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Transmission studies with *Cassava brown streak Uganda virus* (*Potyviridae*: *Ipomovirus*) and its interaction with abiotic and biotic factors in *Nicotiana benthamiana*

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A B S T R A C T

Cassava brown streak disease (CBSD), caused by two distinct species, *Cassava brown streak Uganda virus* (CBSUV) and *Cassava brown streak virus* (CBSV), is a major constraint to cassava (*Manihot esculenta* Crantz) production in Africa. Absence of infectious clones of CBSUV or CBSV and the lack of efficient means of mechanical transmission of CBSD has hampered laboratory studies of this disease. Mechanical transmission, achieved mainly by plant sap inoculation, is a widely used technique for characterizing plant viruses. Efficient sap transmission of CBSUV/CBSV to the common laboratory host *Nicotiana benthamiana* is essential for both basic and applied studies of the virus. We report here the development of an efficient protocol for sap transmission of CBSUV to *N. benthamiana* and *N. debneyi*. Several factors affecting transmission efficiency were identified such as the effects of buffer composition, antioxidants, inoculum concentration, plant age and temperature. Higher temperatures (30 °C) favored rapid symptom initiation compared to lower temperatures (21 °C) when sap prepared in phosphate buffer of pH 7.0 was applied on the leaves of *N. benthamiana* dusted with the abrasive (carborundum). We demonstrated the usefulness of the transmission method in transient evaluation of CBSUV[UG:Nam:04]-derived RNA interference constructs for CBSD resistance and also in studying the interaction of CBSUV[UG:Nam:04] with cassava mosaic geminiviruses, another important group of viruses infecting cassava.

1. Introduction

Cassava (*Manihot esculenta* Crantz, Family *Euphorbiaceae*), which originates in Latin America, is a staple food crop of sub-Saharan Africa and its production is constrained by several pests and diseases (Calvert et al., 2002; Legg and Fauquet, 2004; Legg et al., 2006; Patil and Fauquet, 2009; Thresh, 2006a,b). Recently, cassava brown streak disease (CBSD) has become a serious threat to cassava production in eastern and southern Africa, leading to large scale yield loss and poor tuber quality (Alicai et al., 2007; Hillocks and Thresh, 2000; Hillocks et al., 2001, 2002; Pennisi, 2010). CBSD is caused by at least two distinct virus species, *Cassava brown streak Uganda virus* (CBSUV) and *Cassava brown streak virus* (CBSV), present predominantly in the coastal lowland (Tanzania and Mozambique) and highland (Lake Victoria Basin, Uganda, Kenya, Malawi) regions of East Africa, respectively (Mbanzibwa et al., 2009b; Monger et al., 2010; Patil et al., 2010; Winter et al., 2010). CBSUV and CBSV are positive sense single stranded RNA viruses with a size of ~9069 nt producing a polyprotein of ~2902 amino acids, belonging to the genus Ipomovirus of the family *Potyviridae* (Mbanzibwa et al., 2009a; Monger et al., 2001a, 2010; Winter et al., 2010). CBSUV and CBSV induce a range of symptoms in cassava (Nichols, 1950; Storey, 1936). The foliar symptoms include feathery chlorosis along veins, brown streaks on stem, and stem die-back in severe infections (Bock, 1994; Jennings, 1960; Mbanzibwa et al., 2009b; Winter et al., 2010). The internal root symptoms vary a lot but most often consist of a yellow/brown corky necrosis of the starchy tissue. In susceptible varieties, necrotic lesions spread throughout the starch storage tissue discoloring it and thus making it unsuitable for human consumption (Hillocks and Thresh, 2000). On the outside of the root, symptoms may appear as radial constrictions on the surface bark. CBSD can have a serious impact on food security, as the extent of the loss caused does not become apparent until the crop is harvested (Donald, 2010; Hillocks and Jennings, 2003; Pennisi, 2010).

CBSD is transmitted mainly by the whitefly vector (*Bemisia tabaci*) to cassava (Maruthi et al., 2005), by graft-inoculation from cassava to cassava (Storey, 1936) and also mechanically from cassava to herbaceous host plants (Lister, 1959; Mbanzibwa et

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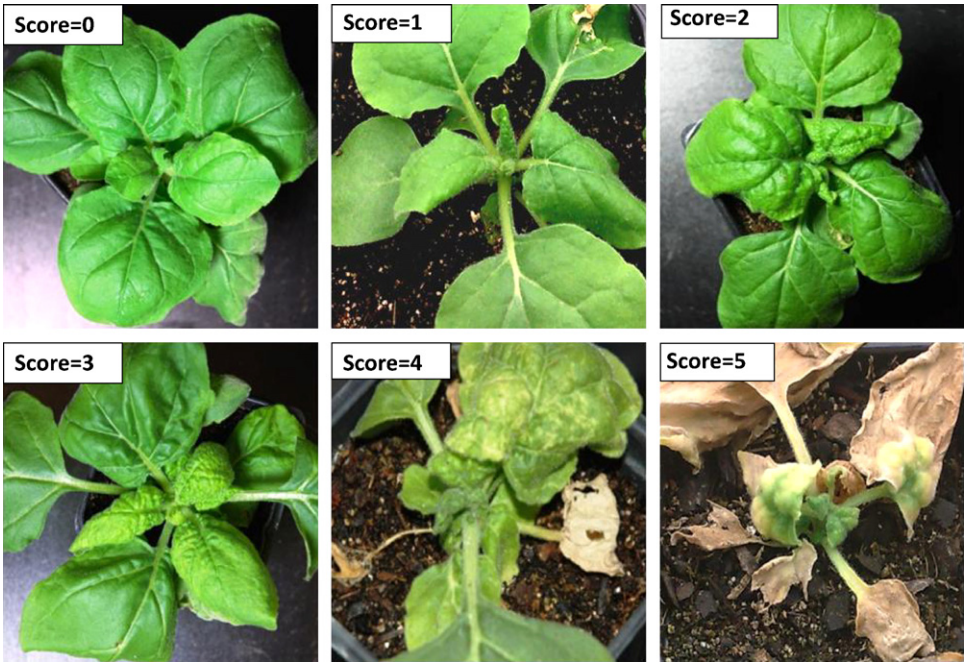

Fig. 1. Symptoms of CBSD on leaves of *Nicotiana benthamiana*. Plants were visually assessed for development of symptoms after inoculation with CBSUV[UG:Namulonge:04] infectious sap. Each plant was scored on a scale of 0–5 where symptom severity score was rated on a 6-point scale: 0 = no symptoms, 1 = 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.

al., 2009b; Monger et al., 2001a). However, CBSD transmission rate by whiteflies is extremely low in laboratory conditions compared to field situation (Maruthi et al., 2005). Thus, a major difficulty in studying CBSD in laboratory conditions so far has been the lack of means of transmitting the causal virus readily. Although recently several complete genome sequences of CBSUV and CBSV have been published, no infectious clone is available yet (Mbanzibwa et al., 2009a; Monger et al., 2010; Winter et al., 2010) (Valkonen, Pers. Commun.). To complement 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 CBSUV or CBSV (Sudarshana et al., 2007; Thomson, 2008). These constructs need to be evaluated transiently for resistance to CBSUV or CBSV initially in a susceptible laboratory host plant prior to transformation into cassava, which is a highly laborious and time consuming task (Johansen and Carrington, 2001; Taylor et al., 2004; Tenllado et al., 2004). Availability of a highly efficient method for mechanical transmission would facilitate further understanding of the etiology and biology of CBSUV or CBSV (Mbanzibwa et al., 2009b). A protocol is described that ensures efficient and reliable mechanical transmission of CBSUV to *Nicotiana benthamiana*, a widely used laboratory host plant for study of many plant viruses. Also demonstrated, is the usefulness of sap transmission for transient evaluation of transgenes derived from CBSUV[UG:Namulonge:2004] (here onwards referred to as CBSUV[UG:Nam:04]), and for studying the interaction between CBSUV[UG:Nam:04] and cassava mosaic geminiviruses (CMGs) that cause cassava mosaic disease (CMD) (Patil and Fauquet, 2009, 2010; Patil et al., 2010; Tenllado et al., 2004).

2. Materials and methods

2.1. Virus source

Stem cuttings from the CBSD infected cassava plants showing characteristic foliar CBSD symptoms were collected from farmers' fields in Mukono district, central Uganda and shipped to Donald Danforth Plant Science Center (DDPSC), St. Louis, where they were propagated by nodal culture and maintained in a growth chamber. CBSUV isolates were tested from four different Ugandan cassava cultivars (I 92/00057, I 95/00087, Ebwanateraka and TME204), which were infectious in *N. benthamiana* with slight variations in symptom expression (data not shown). Further studies used the CBSUV[UG:Nam:04] isolate from the cassava cultivar Ebwanateraka. Total RNA was isolated from symptomatic leaves as described by Patil et al. (2010), cDNA synthesized and subjected to reverse transcription polymerase chain reaction (RT-PCR) using CBSUV/CBSV specific primers (CBSV10 and CBSV11) to confirm presence of CBSUV (Alicai et al., 2007; Monger et al., 2001a; Patil et al., 2010). The RT-PCR studies indicated presence of higher virus titer in the older than in the younger symptomatic leaves (data not shown). Inoculum prepared from infected cassava leaf tissues was used to inoculate a total of 18 *N. benthamiana* plants. The inoculated plants were maintained in the growth chamber at 28 °C and relative humidity (RH) of 70% with a 16 hr light period (with a light intensity of 200 $\mu E\ m^{-2}\ s^{-1}$). Symptoms were recorded daily on a 0–5 scale (Fig. 1) and leaf tissues from symptomatic plants were analyzed for CBSUV by RT-PCR. Further inoculation studies to evaluate the various factors influencing sap transmission of CBSUV to *N. benthamiana* were performed using tissues from the CBSUV positive plants.

2.2. Inoculum preparation and sap inoculation

Infected cassava leaf tissues showing fresh symptoms of CBSD were ground using a pestle and mortar with the aid of carborundum 320 grit (Fisher Scientific). 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-mercapto-ethanol (1:6 [w/v] tissue:buffer) was added to the ground tissue and mixed, transferred to Falcon tube and allowed to stand for 5 min in ice for debris to settle at the bottom of the tube. The sap was kept on ice until inoculation was completed. Sap inoculum was applied to 21-day-old

N. benthamiana plants. Test plants were dusted with carborundum to act as abrasive and drops of inoculum were rubbed gently on the leaf surfaces using gloved fingers. After inoculation, the plants were gently sprayed with water to remove excess carborundum. Inoculation was done in the evening and plants kept under a light intensity of 200 $\mu E\,m^{-2}\,s^{-1}$, 28 °C, 70% RH, with alternating light and dark periods of 16 h/8 h photoperiod.

2.3. Determination of effects of antioxidants, inoculum concentration, temperature, and growth stages of N. benthamiana on CBSUV transmission

To determine the effect of antioxidant on CBSUV[UG:Nam:04] transmission, 3 g of infected *N. benthamiana* leaf tissue were 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-mercapto-ethanol. Eighteen 21-day-old *N. benthamiana* plants were inoculated with inoculum prepared using the above four buffers, with three replications.

To evaluate the effect of inoculum concentration on CBSUV[UG:Nam:04] transmission, 10 g of infected leaf tissue were ground as described and 20 ml of freshly prepared ice-cold 0.01 M potassium phosphate buffer, pH 7.0, containing 0.2% sodium sulfite and 0.01 M β-mercapto-ethanol (1:2 [w/v] tissue:buffer) added, mixed, transferred to Falcon tube, and centrifuged at 4 °C for 5 min to remove the debris. The supernatant (sap) was used to make the following dilutions ([w/v] 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 each inoculum dilution, in three replications.

To identify the optimum age of *N. benthamiana* for efficient CBSUV[UG:Nam:04] sap transmission, 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 [w/v] tissue:buffer) in phosphate buffer (containing 0.2% sodium sulfite and 0.01 M β-mercapto-ethanol). Only the top two completely opened leaves were inoculated. The number of days required for the initiation of symptoms and also the symptom progression were recorded, with three replications of each treatment.

Also, to evaluate the effect of the age of the inoculum source plant on CBSUV[UG:Nam:04] transmission, *N. benthamiana* plants of six different ages were used as source of inoculum. Infected leaf tissues from lower part of the plant were obtained from plants that were 14, 28, 42, 56, 70, and over 80 days old. For each source of inoculum, 18 healthy *N. benthamiana* plants were inoculated in three independent replications with sap prepared (1:6 [w/v] tissue:buffer) in phosphate buffer containing 0.2% sodium sulfite and 0.01 M β-mercapto-ethanol as above.

The effect of temperature on the rate of CBSUV[UG:Nam:04] transmission was assessed at 21 °C, 25 °C, 27 °C, and 30 °C. Sap was prepared in phosphate buffer (1:6 [w/v] tissue:buffer) and *N. benthamiana* plants 21 DAP were inoculated as described above. The inoculated plants were maintained in growth chambers with a light intensity of 200 $\mu E\,m^{-2}\,s^{-1}$, 70% RH, and alternating light and dark periods of 16 h/8 h photoperiod in the four different temperatures mentioned above.

2.4. Assessment of virus-derived siRNA

To determine viral RNA accumulation and virus-derived siRNA (small interfering RNA) levels, symptomatic young leaf samples were collected daily from plants maintained at the four different temperatures, and total RNA and siRNA was isolated and analyzed by northern-blot hybridization (Patil et al., 2010). Small RNA was isolated using the protocol of (Akbergenov et al., 2006) with some modifications. Total RNA was extracted from a single symptomatic leaf of *N. benthamiana* using Trizol reagent (Invitrogen, Carlsbad, CA, USA) per the manufacturer's instructions. The total RNA was fractionated using RNeasy Plant mini kit (Qiagen, Valencia, CA, USA) to remove the large RNA. Ten micrograms of fractionated small RNAs were resolved through a pre-cast 15% TBE Urea gel (Criterion-BioRad, Hercules, CA, USA) at 150 V using the Criterion gel apparatus. The RNA was blotted to the Hybond N+ membrane using the semidry electro blotter at 10 V for 1 h. The membrane was UV cross-linked and pre-hybridized in DIG Easy-hyb buffer (Roche Applied Science, Indianapolis, IN, USA) at 42 °C for an hour. To produce a probe, CBSUV[UG:Nam:04] coat protein sequence was cloned in the *in vitro* transcription vector pSPT19 and subjected to *in vitro* transcription using DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN, USA). The labeled RNA was hydrolyzed for 5–10 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, Indianapolis, IN, USA). Autoradiography was done by exposing the membrane to Amersham high performance chemi-luminescence film (GE Healthcare Biosciences, Pittsburgh, PA, USA) and developed in automated developer (KODAK X-OMAT, Rochester, USA) and the auto-radiograms were scanned and adjusted for clarity.

2.5. CBSD incidence, detection and analysis

Symptoms were recorded daily for a period of 30 days from the day of initiation of the symptoms. We developed our own scale to record the CBSUV symptom severity based on the scoring system developed for cassava mosaic disease by Fauquet and Fargette (1990) and Patil and Fauquet (2010). Each plant was scored for their symptom severity on a scale of 0–5: 0 = no symptoms, 1 = mild chlorosis with slight distortions, 2 = moderate chlorosis with moderate distortion, 3 = severe chlorosis and distortion, 4 = very severe chlorosis with stunting and wilting, 5 = necrosis and death of the plant (Fig. 1). 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 $P = 0.05$.

2.6. Transient protection study of CBSUV[UG:Nam:04]-derived RNAi constructs in N. benthamiana

To evaluate the level of expression of the CBSUV[UG:Nam:04]-derived RNAi (RNA interference) constructs targeting the different regions of coat protein (*CP*) (Callaway et al., 2001) and their potential to protect against CBSUV[UG:Nam:04] in transiently transformed *N. benthamiana*, a transient protection assay protocol developed previously by Wydro et al. (2006) for expressing genes in leaves of *N. benthamiana* was used (Patil et al., 2010). Transgenic *N. benthamiana* for GFP and *A. tumefaciens* strain GV3103 transformed with the recombinant binary vector AKK-1420-RNAi-GFP harboring CBSUV[UG:Nam:04]-derived hairpin gene encoding plasmids pILTAB715, pILTAB716, pILTAB717 or pILTAB721, were used in transient vaccination studies (Collier et al., 2005; Helliwell and Waterhouse, 2005; Patil et al., 2010). Two or three top-leaves of *N. benthamiana* plants at 4–6 leaf stage were infiltrated with culture of recombinant *A. tumefaciens* strain GV3103 on the under-surface of the leaves using a 2-ml syringe without needle. The test plants were sap-inoculated with CBSUV[UG:Nam:04] 3 days 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 described previously.

2.7. Interaction between CBSUV and CMGs

To gain insight into the nature of interaction between CBSUV and different species of CMGs, we initiated dual infection of wild type *N. benthamiana* plants with both CBSUV[UG:Nam:04] and a recovery and non-recovery type CMG species (Patil and Fauquet, 2009). A tray of 18 *N. benthamiana* plants 21 DAP were infiltrated with a culture of *A. tumefaciens* strain GV3103 harboring agro-infectious clones of CMG strain East African cassava mosaic virus-Kenya [Kenya:Msambweni:K201:2002] DNA-A and DNA-B (EACMV-KE[KE:Msa:K201:02]; NCBI Acc. No. AJ717541 and AJ704953) (Bull et al., 2006; Patil and Fauquet, 2010). Two days later, the CMG agro-infiltrated plants were inoculated with CBSUV[UG:Nam:04] infectious sap prepared in phosphate buffer (containing 0.2% sodium sulfite and 0.01 M β-mercapto-ethanol) as previously described. In addition, two trays each of 18 *N. benthamiana* plants 21 DAP were inoculated with either virus. One tray was agro-infiltrated with CMGs EACMV-KE[KE:Msa:K201:02] a non-recovery type CMG, or African cassava mosaic virus-[Kenya:844:1982] a recovery type CMG (ACMV-[KE:844:82]; NCBI Acc. No. J02057 and J02058) DNA-A + DNA-B) alone and another tray was inoculated with CBSUV[UG:Nam:04] infectious sap to act as controls. The test plants were kept in the growth chamber and symptoms of CBSD and CMD on fully expanded leaves were assessed daily after 2 days of sap inoculation for a minimum of 2 weeks on a scale previously described.

3. Results

3.1. Initial attempts of sap transmission to N. benthamiana

Sap prepared from CBSD symptomatic leaves was rubbed on carborundum dusted leaves of test plants. Inoculation of 21-day-old *N. benthamiana* plants with inoculum prepared from topmost symptomatic cassava leaves resulted in 0% ($N = 18$) transmission and from mature symptomatic cassava leaves, 38.9% ($N = 18$) transmission by 3–14 DPI. Infected plants showed chlorotic mosaic on emerging leaflets and were severely stunted compared with non-inoculated control plants. Leaf samples from inoculated plants were tested by RT-PCR and all the symptomatic plants were CBSUV positive. None of the asymptomatic plants showed the presence of CBSUV.

3.2. Effect of antioxidants on the rate of CBSUV transmission

A transmission rate of 100% was achieved at 3–5 DPI when inoculum was prepared in the buffer containing antioxidants Na_2SO_3 and β-mercapto-ethanol (Fig. 2A). 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. 2A).

3.3. Effect of inoculum concentration on CBSUV transmission

The effect of inoculum concentration on CBSUV transmission was studied using different sap dilutions in three replications. The rate of CBSUV[UG:Nam:04] transmission decreased with increasing sap dilution. At dilution 1:5, 61.1% of inoculated plants produced symptoms at 3–5 DPI (Fig. 2B); at dilution 1:10, 1:20, 1:50, 1:100, 1:500, and 1:1000, transmission rates of 72.3%, 50.0%, 13.9%, 11.1%, 5.6%, and 2.8% resulted, respectively (Fig. 2B).

3.4. Effect of age at inoculation and of inoculum source plants on efficiency of transmission of CBSUV to N. benthamiana

A plant's 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 (50–75%) of inoculated plants by 3–5 DPI and in the rest of the plants, symptoms appeared by 7 DPI. For plants inoculated at 21 DAP, a final CBSD incidence of 94.4% resulted. Inoculation of plants at 28 DAP, 35 DAP and 42 DAP resulted in transmission rates of 100% in the three replications; 50–60% of the inoculated plants developed symptoms at 6 DPI, whereas the remaining plants produced symptoms by 10 DPI. The average incubation period increased with the age of plants inoculated. In the three inoculation trials, an average incubation period of 3.3 days, 3.1 days, 4.3 days and 5.0 days were required for the 21-, 28-, 35-, and 42-day-old *N. benthamiana* plants respectively (data not shown). Symptoms in the plants inoculated after flowering stage (42 DAP) were less prominent in the beginning though eventually they became severe. It took an average of 5.0 days for symptoms to develop clearly. When seedlings were inoculated with sap from 14-day-old infected leaf tissues, 19.5% of the inoculated plants became infected (Fig. 2C). However, inoculum from 28-, 42-, 56-, 70- and over-80-day-old infected leaf tissues resulted in 38.9%, 41.7%, 55.6%, 94.5%, and 100% infection respectively in the three trials (Fig. 2C).

3.5. Effect of temperature on CBSUV transmission and expression

The symptom severity of CBSUV[UG:Nam:04] in inoculated plants increased with increase in temperature (Fig. 3A) while the average incubation period decreased with increasing temperature from 21 °C to 30 °C (Fig. 3B). In the three inoculation trials of 21-day-old plants, when seedlings were inoculated and kept in the temperature chamber maintained at 21 °C, symptoms started developing in the majority (>60%) of inoculated plants by 6–8 DPI, and in the rest of the plants, symptoms appeared by 11 DPI, with the average incubation period required 7.1 days (Fig. 3B). 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, with the average incubation period 5.2 days (Fig. 3B). At 27 °C, up to 50% of the inoculated plants developed symptoms at 4 DPI, and by 9 DPI the remaining plants produced symptoms, with an average incubation period of 4.4 days (Fig. 3B). And at 30 °C over 60% of the inoculated plants developed symptoms at 3 DPI, and by 5 DPI, the remaining plants produced symptoms. An average incubation period of 3.6 days was required at 30 °C (Fig. 3B).

3.6. Analysis of virus-derived siRNA in CBSUV[UG:Nam:04] inoculated plants at different temperatures

Analysis of the siRNA levels at 21 °C, 25 °C, 27 °C, and 30 °C showed that plants kept at 30 °C accumulated siRNA faster than plants kept at 21 °C (Fig. 4). The siRNA accumulation corresponded with the days to onset of symptom expression at the respective temperature conditions. This demonstrated that there was a positive correlation between the virus accumulation, assessed by symptom development, and siRNA accumulation. These results indicate that higher temperatures favor CBSUV transmission and expression. Taken together, these results show that buffer composition, inoculum source and concentration, plant age, and temperature of growth environment play vital roles in CBSUV transmission and disease progress.

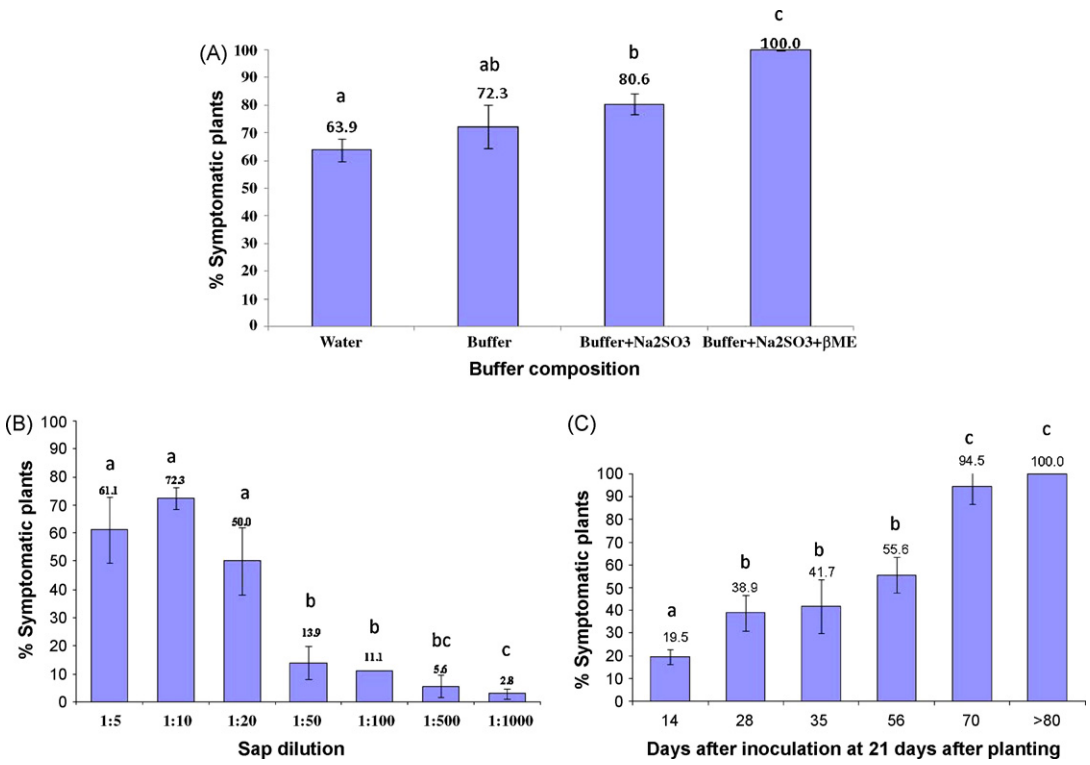

Fig. 2. Sap transmission of CBSUV[UG:Nam:04] to *N. benthamiana* (A) using buffers of different compositions; (B) with sap of varying concentrations; (C) using sap from source plants with varying ages. Bars represent mean incidence from three trials at 14 days post-inoculation. Bars marked with the same letter are not significantly different from each other at $P = 0.05$.

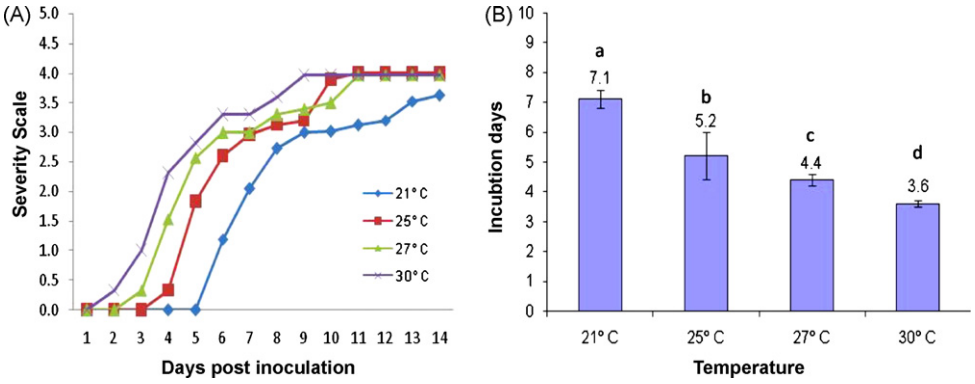

Fig. 3. Progress in expression of symptoms incited by CBSUV[UG:Nam:04] in 21-day-old *N. benthamiana* at different temperatures. (A) Changes in severity over time. (B) Mean number of days to first appearance of symptoms. Bars marked with the same letter are not significantly different from each other at $P = 0.05$.

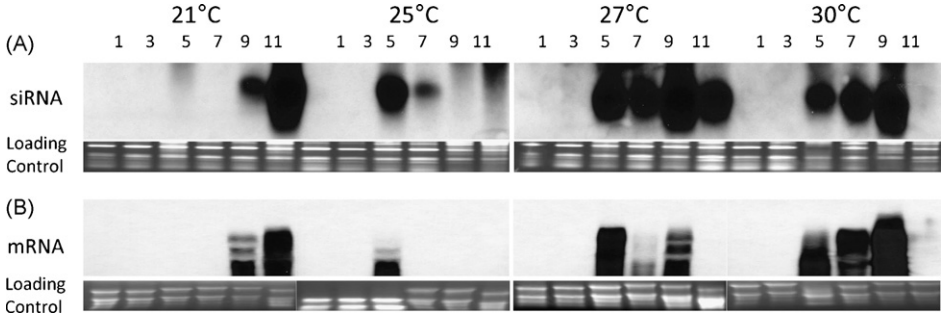

Fig. 4. Molecular analysis of CBSUV[UG:Nam:04] at different temperatures. (A) changes in CBSUV[UG:Nam:04] specific siRNA accumulation over time (1 days, 3 days, 5 days, 7 days, 9 days and 11 days post-inoculation) at 21 °C, 25 °C, 27 °C, and 30 °C. (B) Levels of CBSUV[UG:Nam:04] RNA at 21 °C, 25 °C, 27 °C, and 30 °C over time.

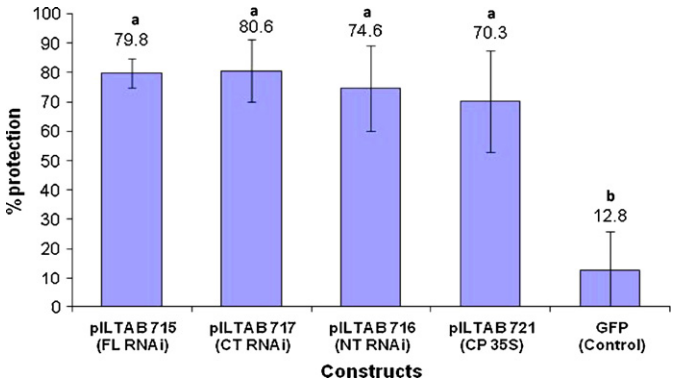

Fig. 5. Level of protection offered by different CBSUV[UG:Nam:04]-derived RNAi constructs against CBSUV[UG:Nam:04] in transient protection assay in *N. benthamiana*. Bars marked with the same letter are not significantly different from each other at $P = 0.05$.

3.7. Transient protection study of CBSUV[UG:Nam:04]-derived RNAi constructs in N. benthamiana

To obtain good protection in transient studies, it is important to determine the gap (number of days) between agro-infiltration of RNAi constructs targeting CBSUV[UG:Nam:04]-CP and the virus challenge with CBSUV[UG:Nam:04]. Thus, the *N. benthamiana* plants (18 plants per treatment) were sap-inoculated for 6 consecutive days and the studies showed that challenging with CBSUV[UG:Nam:04] 3 days after agro-infiltration of the RNAi constructs gave the highest protection. This was confirmed by a time course molecular analysis which showed that siRNA accumulated rapidly with a peak around the third day after agro-infiltration for all the RNAi constructs tested. The percentage of CBSUV[UG:Nam:04] protected plants was determined for each construct in three independent challenging experiments. For each experiment, disease symptoms were recorded daily for 14 consecutive days on plants agro-infiltrated with CBSUV[UG:Nam:04]-derived constructs and inoculated 3 days after agro-infiltration with CBSUV[UG:Nam:04] infectious sap. The results showed very high levels 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. 5).

3.8. Interaction between CBSUV and CMGs

The disease incidence in plants infected with individual or both CBSUV[UG:Nam:04] and CMGs (EACMV-KE[KE:Msa:K201:02] or ACMV-[KE:844:82]) was 100% and plants infected with individual viruses showed distinct and discernible symptoms characteristic of the virus used (Fig. 6A). However, plants infected with both viruses showed symptoms of both viruses and were very severe to the extent that these plants eventually died (Fig. 6B). Molecular analysis revealed that in the presence of CBSUV[UG:Nam:04], the siRNA produced by CMGs (EACMV-KE[KE:Msa:K201:02] or ACMV-[KE:844:82]) is reduced, but not of CMGs (Fig. 6C). These results suggest that CBSUV and CMGs interact synergistically in dual infection and CBSUV reduces the accumulation of siRNA by CMGs.

4. Discussion

Plant viruses are transmitted mostly by insect vectors, by nematodes, by seeds, by cuttings of infected plants or by mechanical means (Harrison, 1981; Patil and Fauquet, in press). Insect vectors transmit viruses by feeding on infected plant tissues and later on uninfected plants (Power, 2000). Mechanical inoculation occurs when sap from infected plant tissues comes into direct contact with tissues of uninfected plants (Lister, 1959). Several factors influence the rate of transmission and ability of the virus to cause infection in a host plant (Nichols, 1950). This study has identified important factors that influence sap transmission of CBSUV to host plants including buffer composition, age of inoculum source plants, age of plants at inoculation, and temperature of growth environment after inoculation (Mandal et al., 2001).

The rate of sap transmission of CBSUV from cassava to *N. benthamiana* was low compared to between *N. benthamiana*. This was reported by Lister (1959) 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. (2001b) only when they detected the virus in radioactively probed dot blots of artificially infected *N. benthamiana* and not in infected cassava.

Use of the two antioxidants Na_2SO_3 and β-mercapto-ethanol together in the extraction buffer improved the transmission rate of CBSUV (Fig. 2A). The β-mercapto-ethanol may have reduced the oxidation process and thus the production of antioxidants such as phenolics. 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 usually 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; at a dilution of 1:1000 the sap was almost non-infective, indicating that the virus titer was below threshold levels that cause infection. Sap dilution up to 1:10 gave highest rate of transmission and therefore it is a good concentration range for mechanical transmission of CBSUV.

Plant age is also a very important criterion for successful virus transmission and infection. The *N. benthamiana* plants of 21 DAP or less developed CBSD symptoms readily, but the sap application caused severe damage when the abrasive was applied on the very tender leaves of these young plants. But, at 28 DAP, with the lamina expanded, it was easier to apply the inoculum by rubbing without 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 period increased slightly with age but was not very significant.

The transmission rate increased gradually up to 100% with increased age of inoculum source plant (Fig. 2C). 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 CBSUV infected plant showed that the older leaves had a higher virus titer compared to the younger symptomatic leaves, thus supporting above observations. This is the opposite of the case of most plant viruses with no obvious explanation and needs further investigation.

Environmental factors such as temperature greatly influence plant virus interactions and experiments indicate that CBSUV infectivity and symptom severity are highly influenced by temperature (Chellappan et al., 2005; Szittya et al., 2003). The average incubation

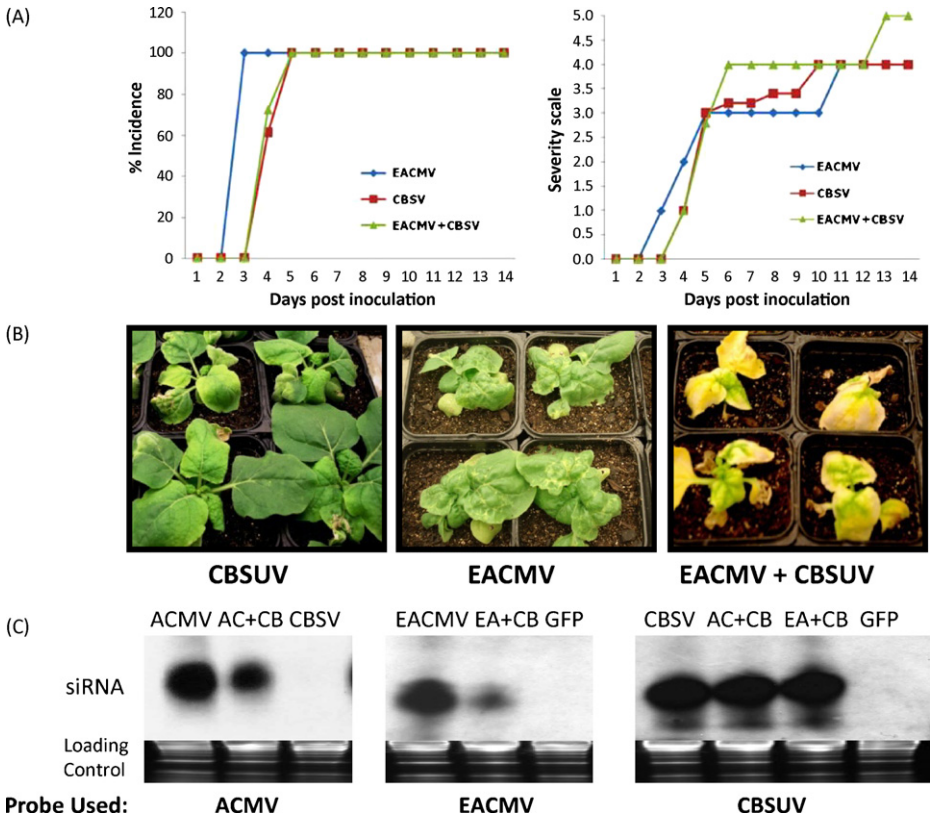

Fig. 6. Expression of CMGs and CBSUV[UG:Nam:04] in 21-day-old *N. benthamiana* in individual and dual infections. (A and B) Symptoms of EACMV-KE[KE:Msa:K201:02] (labeled as EACMV) and both EACMV-KE[KE:Msa:K201:02] + CBSUV[UG:Nam:04] in leaves of *N. benthamiana*. (C) siRNA produced by CMGs (ACMV-[KE:844:82] (labeled as ACMV) or EACMV-KE[KE:Msa:K201:02]), CBSUV[UG:Nam:04], and both CMGs + CBSUV[UG:Nam:04] detected by virus specific probes. AC: ACMV-[KE:844:82]; EA: EACMV-KE[KE:Msa:K201:02]; CB: CBSUV[UG:Nam:04]; GFP: negative control.

period of CBSUV in inoculated plants decreased significantly with increased temperature (Fig. 3A) and symptom severity increased with increasing temperature (Fig. 3B) and this was confirmed by molecular analysis of the virus-derived siRNA accumulation over time (Fig. 4). However, this unusual behavior for plant viruses is not restricted to CBSUV and has been demonstrated also for Cucumber vein yellowing virus (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 (Maruthi et al., 2005). 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 recent years (Alicai et al., 2007; Mbanzibwa et al., 2009b). However, the behavior of CBSUV, CBSV and CVYV seems 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 (Chellappan et al., 2005; Johnson, 1922; Szittya et al., 2003). By contrast, low temperature is often associated with rapid spread of virus diseases and the development of severe symptoms (Gerik et al., 1990). Thermotherapy has been a method of choice to free vegetative material from infected viruses (Manganaris et al., 2003), but whether it can be effective for CBSD control remains unknown given its good response at higher temperatures. The underlying molecular mechanism behind the effect of temperature on CBSUV 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 (Ding and Voinnet, 2007; Szittya et al., 2003). It is probable that other mechanisms control the accumulation of CBSUV at high temperatures and therefore this needs further investigation.

Evaluation of several factors affecting the sap transmission of CBSUV to *N. benthamiana* resulted in development of a highly efficient protocol using infected tissue from different host plants such as cassava and tobacco. Transmission variability may exist among the isolates originating from different places and different plant species, and the present procedure has not been examined for CBSV isolates. The inoculation methods described here would help in rapid evaluation of gene constructs for CBSD resistance in *N. benthamiana* and eventually of cassava breeding lines for sources of resistance, which will eventually accelerate the breeding program for developing CBSUV or CBSV resistant cultivars (Ceballos et al., 2004; Jennings, 2003).

When the transiently transformed plants were challenged with the virus 3 days after infiltration with recombinant *Agrobacterium* harboring CBSUV[UG:Nam:04]-derived RNAi constructs targeting different regions of *CP*, over 70% of plants showed no symptoms of CBSD (Patil et al., 2010). Plants infiltrated with GFP control RNAi construct or non-infiltrated plants challenged with the virus were almost always completely infected (Fig. 5). There was no significant difference in levels of protection offered by the four CBSUV[UG:Nam:04]-derived RNAi constructs suggesting the plants were protected against CBSUV[UG:Nam:04]. However, these constructs need to be tested with different isolates and/or strains of CBSUV and CBSV to check whether they can confer protection against the isolates/strains (Patil et al., 2010). These results demonstrated the usefulness of efficient sap transmission of CBSUV to screen transgenes for downstream applications. Thus far, the four constructs are potentially good for developing transgenic cassava for further evaluation for protection against CBSD (Patil et al., 2010).

The development of very severe disease symptoms in plants infected with both CMGs (EACMV-KE[KE:Msa:K201:02] or ACMV-[KE:844:82]) and CBSUV[UG:Nam:04] suggests that CBSV and CMGs interact synergistically in dual infection with devastating consequences. True synergism between ssDNA and an RNA virus combination has been reported in plants (Wege and Siegmund, 2007). The ssDNA geminivirus, Abutilon mosaic virus (AbMV) in double-infection with Cucumber mosaic virus (CMV), a (+)ssRNA virus resulted in synergistic symptom enhancement in infected *N. benthamiana*, with no significant difference in CMV concentration from plants infected by CMV alone (Wege and Siegmund, 2007). Thus, synergy between CMV and AbMV involved one virus enhancing the symptoms and titer of the other (Wege and Siegmund, 2007). Similarly, the molecular detail of interaction between CMGs and CBSUV[UG:Nam:04] suggest existence of synergism between the viruses in the model host *N. benthamiana*, as in the presence of CBSUV[UG:Nam:04] the siRNA produced by CMGs is reduced (Fig. 6B). However, such studies are not done in cassava and at this moment it would not be logical to extrapolate the observations made in *N. benthamiana* to cassava, as the interaction of viruses is also largely manifested by host plants (Mendez-Lozano et al., 2003). These results once again demonstrated the usefulness of efficient sap transmission of CBSUV to understand its biological properties and its significant implication on developing strategies to control CBSD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jviromet.2010.07.030.

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