

RNAi-mediated resistance to diverse isolates belonging to two virus species involved in Cassava brown streak disease

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SUMMARY

Cassava brown streak disease (CBSD) is emerging as one of the most important viral diseases of cassava (*Manihot esculenta*) and is considered today as the biggest threat to cassava cultivation in East Africa. The disease is caused by isolates of at least two phylogenetically distinct species of single-stranded RNA viruses belonging to the family *Potyviridae*, genus *Ipomovirus*. The two species are present predominantly in the coastal lowland [*Cassava brown streak virus* (CBSV); Tanzania and Mozambique] and highland [*Cassava brown streak Uganda virus* (CBSUV); Lake Victoria Basin, Uganda, Kenya and Malawi] in East Africa. In this study, we demonstrate that CBSD can be efficiently controlled using RNA interference (RNAi). Three RNAi constructs targeting the highland species were generated, consisting of the full-length (FL; 894 nucleotides), 397-nucleotide N-terminal and 491-nucleotide C-terminal portions of the coat protein (CP) gene of a Ugandan isolate of CBSUV (CBSUV-[UG:Nam:04]), and expressed constitutively in *Nicotiana benthamiana*. After challenge with CBSUV-[UG:Nam:04], plants homozygous for FL-CP showed the highest resistance, followed by the N-terminal and C-terminal lines with similar resistance. In the case of FL, approximately 85% of the transgenic plant lines produced were completely resistant. Some transgenic lines were also challenged with six distinct isolates representing both species: CBSV and CBSUV. In addition to nearly complete resistance to the homologous virus, two FL plant lines showed 100% resistance and two C-terminal lines expressed 50–100% resistance, whereas the N-terminal lines succumbed to the nonhomologous CBSV isolates. Northern blotting revealed a positive correlation between the level of transgene-specific small interfering RNAs detected in transgenic

plants and the level of virus resistance. This is the first demonstration of RNAi-mediated resistance to CBSD and protection across very distant isolates (more than 25% in nucleotide sequence) belonging to two different species: *Cassava brown streak virus* and *Cassava brown streak Uganda virus*.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz, Family Euphorbiaceae) is an important subsistence food crop, known for its ability to grow in poor soils, and is cultivated in the tropical regions of Africa, Asia and Latin America (El-Sharkawy, 2003). Cassava is vulnerable to at least 20 different viral diseases, among which cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) are the most important in Africa (Patil and Fauquet, 2009). Although CMD occurs in almost all cassava-cultivating regions of sub-Saharan Africa and has received considerable attention within the research community (Patil and Fauquet, 2009, 2010), CBSD has only recently become a major concern (Hillocks and Jennings, 2003; Pennisi, 2010), but is rapidly emerging as an important disease of this crop, prevalent in eastern Africa and expanding towards Central Africa (Alicai *et al.*, 2007; Mbanzibwa *et al.*, 2009b). CBSD is caused by two different ipomovirus species, *Cassava brown streak virus* (CBSV) and *Cassava brown streak Uganda virus* (CBSUV), both of which are monopartite, positive-sense, single-stranded RNA (ssRNA) viruses encapsidated into flexuous filamentous particles and classified in the genus *Ipomovirus* (Family *Potyviridae*) (Hillocks and Jennings, 2003; Mbanzibwa *et al.*, 2009a; Monger *et al.*, 2001b; Winter *et al.*, 2010).

Recent studies comparing complete sequences of several isolates have clustered them into two distinct clades, the first comprising isolates from Kenya, Uganda, Malawi and north-western Tanzania, which have 87–95% homology, and the second

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containing isolates from coastal regions of Tanzania and Mozambique, which show 70% homology to the first clade and 90–95% homology amongst themselves (Mbanzibwa *et al.*, 2009a; Monger *et al.*, 2010; Winter *et al.*, 2010). Based on a comparison of coat protein (CP) sequences, isolates in East Africa can also be grouped into two phylogenetically distinct clusters: coastal lowland isolates (Tanzania and Mozambique) and highland isolates (Lake Victoria Basin, Uganda, Kenya and Malawi) (Mbanzibwa *et al.*, 2009a,b; Monger *et al.*, 2010c; Winter *et al.*, 2010). The isolates from both clades vary significantly in their sequence (>25%) and possess significant sequence deletions in their *Ham1* (Ham1h-pyrophosphatase) and CP gene sequences. Thus, it has been proposed that two species can be defined: CBSV for the isolates from lowland or coastal regions of East Africa and CBSUV for the isolates from the highland region or Lake Victoria basin (Winter *et al.*, 2010; C. M. Fauquet, unpublished results).

CBSD has been shown to be transmitted by whitefly vectors (Maruthi *et al.*, 2005; Mware *et al.*, 2009) and by mechanical and grafting techniques under laboratory conditions (Lister, 1959; Monger *et al.*, 2001a). Symptoms vary, but are characterized by general chlorosis on the foliage, brown lesions under the bark of the stem and brown streaks on the mature stem of cassava (Fig. S1, see Supporting Information) (Hillocks and Jennings, 2003; Hillocks and Thresh, 2000). Economically, in East Africa, CBSD is considered to be a more threatening disease than CMD, because, in addition to overall yield loss, the pathogen causes severe necrosis within the edible storage roots, making them unfit for marketing or human consumption, thereby reaching 100% losses (Hillocks and Jennings, 2003; Hillocks *et al.*, 2001).

There is some natural source of resistance for CBSD, thus making conventional breeding programmes a possibility for the introgression of CBSD resistance; however, it is a very long process to combine CBSD resistance with good root and harvest qualities (Jennings, 2003). Furthermore, studies have shown that some cassava cultivars that are resistant to one virus species are not resistant to the other virus species causing CBSD (S. Winter, DSMZ, Plant Virus Division, Braunschweig, Germany, personal communication). Biotechnological approaches, in particular, post-transcriptional gene silencing (PTGS) offer significant potential for addressing the control of CBSD (Prins *et al.*, 2008; Reddy *et al.*, 2009; Sudarshana *et al.*, 2007). Transgenically induced PTGS, or RNA interference (RNAi), has been employed to control both DNA and RNA plant viruses (Chellappan *et al.*, 2004; Kamachi *et al.*, 2007; Sudarshana *et al.*, 2007; Tenllado *et al.*, 2004; Tougou *et al.*, 2006). PTGS is a sequence-specific RNA degradation mechanism triggered via a double-stranded (ds)RNA intermediate, with the dsRNAs cleaved by Dicer proteins and processed by the RNA-induced silencing complex (RISC) to produce 21–25-nucleotide (nt) small interfering (si)RNAs (Ding and Voinnet, 2007; Ruiz-Ferrer and Voinnet, 2009). The introduction of an inverted repeat sequence, homologous to part of the

viral genome, through the expression of a hairpin (hp), is an efficient method for inducing gene silencing and conferring viral resistance in plants (Helliwell and Waterhouse, 2005).

The CP of potyviruses plays an important role in the encapsidation of the viral genome and the regulation of viral RNA replication, insect transmission, cell-to-cell and systemic movement in the host, and is also one of the most conserved genes across related viral species (Callaway *et al.*, 2001; Monger *et al.*, 2001b; Urcuqui-Inchima *et al.*, 2001). The CP gene has therefore been an important target for the RNAi strategy designed to control RNA viruses, and has been deployed successfully and commercialized in the case of Papaya ring spot virus and Plum pox virus (Krubphachaya *et al.*, 2007; Scorza *et al.*, 2001).

The goal of the present work was to determine whether the RNAi approach could control CBSD, and thus demonstrate a potential biotechnological solution to tackle the spread of this devastating disease in Africa. As the production of transgenic cassava plants is time consuming (Taylor *et al.*, 2004) and screening for resistance to CBSD on cassava is difficult, we utilized the model host *Nicotiana benthamiana* to assess the efficacy of transgenically induced RNAi to control CBSUV and CBSV. Here, we report the first evidence of effective RNAi-based control of an ipomovirus and demonstrate effective cross-protection against diverse CBSD-causing virus isolates belonging to two distinct species. The potential implications of this work with regard to the introduction of resistance to CBSD in local cassava cultivars for African cassava farmers are discussed.

RESULTS

Analysis of transgenic plant lines and resistance to the homologous virus CBSUV-[UG:Nam:04]

Approximately 20 independent transgenic lines derived from each of the three RNAi constructs, FL (full-length), NT (N-terminal) and CT (C-terminal) regions of CP, with sequences derived from the isolate CBSUV-[UG:Nam:04] (Ugandan isolate of CBSUV), were polymerase chain reaction (PCR) screened for the presence of the transgene using specific primers (Table S1, see Supporting Information). Transgenic, homozygous plants were selected on a medium containing kanamycin in the T₁ and T₂ generations, and seven independent transgenic lines were selected for each RNAi construct to allow the evaluation of resistance to CBSUV-[UG:Nam:04]. Plants were challenged by sap inoculation with CBSUV-[UG:Nam:04] and the resistance levels displayed by each line were analysed statistically (Fig. 1). Most (90–100%) of the transgenic empty vector controls (54 plants in total) developed disease symptoms by about 3 days post-inoculation (dpi), which were characterized by leaf distortion, mosaic and, in extreme cases, necrosis of the whole plant (Fig. 1A). Transgenic plant lines showing different levels of pro-

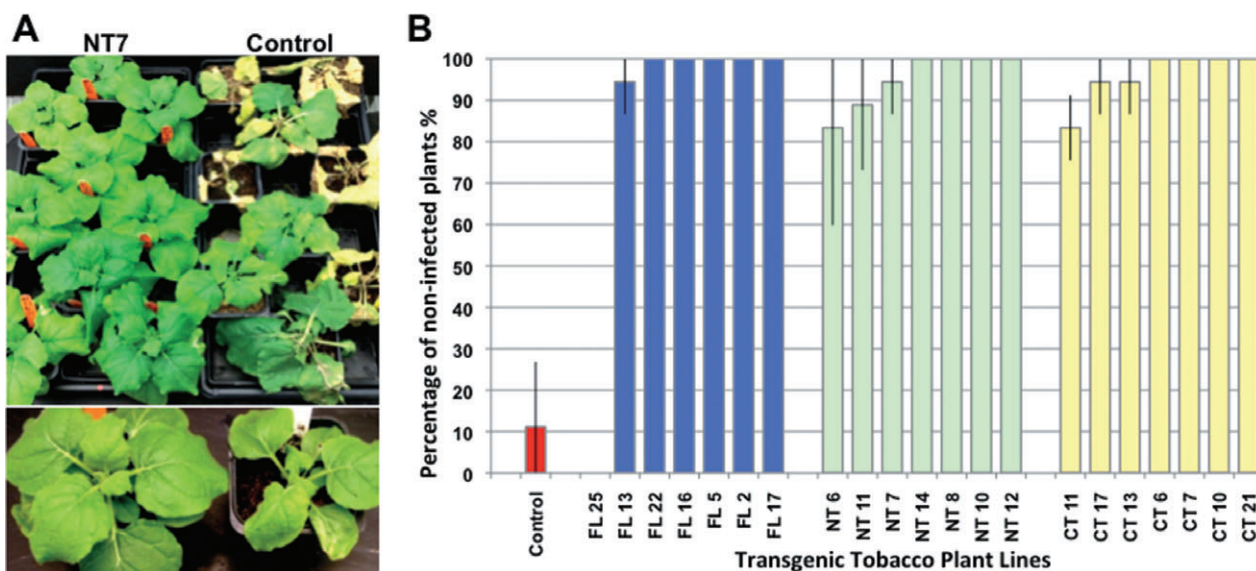


Fig. 1 Screening of *Nicotiana benthamiana* transgenic lines in the T₂ generation for resistance against CBSUV-[UG:Nam:04] (Ugandan isolate of *Cassava brown streak Uganda virus*). (A) Phenotype of resistant line NT7 derived from the RNAi construct targeting the N-terminus of CBSUV-[UG:Nam:04]/CP, showing 100% protection (left) when compared with complete infection in control plants (right). Individual plants are enlarged and shown in the bottom panel. (B) Different levels of resistance obtained in *N. benthamiana* transgenic lines derived from the RNAi constructs targeting full-length (FL; left), N-terminal (NT; centre) and C-terminal (CT; right) parts of CBSUV-[UG:Nam:04]/CP in the T₂ generation after challenge with CBSUV-[UG:Nam:04] at 30 days post-inoculation (dpi).

tection (0–100%) were observed across each RNAi construct studied (Fig. 1B). However, of the three RNAi constructs tested, that derived from FL-CP (894 nts) was the most effective for conferring resistance to CBSUV-[UG:Nam:04], such that five transgenic lines (of the seven tested), FL22, FL16, FL5, FL2 and FL17, showed 100% protection, with no symptoms appearing on any sap-inoculated plant (54 plants for each line over three replicated experiments) over the 30-day observation period (Fig. 1A,B). In the case of the N-terminal construct, four of the seven lines (NT8, NT10, NT12 and NT14) showed 100% resistance and, in the case of the C-terminal construct, four lines (CT6, CT7, CT10 and CT21) also showed 100% protection (Fig. 1B). Thus, among the three RNAi constructs targeting different regions of CBSUV-[UG:Nam:04] CP, FL-CP gave the highest frequency of protection, with 85% of the plant lines produced demonstrating complete resistance to the pathogen.

Analysis of expression levels, siRNA accumulation and correlation with resistance levels

Levels of mRNAs and siRNAs specific to the CP gene, produced by the different transgenic lines displaying varying levels of resistance, were quantified before challenge by the CBSUV-[UG:Nam:04] pathogen, and the blots were scanned and analysed statistically (Figs 2 and S3, see Supporting Information). All transgenic plant lines which subsequently showed a high level of resistance to the pathogen were found to accumulate high levels of siRNA (Fig. 2A). The multiple forms of the transcripts seen in

Northern blot hybridizations (Fig. 2A) of the transgenic lines can be attributed to intermediate products of mRNA as a result of Dicer action during PTGS (Ruiz-Ferrer and Voinnet, 2009). Conversely, no detectable transcript or corresponding siRNAs were detectable in plant lines showing no protection against CBSUV-[UG:Nam:04] infection (Fig. 2A,B). The analysis of these two quantitative criteria across all three constructs showed a strong positive correlation ($R = 92\%$) between the amount of transcript produced by the transgenic lines and the amount of CP-specific siRNA accumulated (Fig. S3A). Similarly, we found a positive correlation of $R = 86\%$ between the amount of CBSUV-[UG:Nam:04] siRNAs present in the transgenic plants before viral challenge and the subsequent level of resistance to CBSUV-[UG:Nam:04] exhibited by each transgenic plant line.

Virus accumulation and correlation with resistance levels

Four weeks after sap inoculation with the virus, all CBSUV-[UG:Nam:04]-resistant and -susceptible transgenic plant lines were analysed for the accumulation of viral RNA by Northern blot hybridization (Fig. 2B). Viral RNA was not detected in resistant plant lines observed to be free of viral symptoms, but viral RNA accumulated to varying levels in symptomatic plant lines and at significant levels in infected, nontransgenic plants (Fig. 2B). The same plant lines were analysed for the accumulation of siRNA in response to virus inoculation, 4 weeks after CBSUV-[UG:Nam:04] inoculation. A positive correlation (85%)

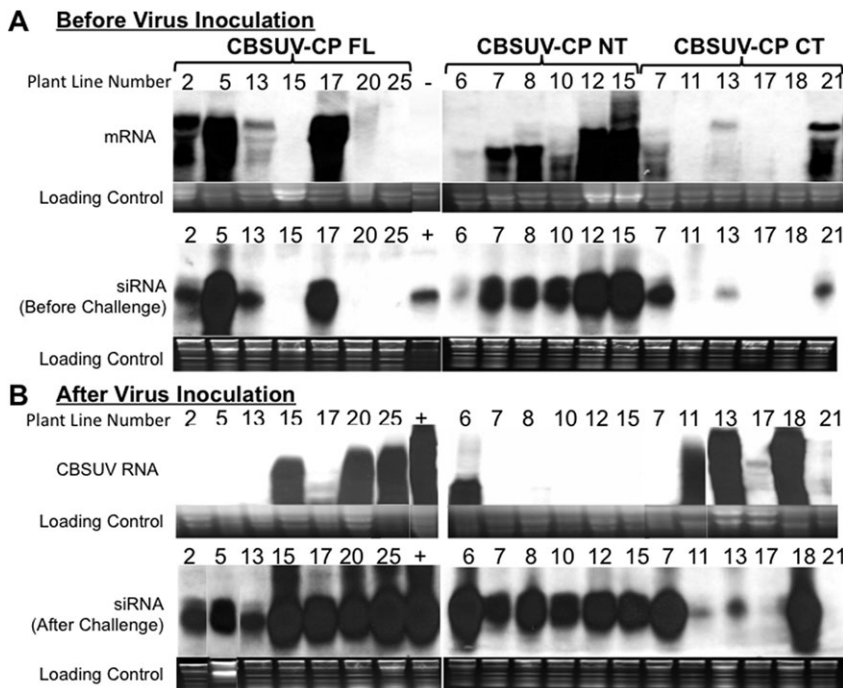


Fig. 2 Molecular analysis of *Nicotiana benthamiana* transgenic plant lines (line numbers indicated for each lane) derived from three RNAi constructs targeting different regions of CBSUV-[UG:Nam:04] (Ugandan isolate of *Cassava brown streak Uganda virus*) coat protein (CP) [N-terminus (NT), C-terminus (CT) and full-length (FL)] in the T₂ generation. (A) Accumulation of mRNAs and siRNA as detected by Northern blot hybridization before CBSUV-[UG:Nam:04] challenge. (B) Accumulation of viral RNA and siRNA after challenge with CBSUV-[UG:Nam:04] in the same transgenic lines. '-' in (A) is a nontransgenic healthy plant; '+' in (A) is a small RNA from transient expression by agroinfiltration of the FL-CP construct; '+' in (B) is a nontransgenic virus-infected control.

was observed between the amount of siRNA produced by the transgenic lines before and after CBSUV-[UG:Nam:04] inoculation (Fig. S2, see Supporting Information).

Resistance of transgenic *N. benthamiana* to diverse, nonhomologous isolates of CBSUV and CBSV

In order to test the resistance of these transgenic tobacco lines to different CBSUV and CBSV isolates, we selected six isolates with different virulence originating from different regions in East Africa. Cloning and sequence analysis of the entire CP genes isolated from CBSUV- and CBSV-infected cassava collected at Kabanyoro in Uganda (CBSUV-[UG:Kab:07]), Kibaha in Tanzania (CBSUV-[TZ:Kib:07]), Mwalumba in Kenya (CBSUV-[KE:Mwa:07]), Zanzibar in Tanzania (CBSV-[TZ:Zan:07]), Naliendele in Tanzania (CBSV-[TZ:Nal:07]) and Nampula in Mozambique (CBSV-[MZ:Nam:07]) revealed that they pertained to both species (Table 1). After initial transmission of these viruses from cassava to the model host *N. benthamiana*, the identity of the original CBSUV and CBSV isolates was re-confirmed by cloning and re-sequencing, thus ruling out the possibility of cross-contamination. Selected transgenic *N. benthamiana* lines derived from each construct were challenged with these six diverse CBSUV and CBSV isolates. Transgenic plant lines for challenge experiments were selected to represent different expression levels of siRNA in FL-CP (FL2, FL5, FL13 and FL17), NT-CP (NT7 and NT8) and CT-CP (CT7 and CT21). The determination of disease development 4 weeks after virus challenge showed that two lines, FL5 and FL17, derived from the

894-nt region of CP, were completely resistant to infection across all six isolates of CBSUV and CBSV, whereas the vector control plants showed 90–100% infection with all six isolates (Fig. 3B,C). Both FL2 and FL13 displayed resistance at approximately 40–95% to the homologous virus CBSUV-[UG:Nam:04] and other isolates most closely related to CBSUV-[UG:Nam:04], namely CBSUV-[UG:Kab:07] (99.4% identity) and CBSUV-[KE:Mwa:07] (94.3%), but showed lower levels of resistance (20–65%) to the CBSV isolates (Fig. 3B,C).

In the case of the transgenic plants expressing CT-CP, CT7 and CT21 showed high resistance (100% and ~80–100%, respectively) to all CBSUV isolates, but had low and varying resistance (20–90%) towards CBSV isolates (Fig. 3B,C). Plant lines derived from NT-CP, NT7 and NT8, showed a high level of resistance (~90–100%) to CBSUV isolates (Fig. 3B,C), but succumbed to the CBSV isolates. In general, the lines derived from FL-CP showed the highest spectrum of resistance to diverse and nonhomologous isolates of CBSUV and CBSV, followed by plant lines derived from the C-terminal region, which is more conserved than the N-terminal region, with the latter showing the least resistance to CBSV isolates (Fig. 3C).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of CBSUV- and CBSV-challenged transgenic *N. benthamiana*

Total RNA was extracted from both symptomatic and nonsymptomatic transgenic plants at 30 dpi, and RT-PCR was performed to detect CBSUV or CBSV CP, using specific primers designed to

Table 1 Percentage identity levels (nucleotides, nts) of coat protein (CP) sequences of *Cassava brown streak Uganda virus* (CBSUV) and *Cassava brown streak virus* (CBSV) isolates used to challenge transgenic plants with the CP of CBSUV-[UG:Nam:04].

Percentage identity with CP fragments of CBSUV-[UG:Nam:04]	CBSUV			CBSV		
	[UG:Kab:07]	[TZ:Kib:07]	[KE:Mwa:07]	[TZ:Zan:07]	[TZ:Nal:07]	[MZ:Nam:07]
Country of origin	Uganda	Tanzania	Kenya	Tanzania	Tanzania	Mozambique
Symptom severity	Mild	Mild	Moderate	Moderate	Severe	Severe
CP-FL (894 nts)	99.4	91.1	94.3	75.8	76.1	75.7
CP-NT (397 nts)	99.0	89.4	93.7	70.5	71.0	70.5
CP-CT (491 nts)	99.8	92.5	95.3	80.7	80.7	80.2

CT, C-terminus; FL, full length; nts, nucleotides; NT, N-terminus.

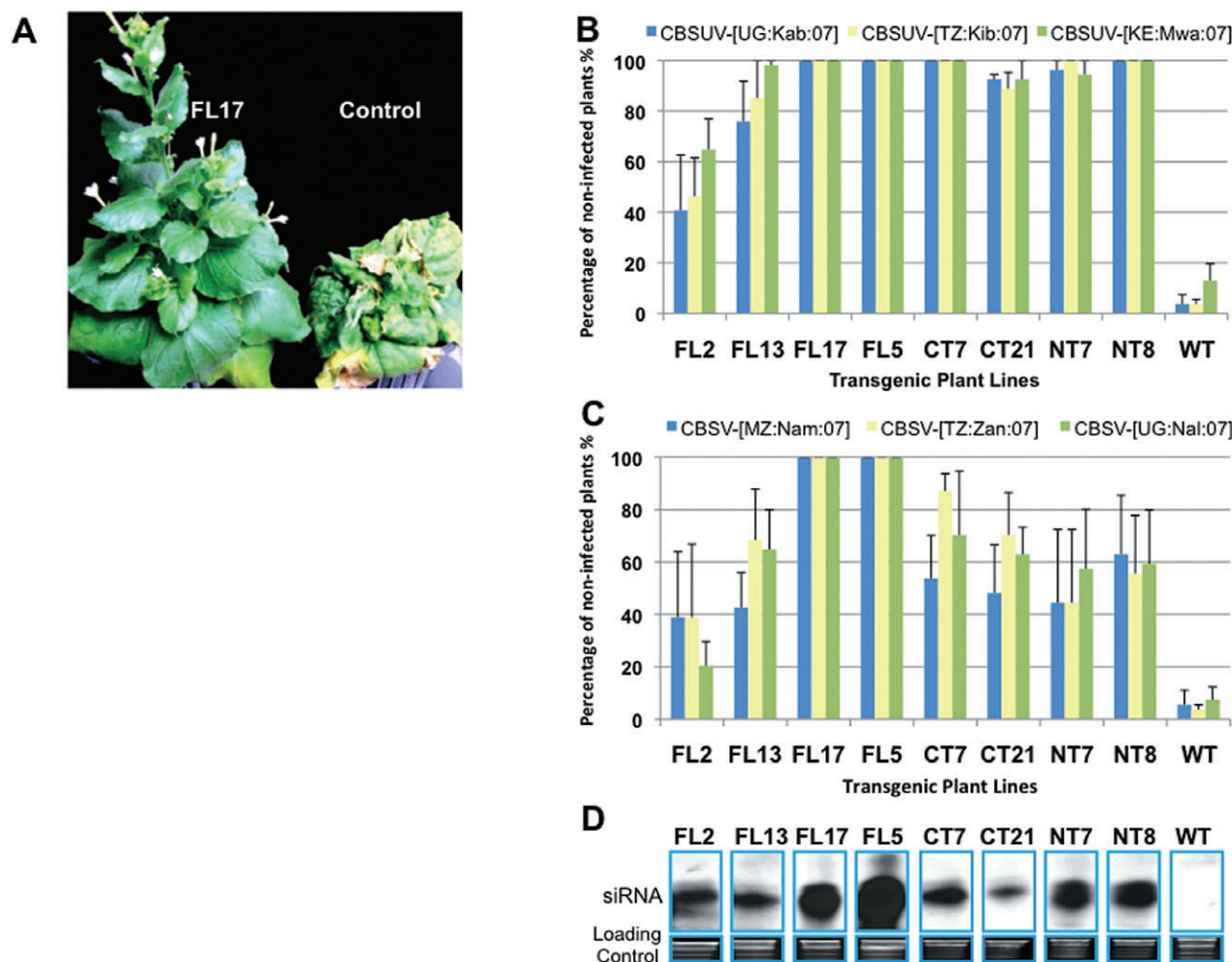


Fig. 3 Screening of *Nicotiana benthamiana* transgenic lines in the T₂ generation for resistance against six distinct *Cassava brown streak Uganda virus* (CBSUV) and *Cassava brown streak virus* (CBSV) isolates. (A) Phenotype of a resistant line FL17 derived from the RNAi construct targeting the 894 nts of CBSV-UG[UG:Nam:04]/CP, showing 100% protection (left) when compared with complete infection in control plants (right) when challenged with CBSV-[TZ:Zan:07]. (B, C) Different levels of protection obtained in *N. benthamiana* transgenic lines derived from RNAi constructs targeting full-length (FL; left), C-terminal (CT; centre) and N-terminal (NT; right) parts of CBSV-UG[UG:Nam:04]/CP in the T₂ generation after challenge with six different isolates of CBSUV and CBSV. (B) shows protection for CBSUV isolates (CBSUV-[UG:Kab:07], CBSUV-[TZ:Kib:07] and CBSUV-[KE:Mwa:07]) and (C) shows protection for CBSV isolates (CBSV-[MZ:Nam:07], CBSV-[TZ:Zan:07] and CBSV-[UG:Nal:07]). Different levels of siRNA expressed by these lines positively correlate ($R = 0.86$) with their resistance levels, as shown in (D).

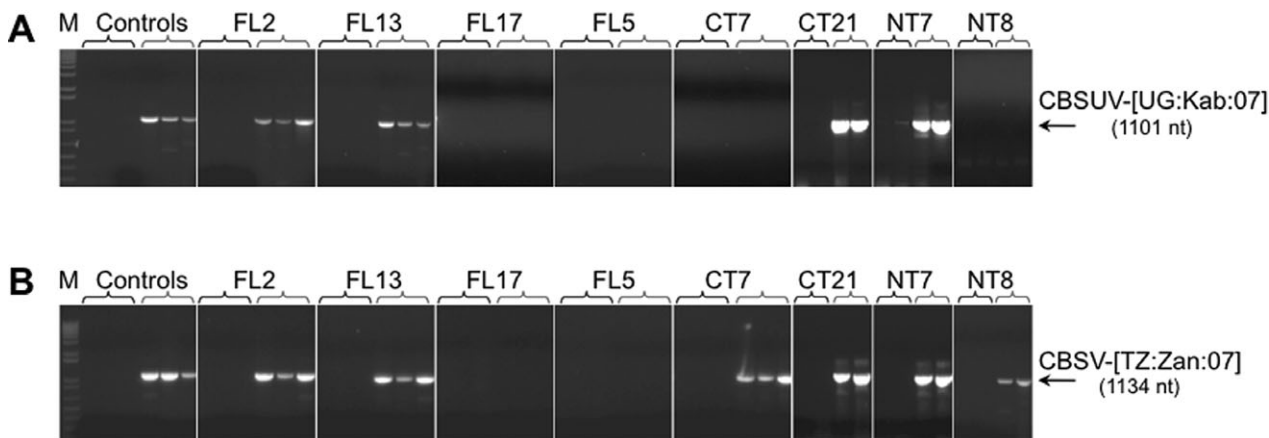


Fig. 4 Reverse transcriptase-polymerase chain reaction (RT-PCR) for the detection of the coat protein (CP) of *Cassava brown streak Uganda virus* (CBSUV) (A) and *Cassava brown streak virus* (CBSV) (B) isolates. Specific primers were used to amplify 1101 nts of CP for the CBSUV isolates (CBSUV-[UG:Kab:07], CBSUV-[TZ:Kib:07] and CBSUV-[KE:Mwa:07]) and 1134 nts of CP for the CBSV isolates (CBSV-[TZ:Zan:07], CBSV-[TZ:Nal:07] and CBSV-[MZ:Nam:07]) from the total RNA extracts for both symptomatic (first three/two lanes for each transgenic plant line) and nonsymptomatic (next three/two lanes) transgenic plants after challenge of transgenic *N. benthamiana* with CBSUV-[UG:Kab:07] and CBSV-[TZ:Zan:07].

amplify 1101 nts (Fig. 4A) of the CBSUV isolates (CBSUV-[UG:Kab:07], CBSUV-[TZ:Kib:07] and CBSUV-[KE:Mwa:07]) and 1134 nts (Fig. 4B) of the CBSV isolates (CBSV-[TZ:Zan:07], CBSV-[TZ:Nal:07] and CBSV-[MZ:Nam:07]). Amplification of viral RNA was seen from both symptomatic control and transgenic plants, but was not detected in extracts from symptom-free transgenic plants after challenge with all six CBSUV and CBSV isolates, indicating that the virus did not move systemically from the inoculation point in such plants, and these plants remained free of the pathogen over the 30-dpi observation period (Fig. 4A,B).

***In silico* analysis for the presence of cross-protecting siRNAs in the CBSUV CP sequence**

In order to determine why effective cross-protection was achieved against all nonhomologous CBSV isolates in the transgenic lines FL5 and FL17, we investigated the number of mismatches among theoretically possible siRNAs in the 894-nt CP sequence (CBSUV-[UG:Nam:04]) using the program MAQ (<http://maq.sourceforge.net>). A total of 875 types of 21-nt siRNAs and 874 types of 22-nt siRNAs were predicted by the analysis. No single siRNA with complete identity to any of the three CBSV isolates (CBSV-[TZ:Zan:07], CBSV-[TZ:Nal:07] and CBSV-[MZ:Nam:07]) used in the present study was found, but 15, 14 and 12 siRNAs with a 1-nt mismatch and 64, 78 and 63 siRNAs with a 2-nt mismatch were identified for CBSV-[TZ:Zan:07], CBSV-[TZ:Nal:07] and CBSV-[MZ:Nam:07], respectively, having nucleic acid identities of 75.8%, 76.1% and 75.7%, respectively, with the CBSUV-[UG:Nam:04] FL-CP transgene sequence; for CT-CP and NT-CP, these numbers were significantly less (Table 2). In the case of the three CBSUV isolates (CBSUV-[UG:Kab:07],

CBSUV-[TZ:Kib:07] and CBSUV-[KE:Mwa:07]), for which CBSUV-[UG:Nam:04] showed 99.4%, 91.1% and 94.3% identities, respectively, 769, 101 and 270 types of siRNA with complete homology were found for FL-CP, in contrast with a significantly lower number of siRNAs with complete homology for CT-CP and NT-CP (Table 2).

DISCUSSION

Of the available biotechnological approaches to control plant viral diseases, RNAi is a very promising strategy that has been successfully employed to control numerous viral diseases caused by both DNA and RNA viruses (Tenllado *et al.*, 2004; Waterhouse and Fusaro, 2006). Of the various viral sequences tested, the CP gene, which has multiple functions in the pathogen's life cycle, has been utilized as a transgene to control plant RNA virus diseases (Callaway *et al.*, 2001; Jan *et al.*, 1999; Ling *et al.*, 2008; Scorza *et al.*, 2001; Urcuqui-Inchima *et al.*, 2001). However, the nature of the virus and its life cycle in the host are important in determining whether it will be subject to, or evade, RNAi, as reported for Turnip mosaic virus (potyvirus; Jan *et al.*, 1999), Citrus tristeza virus (closterovirus; Olivares-Fuster *et al.*, 2003) and Cucumber green mottle mosaic virus (tobamovirus; Kamachi *et al.*, 2007).

Among the few ipomoviruses molecularly characterized today, CBSV and CBSUV are unique in their genome organization because of the absence of the characteristic potyvirus silencing suppressor *HcPro* (Helper component proteinase) and the presence of *HAM1h*, a rare gene among plant viruses, hypothesized to prevent mutations in the viral genome by intercepting the incorporation of noncanonical nucleoside triphosphates (Galp-

Table 2 Number of *in silico*-predicted small interfering (si)RNAs (21 nt and 22 nt) for the three regions of CBSUV-[UG:Nam:04] (Ugandan isolate of *Cassava brown streak Uganda virus*) coat protein (CP) [full-length (894 nt), N-terminal (397 nt) and C-terminal (491 nt)] used to prepare the RNAi constructs against the corresponding regions of the six distinct CBSUV and *Cassava brown streak virus* (CBSV) isolates used in screening the resistance levels.

Size of siRNA	Number of mismatches	CBSUV									CBSV								
		[UG:Kab:07]			[TZ:Kib:07]			[KE:Mwa:07]			[TZ:Zan:07]			[TZ:Nal:07]			[MZ:Nam:07]		
		FL	NT	CT	FL	NT	CT	FL	NT	CT	FL	NT	CT	FL	NT	CT	FL	NT	CT
21 nt	0	769	314	429	101	48	51	270	72	197	0	0	0	0	0	0	0	0	0
	1	105	63	42	255	90	157	272	140	127	15	0	15	14	0	14	12	6	6
	2	0	0	0	312	126	174	244	120	103	64	8	56	78	23	55	63	11	52
	Total	874	377	471	668	264	382	786	332	427	79	8	71	92	23	69	75	17	58
22 nt	0	763	311	426	87	43	43	255	67	188	0	0	0	0	0	0	0	0	0
	1	110	65	44	246	86	152	265	134	127	11	0	11	9	0	9	8	4	4
	2	0	0	0	308	125	171	256	126	109	53	5	48	65	18	47	51	10	41
	Total	873	376	470	641	254	366	776	327	424	64	5	59	74	18	56	59	14	45

nt, nucleotide.

erin *et al.*, 2006; Mbanzibwa *et al.*, 2009a). The only other plant virus in which the presence of a *Ham1h*-like sequence has been reported is Euphorbia ringspot virus (EuRSV; National Center for Biotechnology Information Acc. No. AY397600), which also infects plants of the *Euphorbiaceae* family (Mbanzibwa *et al.*, 2009a). For these reasons, it is important to verify whether RNAi can control ipomoviruses, such as CBSUV and CBSV, and whether the CP gene could be an effective target to achieve this goal via transgenic expression. We employed RNAi to control CBSV in the model host *N. benthamiana*, in which screening of CBSUV and CBSV resistance can be performed easily by mechanical sap inoculation (Mbanzibwa *et al.*, 2009b; Monger *et al.*, 2001a).

To determine the role of the size and sequence of the CBSUV CP required to achieve effective RNAi control of the viral pathogen, three RNAi constructs were designed to target the FL (894 nts), N-terminal (397 nts) and C-terminal (491 nts) regions of the CBSUV-[UG:Nam:04] CP gene. Transgenic lines derived from each of the three constructs showed a diverse range of siRNA accumulation and resistance to CBSUV-[UG:Nam:04], with the lines derived from FL-CP conferring the highest protection (Figs 1B and 2A), followed by similar levels of resistance by lines derived from both N-terminal and C-terminal regions. Similar to the results observed here, comparative studies with the N- and C-terminal halves of the S4 and S12 genes of Rice dwarf virus showed no difference in the efficacy of levels of viral protection through RNAi (Shimizu *et al.*, 2009).

In the present study, a positive and significant correlation ($R = 86\%$) was observed between the presence of siRNAs, the hallmark of RNAi, and the levels of protection against CBSUV-[UG:Nam:04] (Figs 1B and 2A). Most of the transgenic plant lines showing high levels of detectable transcript also produced significant levels of siRNA and displayed strong resistance to CBSUV-[UG:Nam:04] (Fig. 2A). A highly significant positive correlation was also found between the level of CBSUV-[UG:Nam:04]/CP mRNA expression and siRNA accumulation

($R = 92\%$), and between such siRNA levels and CBSUV-[UG:Nam:04] protection ($R = 86\%$), indicating that these molecular criteria could potentially be of value as screening tools in subsequent studies, for example in cassava, to identify transgenic plant lines with high levels of resistance to CBSV viral agents (Figs 2A and S3).

There was a strong positive correlation between the levels of siRNA before and after CBSUV-[UG:Nam:04] inoculation. The increase in siRNA accumulation after virus inoculation was insignificant (less than two-fold), indicating that the highly expressing plant lines efficiently responded to viral inoculum through gene silencing (Fig. 2B). This observation, associated with the very high level of resistance obtained (100%), indicates that this technology is very effective for controlling CBSV in tobacco.

In order to determine whether the plants from transgenic lines that remained asymptomatic after challenge with the pathogen were free of virus, such plants were studied by Northern blot hybridization and RT-PCR. Analysis showed that these highly resistant plants did not contain detectable amounts of CBSUV-[UG:Nam:04] or CBSV-[TZ:Zan:07]. This is considered to be important because the development and deployment of tolerant plants that display no symptoms, but carry a high virus load, would be undesirable with regard to the field level control of the disease, as they could be a potential source of virus acquisition by whiteflies.

Phylogenetic studies from East Africa have shown that there are at least two distinct species of virus causing the same symptomatology, and several different strains associated with CBSV in this region (Mbanzibwa *et al.*, 2009a; Winter *et al.*, 2010). If strategies to deploy improved germplasm are to succeed, they must deliver plant materials possessing high levels of resistance to all CBSVs. An important drawback of RNAi is that it is usually highly sequence specific, and viruses with more than 10% nucleotide diversity are mostly insensitive to this technology; this necessitates the combination of fragments from different virus

species to make an RNAi construct with broad-spectrum resistance (Prins, 2003). The use of such a large set of genes or their fragments in one single construct could be unstable during the cloning and transformation processes and, more importantly, in their expression and ability to form perfect hairpins within the transgenic plant.

However, the challenge of transgenic plants produced in this study with isolates collected from Uganda, Kenya, Tanzania and Mozambique (Table 1) showed significant cross-protection imparted to both virus species. Two transgenic plant lines derived from FL-CP remained completely symptom free and apparently immune against all six diverse isolates of CBSUV and CBSV tested. The lines derived from CT-CP also showed low to high resistance (~20–100%) to these six isolates (Fig. 3). Conversely, the two high-expressing lines derived from NT-CP were susceptible to the nonhomologous isolates from Tanzania (CBSV-[TZ:Nal:07]), Zanzibar (CBSV-[TZ:Zan:07]) and Mozambique (CBSV-[MZ:Nam:07]) (Fig. 3C). Transgenic CT-CP imparted superior resistance to nonhomologous isolates than did NT-CP, most probably because the C-terminal region of CBSUV and CBSV *CP* is more conserved than the N-terminal region. For some of the plant lines, there was a large amount of variation in resistance levels across different experiments, which could be attributed to variation in the quality and amounts of virus inoculum used for each experiment during sap inoculations.

The two FL-CP lines (FL5 and FL17) which exhibited immunity or very strong resistance to all of the CBSUV and CBSV isolates were shown to express the highest levels of mRNA and siRNA of all the FL-CP transgenic plant lines tested (Fig. 2), whereas the two FL-CP lines (FL2 and FL13) that did not show significant resistance were poor expressers of siRNAs (Fig. 3D). It is possible that the FL-CP lines gave the best resistance to all tested viruses because they had the largest fragment of *CP*, capable of producing a diverse and larger population of siRNAs to target both the CBSUV and CBSV isolates, when compared with the smaller sized NT-CP and CT-CP. *In silico* prediction for siRNA across the *CP*s of CBSUV-[UG:Nam:04] and the other three CBSV isolates showed no siRNA with complete identity and only ~14 and ~64 siRNAs with one and two mismatches, respectively (Table 2). These results indicate that complete identity between siRNAs and the target RNA is not essential for the induction of gene silencing. More important is the presence of perfect complementarity with the 6-nt seed region (nts 2–7) of siRNAs for their binding to the target RNA and further processing (Jackson *et al.*, 2006). Previous studies in HeLa cells using synthetic siRNAs have demonstrated that perfect complementarity with a specific target at the 5' end of the antisense strand of siRNA was sufficient to direct siRNA–target binding and cleavage (Haley and Zamore, 2004; Jackson *et al.*, 2006).

It was encouraging to see that, in addition to the absence of disease symptoms, plants challenged with different isolates of

CBSUV and CBSV remained virus free as determined by RT-PCR (Fig. 4). We consider this to be the first demonstration of cross-protection against a plant virus with no single siRNA from the transgene having complete identity to the target virus, and, in addition, the level of cross-protection was positively correlated with siRNA expression levels. These results encourage continued research into the use of RNAi technology as an effective biotechnological tool to control the dramatic impact of CBSV in farmers' fields.

EXPERIMENTAL PROCEDURES

RNAi vector construction

Fragments of the *CP* gene were PCR amplified from CBSUV-[Uganda:Namulonge:2004]-infected cassava plants collected from Namulonge, Uganda (hereafter abbreviated to CBSUV-[UG:Nam:04]) as full-length (FL; 894 nts: nts 208–1101 of *CP*), N-terminal (NT; 397 nts: nts 208–604 of *CP*) and C-terminal (CT; 491 nts: nts 611–1101 of *CP*) fragments. The actual size of the *CP* open reading frame (ORF) for CBSV-UG is 1101 nts, but, when this project was initiated, only the 894-nt sequence of CBSUV-[UG:Nam:04] *CP* was available; thus, for convenience, we refer here to the 894 nts of *CP* as the full-length (FL or FL-CP) sequence. These sequences were cloned in sense orientation between the *Kpn*I and *Xba*I restriction sites and in antisense orientation between the *Bam*HI and *Bst*BI sites on either ends of the Pdk-intron derived from the vector pHELLSGATE (from Dr P. M. Waterhouse, CSIRO, Plant Industry, Canberra, Australia; National Center for Biotechnology Information Acc. No. AJ311874) (Table S1). In order to generate stable transgenic plants, the RNAi cassette driven by the Cassava vein mosaic virus (CsVMV) promoter (Verdaguer *et al.*, 1998) was subcloned into the binary vector pCambia2300 (Acc. No. AF234315) and transformed into *Agrobacterium tumefaciens* strain LBA4404.

Production of transgenic *N. benthamiana*, and PCR analysis

Nicotiana benthamiana plants were transformed by the leaf disc method using *A. tumefaciens* (Horsch *et al.*, 1985), and regenerated putative transgenic plants were transferred to pots and maintained in the glasshouse at 28 °C. T₀ plants were screened at 8 weeks of age by PCR using specific primers (Table S1) and the PCR-positive lines were maintained for the production of the homozygous T₂ generation.

Screening for CBSUV-[UG:Nam:04] resistance by sap inoculation

T₂ transgenic homozygous lines were grown in the glasshouse and sap inoculated with CBSUV-[UG:Nam:04] inoculum

obtained from CBSUV-[UG:Nam:04]-infected *N. benthamiana* plants. Sap extract was prepared in potassium phosphate buffer (0.01 M; pH 7.0) containing antioxidants (0.01 M β -mercaptoethanol and 0.2 M sodium sulphite) and gently rubbed onto two completely opened young leaves with the help of carborundum as an abrasive agent (Mandal *et al.*, 2001; Mbanzibwa *et al.*, 2009b). For each independent transgenic line, 18 plants were challenged and the inoculated plants were maintained in a growth chamber at 28 °C with 200 μ E/m²/s of light and 70% relative humidity, and monitored for symptom development every alternate day for a period of 4 weeks. All viral challenges were performed as three replications. Disease symptom scoring was carried out using a scale of 0–5 (Fargette *et al.*, 1993) and analysed to determine resistance levels for each plant line. Plants transformed with the empty vector pCam-bia2300 were used as negative controls.

CBSUV and CBSV isolates used for screening resistance in transgenic *N. benthamiana*

The six virus isolates collected from different locations in East Africa, namely Kabanyoro in Uganda (CBSUV-[Uganda:Kabanyoro:2007] or CBSUV-[UG:Kab:07]), Kibaha in Tanzania (CBSUV-[Tanzania:Kibaha:2007] or CBSUV-[TZ:Kib:07]), Mwalumba in Kenya (CBSUV-[Kenya:Mwalumba:2007] or CBSUV-[KE:Mwa:07]), Zanzibar in Tanzania (CBSV-[Tanzania:Zanzibar:2007] or CBSV-[TZ:Zan:07]), Naliendele in Tanzania (CBSV-[Tanzania:Naliendele:2007] or CBSV-[TZ:Nal:07]) and Nampula in Mozambique (CBSV-[Mozambique:Nampula:2007] or CBSV-[MZ:Nam:07]), which also represented both lowland and highland regions, were used to challenge the transgenic plants (Table 1). The complete genes of the CP of all isolates (with a size of 1101 nts for CBSUV isolates and 1134 nts for CBSV isolates) were cloned from infected cassava plants, and the sequences were analysed for identity to the CBSUV-[UG:Nam:04]-CP sequence used to generate the transgenic tobacco plants. These isolates were further sap transmitted to *N. benthamiana* plants for screening the transgenic lines, and their identity was confirmed by re-sequencing of CP.

Analyses of plants for viral mRNA and siRNA before and after virus challenge

Transgenic *N. benthamiana* lines, together with controls, were analysed for transcript production, siRNA accumulation and virus accumulation by Northern blot hybridization (Sambrook and Russell, 2001), both before and after challenge with CBSUV-[UG:Nam:04]. Total RNA was isolated from leaves using Trizol (Invitrogen, Carlsbad, CA, USA), and subjected to electrophoresis and Northern blot hybridization for the detection of transcript

and viral RNA. However, for the detection of siRNA, total RNA was fractionated using an RNeasy plant mini kit (Qiagen, Valencia, CA, USA) and the RNA clean-up protocol (Akbergenov *et al.*, 2006). Ten micrograms of small RNAs were electrophoresed on a 15% TBE (Tris Borate EDTA) urea gel (Criterion-Bio-Rad, Hercules, CA, USA), and subjected to Northern blot hybridization with a hydrolysed probe obtained by the *in vitro* transcription of CBSUV-CP using a SP6/T7 Transcription Kit (Roche Applied Science, Indianapolis, IN, USA). The probe used for the detection of transcript and viral RNA was PCR amplified CBSUV-[UG:Nam:04]/CP DNA generated by the DIG High Prime DNA labelling and detection kit (Roche Applied Science). The membranes were processed and the signal was detected using CDP-star (Roche Applied Science), as described in the DIG system and the DIG application manual. The signal intensities were quantified by ImageJ (version 1.36b) software over a uniform selected area for each transgenic line, and analysed statistically to determine the correlation coefficients between mRNA and siRNA, and siRNA and resistance.

RT-PCR analysis of CBSUV- and CBSV-challenged transgenic plants

Using an RNeasy mini kit (Qiagen), total RNA was extracted from transgenic plants challenged with different isolates of CBSUV and CBSV and subjected to RT-PCR (Invitrogen SuperScript® III First-Strand RT-PCR kit) by primers amplifying the 1101/1134 nts of the entire CP of CBSUV/CBSV isolates from highland/lowland regions. A pair of primers to amplify the constitutively expressed tubulin (primers TobefS and TobefA; Table S1) was also used as a control to check the quality of cDNA synthesized in the RT-PCRs.

In silico analysis of possible siRNAs in the CBSUV and CBSV CP sequences

To analyse the theoretical possibility of siRNAs derived from the inverted repeat constructs targeting different regions of CBSUV-CP, the sequence of the transgene was processed *in silico* into every possible 21-, 22- and 24-nt RNA using a custom-prepared script in the Python programming language. The theoretical siRNA libraries derived from the constructs were mapped against sequences of each of the viruses using the program MAQ (<http://sourceforge.net/projects/maq/>), and the results were imported into an Excel file for further analysis. The number and position of theoretical siRNAs homologous to each virus strain with zero, one and two mismatches were tabulated, and the positions of common siRNAs were recorded (Table 2).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Characteristic cassava brown streak disease (CBSD) symptoms visible on different parts of the cassava plant: (A) feathery chlorosis on cassava leaf; (B) root necrosis; (C) brown streaks on young cassava stem.

Fig. S2 Accumulation of small interfering (si)RNA before and after challenge with CBSUV-[UG:Nam:04] (Ugandan isolate of *Cassava brown streak Uganda virus*) in *Nicotiana benthamiana* transgenic lines as detected by Northern hybridization. The relative signal intensities of siRNA against the background of the autoradiograms were quantified by ImageJ software for a uniform selected area for each transgenic line, and analysed statistically to determine the correlation coefficients.

Fig. S3 Linear regression analysis to establish the correlation between small interfering (si)RNA and mRNA (A) and the amount of siRNA and the resistance levels (B) in each transgenic plant line before and after challenge with CBSUV-[UG:Nam:04] (Ugandan isolate of *Cassava brown streak Uganda virus*).

Table S1 Sequences of the primers used in making the RNAi constructs for CBSUV-[UG:Nam:04] (Ugandan isolate of *Cassava brown streak Uganda virus*) coat protein (CP) [full length (FL), N-terminal (NT) and C-terminal (CT)] in sense (*Xba*I and *Kpn*I) and antisense (*Bam*HI and *Bst*BI) orientation; other primers used in this study are also listed.

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