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TRANSIENT STUDIES ON RNA INTERFERENCE AND COAT PROTEIN-MEDIATED RESISTANCE TO CASSAVA BROWN STREAK DISEASE

by

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B.S. Biology
Dip. Biology

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Advisory Committee

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To my late father Mr. Bartholomew Ejura and my mother Mrs. Albetina Ejura

"A wise man will hear, and will increase learning…" (Proverbs 1:5)

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SUMMARY

Cassava (*Manihot esculenta* Crantz) is an important staple and cash crop in Africa, Latin America and Asia. In east and southern Africa, cassava brown streak disease (CBSD) caused by cassava brown streak virus (CBSV) is associated with significant losses in cassava production. Previously, the disease was prevalent only along coastal eastern and southern Africa, but it recently emerged in Uganda and is spreading rapidly in the country as well as in neighboring countries. Apart from a few cultivars that have shown tolerance to CBSV in Tanzania, no effective resistance to CBSV has been developed and deployed to date. The full genome sequence of CBSV is not yet known, but it is thought to be monopartite, linear, positive sense ssRNA, translated into a polyprotein that is further auto-cleaved into functional proteins with the capsid protein (CP) at the C-terminus. The present study aims to develop transient resistance to CBSV through CP-mediated protection and RNA interference (RNAi) strategies. The entire CBSV CP gene was used to express the CP and thereby trigger CP-mediated protection against CBSV. In addition, the full-length CP gene and its N- and C-terminal regions were used to generate three RNAi constructs, with RNAi-GFP as an internal control for transient studies in sap-inoculated GFP transgenic *Nicotiana benthamiana*. An efficient protocol for sap transmission of CBSV to *N. benthamiana* was also developed and used in transient protection studies of the constructs as proof-of-concept for control of CBSV using virus-derived resistance strategies in cassava. The constructs offered high levels of protection against CBSV and are highly recommended for use to transform cassava to generate CBSV resistant cassava plants for the farmers.

Chapter 1

INTRODUCTION

1.1 Origin and importance of cassava

Cassava, *Manihot esculenta* Crantz, is a tropical root crop that originated from the Amazon region of Latin America. In the 16th century, the Portuguese sailors took cassava to West Africa and later to Central and East Africa through the Cape of Hope via Madagascar and Zanzibar (30). Farmers adopted cassava cultivation and the crop was integrated into the farming systems of East Africa (16). Cassava became established in Uganda during the 19th century and became a valuable food security crop in the 20th century. Cassava is cultivated in an area of 16 million hectares, 50% in Africa, 30% in Asia and 20% in Latin America (26). In Africa alone, the largest producing nations are Nigeria (39%), Democratic Republic of Congo (13%), Ghana (8%), Angola (7%), Tanzania (6%), Mozambique (6%), and Uganda (4%) (13).

Cassava is being cultivated both for subsistence and commercial purposes across the world. In sub-Saharan Africa, it is mainly used as a food security crop. In Uganda, cassava ranks second after banana in terms of production and consumption. It is a staple food crop and a major source of income for the poor rural communities (53). Most farmers prefer cassava because of its ability to withstand unreliable rains and drought conditions, it performs relatively well on soils with low fertility compared to other crops, and is flexible in a number of crop production systems. Processed cassava products can be stored for several weeks and the starchy storage roots can be harvested for up to 3 years. All these characteristics make cassava a very important subsistence crop. In addition to the roots, the cassava leaves are also an important source of nutrients and are used both as food and fodder by several Africans, as it contains significant amount of proteins, vitamins and minerals. Cassava also has significant industrial value, as up to 10% of the world production is used to produce a range of products including

starch extracted from the roots and pharmaceutical products, and cassava is also grown as an ornamental plant.

1.2 Constraints to cassava production in Uganda

Despite the long history of cassava research and development in Africa and Uganda in particular, there are persistent major constraints to cassava production. Abiotic factors such as drought, poor soils, and unstable weather conditions limit cassava production. The crop is often grown in poor soils and sometimes in drought-prone regions. Low multiplication rate of improved varieties and long storage periods between planting seasons affect establishment of plants derived from such stem cuttings and compromise their viability. Cassava is also affected by pests, which feed on cassava and reduce its productivity. In Uganda the most common pests include cassava green mite, *Mononychellus tanajoa* and cassava mealybug, *Phenacoccus manihoti* Matt.-Ferr, which were introduced from South America in the early 1970s (16). In addition to these introduced insect pests, whiteflies, which also transmit several viral diseases, have become an important pest of cassava. The whitefly population in Uganda has increased tremendously in the recent past, reaching pest level with several thousands of whiteflies per leaf. The high population of whiteflies has been associated with the cassava mosaic disease (CMD) pandemic, suggesting that other whitefly-transmitted pathogens, such as CBSV, could easily spread to regions where CBSD was non-existent. Cassava bacterial blight, caused by *Xanthomonas axonopodis* pv. *manihotis* occurs in Uganda too. The bacteria can cause devastating effects on yields of cassava and availability of clean planting material (23). Among the several viral diseases of cassava, CMD and CBSD are the two most important viral diseases affecting cassava production in Uganda and in several other African countries. In East Africa, CMD is present everywhere cassava is cultivated. CMD resistant varieties have been developed and CMD-free planting materials distributed. This activity helped to rescue cassava production in Uganda in less than 3 years. However, all the CMD resistant cultivars succumb to the CBSD, making currently CBSD a major disease of cassava in East Africa. The biology of CBSV is not yet

well studied and no reliable CBSD highly resistant cassava variety has been developed so far.

1.3 History of CBSD in Uganda

The first report of CBSD was in 1930s in Tanzania but it is now known to be endemic in Tanzania, Kenya and Mozambique mainly at altitudes below 1000 m (33). Recently, there has also been reports of CBSD in Malawi and Zanzibar (21, 33). In Kenya, CBSD was observed in the coastal areas but at low incidence and severity (7, 71). The disease was first observed in Uganda in 1934 but was efficiently eliminated by destroying all plants showing CBSD symptoms (25). A new outbreak of CBSD in Uganda has been recently reported and the presence of CBSV, which was confirmed by reverse transcription–polymerase chain reaction (RT-PCR) and nucleotide sequence analysis (2). A survey for cassava pests and diseases conducted jointly by International Institute of Tropical Agriculture (IITA) and Uganda's National Agricultural Research Organization (NARO), which incorporated parameters for CBSD symptoms, revealed that CBSD is distributed throughout Uganda with greatest incidence in the south and central regions (52). The re-emergence of CBSD in Uganda shows that other countries in the region may be at high risk of spread and increased prevalence.

1.4 Cassava brown streak virus

CBSV is tentatively assigned to the genus *Ipomovirus* of the family *Potyviridae* (32, 49). The full genome sequence of CBSV is not yet known, but comparison of CBSV CP gene sequence indicates its close association with members of the genus *Ipomovirus* in the family *Potyviridae.* Members of the genus *Ipomovirus* include Sweet potato mild mottle virus (SPMMV), Cucumber vein yellowing virus (CVYV), Squash vein yellowing virus (SqVYV), and a tentative member, Sweet potato yellow dwarf virus (SPYDV). This genus is characterized by flexuous non-enveloped filamentous (650-690 nm) virion particles, presence of inclusion bodies in infected plant cells and by having a polyprotein genome strategy. The *Ipomovirus* genome organization is similar to that of Potato virus Y (PVY) (10). Therefore, CBSV is expected to have the genetic information contained in a

monopartite, linear, single stranded, positive sense RNA (+ssRNA) approximately 10 kilobases (kb) in length coding for a polypeptide of ~3000 – 3400 amino acids. The 3' terminus has a poly (A) tract and the 5' terminus is expected to have a genome-linked protein (VPg). The genetic maps of the sequenced *Ipomovirus* genomes indicate that the virus encodes a single protein that is proteolytically cleaved into smaller functional proteins. The genome map of CVYV and SqVYV reveals that the polyprotein is cleaved into functional proteins designated as P1a, P1b, P3, cylindrical inclusion protein (CI), nuclear inclusion protein a (NIa), viral genome-linked protein (VPg), nuclear inclusion protein b (NIb), capsid protein (CP), and two smaller proteins $6K_1$ and $6K_2$ (35). However, SPMMV has helper component proteinase (HC-Pro) instead of P1b, but the other proteins are the same (35).

1.5 Characterization and variability of CBSV

The first published data on CBSV characterization relates to isolates collected from Tanzania and Mozambique (49). Each of the isolates elicited different symptom phenotypes in *N. benthamiana* and *N. tabaci.* However, comparisons of CP gene sequences revealed only 8% differences in nucleotides (nt) and 6% differences in amino acid (49) sequences (49). In late 2004, leaf samples were collected from plants showing typical CBSD symptoms from Mukono district in central Uganda. Comparisons of the partial CP gene sequence (222 bp) obtained from the Ugandan isolates showed 77.0% to 82.9% nt and 43.9% to 56.8% aa identity with those from Mozambique and Tanzania (2), therefore indicating at least a different strain of the virus (CBSV-UG). There was 95.9% to 99.5% nt and 85.1% to 90.5% aa identity among the Ugandan isolates suggesting that the Ugandan isolates are variants of CBSV-UG (2). More recent data, based on analysis of the complete CP-encoding sequences (1,101 nt) of eight isolates obtained from the Lake Victoria basin in Uganda and the Indian Ocean coastal areas of East Africa, were only 75.8-77.5% nt, and 87.0-89.9% aa identical when compared to the partial CP sequences (714 nt) of the six CBSV isolates previously characterized from

Tanzania and Mozambique (48). These findings further suggest that separate CBSV strains exist in East Africa.

1.6 Host range of CBSV

The host range of CBSV is fairly limited but does include several *Solanaceous* plants such as *Petunia hybrida*, *Datura stromonium* L., *N. tabacum* L., *N. rustica* L., and *N. glutinosa* L.(38).

1.7 Symptoms of CBSD

CBSD show a diversity of symptoms all over the plant but with considerable variation in expression levels depending on variety and growing conditions (19).

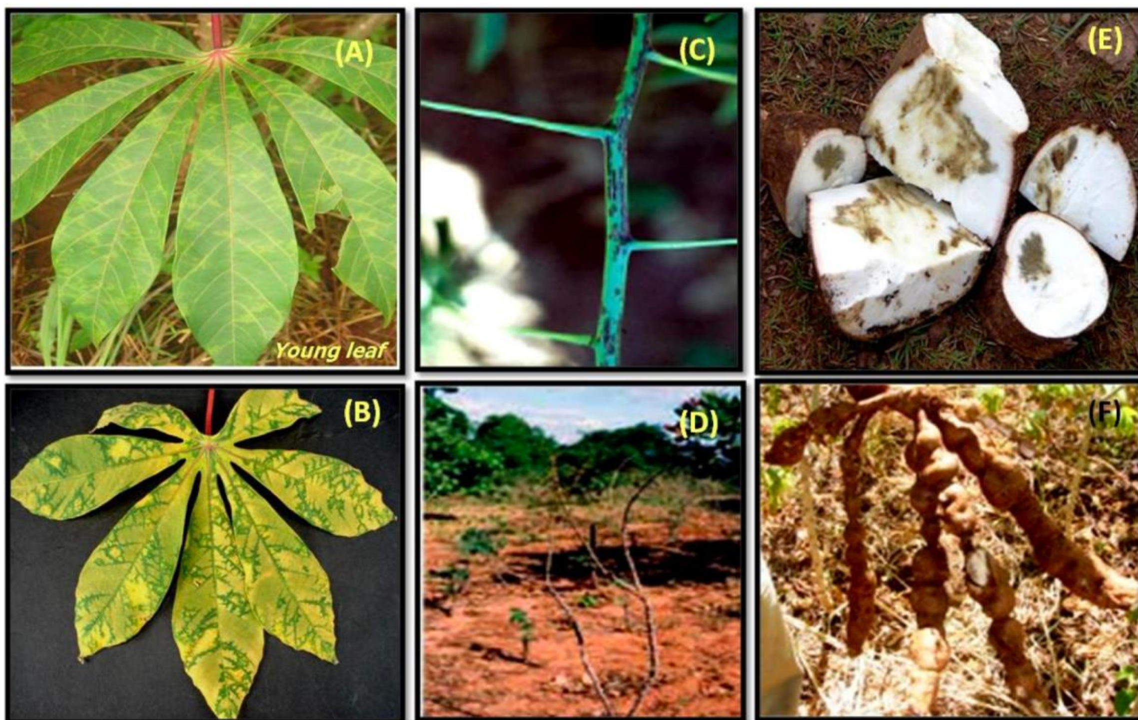


Fig.1: Symptoms of CBSD. (A) Symptoms on younger leaf, (B) symptoms on older leaf, (C) brown streak on young stem, (D) stem dieback, (E) necrotic rot of roots, and (F) root constrictions

Foliar symptoms include feathery chlorosis along veins (Fig. 1A and 1B), brown streaks on stem (Fig. 1C), and stem die-back in severe infections (Fig. 1D) (27). The internal root symptoms vary a lot, but most often consist of a yellow/brown corky necrosis of the starchy tissue (Fig. 1E). In susceptible varieties, necrotic

lesions spread throughout the starch storage tissue discoloring it and thus making it unhealthy for human consumption. Radial constrictions may show outside of the root in the surface bark (Fig. 1F) (19).

1.8 CBSD detection and diagnostics

Due to the variable nature of CBSD symptom expression, it is unreliable to visually inspect and diagnose CBSD. Monger et al (2001) developed a reliable RT-PCR-based diagnostic technique using primers specific to CBSV CP gene (49). The primers were successfully used to amplify CBSV isolates from Uganda (2), nevertheless it is the only CBSV diagnostic method available thus far besides the use of indicator plants. However, most laboratories in the region affected by CBSD lack the required capacity to perform the diagnostics effectively. In addition, there is a need to develop rapid and robust CBSV diagnostics techniques such as real-time PCR and enzyme-linked immunosorbent assay (ELISA) to facilitate disease monitoring and disease control. Developing antibodies against CBSV for use in ELISA would facilitate cheaper and timely CBSD diagnosis.

1.9 Epidemiology of CBSD

CBSV transmission to cassava by whiteflies was recently demonstrated (45). The disease spreads naturally in the field between cassava plants (7, 59). The use of infected cassava stem cuttings by farmers as planting materials provides alternative means of CBSV transmission, perpetuation and spread. In the last five years CBSD invaded Kenya, moved into Uganda, Rwanda, Burundi, Democratic Republic of Congo, and down Zambia, Malawi and Mozambique (9, 52).

In the lab, the disease can be transmitted by graft-inoculation from cassava to cassava (59) and also mechanically from cassava to herbaceous host plants (38). CBSV transmission rate by *B. tabaci* is very low in laboratory conditions compared to field situation (45). This has made studying CBSD in laboratory conditions difficult due to lack of a means of transmitting the causal virus readily, and the absence of an infectious CBSV clone.

1.10 Economic impact of CBSD

Losses due to CBSD depend not only on the susceptibility of the cultivar but also on the time the crop is harvested. Studies in Tanzania demonstrated that more than 90% of plants of CBSD susceptible varieties sprouting from cuttings taken from diseased plants expressed leaf symptoms, and many of the same plants showed root symptoms at harvest and reductions in root weight (20). The losses are worsened by necrosis on roots that render it unsuitable for consumption, prevent harvested roots from being marketed or encourage premature harvesting to avoid complete damage. Thus CBSD is a very important drawback to farmers who rely on cassava as a food security crop. An annual loss of about US $50 million due to CBSD for the farmers in Tanzania alone has been reported (9).

Recent CBSD surveys documented incidences of 90–100% in some fields in northern Mozambique (22, 63), while in Zanzibar and coastal Kenya higher incidences (30-100%) were prevalent along the coastal areas (50, 64); recently in Uganda, CBSD incidence ranged from 0-64% (2). Root necrosis and yield loss increase with crop age (51). Root necrosis in local varieties begins to increase from six months after planting, encouraging farmers to harvest prematurely (20). In Uganda, CBSD incidence is highest and severity greatest in CMD-resistant varieties which are highly popular with farmers and have spread rapidly within and between farming communities (52).

1.11 Management of CBSD

1.11.1 Use of CBSD tolerant/resistant varieties

Surveys conducted in Tanzania and Mozambique indicated that some local cultivars showed tolerance to CBSD (22). Recently in Zanzibar, a CBSV tolerant variety, variety 452, has been developed through conventional breeding (54). In Uganda, conventional breeding for CBSD resistance started recently, yet cassava breeding is time consuming. It is therefore vital that other approaches such as marker assisted breeding and genetic engineering be explored.

1.11.2 **Phytosanitation**

At low disease incidence it may be advisable to remove and replace infected plants with disease-free cuttings, or select and use only disease-free cuttings. However, this has limitations such as having too few disease-free plants to provide sufficient planting material and disease diagnosis is not always straightforward and symptomless plants may be latently infected (27, 59).

1.11.3 **Engineering antiviral resistance in transgenic plant**

Many viruses cause disease in plants with some viruses infecting a huge number of host plants yet sources of resistance genes to such viruses are scarce. Nevertheless, transgenic approaches offer the ability to integrate a target-specific (virus-derived) resistance gene into unlimited elite varieties of a crop, irrespective of their genetic make-up, and avoiding the need for extensive back-crossing (55). Naturally, genes for resistance exist in wild species but are difficult to introgress into farmer preferred cultivars. Many potyviruses have been controlled through pathogen-derived resistance (PDR) approaches, which involve expression of pathogen-derived nucleotide sequences in transgenic plants to confer resistance against the pathogen. Resistance conferred by the expression of CP genes has been described for plant viruses in several different virus groups. Besides, expression of virus-derived nucleic acid sequences in transgenic plants based on the principle of post-transcriptional gene silencing (PTGS) has been useful in developing virus-resistant plants. Although the full genome of CBSV excluding the CP gene sequence is not yet known, being a potyvirus, it has been suggested to be an appropriate target for PDR strategy (34). Several approaches have been used to induce durable and safe resistance to plant viruses in the field (55). Some of the major approaches are discussed.

1.11.3.1 **Coat protein-mediated resistance (CPMR)**

The viral CP plays vital roles in the life cycle of a virus notably genome encapsidation, viral RNA assembly and replication, insect transmission, and systemic viral infection (66). The expression of functional or dysfunctional CP of many plant viruses in transgenic plants results in enhanced plant pathogen

resistance. Though discovered way back in 1986, the molecular mechanisms that modulate CPMR is still poorly understood (55). The expression of viral CP in transgenic plant cells probably disrupts one or more of the essential functions of the CP, or interferes with the transcript and protein levels resulting in poor plant-virus interaction. Some transgenic plants expressing virus-derived CP confer crossprotection to related viruses presumably by stabilizing and preventing virion replication, inhibition of viral uncoating, slower replication, or by interfering with vascular transport. Plants stably transformed with CP genes are inherited across generations and show varying levels of resistance ranging from wild-type phenotype to immunity. Besides CP, other viral proteins such as movement proteins and replicases have also been expressed in transgenic plants to confer virus resistance (61, 62). Classic examples of CPMR include Tobacco mosaic virus (TMV: *Tobamovirus*) (5), Potato virus Y (PVY: *Potyvirus*), Tobacco etch virus (TEV; *Potyvirus*), Tomato spotted wilt virus (TSWV: *Tospovirus*) (36), and Papaya ring spot virus (PRSV; *Potyvirus*) (39).

1.11.3.2 **Post-transcriptional gene silencing**

Post-transcriptional gene silencing, also generally called RNA interference or gene silencing (37) is a defense mechanism in plants that specifically degrades alien RNA molecules. The degradation mechanism is triggered by double-stranded RNA molecules (dsRNA), which are substrates for an RNase III family enzyme Dicer. Sources of dsRNA molecules in plants include replicating viral RNA, single-stranded hairpin RNA (hpRNA) or virus-derived dsRNA transgenes, and transposable elements. In the cell, dsRNA is actively degraded into short 20–25 nt small interfering RNAs (siRNAs) with 2 nt overhangs at the 3’ ends by Dicer(3). The siRNA duplexes are actively unwound and assembled into a nuclease complex called RNA induced silencing complex (RISC), which become activated. The activated RISC complex targets and degrades mRNA transcripts homologous to the RISC-incorporated single-stranded siRNA by complementary base pairing (46). Thus, accumulation of mRNA homologous to the RISC-incorporated siRNA is controlled in the cell. Transgenes encoding dsRNA or hpRNA are very effective

at triggering PTGS of both endogenous and alien genes and can be artificially introduced into cells for this purpose.

1.11.3.3 **Hairpin RNA transgenes silencing**

An hpRNA is a long single-stranded RNA molecule containing inverted repeat regions that folds back and hybridizes with itself to form a single-stranded hairpin loop and a double-stranded stem region (70). The hpRNA structure mimics that of dsRNA because of the base-paired stem region and therefore effectively triggers RNAi (72). Gene fragments in the range of 300 – 800 nts are normally cloned in the sense and antisense orientation across an intron to generate an inverted repeat hpRNA transgenes (72). The hpRNA transgene sequences can be obtained from almost any exon regions and from non-coding regions including the 5'- or 3'- untranslated regions (UTR), although sequences from conserved regions are preferred (70). For unknown reasons, the size of introns influences silencing efficiency of hpRNA transgene. Shorter introns enhances silencing efficiency better than longer introns (12). Depending on the promoter used to control expression, hpRNA can be expressed in specific tissues or in every tissue.

A diversity of genes has been effectively silenced using hpRNA transgenes with stable phenotypes resembling those of null allelic mutants of the target genes (55). Moreover, several genes can be simultaneously silenced if sequences of the individual genes are used in the hpRNA transgene (8). Members of a multi-gene family can also be simultaneously silenced if the hpRNA transgene sequences are derived from a less conserved region of the multi-gene family, usually the 5' or 3' UTR. Several virus-derived transgenes encoding hpRNA have been introduced into plants with exciting results. Among the plant viruses that have been effectively controlled using hpRNA transgene technology include Barley yellow dwarf virus (BYDV) (69) TSWV, Groundnut ring spot virus (GRSV), Tomato chlorotic spot virus (TCSV), Watermelon silver mottle virus (WSMoV), and PVY (8, 44). In a recent study, Bucher et al (2006) fused sequences of four tospoviruses in a single hpRNA transgene and were able to obtain plants with resistance against the multiple viruses (8). Plants transformed with hpRNA constructs generate

independent events with considerable variation in phenotype and target mRNA silencing and are inherited in a Mendelian manner to progeny plants (18).

1.11.3.4 Small interfering RNA and MicroRNA

Small interfering RNAs are 20-25 nucleotide-long double-stranded RNA molecules. An siRNA interferes with expression of a specific gene by binding to complementary sequences on the mRNA transcripts of the gene, which in turn cause cleavage of the mRNA strand by the nuclease activity of RISC (4). Many RNAi-based transgenic plants, particularly those targeting virus resistance, have been derived through expression of long dsRNA derived from hpRNA transgenes or related dsRNAs, which are processed into siRNA. However, siRNA can also be synthesized and delivered into cells through biolistics or *Agrobacterium*-mediated transformation to silence a target gene. In essence, sequences of any known genes can be targeted with an appropriately tailored siRNA. Thus, RNAi is an important tool for analysis of gene function and post-genomic studies (55).

Besides siRNA, microRNAs (miRNAs) are another class of small RNAs, 21-23 nt long that also act as effectors of RNA silencing. The difference between miRNA and siRNA is that miRNA are processed from endogenous single stranded RNA precursors in the nucleus and show only partial complementarities to mRNA targets (41). The miRNAs are first transcribed as primary miRNA and later processed by endonuclease Dicer into mature 21-24 nt siRNA-like molecules. The miRNAs are mainly negative regulators of post-transcriptional gene expression. They guide mRNA cleavage similar to siRNAs in plants where the target sites are typically highly complementary to the miRNA (70). miRNA also silence genes where the sequences are partially complementary by translational attenuation.

1.11.4 Transcriptional gene silencing

Recently, dsRNA have been discovered to induce sequence-specific DNA methylation in plants, referred to as RNA-directed DNA methylation (RdDM) (47). Several transgenes including virus-induced gene silencing (VIGS) transgenes, hpRNA transgenes or viral satellite RNA of potyviruses or potexviruses have been

shown to induce hypermethylation of cytosine residues of homologous nuclear transgene sequences at the CpG or CpNpG sites of the promoter sequences (55). This promoter methylation prevents transcription of the target genes as it presumably alters the chromatin conformation that results in loss of binding of transcription factors and thus loss of transcription of the target gene. Thus, dsRNA might silence genes at transcriptional and post-transcriptional levels.

1.12 Introducing transgenes into plant cells

The silence-inducing dsRNA or hpRNA can be delivered into plant cells by several means including particle bombardment of plants with nucleic-acid-coated gold or tungsten beads, by infiltrating plant cells with *A. tumefaciens* carrying the transgene, or by infecting plants with the virus. The plants can be stably or transiently transformed with the transgenes. Particle bombardment, sometimes called microprojectile bombardment or biolistics has been the main method of plant transformation mainly of monocotyledonous plant species such as rice, maize, wheat and barley (58). The method is also convenient for delivering dsRNA or DNA transgenes that encode hpRNA into plant cells. However, *Agrobacterium*-mediated gene transfer system is another convenient way of delivering transgenes into plant cells for stable or transient transformation (28). Infiltrating plant leaves with a culture of recombinant *A. tumefaciens*, containing a transgene in its T-DNA plasmid through the stomata mediates transfer of the transgene from the T-DNA of the bacteria into plant cells resulting into transient expression of the transgene (57).

1.13 Purpose of the study

In East Africa, CMD is found almost everywhere cassava is cultivated. Varieties resistant to CMD have been developed and widely distributed. This has helped to restore cassava production in Uganda after the severe CMD pandemic that started in the early 1990s (53). The recent outbreak of CBSD constitutes an additional and significant threat to cassava production. For over seven decades since it was first observed, CBSD remained endemic only in the lowland coastal areas of East Africa, from the north-east border of Kenya to Mozambique and the low altitudes in

Malawi. However, over the last five years, CBSD has emerged at several mid-altitude locations in East Africa and is spreading rapidly in Uganda and Northwestern Tanzania. Recent extensive surveys recorded CBSD incidences of 90–100% in some fields in Tanzania and northern Mozambique (22, 63), and 30-100% in Zanzibar (64), and coastal Kenya (50). In Uganda, CBSD incidences of up to 64% in individual fields were recorded (2). Most CMD-resistant varieties succumb to CBSD. There are only a few cultivars with tolerance to CBSD that have been developed through conventional breeding in Tanzania, but these have not been widely adapted and deployed (22, 33). Some cassava varieties begin to show very severe root necrosis that finally cause complete root deterioration six months after planting, forcing farmers to harvest the crop early (20). No effective CBSD resistance has been deployed in the region, yet the disease significantly affects quantitative and qualitative yield of cassava. It is therefore imperative that there is an exploration of alternative sources of resistance to complement conventional and marker assisted breeding. Since CBSD directly affects the yield and quality of storage roots, it is a major threat to food security and incomes of millions of people who largely depend on cassava for their livelihood. Effective management of CBSD will need development and deployment of CBSD resistant cassava. This study aimed to develop resistance to CBSD through pathogen-derived resistance using both CPMR and RNAi approaches.

1.13.1 Goal

The goal of the study is to develop resistance to CBSV using transgenic approaches.

1.13.2 Specific objectives

The specific objectives of the study were to:

1. Develop an efficient protocol for mechanical transmission of CBSV to *N. benthamiana*
2. Generate CBSV-derived RNAi and CP gene constructs using CP gene sequences of a Ugandan CBSV isolate

3. Assess effectiveness of CBSV-derived RNAi and CP gene constructs to confer resistance against CBSV in a transient assay using *N. benthamiana*

1.13.3 Hypothesis

Resistance to CBSV can be achieved through either gene silencing targeting the viral genes or through CPMR strategies**.**

1.13.4 Expected output

- Efficient protocol for mechanical transmission of CBSV to *N. benthamiana* optimized
- Gene constructs conferring resistance to CBSV developed
- Effectiveness of CBSV-derived gene constructs to confer resistance to CBSV determined in a transient assay with *N. benthamiana*

Chapter 2

MATERIALS AND METHODS

2.1 Development of an Efficient System for Mechanical Transmission of CBSV to *N. benthamiana*

2.1.1 Virus source

Stem cuttings were collected from cassava plants showing characteristic foliar CBSD symptoms from farmer's fields in Uganda and shipped to Donald Danforth Plant Science Center (DDPSC), St Louis, USA, where they were propagated and maintained in a growth chamber. CBSV isolates from four different Ugandan cassava cultivars (I 92/0057, I 95/0087, Ebwanatereka and TME 204) were tested to confirm presence of CBSV. All isolates were infectious in *N. benthamiana* with slight variation in symptom expression, but for further studies the CBSV isolate from the cassava cultivar Ebwanateraka was used. Total RNA was isolated from symptomatic cassava leaves following cetyl trimethylammonium bromide (CTAB) protocol originally described for DNA isolation by Lodhi et al. (1994) (40), cDNA was synthesized using SuperScript^{TM} III first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA), and used in RT-PCR with CBSV specific primers (10F and 11R) (49).

2.1.2 Inoculum preparation and sap inoculation

Infected cassava leaf tissues showing fresh symptoms of CBSD were ground using a chilled pestle and mortar with the aid of carborundum 320 grit (Fisher Scientific). Freshly prepared ice-cold 0.01M potassium phosphate buffer (K_2HPO_4 + KH_2PO_4), pH 7.0, containing 0.2% sodium sulfite and 0.01M β-mercaptoethanol (1:6 [wt/vol] tissue:buffer) was added to the ground tissue and mixed well, transferred to Falcon tube, and the tissue debris was allowed to stand for 5 min in ice for debris to settle at the bottom of the tube. The sap was used to inoculate 21-day-old *N. benthamiana* plants. Test plants were dusted with carborundum to act as abrasive and ice-cold drops of inoculum rubbed gently on the leaf surfaces

using gloved fingers. After inoculation, the plants were gently sprayed with water to remove excess carborundum and plant debris. Inoculation was done in the evenings and plants kept in the dark overnight. The growth chamber was maintained at light intensity of 200 μM, 28°C, 70% RH, and alternating light and dark periods of 16 hr/8 hr photoperiod.

2.1.3 Effects of antioxidants, inoculum concentration, temperature, and age of *N. benthamiana* on CBSV transmission

2.1.3.1 Effect of antioxidant on sap transmission of CBSV

To determine the effect of antioxidant on transmission of CBSV, 3 g of CBSV infected *N. benthamiana* leaf tissue was ground in 18 ml of four solutions; (i) sterile distilled water, (ii) 0.01 M potassium phosphate buffer, pH 7.0, (iii) phosphate buffer containing 0.2% sodium sulfite, and (iv) phosphate buffer containing 0.2% sodium sulfite and 0.01 M β-mercaptoethanol. Eighteen 21-day-old *N. benthamiana* plants were inoculated with inoculum prepared using each of the four buffers, with 2 replications. Symptoms were recorded daily, from two days after inoculation for a period of 14 days. A scoring scale to record the CBSV symptom severity was developed based on the scoring system developed for cassava mosaic disease by Fauquet and Fargette (14). Each plant was scored for their symptom severity on a scale of 0–5: 0 = no symptoms, 1 = slight leaf distortion, 2 = moderate leaf distortion, 3 = severe leaf distortion, 4 = very severe leaf distortion, stunting and wilting, 5 = necrosis and death of the plant (Fig. 2). Data were analyzed using GenStat software (GenStat for Windows 11th Edition, VSN International, Hemel Hempstead, UK.). ANOVA was used to obtain least significant difference (l.s.d) values, which were used to separate the means at $P = 0.05$.

2.1.3.2 Effect of inoculum concentration on CBSV transmission

To evaluate the effect of inoculum concentration on CBSV transmission, 10 g of infected leaf tissue was ground as described and 20 ml of freshly prepared ice-cold 0.01 M potassium phosphate buffer (K_2HPO_4 + KH_2PO_4), pH 7.0, containing 0.2%

sodium sulfite and 0.01 M β-mercaptoethanol (1:2 [wt/vol] tissue:buffer) added, mixed well, transferred to Falcon tube, and centrifuged at 4°C for 5 min to remove the debris. The supernatant (sap) was used to make seven dilutions ([wt/vol] tissue:buffer); (i) 1:5, (ii) 1:10, (iii) 1:20, (iv) 1:50, (v) 1:100, (vi) 1:500, and (vii) 1:1000 and 18 plants were inoculated with the different inoculum dilutions, with 2 replications. Data was collected and analyzed as previously described (section 2.1.3.1)

Fig. 2: Symptoms of cassava brown streak disease in leaves of *N. benthamiana*. Plants were visually assessed for development of symptoms after inoculation with CBSV-infectious sap. Each plant was scored on a scale of 0–5 where symptom severity score was rated on a six-point scale: 0 = no symptoms, 1 = slight leaf distortion, 2 = moderate leaf distortion, 3 = severe leaf distortion, 4 = very severe leaf distortion, stunting & wilting, 5 = necrosis and death of the plant

2.1.3.3 **Effect of age at inoculation on transmission of CBSV to *N. benthamiana***

To identify the best age of *N. benthamiana* for efficient sap transmission of CBSV, 18 test plants were inoculated at (i) 21 days after planting (DAP), (ii) 28 DAP, (iii)

35 DAP, and (iv) 42 DAP with sap prepared (1:6 [wt/vol] tissue:buffer) in phosphate buffer as previously described (section 2.1.2). Only the topmost two fully open leaves were inoculated. The number of days required for the initiation of symptoms and also the symptom progression were recorded and analyzed as in section 2.1.3.1. The treatment was replicated 3 times.

2.1.3.4 **Effect of age of inoculum source plant on transmission of CBSV to *N. benthamiana***

To evaluate the effect of the age of inoculum source plant on CBSV transmission, *N. benthamiana* plants of six different ages were used as source of inoculum. Infected leaf tissue was obtained from plants that were 14, 28, 42, 56, 70, and over 80 days old. For each source of inoculum, 18 healthy *N. benthamina* plants were inoculated in three independent replications with sap prepared (1:6 [wt/vol] tissue:buffer) in phosphate buffer containing 0.2% sodium sulfite and 0.01 M β-mercaptoethanol as described in section 2.1.2. Data was recorded and analyzed as previously described (section 2.1.3.1).

2.1.3.5 **Effect of temperature on progress and severity of CBSV**

The effect of temperature on the rate of CBSV transmission was assessed at 21°C, 25°C, 27°C, and 30°C. Sap was prepared in phosphate buffer (1:6 [wt/vol] tissue:buffer) and 21-day-old plants were inoculated as described above. The inoculated plants were maintained in growth chambers with a light intensity of 200 μM, 70% RH, and alternating light and dark periods of 16 hr/8 hr photoperiod at the above four different temperatures. The experiment was repeated three times and data recorded and analyzed as previously described (section 2.1.3.1).

2.1.3.6 **Assessment of virus-derived siRNA**

To determine viral RNA accumulation and virus-derived siRNA levels, symptomatic young leaf samples were collected daily from plants maintained at 21°C, 25°C, 27°C, and 30°C, total RNA and siRNA isolated, and analyzed by northern-blot. Small RNA was isolated using the protocol of Akbergenov *et al* (2006) (1) with some modifications. Total RNA was extracted from a single symptomatic leaf of *N.*

benthamiana using Trizol reagent (Invitrogen) as per the manufacturer's instructions. The total RNA was fractionated using RNeasy Plant mini kit (Qiagen) to remove the long RNAs. Ten micrograms of fractionated small RNAs was run on a pre-cast 15% TBE Urea gel (Criterion-BioRad) at 150V using the Criterion gel apparatus. The RNA was blotted to the Hybond N+ membrane using the semidry electro blotter at 10V for 1 h. The membrane was UV cross-linked and pre-hybridised in DIG Easy-hyb buffer (Roche) at 42°C for an hour. To produce a probe, CBSV CP isolate from the cassava cultivar Ebwanateraka was cloned in the *in vitro* transcription vector pSPT19 and subjected to *in vitro* transcription using DIG RNA labelling kit (Roche). The labelled RNA was hydrolysed for 5-7 min in 50 mM sodium bicarbonate/carbonate at 95°C and denatured before hybridization in DIG Easy-hyb buffer. The hybridization was done for 16 hrs (overnight) at 42°C and the membrane was subjected to different treatments as described in the manual (DIG High Prime DNA labeling and detection kit (Catalogue 11585614910, Roche Applied Science). Autoradiography was done by exposing the membrane to Amersham high performance chemiluminescence film (GE healthcare) and developed in automated developer (KODAK X-OMAT) and the auto-radiograms were scanned and adjusted for clarity. The siRNA titers were estimated by quantifying the signals on autoradiogram by using the IMAGE-J software (National Institutes of Health, USA).

2.2 Construction of CBSV-Derived RNAi and CP Genes

2.2.1 Oligonucleotide primer design

The computer program "Gene Runner" was used to design oligonucleotide primers flanking known sequence regions on the N-terminus, C-terminus, and of the full length CP gene based on available sequences in the database (Table 1). The primer sequences were designed such that the melting temperature (6) values of the primer pairs do not differ by more than 5°C; no inverted repeat sequences or self-complementary sequences more than 3 base pairs are present; the primer length is 18-25 nucleotides long and primer pairs do not differ by more than 3 base pairs in length; the forward and reverse primers are well-matched with similar C +

G content of between 40-60%; and the primers have no obvious tendency to form secondary structures and have no significant homology with other sequences on either strands of the target gene. The 5'-end of the oligonucleotide primer contained enzyme restriction sites and 'clamp' sequences of 2-3 additional bases. The clamp sequences besides protecting the 5'-end of the amplified DNA anchors the restriction enzyme firmly during DNA cleavage.

2.2.2 Extraction of RNA from plant leaves

Total RNA was extracted from infected leaf material using the CTAB protocol originally described for DNA isolation by Lodhi et al., (1994) (40) with slight modifications. About 200 mg of infected symptomatic mature or maturing cassava and/or *N. benthamiana* leaf material were picked, wrapped in aluminum foil and immediately frozen in liquid nitrogen. The working surfaces, pipettes, centrifuges were thoroughly cleaned with RNase Zap to ensure an RNase-free environment and RNase-free tips and tubes were used. Leaf tissues were ground, with the aid of liquid nitrogen, in a sterile pestle and mortar wiped with RNase Zap. The powder was transferred using sterile spatula/pipette tips into 1.5 ml microcentrifuge tube and to it 700 µl of CTAB buffer (containing β-Mercaptoethanol), pre-warmed at 65°C added. The mixture was vortexed to disperse tissue in buffer, incubated at 65°C for 30 min, and mixed by inversion every 10 min. The tubes were kept at room temperature for 10 min before adding an equal volume (700 µl) of chroloform:isoamylalcohol (24:1) and mixing by inversion for 10 min and spinning at 12,000 rpm for the next 10 min to separate the organic and aqueous layers. The upper aqueous phase (~550 µl) was transferred to a clean tube. Chloroform:isoamylalcohol extraction and centrifugation was repeated and about ~490 µl of aqueous phase transferred to a clean tube, 0.7 volume (~343 µl) of cold (-20°C) isopropanol added, gently mixed, followed by centrifugation at 13,000 rpm for 10 min. The isopropanol was decanted to leave behind the nucleic acid pellet to which 500 µl of 70% ethanol was added to wash the pellet. The ethanol was decanted after 10 min centrifugation at 13,000 rpm. The nucleic acids pellet was left to air dry for ~ 40 min and re-suspend in 50 µl of RNase-free water.

Table 1: List of primers sequences and expected fragment sizes. Clamp sequences are in black uppercase, enzymes sites are in blue uppercase and CBSV–CP sequences in lowercase letter

Primer codes	Sequences	Size (bp)
CP-FL-XbaI-F	GCTCTAGAgtggtggatgatgatagtn	908
CP-FL-KpnI-R	GGGGTACCttcaattgcggcaccactn	
CP-FL-BamHI-F	CGCGGATCCgtggtggatgatgatagtn	908
CP-FL-BstBI-R	CGTTCGAAttcaattgcggcaccactn	
CP-NT-BamHI-F	CGCGGATCCgtggtggatgatgatagtn	402
CP-NT-BstBI-R	CGTTCGAAaattgtacgataaaattcctn	
CP-CT-Xba1-F	CCGTCTAGAtgccagcttggattgtgaactgt	503
CP-CT-Kpn1-R	CGCGGTACCttcaattgcggcaccactg	
CP-CT-BamH1-F	CGCGGATCCtgccagcttggattgtgaactgt	503
CP-CT-BstBI-R	CGGTTCGAAttcaattgcggcaccactg	
CP-FL-XhoI-F	CCGCTCGAGgtggtggatgatgatagtn	908
CP-FL-BamHI-R	CGCGGATCCttattcaattgcggcaccacn	

2.2.2.1 Cleaning RNA of DNA contamination

To retain RNA, but eliminate DNA from the total nucleic acids extract, up to 35 µl of extract, 4 µl of 10X DNAse I buffer and 1 µl *DNAse* I enzyme were mixed in 0.5 ml tube and incubated at 37°C for 30 min. After incubation, the *DNAse* I enzyme was inactivated by adding 4 µl of DNAse inactivation reagent and incubation at room temperature for 2 min with occasional mixing (2-3 times) to disperse the DNAse inactivation agent. The RNA was isolated from the mix by centrifugation at 10,000 rpm for 2 min to pellet the DNAse inactivation agent. The supernatant (RNA) was carefully transferred to a fresh tube, avoiding the pellet.

2.2.2.2 Estimation of RNA concentration

The quantity and purity of nucleic acids in solution was estimated spectrometrically by measuring the absorbance at 260 and 280 nm. The RNA concentrations were

calculated by taking 1 OD_{260} unit equal to 40 μg/ml. The purity of RNA was estimated based on the A_{260}/A_{280} ratio.

2.2.2.3 **First strand cDNA synthesis**

To synthesize cDNA, SuperScript^{TM} III first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) was used. In a 0.2 or 0.5 ml tube, 20-μl reaction volume containing up to 5 μg of total genomic RNA, 1 μl of 50 μM oligo$(39)_{20}$ primer, 1 μl 10 mM dNTP mix, and up to 10 μl DEPC-treated water was mixed and briefly centrifuged before incubation at 65°C for 5 min, then place on ice for at least 1 min. In the mean time, the following cDNA synthesis mix was prepared, adding each component in the indicated order; 2 μl 10X RT buffer; 4 μl 25 mM $MgCl_2$; 2 μl 0.1 M DTT; 1 μl RNaseOUT (40 U/μl) and 1 μl SuperScript^{TM} III RT (200U/μl). To each RNA/primer oligo$(39)_{20}$ mix, 10 μl of cDNA synthesis mix was added, gently mixed, collected by brief centrifugation, and incubated at 50°C for 50 min. The reactions was terminated at 85°C for 5 min, chilled on ice and collected by brief centrifugation (picofuge). To each tube was added 1 μl of RNase H and incubated at 37°C for 20 min. The cDNA synthesis reaction was stored at -20°C or used for PCR immediately.

2.2.3 Amplification of cDNA fragments

Double stranded DNA (dsDNA) fragments were amplified using primer sets targeting specific CBSV-CP sequence on the cDNA template. The 25 μl PCR reaction mix contained 16.8 μl of sterile distilled water, 2.5 μl of 10x PCR buffer, 1.5 μl of $MgCl_2$ (25mM), 1 μl dNTPs (10mM), 0.2 μl of Taq polymerase (1u/μl), 0.5 μl of each primer, and 2 μl of cDNA template. Thermal cycling conditions comprised an initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min; a cycle of 72°C for 10 min, and stored at 4°C.

2.2.3.1 **Agarose gel electrophoresis**

DNA samples were resolved by electrophoresis on 1% (wt/vol) agarose gel prepared by dissolving 1 g of agarose in 100 ml of 1x TAE buffer (40 mM Tris, 1 mM EDTA, adjusted to pH 7.6 with glacial acetic acid), heated till agarose

completely dissolves in the buffer, cooled to about 45°C and 2.5 µl of ethidium bromide (10 mg/ml) added. The mixture was cast into a tray and a comb placed in the gel to form wells. The gel was placed in an electrophoresis unit, filled with electrophoresis buffer (1x TAE) to cover the gel and comb carefully removed. 2 µl of gel loading dye was mixed with 25 µl of the PCR product and carefully loaded into the well. 0.7 µg of the DNA size marker (1 kb+) was included in each gel for comparison of DNA fragments. Electrophoresis was performed at 80 V for 40 min to 1 h. Fragments were visualized by UV radiation (302 nm) and gel pictures taken using Alpha Inotech AlphaImager^{TM} 2200 gel documentation system.

2.2.3.2 **Extraction and Purification of DNA fragments from agarose gels**

The desired fragments were identified using standard molecular weight marker (1kb+ ladder) and purified using the QIAquick Gel Extraction Kit (QIAGEN) according to manufacturer instruction. The DNA fragments were excised from the agarose gel with a clean, sharp scalpel and weighed in a colorless tube. Three volumes of buffer QG was added to 1 volume of gel and incubated at 50°C for 10 min (or until the gel slice completely dissolved), or vortexed every 2–3 min during the incubation to help dissolve gel. After the gel slice dissolved completely, 1 gel volume of isopropanol was added to the sample and mixed. The sample was applied to the QIAquick column to bind DNA to the column membrane, centrifuged at 17,900 x g (13,000 rpm) for 1 min, the flow-through discarded and the QIAquick column put back in the same collection tube. Traces of agarose were removed by adding 0.5 ml of buffer QG to QIAquick column and spinning for 1 min (13,000 rpm). To wash the DNA fragment, 0.75 ml of buffer PE was added to QIAquick column and centrifuged for 1 min (13,000 rpm), the flow-through discarded and the QIAquick column centrifuged for an additional 1 min (13,000 rpm) to completely remove residual ethanol from buffer PE. The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube, 30-50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) added to the center of the QIAquick membrane, allowed to stand for 1 min, and centrifuged for 1 min (13,000 rpm) to elute DNA. The DNA was stored at –20°C.

2.2.3.3 **Purification of PCR products**

PCR products used in subsequent analysis were purified using QIAquick PCR Purification Kit (Qiagen, MD, USA) according to manufacturer's instructions. Five volumes of buffer PBI was added to 1 volume of the PCR product, mixed, sample transferred to a QIAquick spin column and centrifuged for 30–60 s in a 2 ml collection tube to bind DNA to the QIAquick column membrane. The flow-through was discarded and the QIAquick column placed back into the same tube. Buffer PE (0.75 ml) was added to wash the QIAquick column. To remove ethanol, the QIAquick column was centrifuged for 30–60 s, flow-through discarded, and QIAquick column placed back in the same tube for an additional 1 min centrifugation to remove completely residual ethanol. The QIAquick column was put in a clean 1.5 ml microcentrifuge tube, 50 µl buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) added to the center of the QIAquick membrane, the column allowed to stand for 1 min, then centrifuged for 1 min to elute DNA.

2.2.4 Construction of hairpin CBSV-CP genes (hpCBSV-CP)

The strategy outlined in Fig. 3 was designed to clone the CBSV fragments in the sense and antisense orientation to generate hairpin gene constructs. The CBSV-CP fragments were amplified with primers containing both enzyme restriction sites and 'clamp' sequences and cloned directly into CGT11003-intron vector after sequential digestion of both PCR product and the vector with the respective enzymes. The resulting plasmids were transformed in *E. coli* strain DH5α.

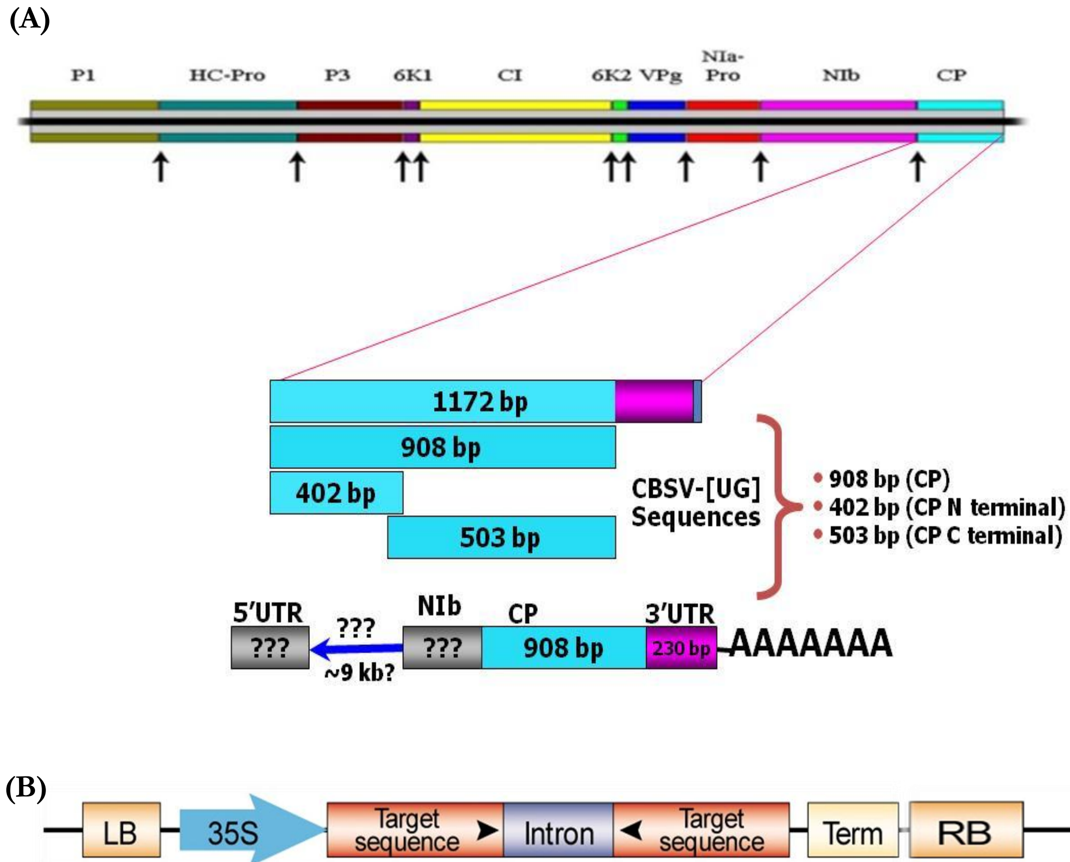


Fig. 3: Cloning strategy to generate CBSV-CP RNAi hairpin constructs. (A) How different CBSV-CP gene fragment were amplified. (B) How the fragments were cloned in the sense and antisense orientation in the shuttle vector. The question marks (?) represent unknown sequences.

2.2.4.1 Restriction digestion of DNA

DNA fragments and cloning vectors were prepared for digestion reaction by mixing up to 30 µl (~30 ng) of DNA with 2-6 µl of appropriate enzyme buffer, 2-6 µl of 1x bovine serum albumin (depending on enzyme requirement), 0.2-4 µl (1u/µl) restriction enzyme, and the volume made up to 20-50 µl with double distilled water. The digested DNA was always separated by agarose gel electrophoresis and the fragments visualized by ethidium bromide staining in UV trans-illuminator. Preparations containing DNA fragments of expected sizes were selected and purified from the gel for further analysis. Digestions were done using appropriate restriction endonucleases. *Xba*I and *Kpn*I were used for cloning gene fragments in the sense orientation, while *Bam*HI and *BstB*I were used for cloning gene fragment in the antisense orientation in the vector CGT11003-intron.

2.2.4.2 Ligation of DNA fragments into CGT11003-intron vector

The vector CGT11003-intron has a Pdk intron derived from the vector pHELLSGATE (from Dr. P. Waterhouse, Accession No. AJ311874). This vector provides a convenient system for cloning PCR products amplified with oligonucleotide primers containing enzyme restriction sites and ‘clamp’ sequences (Table 1). The vector and inserts (FL, CT, and NT) were prepared by digesting with restriction enzymes *Xba*I and *Kpn*I, or *Bam*HI and *Bst*BI, to generate sticky ends for cloning sense and antisense PCR amplified fragments, respectively. The cloning was done sequentially starting with the sense fragment, and later the antisense fragment. Isolated plasmids containing PCR fragments were confirmed by a combination of enzyme digestion, PCR/colony PCR using specific primers, and sequence analysis.

2.2.5 Cloning of CBSV CP gene for expression

For expression of CBSV CP, the full-length CP gene (amplified with a primer pair containing *Xho*I & *Bam*HI sites) was cloned in a pUC vector, digested with the same enzymes, between 35S CaMV promoter and tNOS terminator. The 35S CaMV promoter-FL-CP-tNOS terminator gene cassette was digested and sub-cloned in pCambia2300, to generate a construct pCambia2300-CBSV-CP (pILTAB721), for transient assay in *N. benthamiana.*

2.2.6 Transfer of DNA into *Escherichia coli* DH5α cells

2.2.6.1 Culture media

The bacterial cells used throughout the study were cultured in LB medium (10 g Bacto Tryptone, 5 g Bacto Yeast Extract, and 10 g Sodium Chloride, pH 7.0) from SIGMA.

2.2.6.2 Preparation of competent *E. coli* DH5α cells

A protocol according to Sambrook et al. (2001) (56) was used to prepare competent *E. coli* DH5α with minor changes. Small volume of bacterial cells from a stock culture was grown in 6 ml LB medium incubated at 37°C with shaking

overnight in LB medium. One ml of the overnight culture was transferred to 100 ml of LB to which 1 ml of 10 mM magnesium chloride was added in 500 ml flask and incubated at 37°C with vigorous shaking at 200-250 rpm for 2-4 hrs until the OD_{600} reached between 0.4-0.6. The suspension was transferred to ice-cold 30 ml sterile centrifuge tubes (SS-34 tubes), centrifuged for 10 min at 4000 rpm at 4°C, medium decanted from cell pellet and allowed to stand in an inverted position on a pad of paper towels for 1 min to allow the last traces of media to drain away. The bacterial pellet was resuspended by swirling or gentle votexing first in 1-2 ml of 100 mM ice-cold $CaCl_2$ and then final volume made up to 50 ml and incubated on ice for 1 h. Cells were collected by centrifugation at 4000 rpm for 10 min at 4°C, decanted, tubes allowed to stand in an inverted position on a pad of paper towels for 1 min to allow the last traces of $CaCl_2$ to drain away and resuspended in 2 ml of 100 mM $CaCl_2$, containing 20% glycerol (1.2 ml 100mM $CaCl_2$ + 0.8 ml 100% glycerol) and incubated at 4°C for 2-10 hrs. Aliquots (0.1 ml) of this cell suspension were dispensed into Eppendorf tubes, quick-freezed in liquid nitrogen and stored at -80°C.

2.2.6.3 **Transformation of *E. coli* DH5α cells**

Plasmid DNA (~200 ng) containing gene fragments of interest were gently mixed with 100 µl competent cells (thawed on ice) in a 1.5 ml Eppendorf tube before incubating on ice for 30 min. The cells were subjected to heat shock at 42°C in a water bath for exactly 90 s without shaking tubes and chilled immediately on ice for 1-2 min. To allow transformed cells to recover and express the antibiotic resistance gene/marker encoded by the plasmid, 200-250 µl LB medium was added to the tubes and incubated for 45 min to 1 h at 37°C with slow shaking. 100 µl aliquots of each transformation reaction were plated onto LB agar plates containing 100 mg/ml of ampicillin. The plates were incubated upside down at 37°C overnight.

2.2.6.4 **Colony and plasmid PCR**

Colony PCR using specific primers was used to determine whether a specific colony on a plate had the desired clone. Colonies of transformed *E.coli* cells were

picked, streaked on master plate and a portion resuspended in 100 µl of LB medium, votexed and incubated for 5-10 min at 95°C and 2 µl used for PCR reaction. In addition, plasmid mini preparations were analyzed for the presence of inserts using the vector primers flanking the expected fragment. The PCR product was analyzed for presence of DNA fragments by electrophoresis in a 1% agarose gel.

2.2.6.5 **Isolation of recombinant plasmid DNA from transformed *E. coli***

Plasmid DNA was purified from colonies with desired clones using QIAprep Spin Miniprep kit (Qiagen) according to manufacturer's instruction. A single colony of bacteria was picked from a freshly streaked selective plate to inoculate a starter culture of 5 ml LB medium containing the appropriate selective antibiotic. The culture, in a tube or flask with a volume of at least 4 times the volume of the culture was incubated overnight at 37°C with vigorous shaking (approx. 250-300 rpm). The bacterial cells were harvested by centrifugation at 4,000 rpm for 10 min at 4°C, pellets resuspended in 250 µl of buffer P1 (kept at 4°C) and transferred to a microfuge tube. 250 µl of Buffer P2 added, mixed thoroughly by inverting the sealed tube 4–6 times, and 350 µl of buffer N3 added, mixed immediately and thoroughly by inverting 4–6 times, centrifuged at 13,000 rpm for 10 min, supernatant containing plasmid DNA removed promptly by pipetting and applied to the QIAprep spin column. The QIAquick spin column was centrifuged for 30–60 s in a 2 ml collection tube to bind DNA to the QIAquick column membrane. The flow-through was discarded and the QIAquick column placed back into the same tube. Buffer PE (0.75 ml) was added to wash the QIAquick column and centrifuge for 30–60 s, flow-through discarded, QIAquick column placed back in the same tube and the column centrifuged for an additional 1 min to completely remove residual ethanol. The QIAquick column was put in a clean 1.5 ml microcentrifuge tube, 50 µl buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) added to the center of the QIAquick membrane, the column allowed to stand for 1 min, then centrifuged for 1 min to elute DNA

2.2.6.6 **Sequencing of DNA**

Plasmid DNA samples confirmed by PCR and restriction analysis to have the desired genes were sent to PNACL Washington University sequencing facility, 4559 Scott Avenue Biotech building room 406 Saint Louis, Missouri, USA for sequencing

2.2.6.7 **Preparation of glycerol stock cultures**

Permanent cultures of bacterial cells were prepared for clones containing desired DNA fragments. Well grown bacteria culture (800 µl) in media with appropriate antibiotic was mixed with 200 µl of sterile glycerol by inverting in 2 ml sterile Eppendorf tubes and frozen at -80°C.

2.2.7 Sub-cloning hpCBSV-CP genes into vector AKK-1420-RNAi-GFP

The plasmid CGT 11003-intron containing the sense and antisense gene fragments of FL, CT and NT were digested with the restriction enzyme *Asc*I to release the sense-intron-antisense cassette and the CsVMV promoter and tNOS terminator sequences. The digested plasmids were run on 1% agarose gel and cassette extracted and purified. To clone the cassette in the binary vector AKK-1420-RNAi-GFP (11), the same enzyme *Asc*I was used to digest the vector followed by treatment with calf intestinal alkaline phosphatase (CIP). The cassettes released from CGT 11003-intron vector were ligated into the vector AKK-1420-RNAi-GFP to form the plasmids AKK-1420-RNAi-GFP-CBSV-CP-FL (pILTAB715), AKK-1420-RNAi-GFP-CBSV-CP-CT (pILTAB717), and AKK-1420-RNAi-GFP-CBSV-CP-NT (pILTAB716) harbouring hairpin gene constructs for FL, CT and NT of CBSV-CP respectively, and assigned pILTAB plasmid codes indicated, and also collectively referred to as hpCBSV-CP. The hpCBSV-CP plasmids were transformed directly in *A. tumefaciens* strain GV3103 for transient assay. Presence of respective genes, FL, CT, and NT were confirmed by PCR using gene specific primers, enzyme restriction digestion and sequencing.

2.3 Transient Protection Study of CBSV-Derived RNAi and CPMR Gene Constructs in *N. benthamiana*

2.3.1 Plant material for transient assay

Transgenic *N. benthamiana* for GFP (line 16c) were grown under a 28°C/25°C day/night period and a 16 h/8 h photoperiod. For all assays 3- to 5-week-old *N. benthamiana* plants were used.

2.3.2 Preparation of competent *Agrobacterium tumefaciens* cells

Small volume of *A. tumefaciens* stock culture was used to inoculate 2 ml of LB media with appropriate antibiotics and cultured at 28°C on shaker overnight. The 2 ml of overnight growth was used to inoculate a 50 ml flask of media with antibiotics and incubated on a shaker at 220 rpm, 28°C until OD at 600 nm reached 0.6 – 1. The culture was chilled on ice for 5 min, 30 ml of culture transferred to centrifuge tube (pre-cooled to 4°C) and centrifuged at 7,000 rpm at 4°C for 5 min, supernatant discarded, pellet resuspended in 10 ml of ice-cold 0.15 M NaCl and incubated on ice for 15 min. The bacteria suspension was centrifuged again at 7,000 rpm and pellet resuspended in 1 ml of ice cold 20 mM $CaCl_2$, and dispensed in 100 µl aliquots in sterile, pre-chilled Eppendorf tubes, quick-freezed in liquid nitrogen and stored at -80°C.

2.3.3 Transformation of competent *A. tumefaciens* cells by electroporation

About 50-100 µl aliquots of competent cells were thawed on ice, 10-50 ng of plasmid DNA (either of pILTAB715, pILTAB716, pILTAB717 or pILTAB721) added to cells, mixed gently by tapping tubes, transferred into pre-chilled electroporation cuvettes and incubated on ice for 10-30 min. The Gene Pulser unit set to a voltage of 2.5 kV, capacitance 25 µFD, and resistance low range 200 ohms was used to transform *A. tumefaciens* cells. Immediately after electroporation, 1 ml of LB liquid medium was added to the cell suspension, kept at room temperature for 1-2 min then incubated horizontally on shaker at 28°C for at least one hour (~180 rpm). Cells were centrifuged and 100 µl of pelleted cell suspension spread on LB

solid medium containing 50 mg/l kanamycin and 100 mg/l rifamycin (for plasmid + insert selection). Cell were incubated at 28°C and allowed to grow for 2- 3 days.

2.3.4 **Confirmation of successful plasmid transfer into *A. tumefaciens***

Transformed *A. tumefaciens* that grew to colonies were sampled and transferred into tubes containing 2 ml of LB medium and the relevant antibiotics and incubated at 28°C on a shaking platform overnight. Plasmid DNA was isolated from the cell culture using "QIAprep Spin Miniprep Kit" (Qiagen) following manufacturer's instructions. The isolated plasmid DNA was used to transform competent *E. coli* cells to quickly multiply the DNA, and were later isolated from the *E. coli* colonies for confirmation of clone by restriction analysis and PCR.

2.3.5 **Preparation of *A. tumefaciens* cultures for agro-infiltration**

Agrobacterium tumefaciens strain GV3103 containing the hpCBSV-CP constructs and pILTAB721 were grown overnight at 28°C in 100 ml conical flask containing 10 ml of LB medium supplemented with 50 µg kanamycin per ml on a shaker (240 rpm). Aliquot of 50 µl of this overnight culture was used for inoculation of 10 ml of LB medium supplemented with 10 mM MES buffer, pH 5.7, 50 µg kanamycin per ml and 150 µM acetosyringone (3,5-dimethoxy-4'-hydroxy-acetophenone) (68) and cultured for 12-20 hrs to an OD_{600} of 0.5-1.0. The bacterial cells were centrifuged at 6,000 rpm at 4°C for 10 min, washed once with an equal volume of liquid MS medium (pH 5.3) and cells pelleted again by centrifugation at 4°C for 10 min. The bacterial pellet was finally resuspended to a final concentration corresponding to an optical density of 1.0 (67) at 600 nm in a solution containing 10 mM $MgCl_2$, 10 mM MES pH 5.7, and 150 µM acetosyringone. Cultures were incubated at room temperature for 2-5 hrs before infiltration.

2.3.6 **Agro-infiltration procedure**

Eighteen *N. benthamiana* plants at 4-6 leaf stage (21 days old) were infiltrated. Fully open 2-3 top-leaves per plant were infiltrated with the culture of recombinant *A. tumefaciens* strain GV3103 on the undersurface of the leaves using a 2-ml syringe without a needle.

2.3.7 GFP imaging

Visual detection of GFP fluorescence in whole transient transformed leaves was performed using a hand-held long-wavelength ultraviolet lamp. The transiently transformed leaves were photographed with a Digital Camera Nikon Coolpix 995 Ultra Zoom through a Yellow filter.

2.3.8 Detection of short RNAs in agro-infiltrated *N. benthamiana* plants

To check expression of constructs and determine siRNA levels, leaf samples were collected daily from agro-infiltrated plants, siRNA isolated, and analyzed by northern-blot as previously described (section 2.1.3.6).

2.3.9 Virus challenge of agro-infiltrated plants

Test plants were inoculated with CBSV infectious sap 3 days after agro-infiltration and kept in the growth chamber (16 hrs day 28°C; 8 hrs night 25°C; RH 70%). Symptom development was monitored daily from 2 days after inoculation for 14 days. Disease symptom severity on fully expanded leaves was recorded on a 0-5 scale as previously described (section 2.1.3.1).

Chapter 3

RESULTS

3.1 Development of an Efficient System for Mechanical Transmission of CBSV to *N. benthamiana*

3.1.1 Overview

To complement the ongoing efforts for CBSD control via conventional breeding, cassava cultivars with broad-spectrum resistance to CBSD can be developed through genetic engineering using transgene sequences derived from CBSV. These constructs need to be transiently evaluated for resistance to CBSV initially in a susceptible laboratory host plant prior to transformation into cassava, which is a highly laborious and time consuming task. To develop and deploy CBSD resistant germplasm, inoculation techniques that consistently separate resistant from susceptible cultivars are a prerequisite. CBSV transmission rate by *Bemisia tabaci* is very low in laboratory conditions when compared to the field situation (45). Availability of a highly efficient method for mechanical transmission would facilitate further understanding of the etiology and biological properties of CBSV. The objective of this study was to develop an efficient protocol that ensures reliable mechanical transmission of CBSV to *N. benthamiana*, a widely used laboratory host plant for study of several plant viruses.

3.1.2 Sap transmission of CBSV from cassava to *N. benthamiana*

CBSV isolates from four different Ugandan cassava cultivars (I 92/0057, I 95/0087, Ebwanateraka and TME 204) were used as source of inoculum for transmission of CBSV to *N. benthamiana.* The presence of CBSV was confirmed by RT-PCR as previously described (section 2.1.1) (Fig. 4). Symptomatic leaf tissues were obtained from CBSV positive plants and used to prepare infectious sap as previously described. The sap was used to inoculate 21-day-old *N. benthamiana* plants. Inoculums from all the cassava cultivars were infectious in *N. benthamiana* but the rate of transmission was low. For further studies the CBSV isolate from the cassava cultivar Ebwanateraka was used to ensure consistency. Leaf samples

from inoculated plants were tested by RT-PCR to confirm transmission of CBSV. The CBSV positive plants were maintained as live culture in the growth chamber for subsequent sap transmission.

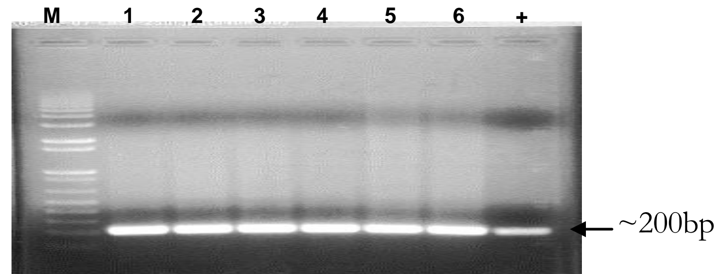


Fig. 4: CBSV-CP DNA fragment amplified by RT-PCR. Lane M, 2 kb+ DNA size marker. Lanes 1-6, cDNA synthesized from CBSV-infected cassava leaf samples from different plants. Lane +, CBSV positive control

3.1.3 Effect of antioxidants on rate of CBSV transmission to *N. benthamiana*

To offset the oxidation process and thus the production of antioxidants like phenolics, which degrade RNA, the antioxidants β-mercaptoethanol and sodium sulphite were added to the phosphate buffer. A transmission rate of 100% (N = 18) was achieved at 3 to 5 DPI when inoculum was prepared in buffer containing the antioxidants, Na_2SO_3 and β-mercaptoethanol. By 7 DPI, the final rates of transmission were 63.9%, 72.3%, and 80.2% with water, buffer, and buffer containing Na_2SO_3, respectively (Fig. 5).

3.1.4 Effect of inoculum concentration on CBSV transmission

The concentration of sap is another important factor that determines successful transmission of the virus. The effect of inoculum concentration on CBSV transmission was studied using different sap dilutions. The rate of CBSV transmission decreased with increasing sap dilution. At dilution 1:5, 61.1% of inoculated plants (N = 18) produced symptoms at 3 to 5 DPI (Fig. 6); at dilution 1:10, 1:20, 1:50, 1:100, 1:500, and 1:1000, transmission rates of 72.3%, 50%, 13.9 %, 11.1 %, 5.6 %, and 2.8 % resulted, respectively (Fig. 6).

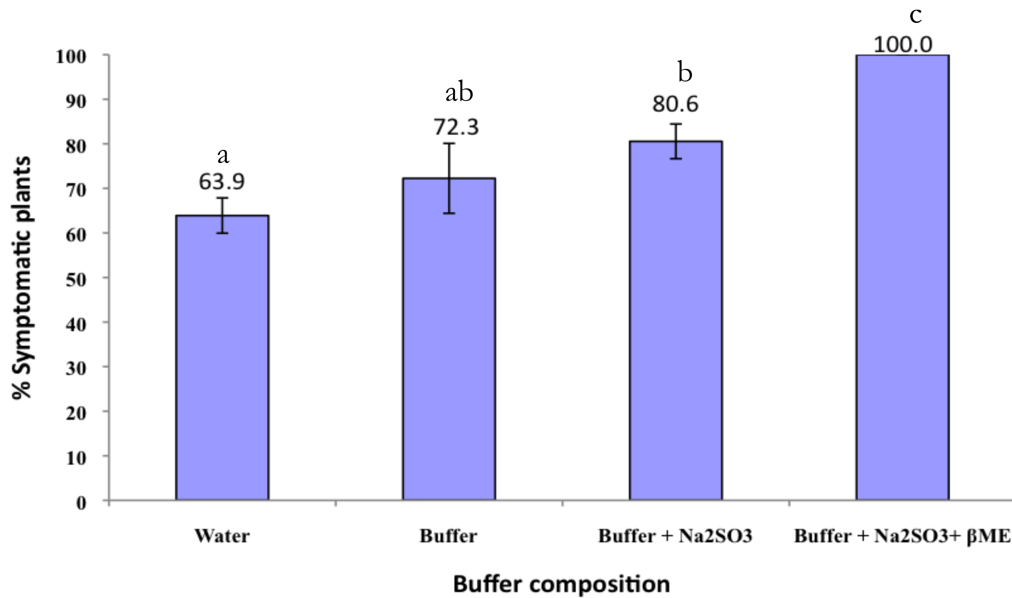


Fig. 5. Sap transmission of CBSV to *N. benthamiana* using buffers of different compositions. Bars represent mean incidence from two trials at 14 days post-inoculation. Bars marked with the same letter are not significantly different from each other at *P = 0.05*.

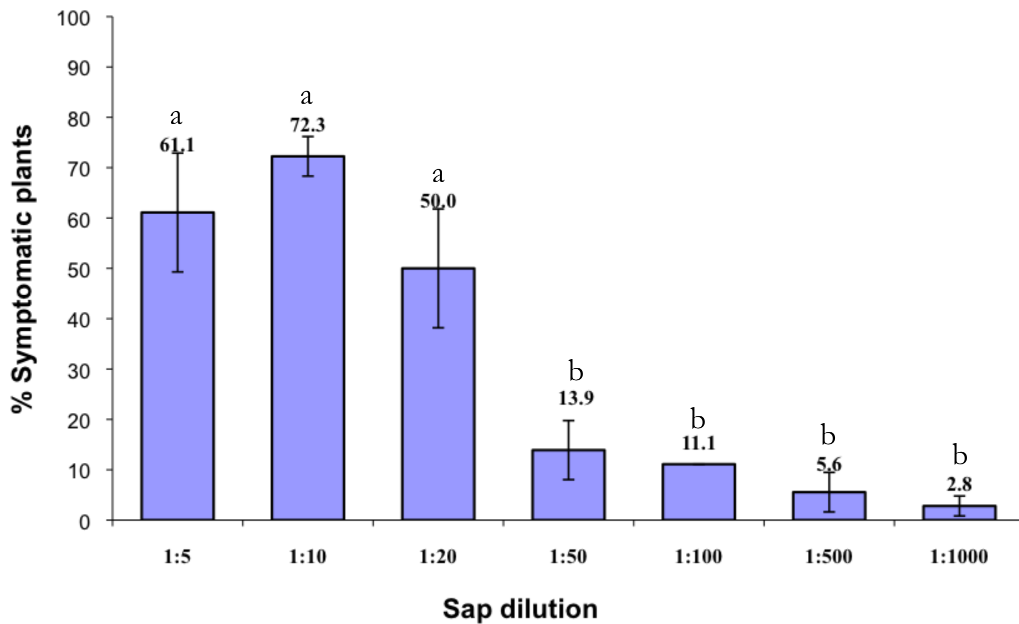


Fig. 6: Transmission of CBSV to *N. benthamiana* with sap of varying concentrations. Bars represent mean incidence from two trials at 14 days post-inoculation. Bars marked with the same letter are not significantly different from each other at *P = 0.05*.

3.1.5 Effect of age at inoculation on efficiency of transmission of CBSV to *N. benthamiana*

A plants' age determines the success of virus transmission and infection. When seedlings at different growth stages were inoculated (N = 18), symptoms started developing in the majority (50 to 75%) of inoculated plants by 3 to 5 DPI and in the rest of the plants symptoms appeared by 7 DPI. For plants inoculated at 21 days after planting (DAP), a final CBSD incidence of 94.4% resulted. Inoculation with plants 28, 35 and 42 DAP resulted into transmission rate of 100% in the three trials and 50 to 60% of the inoculated plants developed symptoms at 6 DPI, and by 10 DPI, the remaining plants produced symptoms. The average incubation period increased with the age of plants inoculated. In the three inoculation trials, an average incubation period of 3.3, 3.1, 4.3 and 5.0 days were required for the 21, 28, 35, and 42 days old plants respectively (Fig. 7). Symptoms in the plants inoculated after flowering stage (42 DAP) were less prominent in the beginning though eventually it became severe. It took an average of 5.0 days for symptoms to clearly develop.

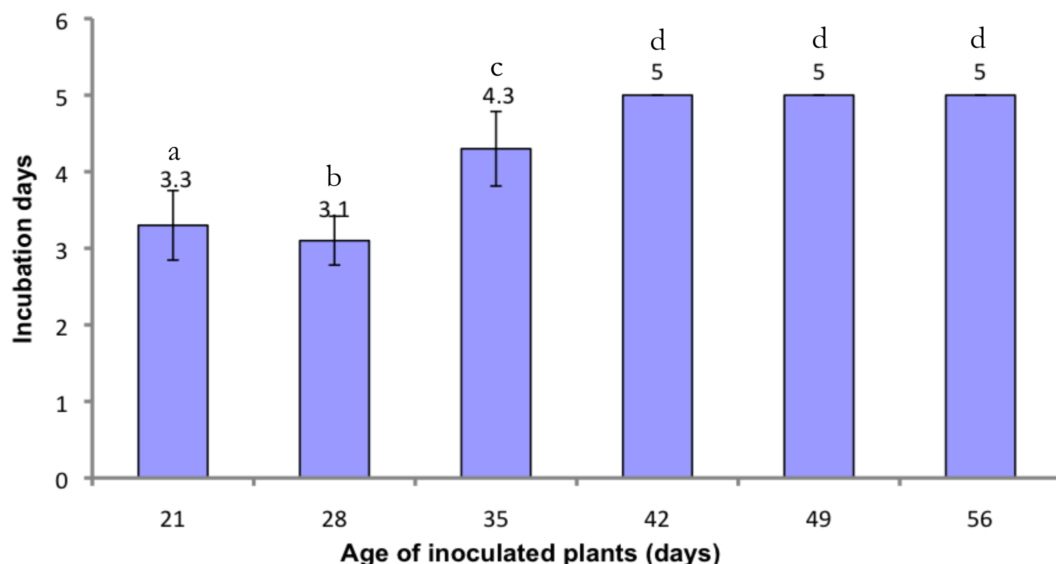


Fig. 7: Transmission of CBSV to *N. benthamiana* plants of varying age. Bars represent mean incubation days from three trials (N = 18). Bars marked with the same letter are not significantly different from each other at $P = 0.05$.

3.1.6 Effect of age of inoculum source plants on transmission of CBSV to *N. benthamiana*

When seedlings were inoculated with sap from 14-day-old infected leaf tissues, 19.5% of the inoculated plants were infected (Fig. 8). However, inoculum from 28, 42, 56, 70 and over 80 days old infected leaf tissues resulted in 38.9, 41.7, 55.6, 94.5, and 100% infection respectively, in the two trials (Fig. 8).

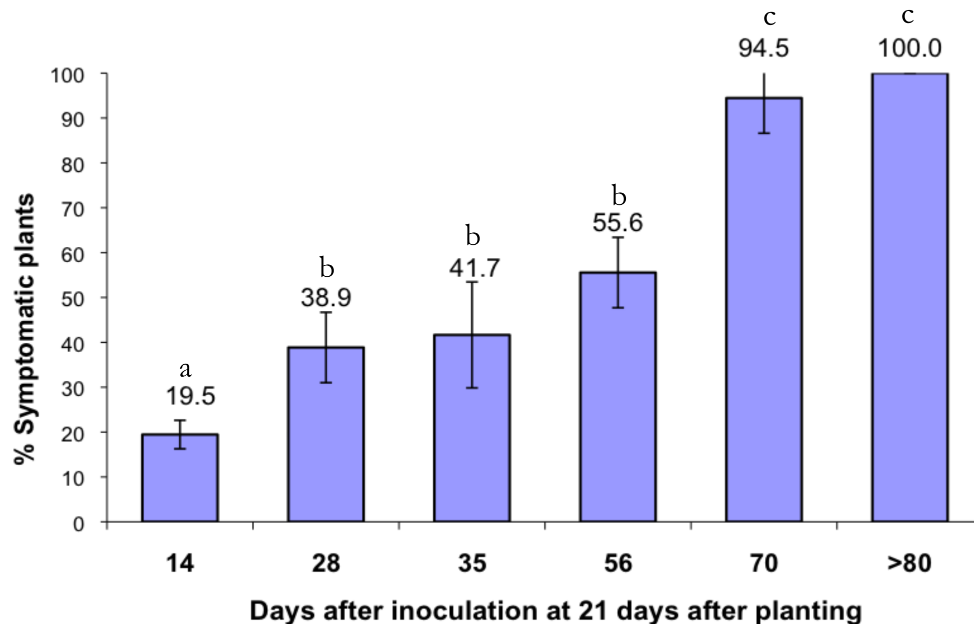


Fig. 8: Transmission of CBSV to *N. benthamiana* from inoculum source plants varying in age. Bars represent mean incidence from two trials at 14 days post-inoculation. Bars marked with the same letter are not significantly different from each other at $P = 0.05$.

3.1.7 Effect of temperature on CBSV transmission and expression

Temperature is known to greatly influence plant virus interactions. To determine its effect on CBSV infectivity and symptom severity, *N. benthamiana* plants inoculated with CBSV infectious sap were kept at varying temperature conditions while keeping the other factors fairly constant. The symptom severity of CBSV in inoculated plants increased with increase in temperature (Fig. 9A) while the average incubation period decreased with increasing temperature (Fig.10B). When seedlings were inoculated and kept in the temperature chamber maintained at 21°C, symptoms started developing in the majority (>60%) of plants by 6 to 8

DPI and in the rest of the plants symptoms appeared by 11 DPI. The average incubation period was 7.1 days (Fig. 9B). When plants were inoculated and kept at 25°C, over 60% of the inoculated plants developed symptoms at 5 DPI, and by 10 DPI, the remaining plants produced symptoms. The average incubation period was 5.2 days (Fig. 9B). At 27°C, up to 50% of the inoculated plants developed symptoms at 4 DPI, and by 9 DPI, the remaining plants produced symptoms. The average incubation period was 4.4 days (Fig. 9B). At 30°C over 60% of the inoculated plants developed symptoms at 3 DPI, and by 5 DPI, the remaining plants produced symptoms. The average incubation period was 3.6 days (Fig. 9B).

3.1.8 **Analysis of virus-derived siRNA in CBSV inoculated plants at different temperatures**

To determine how fast CBSV-derived siRNA accumulate at 21, 25, 27, and 30°C, leaf samples were collected from the inoculated plants of the previous temperature experiment (section 2.1.3.5) for a period of 14 days commencing a day after inoculation. Analysis of the siRNA levels showed that plants kept at 30°C accumulated siRNA faster than plants kept at 21°C (Fig. 10). The siRNA accumulation corresponded with the days to onset of symptom expression at the respective temperature conditions. These results indicate that higher temperature favor CBSV transmission and expression. Taken together, these results show that buffer composition, inoculums source and concentration, plant age, and temperature of growth environment play vital roles in CBSV transmission and disease progress.

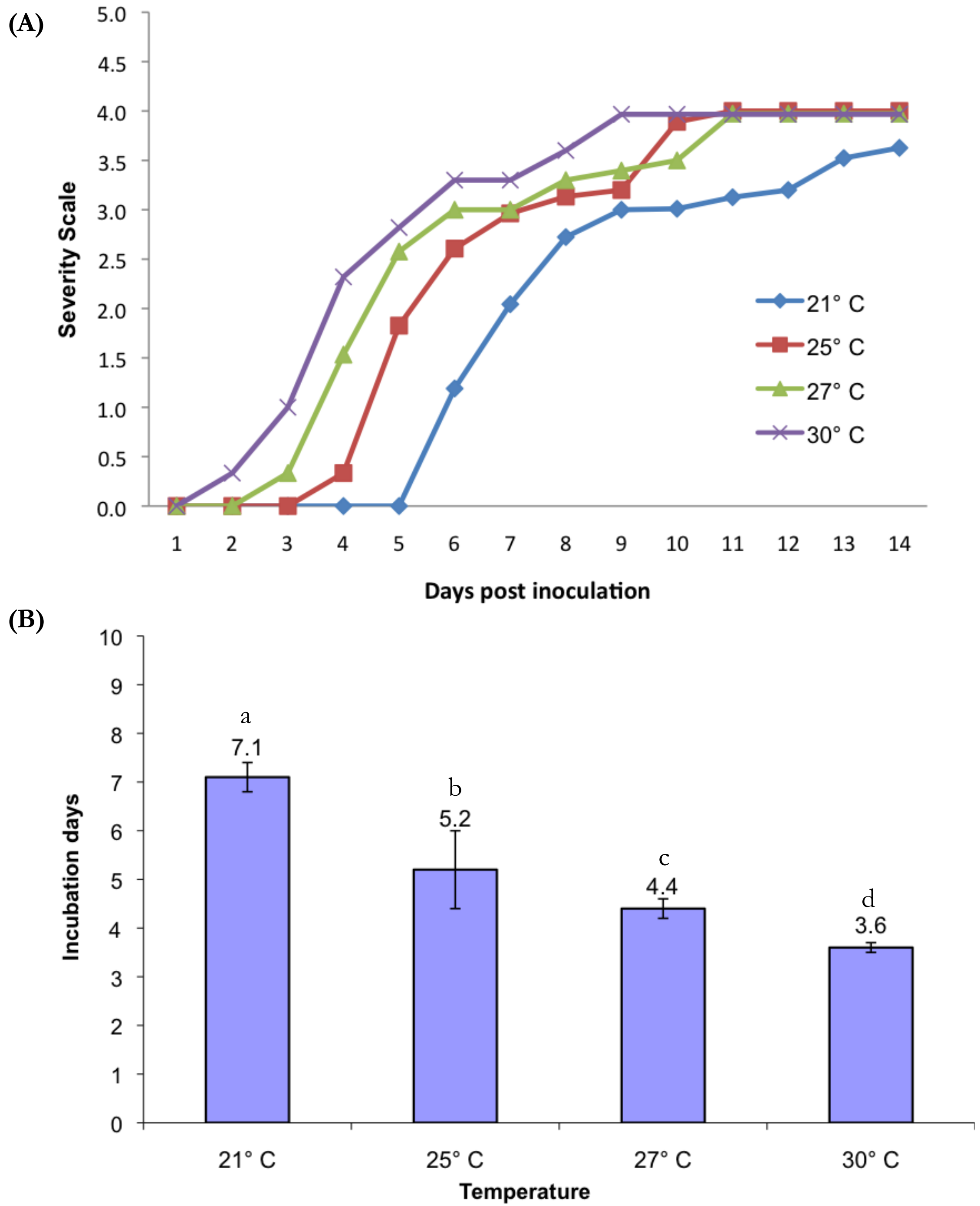


Fig. 9: Progress in expression of symptoms incited by CBSV in 21-day-old *N. benthamiana* at different temperatures. (A), changes in severity over time. (B), mean number of days to first appearance of symptoms. Bars marked with the same letter are not significantly different from each other at $P = 0.05$.

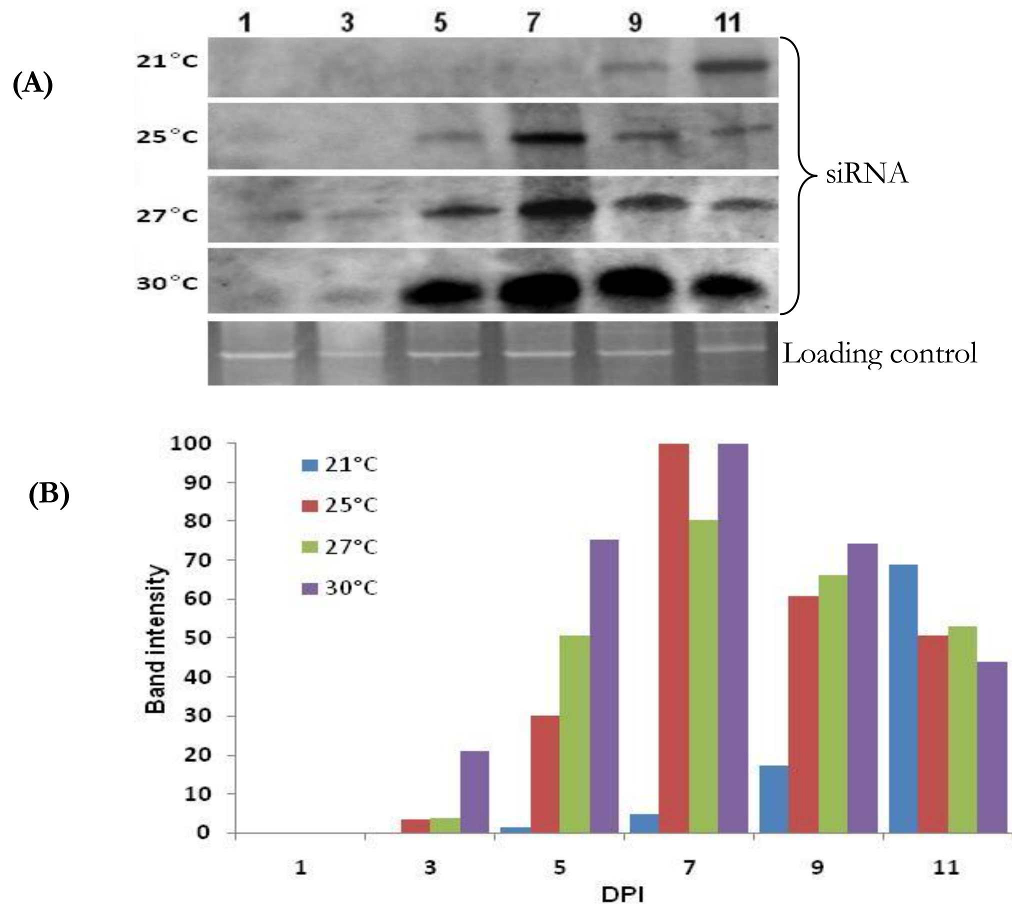


Fig. 10: The levels of viral siRNA accumulation in infected tobacco plants in four temperature conditions: 21°C, 25°C, 27°C, and 30°C. (A) Northern blots using CBSV CP specific probe. (B) Graphical representation of densitometric values of siRNA band intensity in (A) obtained using ImageJ software.

3.2 Construction of CBSV-Derived RNAi and CP Genes Constructs

3.2.1 Overview

Pathogen-derived resistance approaches have been used to develop to commercial level virus resistant crop plants such as squash, potato and papaya. Many of the viruses controlled by PDR approaches such as TSWV, TEV, TMV, PVY, and PRSV belong to the family *Potyviridae*, to which CBSV is a member (36). Thus, CBSV is an appropriate target for PDR strategy. The aim of this study was to develop gene constructs tailored to confer resistance to CBSD through RNAi and CPMR. The full-length CP gene and its N-and C-terminal regions were used to generate three RNAi (hpRNA) constructs in addition to a construct for

expression of full-length CP gene for CPMR studies. Plants transformed with hpRNA constructs produce visible or measurable silencing effect in 70–100% of the resulting plants (18, 72), and are stably inherited from generation to generation, thereby enabling the continued study of a phenotype.

3.2.2 Amplification of CBSV-CP fragments by RT-PCR

Several sets of primers were designed from the published CBSV sequence (GenBank accession No. AY007597) using the Gene Runner computer program (Table 1). The primers, CBSV 10F (5'-ATC AGA ATA GTG TGA CTG CTG G-3') and CBSV 11R (5'-CCA CAT TAT TAT CGT CAC CAG G-3') previously designed by Monger et al (2001) were used as control primers to confirm presence or absence of CBSV in samples (49) (Fig. 4).

The full-length, N- and C-termini of CBSV CP were amplified in the sense orientation using the primer sets CP-FL-XbaI-F and CP-FL-KpnI-R, CP-CT-XbaI-F and CP-CT-KpnI-R, and CP-NT-XbaI-F and CP-NT-KpnI-R that introduced an *Xba*I site and an *Kpn*I site at the 5'-ends and the 3'-ends, respectively. The antisense fragments were amplified using the primer sets CP-FL-BamHI-F and CP-FL-BstBI-R, CP-CT-BamHI-F and CP-CT-BstBI-R, and CP-NT-BamHI-F and CP-NT-BstBI-R that introduced a *Bam*HI site and a *Bst*BI site at the 5'-ends and the 3'-ends, respectively. The expected fragment sizes were 908 bp, 503 bp, and 402 bp for the full-length, C- and N-termini of CBSV CP, respectively. The amplified fragments were resolved by electrophoresis in 1% agarose gel. A DNA size marker (1 kb+, Invitrogen) was included in each gel for comparison of DNA fragment sizes. Fragments were visualized by UV irradiation (302 nm) and gel pictures taken using Alpha Inotech AlphaImager™ 2200 gel documentation system. The observed fragment sizes matched with the expected sizes (Fig. 11). For expression of CBSV CP, the primer pair CP-FL-XhoI-F and CP-BamHI-R was used and introduced *Xho*I and *Bam*HI restriction sites at the 5'-end and the 3'-end, respectively.

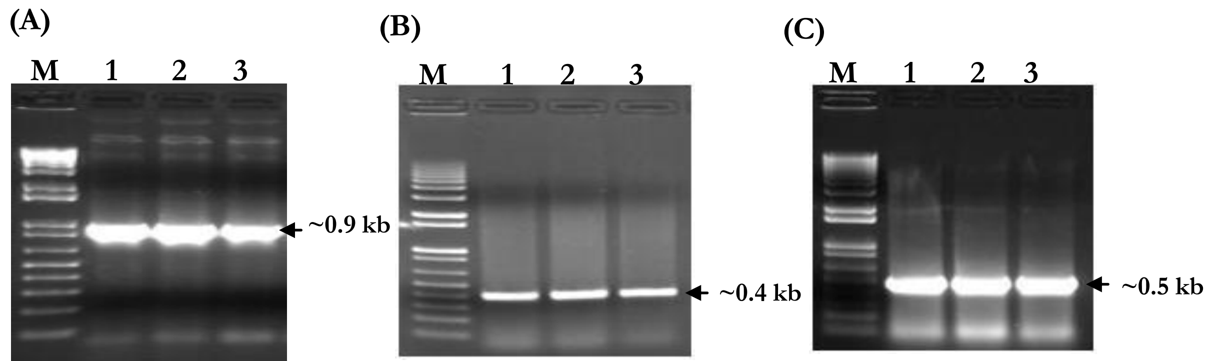


Fig. 11: Amplification of CBSV-CP gene fragments using specific primers. Lane M, 2 kb+ DNA size marker. Lanes 1-3, cDNA synthesized from CBSV-infected cassava leaf samples from cultivar Ebwanateraka. (A) Full-length CP gene fragment, (B) N-terminal, and (C), C-terminal gene fragment.

3.2.3 Construction of the hairpin CBSV-CP gene (hpCBSV-CP)

The resulting PCR fragments were run in 1% agarose gel, extracted from the gel, purified and digested with *XbaI* and *KpnI*, or *BamHI* and *BstBI* for the sense and antisense fragments, respectively. The cloning strategy shown in figure 12 was followed. The fragments were sequentially cloned, one fragment at a time, into CGT11003-intron vector at the corresponding sites, forming the sense-intron-antisense cassettes for the full-length, N- and C-termini of CBSV-CP, respectively (Fig. 13 A-C). The restriction enzyme *Asc*I was then used to digest and release the cassette, including the CvMV promoter and tNOS terminator regions, from the plasmid. The entire cassettes containing the promoter and terminator regions were cloned into the vectors AKK-1420, into the *Asc*I site (Fig. 13 D-F), and into *SmaI* site in pCambia2300 after blunt ligation of the cassette using Klenow DNA polymerase (Fig. 13 G-I). A total of seven different constructs were therefore generated, three hairpin constructs in AKK-1420 (pILTAB715, pILTAB716, and pILTAB717, corresponding to the plasmids AKK-1420-RNAi-GFP-CBSV-FL, AKK-1420-RNAi-GFP-CBSV-CT, AKK-1420-RNAi-GFP-CBSV-NT, respectively) (Fig. 13 D-F), and four (three hairpin constructs, and a CP expression construct) in pCambia2300 (pILTAB718, pILTAB719, pILTAB720, and pILTAB721, corresponding to the plasmids pCambia2300-RNAi-CBSV-FL, pCambia2300-

RNAi-CBSV-CT, pCambia2300-RNAi-CBSV-NT, and pCambia2300-CBSV-CP, respectively) (Fig. 13 E-I and Fig. 14). Presence of inserts was confirmed by PCR, restriction analysis, and sequencing.

3.2.4 Construction of the CBSV-CP expression cassette

The RT-PCR fragments amplified using the primer pair CP-FL-XhoI-F and CP-BamHI-R was purified from the gel, digested with restriction enzymes *Xho*I and *Bam*HI, and cloned in a pUC19 vector at the corresponding sites, between CaMV35S promoter and tNOS terminator, forming the CaMV35S-promoter-FL-CP-tNOS-terminator gene cassette (Fig. 14). The restriction enzymes *Hind*III and *Sac*I were used to digest and release the cassette, including the CaMV35S promoter and tNOS terminator regions, from the plasmid. The released cassette was cloned in pCambia2300, in the corresponding restriction sites (*Hind*III and *Sac*I sites), to generate a construct pCambia2300-CBSV-CP (pILTAB721) (Fig. 14).

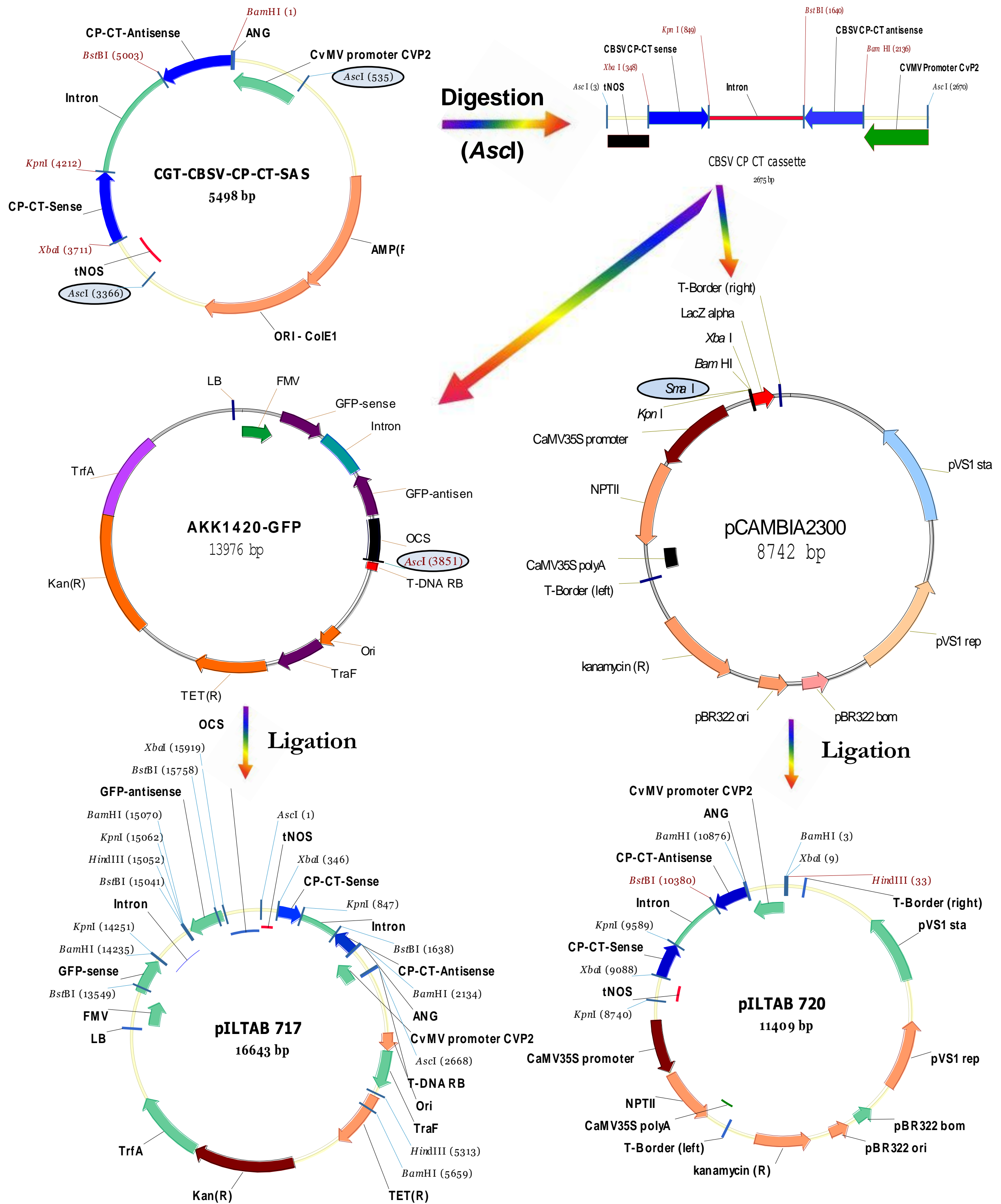
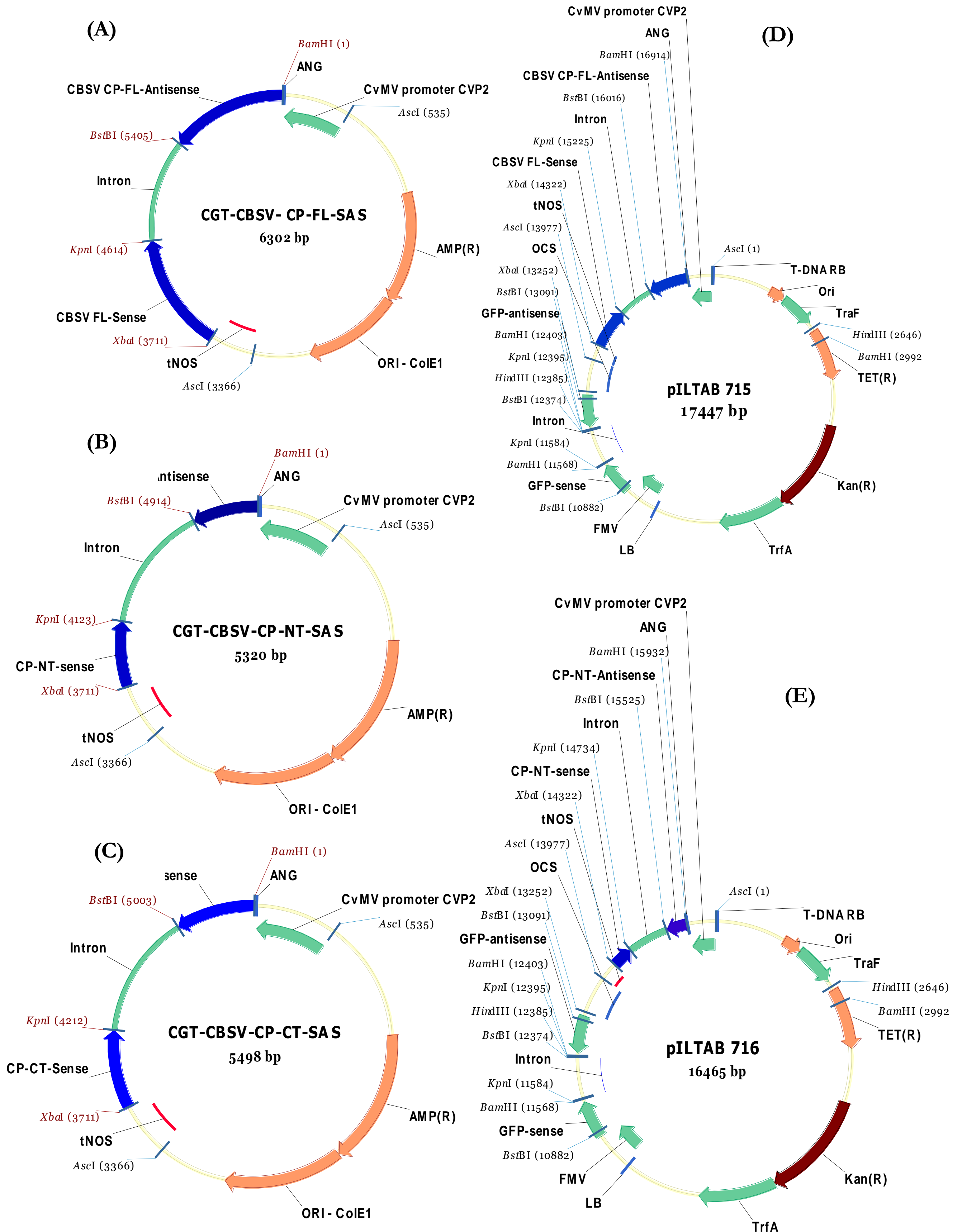


Fig.12: Flow diagram showing vector maps and approach used to clone hpCBSV-CP genes (using CBSV-CP-CT as example)



(A)
CGT-CBSV- CP-FL-SAS
6302 bp
BamHI (1)
ANG
CBSV CP-FL-Antisense
CvMV promoter CVP2
AscI (535)
BstBI (5405)
Intron
AMP(R)
KpnI (4614)
CBSV FL-Sense
XbaI (3711)
tNOS
AscI (3366)
ORI - ColE1
(B)
CGT-CBSV-CP-NT-SAS
5320 bp
BamHI (1)
ANG
BstBI (4914)
CvMV promoter CVP2
AscI (535)
Intron
KpnI (4123)
CP-NT-sense
XbaI (3711)
AMP(R)
tNOS
AscI (3366)
ORI - ColE1
(C)
CGT-CBSV-CP-CT-SAS
5498 bp
BamHI (1)
ANG
BstBI (5003)
CvMV promoter CVP2
AscI (535)
Intron
KpnI (4212)
CP-CT-Sense
XbaI (3711)
AMP(R)
tNOS
AscI (3366)
ORI - ColE1
(D)
pILTAB 715
17447 bp
CvMV promoter CVP2
ANG
BamHI (16914)
CBSV CP-FL-Antisense
BstBI (16016)
Intron
KpnI (15225)
CBSV FL-Sense
XbaI (14322)
tNOS
AscI (13977)
OCS
XbaI (13252)
BstBI (13091)
GFP-antisense
BamHI (12403)
KpnI (12395)
HindIII (12385)
BstBI (12374)
Intron
KpnI (11584)
BamHI (11568)
GFP-sense
BstBI (10882)
FMV
LB
TrfA
Kan(R)
AscI (1)
T-DNA RB
Ori
TraF
HindIII (2646)
BamHI (2992
TET(R)
(E)
pILTAB 716
16465 bp
CvMV promoter CVP2
ANG
BamHI (15932)
CP-NT-Antisense
BstBI (15525)
Intron
KpnI (14734)
CP-NT-sense
XbaI (14322)
tNOS
AscI (13977)
OCS
XbaI (13252)
BstBI (13091)
GFP-antisense
BamHI (12403)
KpnI (12395)
HindIII (12385)
BstBI (12374)
Intron
KpnI (11584)
BamHI (11568)
GFP-sense
BstBI (10882)
FMV
LB
TrfA
Kan(R)
AscI (1)
T-DNA RB
Ori
TraF
HindIII (2646)
BamHI (2992
TET(R)

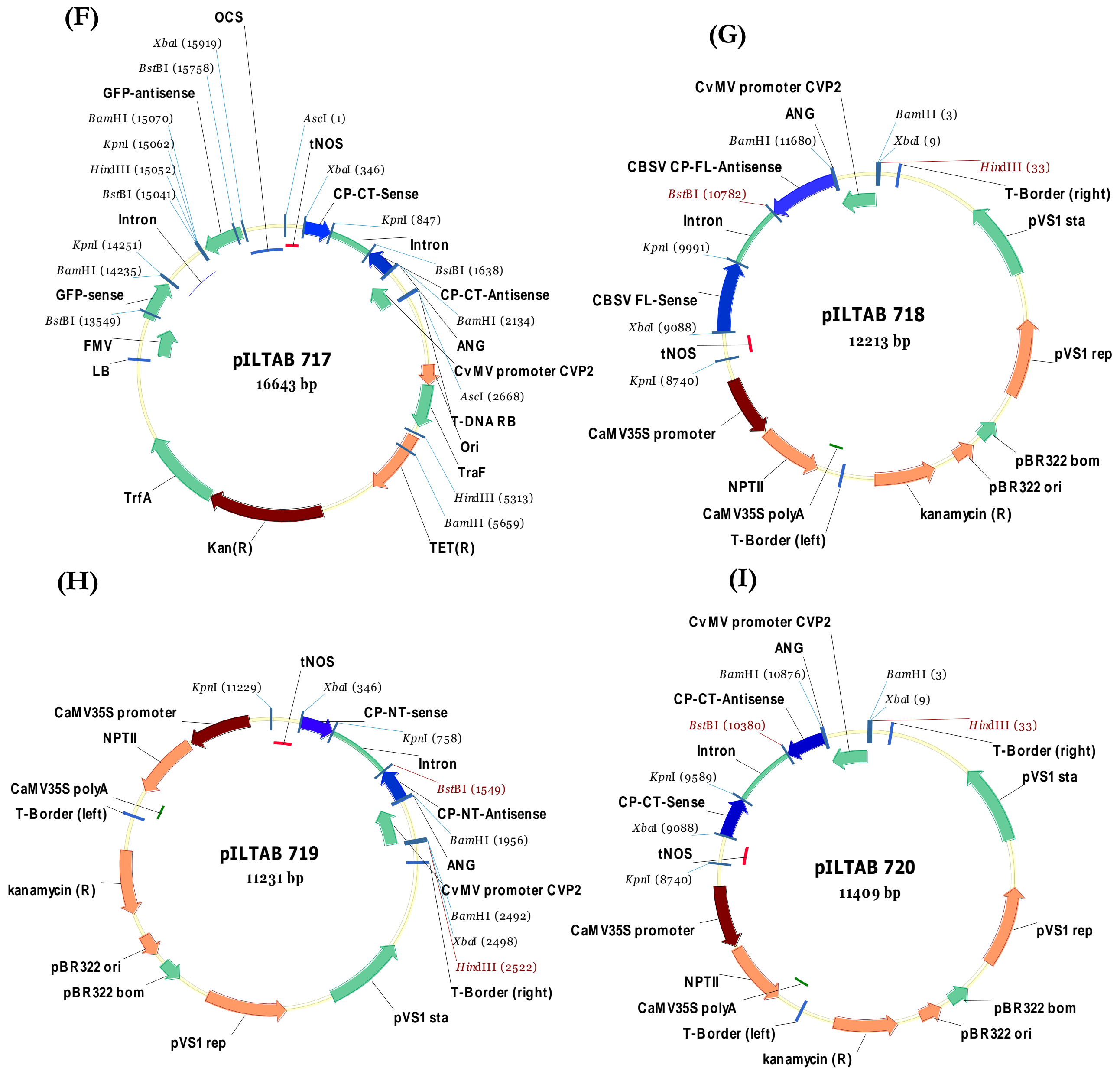


Fig. 13: Vector maps showing positions of cloned CBSV-CP genes. (A) Full-length CBSV-CP cloned in sense and antisense orientation in vector CGT11003-intron. (B) N-terminal CBSV-CP gene fragment cloned in sense and antisense orientation in vector CGT11003-intron. (C) C-terminal CBSV-CP gene fragment cloned in sense and antisense orientation in vector CGT11003-intron. (D) Full-length CBSV-CP cassette sub-cloned in vector AKK-1420. (E) N-terminal CBSV-CP cassette sub-cloned in vector AKK-1420. (F) C-terminal CBSV-CP cassette sub-cloned in vector AKK-1420. (G) Full-length CBSV-CP cassette sub-cloned in vector pCAMBIA2300. (H) N-terminal CBSV-CP cassette sub-cloned in vector pCAMBIA2300. (I) C-terminal CBSV-CP cassette sub-cloned in vector pCAMBIA2300.

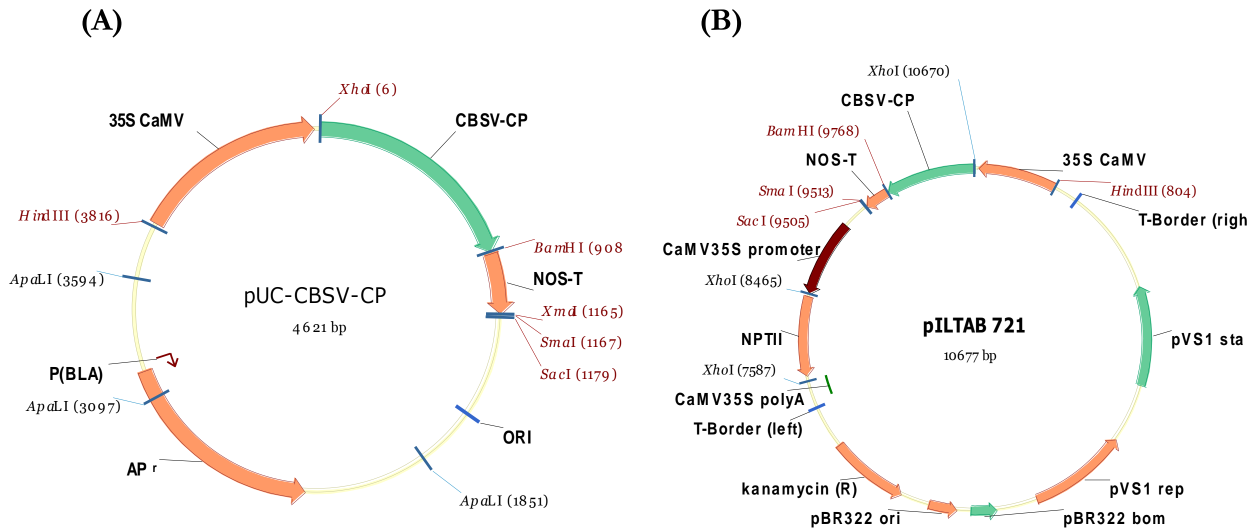


Fig. 14: Vector maps showing cloning of CBSV CP for CP expression. (A) Cloning CBSV CP in vector pUC19. (B), cloning CBSV CP expression cassette in vector pCambia2300

3.3 Transient Protection Study of CBSV-Derived RNAi and CP Gene Constructs in *N. benthamiana*

3.3.1 Overview

Transgenes can be introduced into plant cells by either microprojectile bombardment, or through Agrobacterium-mediated gene expression system (65, 70) to generate stably or transiently transformed plants (section 1.12). For this study, a transient protection assay protocol previously developed by Wydro et al (73) for expressing genes in leaves of *N. benthamiana* was used. Transgenic *N. benthamiana* for GFP and *A. tumefaciens* strains GV3103, transformed with the recombinant binary vector, AKK-1420-RNAi-GFP, harboring hairpin gene encoding plasmids pILTAB715, pILTAB716, pILTAB717 or pILTAB721 were used in transient vaccination studies. Two to three top-leaves of *N. benthamiana* plants at 4-6 leaf stage were infiltrated with culture of recombinant *A. tumefaciens* strain GV3103 on the undersurface of the leaves using a 2-ml syringe without needle. The test plants were sap-inoculated with CBSV after agro-infiltration and kept in the growth chamber. CBSD symptoms on fully expanded leaves were assessed daily after 2 days of sap inoculation for a minimum of 2 weeks on a scale

previously described (section 2.1.3.1). The aim of this study was to evaluate the level of expression of the CBSV-derived gene constructs and their potential to protect against CBSV in transiently transformed *N. benthamiana* before stable transformation into cassava which is a time consuming and labor intensive task.

3.3.2 Plant material for transient assay

N. benthamiana plants which have been stably transformed with the sense and antisense genes for GFP (line 16c) were grown as previously described and used as control to check for GFP silencing by the hpCBSV-CP constructs.

3.3.3 Preparation of *A. tumefaciens* cells for Agro-infiltration

Suspensions of competent *A. tumefaciens* strain GV3103 were prepared as described (section 2.3.2). The cells were transformed with the plasmid DNAs of pILTAB715, pILTAB716, pILTAB717 or pILTAB721 as described (section 2.3.3). Plasmids were isolated from transform cells and analyzed by PCR and restriction digestion to confirm presence of target genes before use in transient assay (Fig.15). Suspensions of recombinant *A. tumefaciens* cells containing plasmids pILTAB715, pILTAB716, pILTAB717 or pILTAB721 were prepared (section 2.3.5) and infiltrated into leaves of test plants.

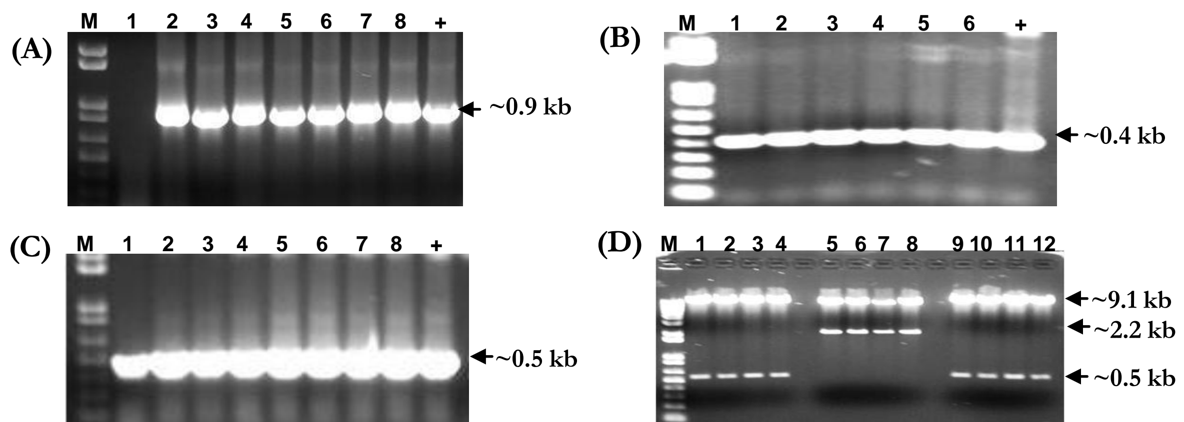


Fig. 15: PCR and Restriction analysis to confirm presence of pILTAB plasmids in transformed *A. tumefaciens* strains GV3103 and LBA4404. (A, B and C) PCR reactions using primers specific to FL (A), NT (B) and CT (C) of CBSV-CP. (D) Restriction digestion of cassettes in vector pCambia2300 using *Xba*I. Lanes M =1kb+ DNA marker, lanes + = positive controls. (A) Lanes 1-8 = pILTAB715; (B) lanes 1-6 = pILTAB716; (C) lanes 1-8 = pILTAB717; (D) Lanes 1-4 = pILTAB720, lanes 5-8 = pILTAB719, lanes 9-12 = pILTAB718.

3.3.4 **Agro-infiltration**

Suspensions of recombinant *A. tumefaciens* strain GV3103 containing ~150 μM acetosyringone (to induce virulence) were used to infiltrate 2-3 fully open top-leaves per test plant using a 2-ml syringe without a needle.

3.3.5 **GFP imaging**

The agro-infiltrated plants were visually checked for GFP fluorescence in whole transient transformed leaves with the aid of a hand-held long-wavelength ultraviolet lamp. The transiently transformed leaves were photographed with a Digital Camera Nikon Coolpix 995 Ultra Zoom through a Yellow filter (Fig. 16). Non-infiltrated plants showed no GFP silencing while plants infiltrated with either GFP control construct or either of the hpCBSV-CP constructs showed clear GFP silencing. This indicated that the constructs were being expressed.

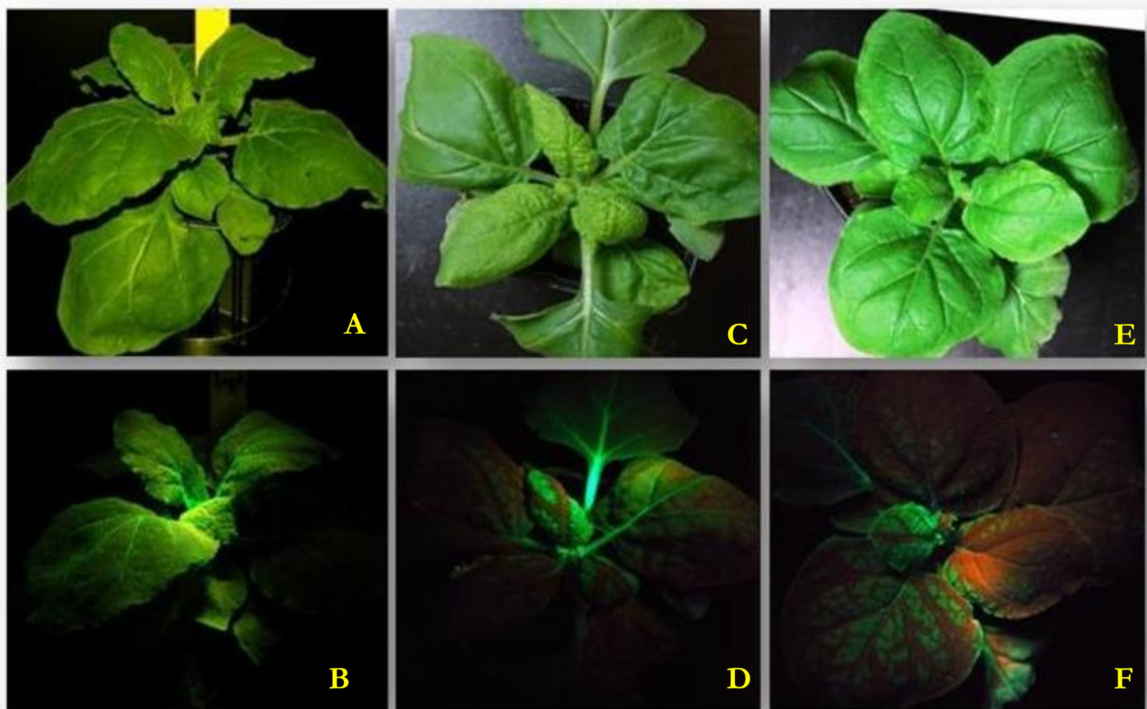


Fig. 16:GFP visualization of *N. benthamiana* in white light and UV illumination. (A) Non-infiltrated transgenic *N. benthamiana* in white light. (B) Non-infiltrated *N. benthamiana* in (A) under UV illumination. (C) *N. benthamiana* infiltrated with GFP construct and challenged with CBSV-infectious sap 3 DPI as seen in white light condition. (D) *N. benthamiana* in (C) under UV illumination. (E) *N. benthamiana* infiltrated with pILTAB715 and challenged with CBSV-infectious sap 3 DPI seen in white light. (F) *N. benthamiana* plant in (D) under UV illumination

3.3.6 siRNAs accumulation in Agro-infiltrated plants

To further check expression of constructs and determine siRNA levels, leaf samples were collected daily from agro-infiltrated plants, siRNA isolated, and analyzed by northern-blot as previously described (section 2.1.3.6). The results revealed that siRNA levels accumulated fairly rapidly in the first three days and then stabilized (Fig. 17B).

3.3.7 Virus challenge of agro-infiltrated plants

To determine the best day for challenging the plants after agro-infiltration, preliminary challenging of test plants was performed. Six trays each containing 18 *N. benthamiana* plants were separately infiltrated with recombinant *A. tumefaciens* harboring GFP (control) or pILTAB715 plasmids. The agro-infiltrated plants were inoculated with CBSV infectious sap, one tray a day for six consecutive days beginning a day after agro-infiltration. Symptom development was monitored daily from 2 days after inoculation for 14 days. Disease symptom severities on fully expanded leaves were recorded on a 0-5 scale as previously described (section 2.1.3.1). The results indicated that the third day offered higher level of protection against the virus, corresponding to the siRNA analysis results (Fig. 17A). The subsequent challenging experiments were therefore performed three days after agro-infiltration of plants with the constructs, for all four constructs at a time. Symptom development was monitored daily commencing two days after virus challenge and the percentage of CBSV-protected plants for each construct determined. The results of three independent experiments showed very high level of protection for all the constructs with 12.8% escape plants (GFP, control) and up to 79.8%, 80.6% 74.6% and 70.3% protection for the pILTAB715, pILTAB716, pILTAB717 and pILTAB721 constructs, respectively (Fig. 18).

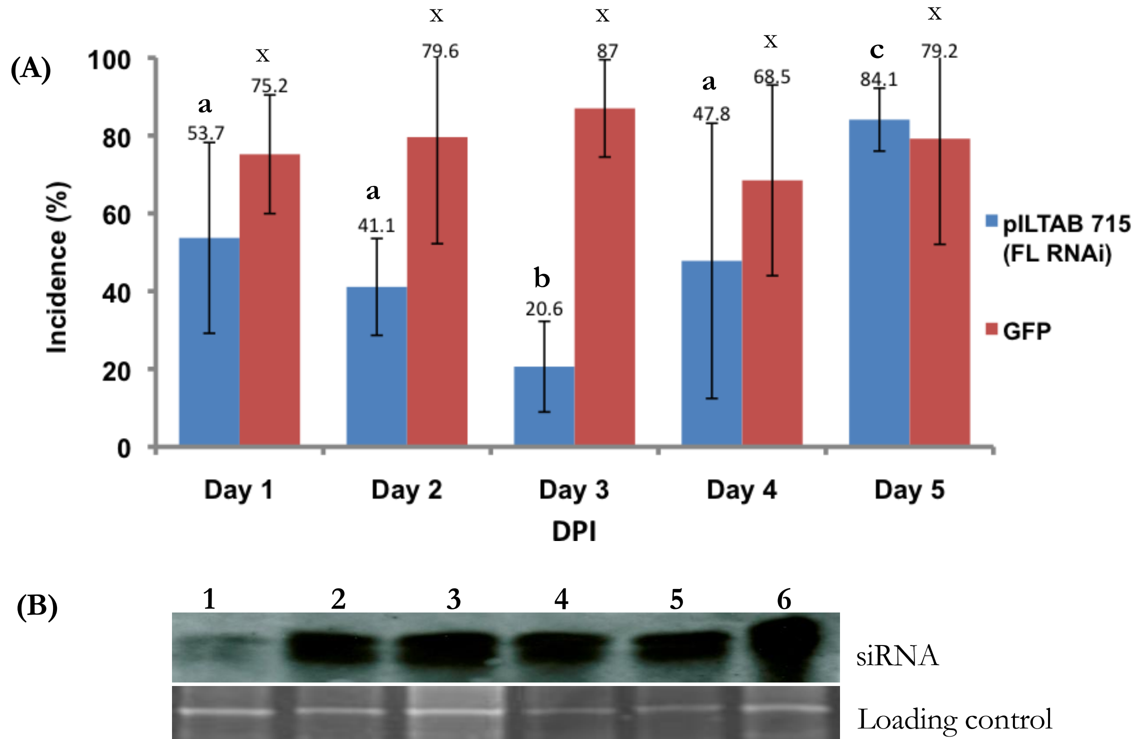


Fig. 17: Preliminary challenging of test plants with recombinant *A. tumefaciens* harboring GFP (control) or pILTAB715 (31) plasmids. (A) Mean CBSD incidence at different days after challenge. Bars marked with the same letter (a-c, or x) are not significantly different from each other at $P = 0.05$. (B) siRNA accumulation over time (1-6 days) after agro-infiltration of test plants with constructs.

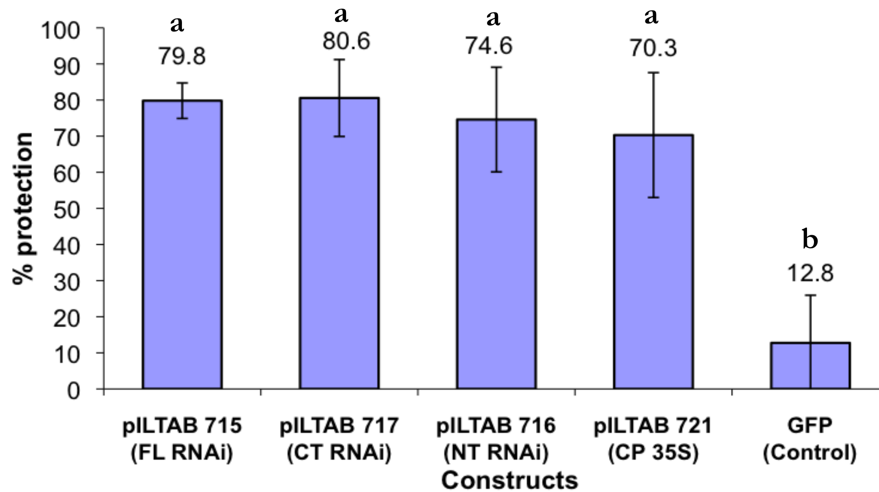


Fig. 18: Level of protection offered by different CBSV-derived constructs against CBSV in transient protection assay in *N. benthamiana*. Bars marked with the same letter are not significantly different from each other at $P = 0.05$.

Chapter 4
DISCUSSION

4.1 Sap Transmission of CBSV to *N. benthamiana*

Plant viruses are transmitted mostly by insect vectors, in seeds, or by mechanical means. Insect vectors transmit viruses by feeding on infected plant tissues and later on uninfected plants. Mechanical inoculation occurs when sap from infected plant tissues come in direct contact with tissues of uninfected plants. Several factors influence the rate of transmission and ability of the virus to cause infection in a host plant. This study has identified important factors that influence sap transmission of CBSV to host plants including buffer composition, age of inoculum source plants, age of plants at inoculation, and temperature of growth environment after inoculation (42).

The rate of sap transmission of CBSV from cassava to *N. benthamiana* was low compared to between *N. benthamiana*. This was reported by Lister (38) who showed that within cassava plant the virus is at low concentration (especially in developing leaves), a situation that was confirmed by Monger et al when they only detected the virus in radioactively probed dot blots of artificially infected *N. benthamiana* and not in infected cassava (49).

Use of the two antioxidants Na_2SO_3 and β-mercaptoethanol together in the extraction buffer improved the transmission rate of CBSV. The β-mercaptoethanol may have reduced the oxidation process and thus the production of antioxidants like phenolics, which degrade RNA. Carborundum is a commonly used abrasive for sap transmission of plant viruses to produce sub lethal injury and to overcome the physical barrier on the leaf lamina. Gentle rubbing of inoculum with the abrasive did not cause lethal damage to the assay plants.

The concentration of sap determines its infectivity, which reflects that high virus titers are required for successful transmission of the virus. Infectivity was high at higher concentration but gradually reduced on dilution and by dilution 1:1000 the

sap was almost non-infective, thus indicating that the virus titer was not above the threshold levels to cause infection. Sap dilution up to 1:10 gave highest rate of transmission and therefore, it is good concentration range for mechanical transmission of CBSV.

The age of the plant is also a very important criterion for successful virus transmission and infection. The *N. benthamiana* plants of 21 days old or less were readily infected, but the sap inoculation caused severe damage when the abrasive was applied on the very tender leaves of these very young plants. But, at 28 DAP, with the lamina expanded, it was easier to apply the inoculum by rubbing without causing much mechanical injury, and thus a higher transmission rate was achieved. Inoculation of older *N. benthamiana* plants also resulted in higher transmission rates, but again application of sap was not convenient due to very thick lignified leaves, which hindered smooth sap application. Therefore, 28-day-old *N. benthamiana* plants were found to be ideal for mechanical inoculation, considering the transmission level and the ease with which sap inoculation can be done. Evaluation of susceptibility of *N. benthamiana* at various growth stages (pre- and post flowering stages) revealed that infection at early stages of growth produced severely stunted plants, whereas inoculation at post flowering stages did not cause obvious reduction in plant height, which is also true for other plant viruses. The rate of transmission and average incubation days increased significant with age.

The transmission rate increased gradually up to 100% with increased inoculum source plant age. Inoculation of test plants with sap prepared from younger plants gave lower rate of infection compared to sap prepared from older plants although both plants showed severe CBSD symptoms. The RT-PCR analysis of individual leaves of a CBSV infected plant showed that the older leaves had a higher virus titer compared to the younger symptomatic leaves, thus supporting above observations. This is opposite of most plant viruses with no obvious explanation and require further investigation.

Environmental factors greatly influence plant virus interactions. This study indicates that infectivity and symptom severity of CBSV are highly influenced by temperature. The average incubation period of CBSV in inoculated plants decreased significantly with increased temperature and symptom severity increased with increasing temperature and this was confirmed by molecular analysis of the virus-derived siRNA accumulation over time. This unusual behavior for plant viruses is however not restricted to CBSV and has also been demonstrated for CVYV, another ipomovirus, which was reported to have infected all tobacco plants exposed to 37°C, while plants kept at 16°C were not infected (45). Thus, it is not surprising that the re-emergence of CBSD in Uganda could have been due to changes in weather, especially the frequent occurrence of the unusually prolonged hot and dry seasons in the recent years (2). However the behavior of CBSV and CVYV seem to be contrary to other viruses earlier investigated. In virus-infected plants, high temperature has been frequently associated with attenuated symptoms (heat masking) and with low virus content (29). By contrast, low temperature is often associated with rapid spread of virus diseases and the development of severe symptoms (15, 24). Thermotherapy has been a method of choice to free vegetative material from infected viruses (43), but whether it can be effective for CBSV control remains unknown given its response at higher temperatures. The underlying molecular mechanism behind the effect of temperature on CBSV is not yet understood. For instance, increasing temperature dramatically elevated virus-derived siRNA accumulation of Cymbidium ring spot virus (CymRSV; a positive sense ssRNA virus), resulting in less symptom development (60). It is probable that other mechanisms control the accumulation of CBSV at high temperatures and therefore this needs further investigation.

Evaluation of several factors affecting the sap transmission of CBSV to *N. benthamiana* resulted in development of a highly efficient protocol using infected tissue from different host plants such as cassava and tobacco. In the present study, a single isolate of CBSV was used to evaluate the inoculation procedure. Transmission variability may exist among the isolates originating from different

places and different plant species, and the present procedure has not been examined for a lot of other CBSV isolates. The inoculation methods described here was used to rapidly evaluate CBSV-derived gene constructs for CBSD resistance in *N. benthamiana* and can be used to evaluate other gene constructs for virus resistance to accelerate breeding programs for developing CBSV resistant cultivars.

4.2 Construction of CBSV-Derived hairpin genes

PTGS using gene constructs encoding self-complementary hairpin RNA of viral origin have been demonstrated to efficiently control viral infection in plants (8, 72). The double-stranded RNA produced by the self-complementary single stranded hairpin RNA in which the double stranded region has the same sequence as part of the target viral mRNA incite sequence specific RNA degradation (17). The degradation results in silencing of all targeted genes irrespective of whether it is viral gene, transgene or endogenous gene, and the silencing is usually uniform within tissues in which the hpRNA is expressed (72). Intron-spliced hpRNA constructs in particular have been shown to offer higher proportion of silencing in transformed plants than intron-free hpRNA constructs (72). In an attempt to generate plants with protection against CBSV, a very serious and rapidly spreading virus of cassava in Africa, CBSV-derived sequences were used to generate hairpin RNA constructs consisting of an inverted repeat of the full-length, N- and C-terminal fragments of the CP gene sequence separated by an intron.

The primer design and use of the vector CGT-intron eased cloning to produce CBSV-derived hairpin RNA constructs for all the amplified gene fragments. Addition of the clamp sequences and restriction sites to primer sequences did not affect amplification of the PCR products as these were of good yield and expected sizes as compared to the molecular size marker. The length of amplified fragments (402 bp, 503 bp and 908 bp) were decided based on the recommendation that gene fragments ranging from 50 bp to 1kb (especially between 300 bp to 800 bp) are suitable for successful silencing of genes (17), and also the published CBSV-CP sequence excluding the poly(A) region fall within this

range (~900 bp). The vector CGT-intron have been designed to allow directional insertion of gene fragments into an inverted repeat conformation separated by an intron into the multiple cloning sites located on either sides of the intron. Similar cloning strategies have been used before with convincing results (8, 72). Gene fragments containing the hairpin RNA cassettes, including the promoter and terminator regions were digested using the restriction enzyme *Asc*I and sub-cloned into the binary vectors AKK-1420 and pCambia2300 and used for transformation.

4.3 Transient assay of CBSV-Derived Gene Constructs in *N. benthamiana*

Since natural resistance against CBSV gives inadequate control of the disease and cassava breeding takes a long time, it is imperative that alternative approaches be used to deal with the problem. The improvement of *Agrobacterium*-mediated transient expression system is an important tool to test expression of constructs before generation of transgenic plants, which is difficult for many plant species and notably cassava (73). In this study, gene constructs for CBSV resistance developed as discussed above were tested in *N. benthamiana* using the *Agrobacterium*-mediated transient transformation system. The GFP gene was used as a sensitive internal control to visualize transient expression in transformed tissues. The plasmids pILTAB715, pILTAB716, pILTAB717, and pILTAB721 were introduced into *A. tumefaciens* strain GV3103 by direct transformation. When suspensions of the recombinant *A. tumefaciens* was infiltrated into leaves of *N. benthamiana* and check for GFP silencing 3-4 days later, plants infiltrated with either GFP control construct or hpRNA constructs showed clear GFP silencing compared to non-infiltrated plants. Similar GFP silencing results were reported by Wydro et al (2006) (73).

When the transiently transformed plants were challenged with the virus three days after infiltration with recombinant *Agrobacterium* harboring CBSV-derived hpRNA constructs, over 70% of plants showed no symptoms of CBSD. Plants infiltrated with GFP control construct or non-infiltrated plants challenged with the virus were almost always totally infected. There was no significant difference in levels of protection offered by the four CBSV-derived constructs. These results suggest

that the plants were protected against CBSV. However, these constructs need to be tested with different isolates and/or strains of CBSV to check whether they can confer protection against the isolates/strains. Thus far, the four constructs are potentially good for developing transgenic cassava for further evaluation for protection against CBSD.

4.4 Conclusion

In this study a non-vector based method of CBSV transmission to *N. benthamiana*, a laboratory host plant for many plant viruses has been optimized. Besides, a transient assay protocol that effectively works to test transgenes targeting CBSV resistance in the host plant *N. benthamiana* has been developed. It is hoped that the optimization of the techniques reported here could stimulate focused research to better understand and manage CBSV. In addition, CBSV-derived constructs have been generated and tested using these techniques with exciting results. The four constructs designed to target mRNA of CBSV provided high levels of protection against CBSV and are therefore highly recommended for further evaluation and eventually for use to improve farmer preferred cassava landraces. The constructs may be stably transformed in to cassava tissues through *Agrobacterium*-mediated gene transfer system. Hopefully, this may translate into development of CBSV immune or resistant transgenic lines of farmer preferred cassava landraces which succumbed to CBSD and lead to improved food security and economic status of the rural communities that majorly rely on cassava.

Chapter 5

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