–001 showing no CBSD root damage. (c) Transgenic line p718–002 showing moderate damage due to CBSD. (d) Transgenic line p718–005 showing significant damage due to CBSD.

To determine presence of CBSV and UCBSV in storage roots, 1-2 cm thick root discs were obtained at harvest 11 MAP. Total RNA was isolated and subjected to RT-PCR (139). As in the case of leaf tissues, root tissues from plants of p718-001 were found free of UCBSV and CBSV (n = 10) (Table 5.4).

No UCBSV was detected in lines p718-002 or p718-005, but roots from these events were found to be positive for presence of CBSV at 60-80%.

Table 5.3: Presence of CBSV and UCBSV in leaves of plants 11 MAP determined by reverse-transcription polymerase chain reaction

Plant line	Number of leaf samples analyzed	No. of leaf samples virus positive (%)	Virus species detected (%)		
			CBSV only	UCBSV only	CBSV + UCBSV
p718-001	30	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
p718-002	30	26 (87.7)	26 (100)	0 (0.0)	0 (0.0)
p718-005	30	16 (53.3)	16 (100)	0 (0.0)	0 (0.0)
60444	30	26 (87.7)	4 (15.4)	1 (3.8)	21 (80.8)
TMS 30572	15	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
TME 204	15	14 (93.3)	2 (14.3)	0 (0.0)	12 (85.7)
Total	150	82 (54.7)	48 (58.5)	1 (1.2)	33 (40.2)

MAP: months after planting. p718: plant lines of 60444 transgenic for inverted repeat construct of 894 bp of UCBSV CP; 60444: wild type non-transgenic plants of cultivar 60444.

Table 5.4: Presence of CBSV and UCBSV in storage root tissues of 11 MAP determined by reverse-transcription polymerase chain reaction

Plant line	Number of root samples analyzed	Number of root samples virus positive (%)	Virus species detected (%)		
			CBSV only	UCBSV only	CBSV + UCBSV
p718-001	10	0 (0)	0 (0.0)	0 (0.0)	0 (0.0)
p718-002	10	8 (80)	7 (87.5)	0 (0.0)	1 (12.5)
p718-005	10	6 (60)	6 (100.0)	0 (0.0)	0 (0.0)
60444	10	9 (90)	7 (77.8)	1 (11.1)	1 (11.1)
TME 204	20	17 (85)	2 (11.8)	1 (5.9)	14 (82.3)
Total	60	40 (66.7)	22 (55.0)	2 (5.0)	16 (40.0)

MAP: months after planting. p718: plant lines of 60444 transgenic for inverted repeat construct of 894 bp of UCBSV CP; 60444: wild type non-transgenic plants of cultivar 60444.

5.5 Discussion

This study has shown that plants derived from stem cuttings of cassava cultivar 60444 transgenic for the RNAi construct p718 (carrying an inverted repeat sequence of a truncated full-length UCBSV CP) remained highly resistant to CBSD across a second planting cycle of 11-month duration. Molecular analysis revealed that all RNAi-derived transgenic plants remained resistant to the homologous virus (UCBSV), and that plant line p718-001 continued to be protected against both CBSV and UCBSV. Such performance demonstrates that siRNA-imparted resistance to CBSD is durable over time and across a vegetative cropping cycle. Importantly, CMD incidence and severity was high across all the transgenic plants in this field trial, indicating that presence of replicating *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV), known to encode the gene silencing suppressor proteins AC4 and AC2 respectively, did not cause significant suppression of transgenic RNAi-mediated resistance to CBSD.

The observed field resistance of transgenic cassava to CBSD through a vegetative cropping cycle as described here confirms previous greenhouse studies (147, 203) and an earlier field experiment (139), and provides proof of concept that RNAi technology can provide effective resistance to CBSD under conditions similar to that encountered in farmers' fields. Combined with the recent, independent report of RNAi-mediated control of CBSD (189), the present study provides strong encouragement to further develop this technology to improve CBSD resistance in cultivars preferred by cassava farming communities in East Africa (173).

Chapter 6

Summary and Future Recommendations

6.1 Results summary and discussion

Cassava brown streak disease (CBSD) ranks among the seven most important threats to global food security (148). CBSD is caused by two distinct virus species: *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV). All attempts towards understanding and combating the impact of CBSD, either through genetic modification, resistance breeding or phytosanitation are of paramount importance. There are so far no known CBSD immune cassava genotypes, and natural sources of resistance remain unclear (76, 189). To facilitate control of CBSD, it is important to understand host-virus interaction mechanisms in order to design appropriate strategies aimed at conferring resistance to the viral pathogens. The work presented in this thesis was aimed at increasing our understanding of the interaction between the viruses and their natural host, cassava, and to assess whether RNAi technology has potential to control CBSD in the field environment.

The study reported here on distribution and accumulation levels of CBSV and UCBSV present in field grown, infected cassava genotypes with different levels of resistance or susceptibility yielded noteworthy information. It was clear that CBSV is the more aggressive virus species compared to UCBSV. Irrespective of the cassava genotype studied, CBSV was detected in 100% of field samples showing CBSD symptoms. Both CBSV and UCBSV were detected in 60444, TME 204, TME 14, and Ebwanateraka. UCBSV was not detected in NASE 3, NASE 14, NASE 16 Nyaraboke and MH97/2961. CBSV-infected plants showed typical CBSD symptoms earlier and more severely, than plants infected with UCBSV alone.

Recently, Kaweesi et al (2014) reported similar findings from field evaluation of eleven selected cassava genotypes. Both CBSV and UCBSV were detected in all the cassava genotypes tested, though CBSV was detected at three months after planting, two months earlier compared to UCBSV,

which was detected from five months after planting (76). Our studies had similarities. For example, the cassava genotypes TME 204 and NASE 14 were common in both studies. Apart from Kaweesi et al. (2014) being able to detect UCBSV in NASE 14, the results of both studies tally. For example, in both cases, TME 204 showed very severe CBSD shoot and root symptoms and mean disease incidence of 100%. Similarly, NASE 14 plants showed mild shoots and root symptoms. Given that NASE 14 and NASE 3 are improved CBSD-tolerant varieties and TME 204 is CBSD-susceptible, results of the two studies corroborate each other. There were however differences in experimental design. For instance Kaweesi and associates monitored disease progress in a planned experiment in a high CBSD pressure area, and sampled at different time points and pooled the samples. In contrast, data presented in this thesis is based on eight samples from different parts of the same plant collected from ten genotypes at one time point.

Results from analysis of field samples were verified for the CBSD-tolerant NASE 3 and susceptible varieties TME 204 and 60444 by grafting experiments with tissue culture-derived plants. These experiments eliminated the complex environmental variables present in the field such as rainfall and temperature fluctuations, and presence of other pests and pathogens, and facilitated study of single virus/genotypes interactions on their own. The results confirmed that NASE 3 was resistant to UCBSV but susceptible to CBSV, whereas the susceptible genotypes TME 204 and 60444 were susceptible to both viral pathogens. CBSV-infected NASE 3 plants showed reduction in growth, stem dieback and maximum root severity of 5. Thus, glasshouse data corroborated field data. The response of NASE 3 to UCBSV therefore indicated the possibility of genotype-specific resistance to this viral pathogen.

The levels of virus accumulation were correlated to disease phenotype in infected plants. Tolerant cassava genotypes were found to have accumulated lower virus titer compared to susceptible genotypes. Additionally, the susceptible genotypes were infected by both CBSV and UCBSV. Similarly,

Kaweesi et al (2014) (76) also found that virus load was higher for CBSV than UCBSV, and susceptible genotypes, particularly TME 204, Albert, and NDL06/132 were found to have accumulated higher virus titer than the tolerant genotypes NASE 1, NASE 14, Kiroba and AR40-6. Storage roots and mature non-senescing leaves accumulated the highest amount of viral RNA and showed more severe CBSD symptoms irrespective of genotype, whereas, younger leaves without CBSD symptoms accumulated less viral RNA. Leaves or branches of tolerant cassava genotypes NASE 3 and NASE 16 without CBSD symptoms accumulated negligible amounts of viral RNA. NASE 3, in particular, was found to be resistant to UCBSV based on samples obtained from both field and glasshouse experiments. This is useful information for breeders and farmers. Breeders could use NASE 3 as a source of resistance to UCBSV, whereas farmers could be advised to select cassava plants or branches of cassava stems without CBSD symptoms for planting the next crop with the aim of cultivating CBSD-free plants.

The level of virus accumulation in highly susceptible, moderately susceptible or tolerant cassava genotypes also has implication on the rate at which the viruses are transmitted by the whitefly vectors (*B. tabaci*). Higher virus titer suggests the viruses are readily available to feeding insects, although no evidence is available. Susceptible varieties are, therefore, a superior source of CBSV and UCBSV for feeding vectors than tolerant varieties, which tend to accumulate more CBSV RNA in the storage roots than in the aerial tissues (138). Although CBSD spread through stem cuttings is presumably more prevalent than vector transmission, identification of cassava plants with resistance to CBSV and UCBSV is important in order to restrict vector transmission and mitigate the impact of CBSD. In addition, high accumulation of CBSV viral RNA in infected cassava suggests that CBSV is more readily available for vector transmission, and might be more widespread compared to UCBSV. Glasshouse experiments indicated that the CBSD outbreak in Uganda is most likely not a result of an emergent super-virulent species or strain of CBSD-causing viruses identified in Uganda, which showed milder

CBSD symptoms to CBSV isolates obtained from coastal Tanzania and Mozambique (118). Since this study considered ten different cassava genotypes and identified at least one genotype with resistance to UCBSV, it is envisioned that screening a wide collection of cassava germplasm for genotype-specific resistance to the CBSD causing viruses would be a reasonable and practical next step in identifying sources of resistance to these viruses for use in breeding programs (76, 89).

Disease development in plants due to viral infection differs among viruses and strongly depend on the mode of virus transmission, plant tissues that the viruses infect and replicate within, and the mode of spread within host plants (57). Potyviruses in particular, induce formation of 200-1000 nm long cytoplasmic inclusion proteins in infected plant cells (80). These proteins have an RNA helicase and ATPase enzymes activity, and are involved in viral cell-to-cell movement across plasmodesmata. Accumulation of cytoplasmic inclusion bodies in the cell causes disorder of normal cell morphology, rearrangement of internal structures of mitochondria and chloroplast, and changes in the sizes of plasmodesmata (80, 162). The changes are reflected in the form of disease symptom appearance or extent of viral RNA and viral particles accumulation in infected cells.

To obtain a complete picture of the distribution and accumulation of the viral RNA within host plants, a comprehensive approach would include identification of sites of virus accumulation at the cellular and sub-cellular level using various techniques. For instance, Kogovsek *et al* (2011) used a combination of RT-qPCR, transmission electron microscopy (TEM) and fluorescent *in situ* hybridization (FISH) methods to study the effect of potyviral infection at sub-cellular levels in PVY-infected potato (80). They observed cytoplasmic inclusion bodies, which appeared as pinwheels, scrolls or laminated aggregates in the cytoplasm at the sub-cellular level in parenchyma, epidermal and vascular tissues. There were particularly high levels of viral RNA and viral particles in chlorotic leaves and trichromes of PVY-infected potato, compared to asymptomatic leaves. This reflected the high rate of viral replication and

assembly in the chlorotic leaves, and supported the idea of unidirectional flow of solutes towards trichromes, since virus movement from trichromes to mesophyll cells has not been described (80).

There is evidence that all other Ipomoviruses (family *Potyviridae*) produce pinwheels in the cytoplasm of infected cells (38), and PVY being a potyvirus, shares similar characteristics with CBSV and UCBSV. For example, CBSV, UCBSV and PVY possess ssRNA genomes, encode similar proteins, and cause tissue necrosis and chlorosis on affected plants. However, no information regarding accumulation of cytoplasmic inclusion bodies is available for CBSV- or UCBSV-infected cassava or in any other plant species. Data presented in this thesis showed that CBSV and UCBSV have a similar pattern of distribution and accumulation in CBSD affected plants of different genotypes. Storage roots and mature non-senescing leaves accumulated higher levels of viral RNA compared to young asymptomatic leaves (138). These results support the aforementioned hypothesis of unidirectional flow and accumulation of solutes including viral RNA and viral particles in the storage root tissues. Importantly, since CBSV and UCBSV are very closely related, it is worth probing whether the viral particles or viral gene products co-exist in the same cell or whether the viruses exhibit spatial separation. Localization studies of CBSV and UCBSV or their viral transcripts are therefore important for the near future. Such investigations would determine the recombination potential and elucidate occurrence of numerous viral variants within species, trace the pathways of viral pathogenicity including virus movement and accumulation from shoots to roots, and establish association of viral particles with host tissues. Importantly, it would help resolve the distribution of viral compounds in the root system to better understand the phenomenon of necrosis in the cassava storage root tissues.

To further comprehend the molecular interaction between CBSD tolerant and susceptible cassava genotypes and CBSD causing viruses, deep sequencing was used to compare the profiles of vsRNAs in CBSV and UCBSV graft-inoculated plants of cassava genotypes NASE 3, TME 204 and 60444

maintained in the glasshouse, during separate CBSV and UCBSV infections. Information about the biogenesis and composition of vsRNAs in CBSV- and UCBSV-infected cassava is important for understanding the molecular mechanisms underlying the aggressiveness and pathogenicity of CBSV and UCBSV, and the response of cassava genotypes to CBSD. Results presented here indicated that plants infected with CBSV accumulated maximum populations of small RNAs that mapped to the virus genome across genotypes compared to UCBSV infected plants. The latter accumulated moderate levels of UCBSV-derived small RNAs in TME 204 and 60444, respectively, but not in NASE 3. This further reflects the more aggressive nature of CBSV and that host genotype plays a critical role in determining the extent of accumulation of UCBSV RNA, including UCBSV-derived small RNAs, compared to CBSV-derived RNAs. The levels of virus-derived small RNA accumulation correlated with disease phenotype in infected plants. Irrespective of the genotype, CBSV-derived small RNAs accumulated to higher levels and CBSV-infected plants showed more severe CBSD symptoms. This varied considerably for UCBSV-infected plants across genotypes. The highly CBSD susceptible cultivar 60444 accumulated higher levels of UCBSV-derived small RNAs and showed more severe CBSD symptoms due to UCBSV-infection compared to TME 204, which accumulated moderate amounts of UCBSV-derived small RNAs and showed mild CBSD symptoms due to UCBSV infection. In contrast, NASE 3 did not accumulate UCBSV-derived small RNAs and showed no CBSD symptoms after graft inoculation with UCBSV. RT-qPCR analysis of UCBSV viral RNA in NASE 3 samples obtained from field grown plants and UCBSV graft-challenged plants in the glasshouse also revealed accumulation of insignificant UCBSV viral RNA (76, 138). Together, data obtained from NASE 3 plants grown in artificial conditions corroborate with data from field-grown plants and shows promise for the occurrence of cassava germplasm with natural resistance to CBSD viruses.

The production of varying CBSD symptoms further indicates that CBSV and UCBSV interact differently in different cassava genotypes. The tolerant cassava genotype, NASE 3, was seen to restrict virus replication and movement within the plant system since CBSV-infected plants showed localized stem lesions and root damage while leaf tissues were largely free of CBSD symptoms and were RT-PCR undetectable. Similarly, UCBSV failed to successfully replicate in UCBSV-challenged NASE 3, or if it did replicate, encountered restricted cell-to-cell movement in NASE 3 plants, in a manner not seen in susceptible genotypes. The behavior of NASE 3 should be further investigated under field conditions in a high UCBSV pressure area to determine whether the resistance to UCBSV is durable. This could also establish whether a threshold exists beyond which the virus overwhelms the plant defense mechanism to cause disease.

Compatible virus-host interactions usually trigger RNA silencing, a defense mechanism against invading nucleic acids, leading to transcriptional gene silencing (TGS), or post-transcriptional gene silencing (PTGS) (115). The gene-silencing pathway is mediated by multi-protein families, which include Dicers (DCLs), Argonautes (AGO) and RNA-dependent RNA polymerases (RDR) (14, 43). The silencing is triggered by dsRNAs, which are cleaved by DCLs to generate 21-24 nt virus-derived small RNA duplexes, resulting in accumulation of vsRNAs in infected plant cells. One strand of the duplexes called ‘guide’ strand assembles with distinct AGO-containing effector complexes (RISC). The AGO-small RNA complex functions to modify specific chromatin structure (TGS) or repress translation (PTGS) in a sequence-specific manner.

The generation of CBSV- and UCBSV-derived small RNAs in virus-infected cassava plants signifies that these viruses were able to trigger gene-silencing pathways in all the cassava host genotypes studied. Specifically, the predominance of 21-22-nt vsRNAs, mostly with uracil and adenine at the first 5’-end, implied that the viral genomes were processed by cassava homologues of Dicer enzymes,

particularly DCL2 and DCL4, and were preferentially loaded into effector complexes containing cassava homologues of AGO1 and/or AGO2 proteins (115, 169). Interestingly, CBSV and UCBSV behaved differently in the same host genetic background. The expression of cassava homologues of DCL2 and DCL4 was examined and were found to be down-regulated in CBSV-infected 60444 and NASE 3 libraries, though DCL4 was slightly up-regulated in CBSV-infected TME 204. Both DCL2 and DCL4 were found to be up-regulated in UCBSV-infected libraries genotypes 60444 and NASE 3. Similarly, there was induced accumulation of AGO2 mRNA in UCBSV-infected libraries across genotypes. CBSV-infected plants of 60444 showed no fluctuation in AGO2 expression. This suggests a difference in the mechanism of interaction between CBSV and UCBSV and their cassava host. Together, the results of deep sequencing, virus distribution and accumulation and CBSD symptom development portray CBSV as the stronger virus capable of overwhelming host defense system.

Hitherto, viral suppressor proteins have been shown to employ a multitude of mechanisms (37, 92, 164). However, information describing the effectiveness of the CBSV or UCBSV putative RNA silencing suppressor protein (P1) is insufficient (111). For example, the P0 protein of TCV has been shown to target AGO1, leading to its degradation though it does not interfere with siRNA-RISC assembly. Similarly, potyviral HC-Pro and ipomoviral P1b protein of CVYV have been shown to suppress the plants silencing machinery through siRNA sequestration thereby interfering with viral RNA degradation (187). In addition, although the ipomoviruses SPMMV and TomMMV both encode HC-Pro, which is related to potyviral HC-Pro, the HC-Pro of SPMMV lacks RNA-silencing suppressor activity. Instead the P1 protein act as the RNA-silencing suppressor by binding with AGO1 thereby interrupting RISC assembly (38). Studies tailored towards understanding the role of P1 protein of CBSV and UCBSV could provide an insight into the observed disparity between CBSV and UCBSV pathogenicity.

From an evolutionary perspective, the occurrence of CBSV and UCBSV together in CBSD-affected areas of East Africa (110, 113) indicate a clear case of virus evolution. Probably virus density was low in the 1930s and plants were infected by a single virus species (CBSV) at that time. Subsequently, virus density increased in the host populations creating an opportunity for competition for host resources, differences in pathogenicity and host genotype-specific reaction to each virus with concomitant evolution of viral variants (167). This could have led to the evolution of CBSV and UCBSV virus species and strains, with CBSV being relatively more fit compared to UCBSV. Indeed, recent reports supports widespread occurrence of CBSV-infected cassava genotypes considered as tolerant and susceptible in Uganda and Tanzania (76, 138). The studies also revealed the absence of cassava genotypes with immunity to CBSV infection, and showed that CBSV-infected cassava plants accumulated greater amounts of viral RNA and showed more severe CBSD symptoms than UCBSV-infected plants of the same genetic background (76, 138, 189). Subsequently, CBSV seems to be more virulent than UCBSV, though movement of infected planting materials within and across geographical boarders, especially while addressing the challenges of CMD, and the new occurrence of super-abundant populations of *B. tabaci* whiteflies might have driven the occurrence of the new CBSD epidemic in the East African region (72).

To date, CBSD management relies on the use of disease-free planting materials and dissemination of CBSD tolerant varieties (90, 91). No robust resistance to CBSD has been identified, and introgression of multiple disease resistance traits into farmer-preferred cassava germplasm is challenging (69). RNAi technology has been exploited to control plant viruses in numerous crop species. Examples include CMV, ZYMV and WMV in squash (79, 184), PLRV, PVY and PVX in potato (179), PRSV in papaya (82) and PPV in plum trees (66). Recently, Vanderschuren *et al.,* (2012) (189) demonstrated that CBSD can be controlled by RNAi-mediated approach. Cassava cultivars 60444 and TME 7 stably transformed

with a CBSV CP-derived inverted repeat construct targeting the C-terminal portion of the CBSV CP sequence conferred very high level of resistance to both CBSV and UCBSV (189).

We recently reported that RNAi transgenes developed to impart resistance to UCBSV controlled CBSD in *N. benthamiana* (147) and cassava (203) under controlled growth chamber and greenhouse environments. Three RNAi (inverted repeat) constructs p718 (894 nt), p719 (402 nt) and p720 (491 nt) were prepared targeting near full-length coat protein (CP), N-terminal and C-terminal regions of the UCBSV CP gene, respectively and stably transformed into *N. benthamiana* and cassava cultivar 60444. In *N. benthamiana* p718 conferred complete resistance in 85% of the transgenic plant lines challenged by sap inoculation with UCBSV (147). Similarly, none of the transgenic cassava expressing p718 tested RT-PCR positive for presence of UCBSV across replicated graft challenge experiments (203). This demonstrated that RNAi-mediated resistance could be used to effectively control CBSD in cassava.

The data on CBSD transgenic resistance presented above is lab-based and needed to be confirmed by field-testing selected transgenic cassava lines in a high CBSD pressure area. Seven transgenic lines from each of construct p718 and p719 that showed high accumulation of transgenically-derived siRNA, and non-transgenic 60444 were selected and field-tested for CBSD resistance in a confined field trial at Namulonge in Uganda. By six months after planting all non-transgenic 60444 had developed typical CBSD shoot symptoms, whereas transgenic plants expressing p718 showed a 3-month delay in CBSD symptom development. Up to 98% of clonal replicates of line p718-001 showed no CBSD shoot symptoms over the 11-months duration of the field trial. Only 0.5% of 413 transgenic plants tested were RT-PCR positive for presence of UCBSV within the leaf tissues compared to 57% of non-transgenic 60444. However, CBSV was detected in all plants with CBSD symptoms, except for line p718-001 in which both CBSV and UCBSV were not detected in 93% of the plants, and up to 95% of the storage

roots from plants of this line remained free of necrosis. Line p718-005 also showed significant suppression of both viral pathogens. These results not only indicated the effectiveness of UCBSV CP-derived inverted repeat constructs to confer resistance to the homologous virus, but also showed evidence of cross-protection against CBSV, which was the non-homologous, non-target virus.

The efficacy of CBSV-CP derived inverted repeat construct to control both CBSV and UCBSV has also been demonstrated in cultivar 60444 under controlled growth conditions (189). Transgenic 60444 plant lines expressing CBSV-CP derived siRNAs were highly resistant to both CBSV and UCBSV, with no viral replication observed in the resistant transgenic lines at seven months post-inoculation. Absence of viral replication means reduction in recombination potential and reduced chances for evolution of resistant breaking virus variants. The data suggests that to achieve silencing of target mRNAs, similarity between the siRNAs and their targeted viral sequences are not strictly reliant on 100% sequence homology. This also provides strong evidence for the suitability of the viral CP-derived siRNAs to confer resistance to potyviruses in cassava as previously reported for PRSV resistant papaya (59), BNYVV resistant sugar beet (93), BYDV resistant barley and wheat (194, 204), and ZYMV resistant melon. Together, the field trial results were consistent with results of glasshouse experiments and provided proof of concept that transgenic RNAi-mediated resistance technology holds promise for effective control of CBSD in farmers' fields. Importantly, the correlation of siRNA accumulation with field resistance in transgenically modified plants is important as selection of transgenic events to identify resistant lines for advancement to the field could be done at *in vitro* and greenhouse stages with the confidence of predicting successful field performance.

Given that cassava is vegetatively propagated to establish the next season crop, we tested whether the observed resistance to CBSD conferred by the CP-derived inverted repeat construct would be durable under high disease pressure in the field. Stem cuttings were obtained from three transgenic lines

(p718-001, p718-002 and p718-005) that showed complete resistance to UCBSV and partial cross-protection to CBSV from the previous trial and replanted alongside non-transgenic 60444, a farmer-preferred CBSD susceptible cassava genotype TME 204 and a CBSD-tolerant cultivar TMS 30572. Data was collected throughout the 11-months trial duration. The results indicated that plants of line p718-001 were free of CBSD shoot and root symptoms compared to non-transgenic 60444 and TME 204, which showed 100% CBSD shoot symptoms and roots necrosis. Plants of line p718-002, p718-005 and TMS 30572 showed mild to moderate shoot and root CBSD symptom severity compared with non-transgenic 60444 and TME 204. No UCBSV was detected in all transgenic plants by RT-PCR analysis, and plants of lines p718-002 and p718-005 were infected by only CBSV. Both CBSV and UCBSV were not detected in samples obtained from plants of line p718-001. The results of both field trials indicated that RNAi-mediated resistance is durable and therefore should be utilized to improve CBSD resistance in farmer-preferred cassava genotypes. This is very new and important data for RNAi-mediated resistance in cassava under field conditions for more than one cropping cycle. Subsequently, transgenic plants of a Ugandan farmer-preferred, CMD-resistant cassava genotype, TME 204 co-expressing CP-derived siRNAs from both CBSV and UCBSV were generated and field trials are underway in Uganda and Kenya to evaluate the plants for robust field resistance to both viral pathogens in cassava (174). The overall goal is to generate virus-resistant cassava, which will be subjected to regulatory assessment and eventually released to farmers.

In conclusion, the work presented in this thesis addressed the questions that were set out for the PhD study. This work contributes significantly to the existing limited knowledge underlying CBSV- and UCBSV-host interactions and control. The findings presented in this thesis will therefore contribute to the long-term goals of devising new methods of CBSD control and understanding the complex interconnected mechanisms involved in virus-host interactions.

6.2 Future recommendations

To fully exploit the data from this work, a number of follow-up studies are recommended. These include: (i) screening a wide collection of cassava germplasm for genotype-specific resistance to CBSD causing viruses; (ii) localization studies of CBSV and UCBSV or their viral transcripts in infected cassava to determine the recombination potential and elucidate occurrence of numerous viral variants within species, trace the pathways of viral pathogenicity including virus transport and accumulation from foliage to stems to roots, establish association of viral particles with host tissues and to resolve the distribution of viral compounds in the root system to better understand the necrosis phenomenon in the cassava storage roots; (iii) study the diversity and specific functions of the components of gene silencing pathway in cassava; (iv) comparative study of the gene functions of CBSV and UCBSV, especially of the effectiveness of the putative RNA silencing suppressor protein (P1); and (v) to utilize RNAi-mediated resistance to improve CBSD resistance in farmer-preferred cassava genotypes.

Chapter 7

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