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RNAi-mediated resistance to *Cassava brown streak Uganda virus* in transgenic cassava

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SUMMARY

Cassava brown streak disease (CBSD), caused by *Cassava brown streak Uganda virus* (CBSUV) and *Cassava brown streak virus* (CBSV), is of new epidemic importance to cassava (*Manihot esculenta* Crantz) production in East Africa, and an emerging threat to the crop in Central and West Africa. This study demonstrates that at least one of these two ipomoviruses, CBSUV, can be efficiently controlled using RNA interference (RNAi) technology in cassava. An RNAi construct targeting the near full-length coat protein (FL-CP) of CBSUV was expressed constitutively as a hairpin construct in cassava. Transgenic cassava lines expressing small interfering RNAs (siRNAs) against this sequence showed 100% resistance to CBSUV across replicated graft inoculation experiments. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed the presence of CBSUV in leaves and some tuberous roots from challenged controls, but not in the same tissues from transgenic plants. This is the first demonstration of RNAi-mediated resistance to the ipomovirus CBSUV in cassava.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz, Euphorbiaceae) is a hardy tropical plant grown for its starchy storage roots and plays a key role in the livelihood of resource-limited farmers in tropical Africa (El-Sharkawy, 2003). Cassava production in East Africa is being increasingly affected by cassava brown streak disease (CBSD), an emerging viral disease that is suppressing yields by 30%–85% (Hillocks and Jennings, 2003; Pennisi, 2010). Recently, CBSD was identified as among the seven most serious threats to world food security (Pennisi, 2010). Field surveys conducted since 2005 have revealed that CBSD is spreading to new regions. Previously restricted to lowland coastal regions of Mozambique and Tanzania, the disease has now been confirmed as present inland, in Kenya, Uganda, Tanzania, Malawi (Winter *et al*., 2010) and Central Africa (Alicai *et al*., 2007; Ntawuruhunga and Legg, 2007) (FAO database, 2010: http://www.fao.org/fileadmin/templates/fcc/documents/CaCESA_EN.pdf).

CBSD is caused by the two viruses, *Cassava brown streak virus* (CBSV) and *Cassava brown streak Uganda virus* (CBSUV), both of which are monopartite, positive-sense, single-stranded RNA viruses encapsidated within flexuous rod particles, classified in the genus *Ipomovirus*, family *Potyviridae* (Hillocks and Jennings, 2003; Mbanzibwa *et al*., 2009; Monger *et al*., 2001b; Winter *et al*., 2010). Different isolates of CBSV and CBSUV show up to 30% variability with each other at the nucleotide level, with major differences located in their *P1* and *Ham1h* (Ham1h-pyrophosphatase) gene sequences (Mbanzibwa *et al*., 2009; Monger *et al*., 2010; Winter *et al*., 2010). CBSUV isolates show 86%–99% homology amongst themselves at the coat protein (CP) gene level and are prevalent in Kenya, Uganda, Malawi and northwestern Tanzania. CBSV isolates are mostly distributed along the coastal regions of Tanzania and Mozambique (Mbanzibwa *et al*., 2009; Monger *et al*., 2010; Winter *et al*., 2010).

CBSD is transmitted naturally to cassava by the two whitefly species, *Bemisia tabaci* and *Aleurodicus disperses* (Maruthi *et al*., 2005; Mware *et al*., 2009), and via stem cuttings to subsequent cropping cycles. In the absence of the natural vector, or when virus pressure is low, grafting is also an effective transmission method, allowing test material to be assessed for disease development (Lister, 1959; Monger *et al*., 2001a). CBSD is symptomless on young leaves, but causes small veinal or blotchy yellow chlorosis on older leaves and dark brown lesions that can fuse to form brown streaks on the stem and reduce the vigour of the planting material for the next cropping season. Brown to dark brown necrotic lesions can also develop in the tuberous roots (Hillocks and Jennings, 2003; Hillocks and Thresh,

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[Correction added after online publication 23 February 2011: citations of Patil *et al*. 2010 were updated to 2011]

2000), making them unfit for human or animal consumption (Hillocks and Jennings, 2003; Hillocks *et al*., 2001).

CBSD is currently managed by multiplication and distribution of disease-free stem cuttings and by dissemination of CBSD-tolerant varieties (FAO, 2006). However, to date, no source of robust resistance has been identified. Difficulties in introgressing resistance traits into germplasm preferred by farmers and consumers (Jennings, 2003) makes breeding for CBSD resistance a challenging and lengthy process (Jennings, 2003). As a result, new strategies to control CBSD are desirable. Post-transcriptional gene silencing (PTGS) and RNA interference (RNAi) are technologies that offer significant potential to control plant viral pathogens, particularly RNA viruses. RNAi has been applied to generate resistance to *Cucumber mosaic virus*, *Zucchini yellow mosaic virus* and *Watermelon mosaic virus 2* (Klas *et al*., 2006; Tricoll *et al*., 1995), *Potato leaf roll virus*, *Potato virus Y* and *Potato virus X* (Thomas *et al*., 2000), *Papaya ring spot virus* (Krubphachaya *et al*., 2007) and *Plum pox virus* (Hily *et al*., 2004; Kundu *et al*., 2008).

RNAi is a sequence-specific RNA degradation mechanism triggered by a double-stranded (ds) RNA intermediate. The dsRNAs are cleaved by Dicer proteins and processed by the RISC complex to produce 21–25-nucleotide small interfering RNAs (siRNAs) (Ding and Voinnet, 2007; Ruiz-Ferrer and Voinnet, 2009). The introduction and expression of an inverted repeat sequence homologous to part of the targeted viral genome is an efficient method for the induction of gene silencing and for conferring viral resistance in plants (Helliwell and Waterhouse, 2005).

Recently, we have demonstrated the application of PTGS to control CBSD in *Nicotiana benthamiana* (Patil *et al*., 2011). Of the three RNAi constructs targeting different regions of the CP gene of CBSUV [i.e. 894-nucleotide near full-length coat protein (FL-CP), 397-nucleotide N-terminal and 491-nucleotide C-terminal portions), FL-CP imparted the highest resistance, with 85% of transgenic tobacco lines tested showing complete resistance to infection by CBSUV after sap inoculation (Patil *et al*., 2011). At that time, this technology was not tested in cassava because of the lengthy process of cassava genetic transformation (Taylor *et al*., 2004) and limitations with the method of evaluating CBSD resistance in cassava under controlled plant growth conditions.

The aims of this study were to evaluate CBSD resistance by graft inoculation of transgenic cassava and to determine whether RNAi-mediated control of CBSUV, as demonstrated in *N. benthamiana*, could be applied to control CBSUV in this crop species.

RESULTS

Analysis of transgenic cassava lines

Thirty-eight independent transgenic cassava lines of cv. 60444 derived from the RNAi construct FL-CP (Patil *et al*., 2011), generated from the isolate CBSUV-[UG:Nam:04], were screened for the presence of one to two copies of the T-DNA and for the accumulation of siRNAs specific to the CP of CBSUV. Eight independent transgenic plant lines satisfying these criteria were selected for further study (Table 1).

Table 1 Molecular analysis of RNA interference full-length coat protein (RNAi FL-CP) transgenic cassava lines of cv. 60444.

RNAi FL-CP transgenic cassava cv. 60444	T-DNA copy no.	Relative siRNA expression
718-001	1	+++
718-003	1	+
718-004	1	++
718-007	1	+
718-008	1	++
718-009	2	+
718-010	1	+
718-012	2	++

Plant lines with the prefix 718 are independent transgenic events for the construct RNAi FL-CP from *Cassava brown streak Uganda virus* (CBSUV). The T-DNA copy number and small interfering RNA (siRNA) expression were determined by dot blot and Northern blot hybridization, respectively, performed on leaves from *in vitro* transgenic events before challenge with CBSUV.

Optimization of a grafting method for efficient CBSD transmission to cassava

A grafting method was optimized to allow testing of CBSUV resistance in transgenic cassava plants. A biodiversity study of CBSD causing viruses in Uganda revealed that CBSUV is widespread in Uganda [National Agricultural Research Organization/Donald Danforth Plant Science Center (NARO/DDPSC) Survey on the Biodiversity of CBSD Viruses in Uganda, unpublished data]. CBSUV isolate [Uganda:TO4-42:2004] was imported from Uganda to DDPSC and confirmed to be infected only with CBSUV by reverse transcriptase-polymerase chain reaction (RT-PCR) and viral sequence analysis. The sequence was deposited in the National Center for Biotechnology Information (NCBI) gene bank as accession number HM171316.1. The CBSUV isolate [Uganda:TO4-42:2004] showed 99% identity at the nucleotide level to the CBSUV-[UG:Nam:04] CP gene used to generate the transgenic cassava plants tested in this study.

Infected scions were grafted onto virus-free, nontransgenic stems of cv. 60444 (the stock) with the objective of transmitting CBSUV to the latter (Fig. 1). Shoot growth from the stock and scion within 10–12 days after grafting indicated a good union and effective contact between the vascular systems of the stock and scion. Three separate grafting experiments were performed with an average graft success rate of 84.2% (Table 2). In the first two experiments, at least 75% of nontransgenic cv. 60444 plants developed CBSD symptoms within 8 weeks after grafting, whereas, in the third experiment, 100% of the tested plants

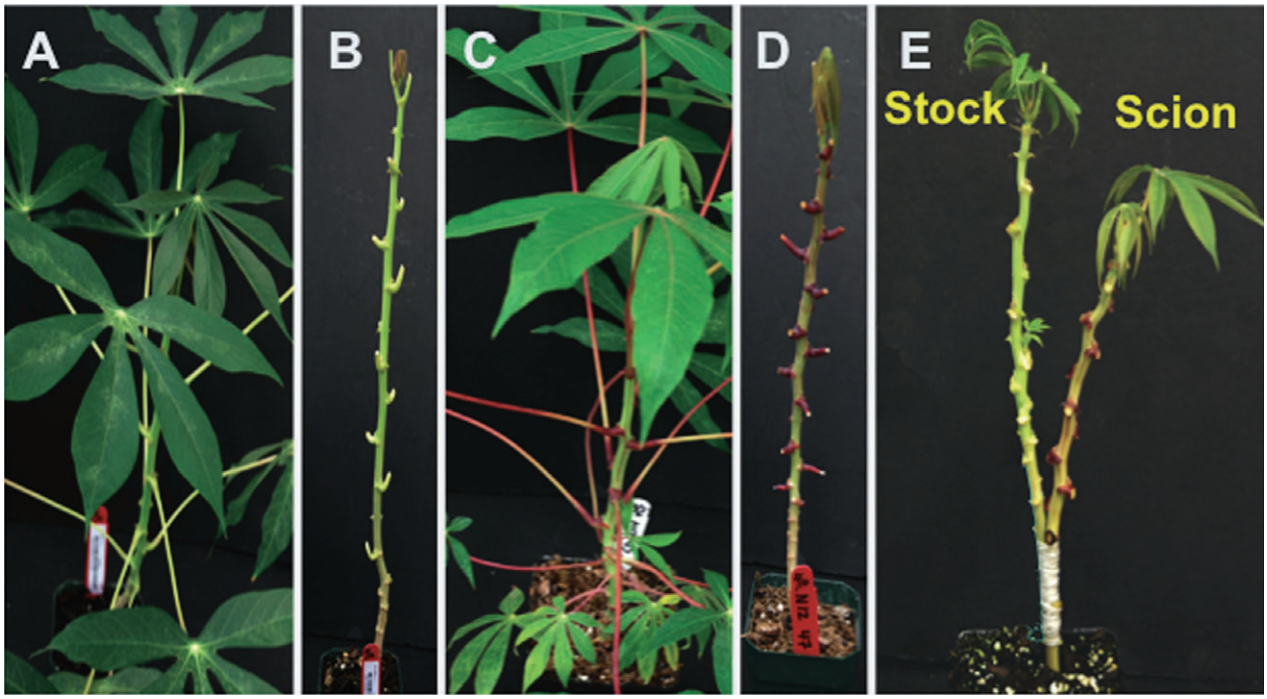


Fig. 1 *Cassava brown streak Uganda virus* (CBSUV) graft inoculation method. (A) Nontransgenic control or transgenic tested plant. (B) Nontransgenic control or tested transgenic plant prepared for grafting. (C) Cassava infected with CBSUV. (D) Cassava infected with CBSUV prepared for grafting. (E) Graft between nontransgenic cv. 60444 or tested transgenic plant and CBSUV-infected cassava plant: left, stock (control or plant under test for virus resistance); right, Scion (source of virus).

Table 2 Development of *Cassava brown streak Uganda virus* (CBSUV) graft inoculation on nontransgenic cassava cv. 60444.

Grafting experiment	No. of plants tested	No. of successful graft unions	No. of stock plants showing CBSD symptoms	Success for CBSUV transmission (%)
1	10	8	6	75.0
2	10	9	7	77.8
3	9	9	9	100.0
Total	29	26	22	84.2±13.6

Plants of cv. 60444 were challenged by grafting with cassava infected with CBSUV and data were recorded 60 days later. Cassava brown streak disease (CBSD) transmission was determined from visual CBSD symptoms on mature leaves. ±, standard deviation.

developed CBSD over the same time period (Table 2). Nontransgenic cv. 60444 developed visibly distinct CBSD symptoms primarily on lower, mature leaves (Table 2, Fig. 3A,C–E). RT-PCR analysis confirmed that these plants were successfully infected only with CBSUV and were free of CBSV when grafted with cassava scions carrying CBSUV (data not shown).

Analysis of siRNA expression in plants from different transgenic lines

Prior to challenging transgenic plants by graft inoculation with CBSUV-infected scions, levels of CP-specific siRNAs were analysed in at least seven clonal plants from each of four independent events transgenic for the siRNA-generating FL-CP hairpin sequence. This was performed: (i) to determine whether siRNA molecules specific to CP of CBSUV were present; (ii) to assess the differences in the levels of siRNA expression from different transgenic events; and (iii) to confirm that individual clonal plants derived from one transgenic event expressed siRNAs at similar levels. Northern blot analysis revealed that all RNAi FL-CP transgenic lines accumulated CP-specific siRNAs (Fig. 2). Similar siRNA accumulation was observed in different clonal plants derived from transgenic lines 718.001, 718.004 and 718.008 (Fig. 2). However, clonal plants from line 718.003 showed variability in siRNA levels (Fig. 2). In addition, the three transgenic lines 718.001, 718.004 and 718.008 accumulated higher levels of siRNA compared with line 718.003 (Fig. 2).

Graft inoculation to determine CBSUV resistance in transgenic cassava

Plants from eight independent transgenic RNAi FL-CP events and nontransgenic controls were tested for resistance to CBSUV isolate [Uganda:TO4-42:2004] by graft inoculation. Cassava scions infected with CBSUV were grafted onto transgenic stock plants or nontransgenic control plants (Fig. 1E) and assessed for visible CBSD symptom development and by RT-PCR 60 days later. In two independent experiments, all nontransgenic control plants (total, 18) developed CBSD symptoms (Table 3, Fig. 3A,C–E), whereas no disease was observed on leaves of transgenic plants (total, 72) tested in this manner (Table 3, Fig. 3B,F–H). Leaf symptoms were displayed as leaf chlorosis along small veins in mature leaves to form feathery chlorosis, followed by blotchy chlorosis on the oldest leaves (Fig. 3C–E).

RT-PCR analysis produced bands specific to the 1101 nucleotides of the CBSUV CP gene from leaves of symptomatic nontransgenic controls, but not from any of the 72 asymptomatic transgenic plants tested (Fig. 4A), indicating that the virus did not infect cassava transgenic for the FL-CP hairpin construct over the 60-day monitoring period (Fig. 4A). It was also apparent that the different levels of siRNA accumulation displayed by the four lines studied, including the lower levels evident in line 718.003 (Fig. 2), were sufficient to provide 100% resistance to CBSUV (Table 3), and to suppress viral transmission/replication to below detectable level by RT-PCR in this study (Fig. 4A).

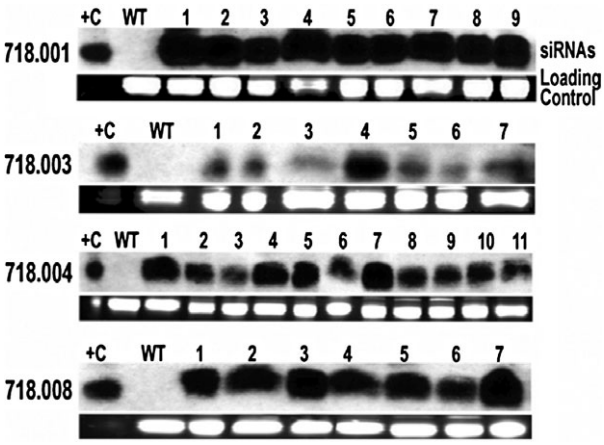


Fig. 2 Analysis of small interfering RNA (siRNA) expression in plants from four transgenic cassava lines derived from the RNA interference full-length coat protein (RNAi FL-CP) construct targeting the CP (CBSUV-[UG:Nam:04]) by Northern blot hybridization before grafting with *Cassava brown streak Uganda virus* (CBSUV)-infected cassava. The numbers on top of the blots show the plant numbers tested per line. +C is a CBSUV-infected cassava plant and WT is a nontransgenic healthy plant.

CBSD detection in new growth from stock plants after cut back

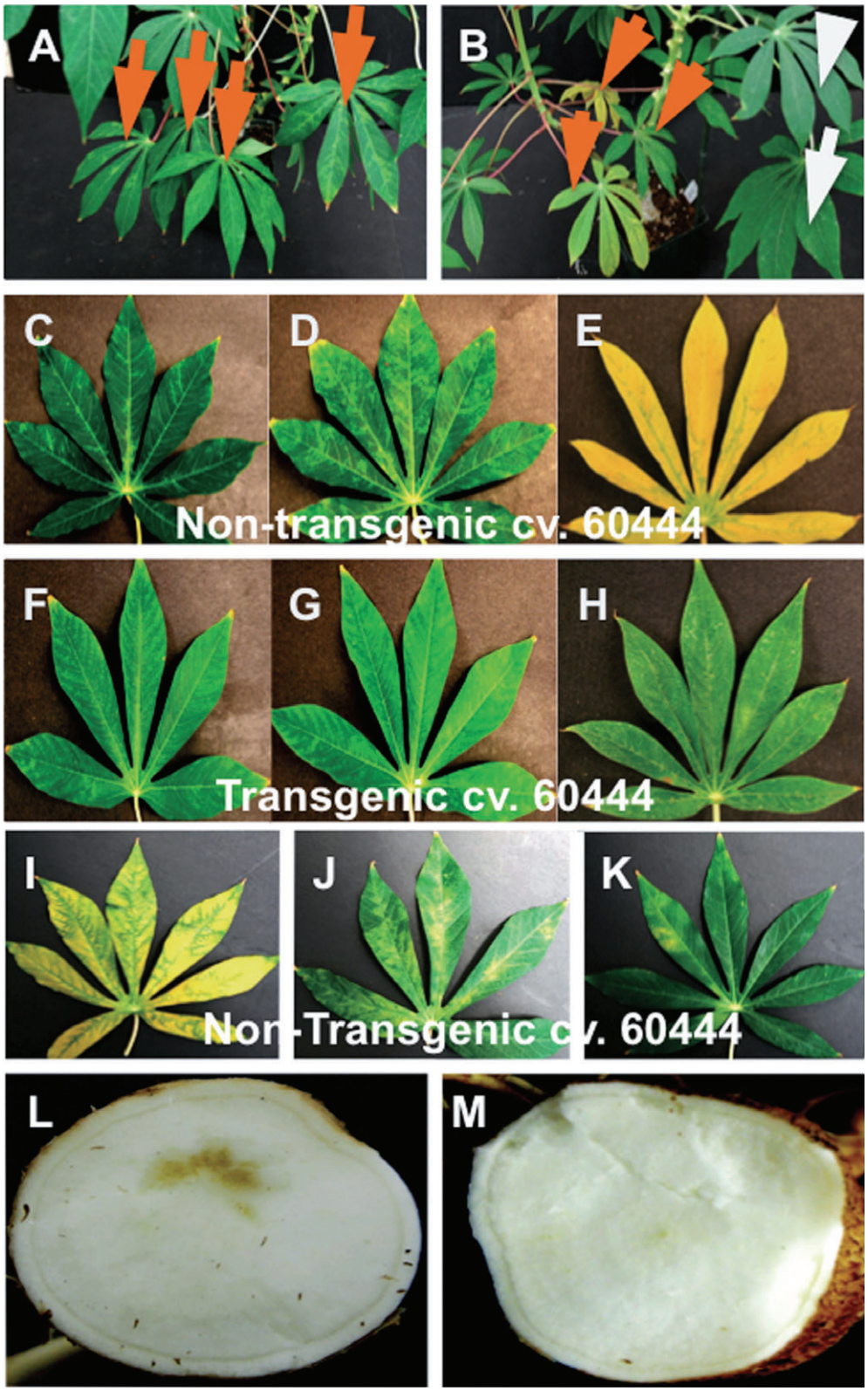
Sixty days after graft inoculation, shoots of scions and stocks were cut back above the graft union. The development of CBSD was assessed in leaves of new shoots developing from symptomatic nontransgenic control plants and asymptomatic transgenic lines over a further 60-day period (120 days after graft inoculation). These experiments facilitated the further determination of siRNA imparted resistance over time and allowed the development of tuberous roots, providing the opportunity to examine CBSD symptoms in these sink organs.

Table 3 Cassava brown streak disease (CBSD) development in RNA interference full-length coat protein (RNAi FL-CP) transgenic cassava after graft inoculation.

	Plants showing CBSD symptoms/plants challenged with CBSUV (resistance %)	
Plant lines tested	Experiment 1	Experiment 2
Nontransgenic 60444	8/8 (0%)	10/10 (0%)
718.001	0/8 (100%)	0/8 (100%)
718.003	0/3 (100%)	0/7 (100%)
718.004	0/9 (100%)	0/8 (100%)
718.008	0/6 (100%)	0/8 (100%)
718.007	0/6 (100%)	NT
718.009	0/4 (100%)	NT
718.010	0/3 (100%)	NT
718.012	0/2 (100%)	NT
Total no. of transgenic plants tested	0/41 (100%)	0/31 (100%)

Plants were challenged by graft inoculation with cassava infected with *Cassava brown streak Uganda virus* (CBSUV) and data were recorded 60 days later. Plant lines with the prefix 718 are independent events transgenic for the RNAi construct of FL-CP from CBSUV. CBSD was determined from visual CBSD symptoms on mature leaves and confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) for CP of CBSUV on RNA extracted from the first fully expanded leaf. NT, not tested.

Fig. 3 Nontransgenic cassava infected with *Cassava brown streak Uganda virus* (CBSUV) grafted onto nontransgenic cassava cv. 60444 (A) and transgenic cassava cv. 60444 (B) free of CBSUV. Orange arrows indicate old leaves showing CBSUV symptoms and white arrows indicate old leaves of transgenic stock free of CBSUV symptoms 60 days after grafting. Close-up of three oldest leaves of nontransgenic cassava cv. 60444 (C–E) and of three oldest leaves of transgenic cassava cv. 60444 (F–H) grafted with CBSUV-infected cassava. Cassava brown streak disease (CBSD) symptoms were observed in growth after cut back of grafted plants: (I–K) characteristic CBSD symptoms visible on oldest leaves of nontransgenic cassava cv. 60444. CBSD necrosis symptoms on tuberous roots of symptomatic nontransgenic cassava cv. 60444 (L) and absence of necrosis in tuberous roots of nonsymptomatic transgenic cassava cv. 60444 (M), 120 days after grafting with CBSUV-infected cassava scion.



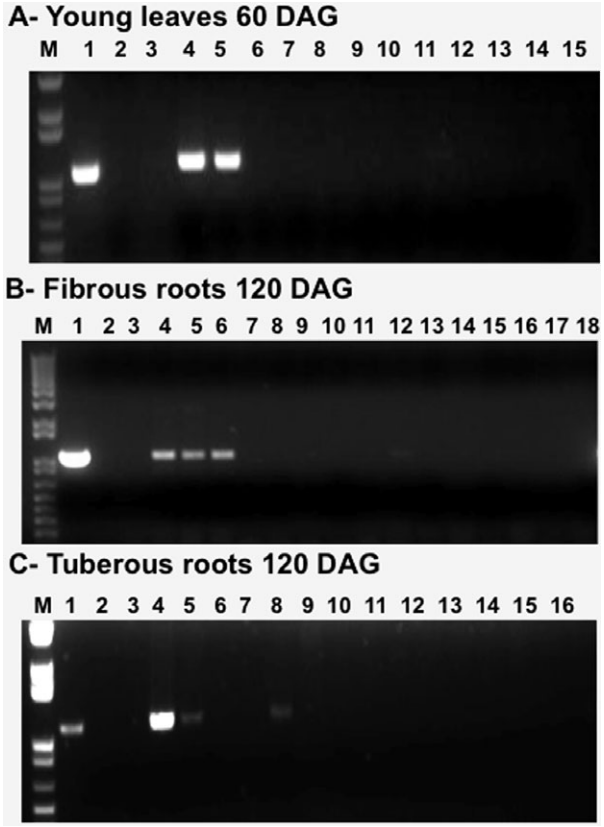


Fig. 4 Reverse transcriptase-polymerase chain reaction (RT-PCR) to detect the 1101-nucleotide *Cassava brown streak Uganda virus* coat protein (CBSUV CP) using specific primers from the total RNA extracts of first open leaves, 60 days after grafting (A), fibrous roots (B) and tuberous roots (C) 120 days after grafting (DAG) of nontransgenic and transgenic cv. 60444 cassava plants with CBSUV-infected cassava scions. (A) Lane 1, virus source plant; lane 2, RT-PCR negative control; lane 3, nontransgenic healthy cassava cv. 60444 negative control; lanes 4–5, symptomatic nontransgenic cassava cv. 60444; lanes 6–15, asymptomatic transgenic cassava grafted with CBSUV-infected scions. (B) Lane 1, virus source plant; lane 2, RT-PCR negative control; lane 3, nontransgenic healthy cassava cv. 60444 negative controls; lanes 4–6, symptomatic nontransgenic cassava cv. 60444; lanes 7–18, asymptomatic transgenic plants grafted with CBSUV-infected scions. (C) Lane 1, virus source plant; lane 2, RT-PCR negative control; lane 3, nontransgenic healthy cassava cv. 60444 negative control; lanes 4–8, symptomatic nontransgenic cassava cv. 60444; lanes 9–16, asymptomatic transgenic cassava plants grafted with CBSUV-infected scions.

Fifteen of 18 symptomatic nontransgenic controls and 49 of 57 asymptomatic transgenic plants from two independent grafting experiments re-established vigorous new growth after cut back (Table 4A). In all grafted plants, the scions (original source of CBSUV) developed CBSD symptoms on newly formed lower leaves after 4 weeks of re-growth, regardless of whether they were grafted onto susceptible control or resistant transgenic stock plants. Stock from nontransgenic controls also showed CBSD symptoms on leaves after 4–5 weeks of re-growth that became more severe over time (Table 4A, Fig. 3C–E,I–K). Conversely, new growth occurring from transgenic stocks showed no CBSD symptom development on leaves at any time during this study (Table 4A, Fig. 3F–H).

As CBSD has a direct impact on cassava yield because of necrosis in storage roots, disease development and the presence of CBSUV in tuberous and fibrous roots of all symptomatic nontransgenic control plants and asymptomatic transgenic plants was determined 60 days after cut back. No differences were visible between fibrous roots of symptomatic controls and asymptomatic transgenic plants. However, of the nontransgenic control plants showing CBSD leaf symptoms, 33% also displayed characteristic brown necrosis within tuberous roots (Table 4A, Fig. 3L). In contrast, leaves and tuberous roots of all transgenic plants from the four RNAi FL-CP lines remained symptom-free over the same period (Table 4A; Fig. 3F–H,M).

RT-PCR was performed on total RNA extracted from leaves, fibrous roots and tuberous roots of all controls and transgenic plants 60 days after cut back. A CBSUV CP-specific band of 1101 nucleotide in size was detected from RNA extracts of leaves and fibrous roots collected from all symptomatic nontransgenic control plants (Table 4A; Fig. 4A,B). Only those tuberous roots that displayed visual CBSD symptoms yielded a CBSUV-specific signal by RT-PCR, whereas storage roots that lacked CBSD symptoms proved negative for the presence of CBSUV, even in plants in which leaf and fibrous root samples were shown by RT-PCR to be infected with CBSUV (Fig. 4C). One sample of fibrous roots collected from asymptomatic transgenic plants showed a weak band on RT-PCR (Table 4A, Fig. 4B, lane 12). No leaves or tuberous roots from 49 asymptomatic transgenic plants tested showed the presence of CBUV either symptomatically or by RT-PCR (Table 4A, Figs 3F–H,M and 4C).

CBSUV detection in stem cuttings re-established from successful grafts

As cassava is vegetatively propagated, viral pathogens are transmitted through cropping cycles via infected stem cuttings. Therefore, it was important to determine whether CBSUV could be detected in plants established from stem cuttings derived from materials challenged with CBSUV by graft inoculation. Stem cuttings were taken from CBSUV-challenged symptomatic nontransgenic control plants and asymptomatic transgenic plants 60 days after graft inoculation and rooted in soil. Fourteen symptomatic controls and 44 asymptomatic transgenic plants were produced in this manner and assessed for CBSD development visually and by RT-PCR after 60 days of growth (120 days after grafting) (Table 4B). All plants generated from stem cuttings of symptomatic nontransgenic controls developed visibly recognizable CBSD symptoms on the lower mature leaves after 4 weeks of growth, with disease becoming more severe with time. Over

Table 4 Cassava brown streak disease (CBSD) detection by visual symptoms and reverse transcriptase-polymerase chain reaction (RT-PCR) on new growth from stock of successful grafts, 60 days after cut back, and in stem cuttings taken from symptomatic nontransgenic cassava control and asymptomatic transgenic plants.

A. Cut back plants obtained from scion and stock of primary grafted plants of symptomatic nontransgenic control and asymptomatic transgenics, 60 days after graft inoculation

Line tested	No. of grafted plants re-establishing growth after cut back	No. of plants displaying CBSD symptoms on new growth after cut back	CBSUV infection on new growth as detected by RT-PCR		
			Leaves	Fibrous root	Tuberous root
60444	15/18	15/15 (100%)	5/5 (100%)	5/5 (100%)	5/15 (33%)
718.001	13/16	0/13 (0%)	0/13 (0%)	0/13 (0%)	0/13 (0%)
718.003	09/10	0/09 (0%)	0/09 (0%)	0/09 (0%)	0/09 (0%)
718.004	14/17	0/14 (0%)	0/14 (0%)	1/14 (0%)	0/14 (0%)
718.008	13/14	0/13 (0%)	0/13 (0%)	0/13 (0%)	0/13 (0%)
Total transgenic plants tested	49/57	0/49 (0%)	0/49 (0%)	1/49 (0%)	0/49 (0%)

B. Stem cuttings taken from stock of successfully grafted control and transgenic plants above the graft union, 60 days after graft inoculation

Line tested	No. of plants re-establishing from stem cuttings	No. of plants displaying CBSD symptoms on new growth from stem cuttings	CBSUV infection on new growth as detected by RT-PCR	
			Leaves	Fibrous root
60444	14/18	14/14 (100%)	5/5 (100%)	5/5 (100%)
718.001	11/16	0/11 (0%)	0/11 (0%)	0/11 (0%)
718.003	09/10	0/09 (0%)	0/09 (0%)	0/09 (0%)
718.004	11/17	0/11 (0%)	0/11 (0%)	0/11 (0%)
718.008	13/14	0/13 (0%)	0/13 (0%)	0/13 (0%)
Total transgenic plants tested	44/57	0/44 (0%)	0/44 (0%)	0/44 (0%)

Shoots from cut back plants and stem cuttings obtained from graft inoculations were re-grown for 60 days and monitored for CBSD symptoms and the presence of *Cassava brown streak Uganda virus* (CBSUV) by RT-PCR.

the same period, leaves of plants produced from asymptomatic transgenic stem cuttings developed no visible CBSD leaf symptoms (Table 4B). RT-PCR performed on total RNA extracted from the leaves and fibrous roots of all plants re-established from stem cuttings of symptomatic control plants showed a CP-specific band of 1101 nucleotides for CBSUV, whereas samples from the same tissues of transgenic plants were negative for the presence of CBSUV (Table 4B).

DISCUSSION

PTGS has been applied successfully to protect plum, papaya, watermelon and potato from RNA viruses, and has led to the development of virus-resistant transgenic crops (Callaway *et al*., 2001; Jan *et al*., 1999; Ling *et al*., 2008; Scorza *et al*., 2001; Urcuqui-Inchima *et al*., 2001). Among the different viral genes used to control plant RNA viruses, CP is the most commonly employed because of its conserved nature and multifaceted role in the life cycle of these viral pathogens (Callaway *et al*., 2001; Monger *et al*., 2001b; Urcuqui-Inchima *et al*., 2001). Recently, we have demonstrated that a hairpin construct expressing an 894-nucleotide sequence of CP from CBSUV can control CBSD-causing viruses in the model host *N. benthamiana* (Patil *et al*., 2011), with complete resistance to CBSUV and CBSV in two of eight transgenic events tested. The presence of CBSUV could not be detected by RT-PCR in these plants after sap inoculation, indicating that they may be immune to this pathogen as a result of PTGS.

As our goal is to develop the effective control of CBSD in cassava, the RNAi technology had to be assessed for efficacy in the crop itself, requiring the production of transgenic cassava plants with the same gene construct, and the development of a method to challenge the resulting plants with the pathogen. Sap inoculation is not effective in cassava and whitefly inoculation is problematic and inefficient under controlled plant growth conditions (Maruthi *et al*., 2005), necessitating that a grafting method be utilized for CBSD transmission. In East Africa, where CBSD is endemic, cassava breeders employ grafting in the field to challenge candidate varieties for resistance to CBSD when the natural virus pressure is low at a given time and location. Grafting provides continuous virus pressure from the infected to the test material and is therefore regarded as a stringent test for virus resistance (http://www.scienceinafrica.co.za/2009/september/cassava.html; Mwanga *et al*., 2002). We developed a grafting method effective for plants of the age and size that can be produced in a glasshouse, such that greater than 80% of these grafts were successful (Table 2). In addition, when employed to graft plants infected with CBSUV, the pathogen was transmitted to 75%–100% of the nontransgenic controls across three independent experiments (Table 2), demonstrating an effective graft inoculation protocol for use in the evaluation of CBSD resistance in RNAi FL-CP transgenic cassava.

Graft inoculation of eight RNAi FL-CP transgenic cassava lines with cassava scions infected with CBSUV demonstrated 100% protection to CBSUV isolate (CBSUV-[Uganda:TO4-42:2004], accession number HM171316) and CBSD development, as determined by visible symptom development and RT-PCR (Table 3; Figs 3B,F–H and 4A). None of the RNAi FL-CP transgenic lines, previously confirmed to be accumulating CP-CBSUV-specific siRNAs (Fig. 2), showed CBSD symptoms on leaves 60 days after grafting, whereas nontransgenic control plants became 100% infected with CBSUV (Table 3, Figs 3 and 4A) over the same time period. To further determine the robustness and durable nature of this resistance, plants were cut back and allowed to re-grow. Stem cuttings were also taken from challenged material and re-planted in a manner analogous to vegetative propagation performed by farmers. Both processes were intended to allow disease development to proceed from even the lowest level of infection by CBSUV. The absence of CBSD symptom development and RT-PCR determination in the leaves, fibrous and tuberous roots across 49 plants tested (except for one fibrous root sample), and in 41 transgenic plants established from stem cuttings (Table 4A, Fig. 4B), confirmed very high levels of resistance imparted by siRNAs produced from the FL-CP construct. In this study, we also demonstrated, for the first time, that CBSD symptoms could be induced in tuberous roots of nontransgenic cassava growing under glasshouse conditions and that, in plants transgenic for the FL-CP hairpin construct, no such root symptoms developed (Table 4A, Figs 3L,M and 4C).

In the field, asymptomatic plants can carry virus and serve as a source of inoculum for subsequent dissemination via whitefly vectors and stem cuttings. Such germplasm is therefore undesirable for deployment to control CBSD. As such, it was important to confirm that asymptomatic RNAi FL-CP transgenic cassava plants actually lacked detectable CBSUV in their tissues. RT-PCR data correlated with the visible symptom data in all cases but one, with asymptomatic transgenic plants showing the absence of detectable CBSUV. Conversely, symptomatic control plants always showed amplification of the CBSUV CP-specific band (Fig. 4).

In the present study, all eight RNAi FL-CP lines tested exhibited very strong resistance, or immunity, to CBSUV. Of these, three lines (718.001, 718.004 and 718.008) expressed high levels of siRNA (Fig. 3) and a fourth (718.003) did so at a lower level (Fig. 2). These data contrast somewhat with our previous results in tobacco, where a highly significant positive correlation was found between the level of CBSUV-[UG:Nam:04] CP-specific siRNA accumulation (R = 92%) and CBSUV-[UG:Nam:04] protection (Patil *et al*., 2011). This suggested that a high level of siRNA accumulation could act as a molecular marker for the identification of plant lines resistant to CBSUV via this technology. The difference between cassava and tobacco data may be a result of the limited number of transgenic lines tested in the present study. Although the grafting method reported is effective for the transmission of CBSUV (Table 2), it is time consuming and space intensive. Therefore, further work is required to determine the minimum siRNA levels required to impart high-level resistance to CBSUV, and how this correlates with resistance in the field under pressure from transmission by the natural whitefly vector.

The results reported here confirm previous data in *N. benthamiana* (Patil *et al*., 2011) and show that siRNAs generated from the FL-CP hairpin construct of CBSUV impart strong protection or immunity to this pathogen in cassava. It remains to be demonstrated whether siRNA-accumulating transgenic cassava lines could also be resistant to CBSV, the second virus causing CBSD in East Africa. This demonstration of RNAi-mediated protection in cassava against the ipomovirus CBSUV encourages continued research into the use of RNAi technology as a potentially effective method to control the spread of CBSD and reduce its impact on cassava production in East and Central Africa.

EXPERIMENTAL PROCEDURES

Production of RNAi vector

The CP sequence used to generate the RNAi full-length CP construct (RNAi FL-CP) and the construction of RNAi FL-CP have been described previously (Patil *et al*., 2011).

Production of transgenic cassava plants

The RNAi hairpin construct was integrated into the CBSD-susceptible West African cassava cv. 60444 by co-culture of *Agrobacterium tumefaciens* strain LBA4404 harbouring the RNAi FL-CP construct with friable embryogenic tissues and subsequent plant regeneration via somatic embryogenesis (Schopke *et al*., 1996; Taylor *et al*., 2001). Putative transgenic cassava plantlets were recovered on medium supplemented with 45 μM paramomycin and micropropagated *in vitro* for subsequent molecular analysis and transfer to soil.

Dot blot analysis was used to estimate the T-DNA copy number in transgenic cassava cv. 60444. DNA was isolated from two leaves of *in vitro* cassava plants using a Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions, and quantified using a UV spectrophotometer. One hundred nanograms of genomic DNA were aliquoted in triplicate into a 96-well plate, denatured with 0.4 M NaOH at 98 °C for 5 min and immobilized on nylon membranes in dots using a 96-well vacuum manifold. Control transgenic lines with inserts of known copy number (one, two and three) were included as references. Blotted membranes were hybridized to a digoxygenin (DIG)-labelled probe consisting of the 35S promoter fragment and processed for signal detection using CDP-star (Roche Applied Science, Indianapolis, IN, USA), as described in the DIG system and the DIG application manual. The signal intensities were quantified by ImageJ (version 1.36b,

National Institutes of Health, Bethesda, MD, USA) software. Imaging of the membrane and comparison of experimental samples with known references yielded both visual and densitometry data that were used to assign a transgene copy number to each transgenic plant line.

Analyses of transgenic plants for the accumulation of viral siRNA before virus challenge

Transgenic plants possessing one to two copies of T-DNA were analysed for CBSUV-specific siRNA accumulation by Northern blot hybridization (Sambrook and Russell, 2001). Total RNA was isolated using TriZol (Invitrogen, Carlsbad, CA, USA) from six to eight leaves of *in vitro* plantlets, and a young leaf of 6-week-old transgenic plants grown in the glasshouse, frozen and fractionated using the miRNA isolation kit (Version 1.0) (IBI Scientific, Peosta, IA, USA). Ten micrograms of small RNAs were electrophoresed on a 15% TBE urea gel (Criterion-Bio-Rad, Hercules, CA, USA) and subjected to Northern blot hybridization with a hydrolysed probe obtained by *in vitro* transcription of CBSUV-CP using a SP6/T7 Transcription 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 DIG application manual.

Source material for viral challenge of transgenic cassava

The CBSUV isolate (CBSUV-[Uganda:TO4-42:2004]) was collected as stem cuttings of cassava grown at Namulonge, Uganda, and transported to and propagated in growth chambers at DDPSC under quarantine conditions. Total RNA was extracted by an RNeasy Mini Kit (Qiagen) from young, fully expanded leaves of CBSUV-[Ug:T04-42:04]-infected cassava and subjected to cDNA synthesis with an Invitrogen SuperScript® III First-Strand RT-PCR Kit according to the manufacturer's instructions using oligo(dT) primers. PCR was carried out on cDNA with high-fidelity DNA polymerase Phusion (Finnzymes, Woburn, MA, USA) using primers specific to CBSUV and CBSV (Patil *et al*., 2011), which amplified 1101 and 1134 nucleotides of the CPs of CBSUV and CBSV, respectively.

A PCR product of 1101 nucleotides specific to CP of CBSUV was obtained and cloned into the TOPO vector (Invitrogen) by blunt-end ligation according to the manufacturer's instructions, and sequenced in both directions. Plants positive for CBSUV-[Uganda:TO4-42:2004] were used as the source of CBSUV for graft inoculations.

Propagation and establishment of cassava for graft inoculation experiments

Nontransgenic controls and transgenic plants of cv. 60444 were micropropagated by nodal culture *in vitro* on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with MS vitamins, 2% w/v sucrose and solidified with 2.2 g/L phytogel (Sigma Aldrich, St. Louis, MO, USA). Three- to four-week-old plantlets rooted *in vitro* were planted in 10-cm pots containing Fafard 51 potting compost. Plants were watered with fertilizer (15–16–17 at 200 ppm nitrogen), applied as a liquid feed two to three times per week, and grown under conditions of 16-h light at 28 °C, 200 $\mu E/m^2/s$ and 50% relative humidity.

CBSUV-infected plants were clonally multiplied before use as source material for graft inoculations. Stem cuttings of 2.5 cm in diameter and 20 cm in length, consisting of a minimum of four nodes, were taken from the base of actively growing woody stems of CBSUV-infected cassava plants. One stem cutting in the upright position was planted per pot (10.5 cm $\times$ 12.5 cm), filled with Fafard 51 potting mix with half the nodes below and half above the soil surface, and grown under the conditions described above. Fertilizer was applied with water after axillary buds had sprouted.

Grafting of cassava for inoculation with CBSUV

Cassava plants infected with CBSUV-[UG:T04-42:04], exhibiting CBSD symptoms on the lower leaves (Fig. 1B), were utilized as the virus source and used as scions for grafting onto healthy stock (test) material. Stock plants being evaluated for virus resistance were either nontransgenic cv. 60444 or RNAi FL-CP transgenic cv. 60444. Two-month-old scion and stock plants were prepared for grafting by removal of all leaves below the two youngest leaves (Fig. 1B,D). Shoots, 18–20 cm in length, consisting of 10–12 nodes, were cut from CBSUV-infected plants with a 5-cm-long protruding 'V' shape at the basal end, using a sharp grafting knife. The vasculature of stock plants to be graft inoculated was opened by making a 5-cm-deep cut in the centre of the stem; the protruding end of the CBSUV-infected scion was inserted into the stem of the stock plant and secured by wrapping with Parafilm M (Pechiney Plastic Packaging Company, Chicago, IL, USA). To avoid dehydration, the newly grafted plants were placed on an enclosed bench for 10–12 days with 14-s misting every hour, before moving to the open bench maintained at 28 °C with a 16-h daylight period at 200 $\mu E/m^2/s$ and 50% relative humidity.

Visual assessment of CBSD symptom development on graft-inoculated plants

Four weeks after graft inoculation, and every week thereafter, grafted plants were observed for the development of typical CBSD symptoms (Fig. 3). Plants showing yellow chlorosis around secondary veins, and/or blotchy chlorosis over part of the mature

leaves, were determined to be infected with CBSUV and scored positive for disease establishment.

Analysis of CBSUV in stem cuttings, re-growth from cut-back scions and stocks of successful grafts

Sixty days after grafting, scion and stock from grafted plants of both symptomatic nontransgenic controls and asymptomatic transgenics were cut two nodes above the graft union and allowed to re-grow. After cut back, leaves from new growth on stock and tuberous roots were evaluated for CBSD symptoms. Starch-bearing tuberous roots were observed for the appearance of yellowish-brown, corky specks 60 days after cut back, a total of 120 days after grafting. The fully expanded uppermost leaf, fibrous and tuberous roots were also sampled after 60 days of re-growth to determine the presence of CBSUV by RT-PCR.

Stem cuttings were taken from the base of actively growing woody stems of CBSUV-challenged stocks above the graft union 60 days after the establishment of graft inoculations. Stem cuttings were approximately 20 cm in length, 2 cm in diameter and contained a minimum of four nodes. One stem cutting was planted per pot and cultivated in the manner described above. Leaves from re-established cuttings were evaluated for CBSUV by visual CBSD symptom assessment, and leaves and fibrous roots were sampled after 60 days to confirm the presence of CBSUV by RT-PCR.

RT-PCR analysis for the presence of CBSUV in graft-inoculated plants

Total RNA was extracted from 100 mg of tissue of fully expanded young leaves and fibrous roots and from 500 mg of starchy tuberous root from nontransgenic cv. 60444 and transgenic plants using an RNeasy Mini Kit (Qiagen). RNA was subjected to CBSUV detection by RT-PCR, as described by Patil *et al*. (2011). Primers to amplify the constitutively expressed ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Mak and Ho, 1995) were used as controls to check the quality of synthesized cDNA.

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