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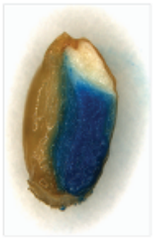

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RESEARCH PAPER

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

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Keywords: cassava brown streak disease, RNAi-mediated resistance, cassava, transgenic, confined field trial, Uganda

Abbreviations: CBSD, Cassava brown streak disease; CBSV, Cassava brown streak virus; CFT, confined field trial; CMD, cassava mosaic disease; CP, coat protein; MAP, months after planting; NaCRRI, National Crops Resources Research Institute; RT-PCR, reverse transcription polymerase chain reaction; siRNA, small interfering RNA; ssRNA, single-stranded RNA; UCBSV, Uganda Cassava brown streak virus

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

Cassava brown streak disease (CBSD) is currently considered the most important disease of cassava in East Africa and a significant threat to food security in the region.[1] CBSD is caused by two distinct virus species: Cassava brown streak virus (CBSV) and Ugandan Cassava brown streak virus (UCBSV), both of which belong to the genus *Ipomovirus*, family *Potyviridae*, and possess a ssRNA genome of messenger sense.[2,3] Both viruses are transmitted by whiteflies (*Bemisia tabaci*), resulting in CBSD-symptomatic plants carrying infections by one or both viral species. Since its re-emergence in Uganda in 2004,[4] CBSD has become prevalent in East Africa, with new outbreaks of the disease resulting from dissemination of infected cassava planting materials and high whitefly vector populations.[4,5]

Genetically engineered resistance to CBSD was reported recently in tobacco and cassava plants under greenhouse conditions[6,7] and in cassava in a confined field trial (CFT) in Uganda.[8] Two RNAi constructs targeting UCBSV were tested in the field: p718 consisting of an 894 bp inverted repeat sequence (nts 208–1101) of a truncated version of the full-length UCBSV coat protein (CP), and p719 consisting of a 397 bp portion (nts 208–604) of the UCBSV CP N-terminal.[7] Tissue culture-derived cassava plants of cv 60444 shown to be accumulating transgenically derived UCBSV CP specific siRNAs, were planted in the field and proved to be highly resistant to whitefly transmitted UCBSV. Fourteen transgenic lines were tested, seven of each of the two constructs. Across all 14 lines, greater than 98% of the plants (n = 60) remained free of UCBSV 11 months after planting (MAP), as determined by RT-PCR. Protection against the non-targeted CBSV was observed in two transgenic lines of the p718 construct (carrying the full length CP sequence). This was most striking in line p718–001 in which 54 out of 60 plants (90%) remained CBSD symptom free and RT-PCR negative for

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presence of UCBSV and CBSV in leaf and storage root tissues.[8]

Farmers propagate cassava vegetatively from field-derived stem cuttings, not from tissue culture-derived plantlets. Demonstrating proof of concept for resistance to CBSD across the vegetative cropping cycle is an essential step within the product development process to develop and deliver resistant planting materials for farmers in East Africa. A field trial was therefore established to evaluate efficacy of the RNAi-mediated resistance to CBSD through a typical vegetative propagation cycle. Woody stem cuttings measuring 25 to 30 cm in length were obtained from plants within the CFT harvested in November 2011, as previously described by Ogwok et al.[8] Three lines transgenic for p718 (p718–001, p718–002, and p718–005) that provided complete resistance to UCBSV and differing levels of protection to CBSV in the original field experiment were re-planted, in addition to stakes of the non-transgenic control cv 60444 and the CBSD tolerant cv TMS30572. Stem cuttings of the highly susceptible Ugandan farmer-preferred cv TME204 were also obtained from an experimental field at the National Crops Resources Research Institute (NaCRRI). A CFT at NaCRRI, Namulonge, Uganda was established using 36 stem cuttings per entry per plot, planted at a spacing of 1 m × 1 m in a randomized complete block design with three replications. Each plot was surrounded by a row of CBSD symptomatic plants of TME204 to act as a supplemental source of virus inoculum. The plants were grown with no agrochemical or fertilizer applications and with hand weeding performed as necessary. Plants were visually assessed on a monthly basis for CBSD and CMD incidence and severity, and for the number of adult whiteflies (*B. tabaci*) on the underside of the uppermost five fully open leaves, as described previously by Ogwok et al.[8] Plants were harvested at 11 MAP.

Adult whitefly populations showed a steady increase, ranging from 152 to 212 per plant by four MAP (data not shown). As expected, CMD observed on plants of cv 60444 within the previous CFT[8] was carried across the vegetative cropping cycle. Severe CMD symptoms were therefore seen immediately on sprouting leaves of all transgenic and non-transgenic plants of cv 60444, with plants reaching a maximum severity score of 5 (1–5 scale) by seven MAP. In contrast, and as expected, plants of TME204 and TMS30572 exhibited very mild to moderate CMD throughout the experiment. While foliar CBSD symptoms were obscured due to CMD in some cases, typical CBSD symptoms were clearly visible on the stems.[8,9] CBSD symptoms were first seen at one MAP on plants of TME204 and non-transgenic 60444 controls, and by three and four MAP in plants of p718–005 and p718–002, respectively. Shoot CBSD symptom severity remained mild to moderate until five MAP in the 60444 controls and all plants of p718–002 and p718–005.

Table 1. CBSD foliar incidence and severity at 11 MAP

Plant line	Incidence (%)	Mean severity (1–5)
p718–001	0.0	1.0
p718–002	46.8	3.0
p718–005	23.7	2.6
60444	100.0	3.5
TME204	100.0	3.4
TMS30572	0.0	1.0

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

Table 2. CBSD storage root incidence and severity at 11 MAP

Plant line	CBSD necrosis incidence (%)	Mean CBSD necrosis severity (1–5)
p718–001	0.0	1.0
p718–002	28.7	2.5
p718–005	100	3.5
60444	61.4	3.9
TME204	100	4.8
TMS30572	2.0	3.0

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

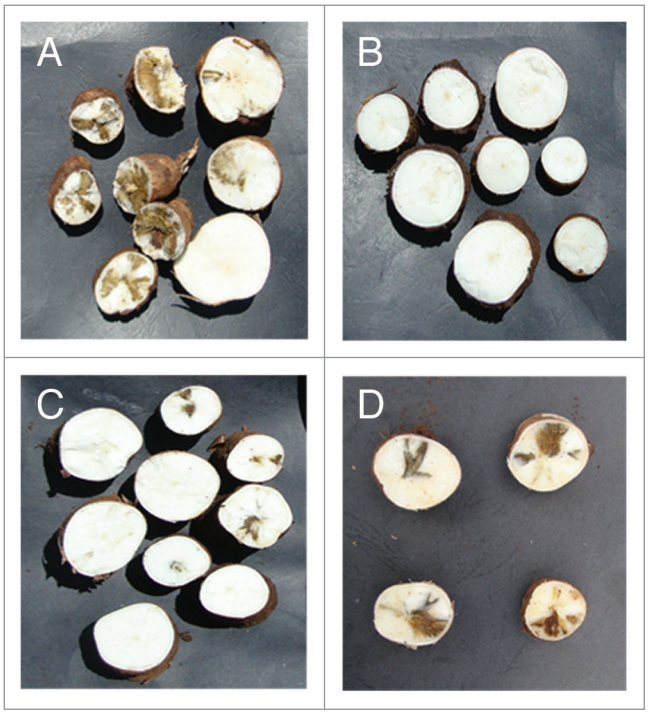

Figure 1. CBSD symptoms in storage roots derived from stem cuttings at harvest 11 months after planting. (**A**) Non-transgenic 60444 showing severe damage due to CBSD. (**B**) Transgenic line p718–001 showing no CBSD root damage. (**C**) Transgenic line p718–002 showing moderate damage due to CBSD. (**D**) Transgenic line p718–005 showing significant damage due to CBSD.

Table 3. Presence of CBSV and UCBSV in leaves of plants 11 MAP determined by reverse-transcription polymerase chain reaction

Plant line	Number of leaf samples analyzed	Number of leaf samples virus positive (%)	Virus species detected (%)		
			CBSV only	UCBSV only	CBSV + UCBSV
p718–001	30	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
p718–002	30	26 (87.7)	26 (100)	0 (0.0)	0 (0.0)
p718–005	30	16 (53.3)	16 (100)	0 (0.0)	0 (0.0)
60444	30	26 (87.7)	4 (15.4)	1 (3.8)	21 (80.8)
TMS30572	15	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
TME204	15	14 (93.3)	2 (14.3)	0 (0.0)	12 (85.7)
Total	**150**	**82 (54.7)**	**48 (58.5)**	**1 (1.2)**	**33 (40.2)**

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

Table 4. Presence of CBSV and UCBSV in storage root tissues of 11 MAP determined by reverse-transcription polymerase chain reaction

Plant line	Number of root samples analyzed	Number of root samples virus positive (%)	Virus species detected (%)		
			CBSV only	UCBSV only	CBSV + UCBSV
p718–001	10	0 (0)	0 (0.0)	0 (0.0)	0 (0.0)
p718–002	10	8 (80)	7 (87.5)	0 (0.0)	1 (12.5)
p718–005	10	6 (60)	6 (100.0)	0 (0.0)	0 (0.0)
60444	10	9 (90)	7 (77.8)	1 (11.1)	1 (11.1)
TME204	20	17 (85)	2 (11.8)	1 (5.9)	14 (82.3)
Total	**60**	**40 (66.7)**	**22 (55.0)**	**2 (5.0)**	**16 (40.0)**

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

No CBSD was observed on leaves or stems of plants of p718–001 and TMS30572 throughout the 11-month duration of the experiment (**Table 1**).

Damage due to CBSD-induced necrosis in storage roots was assessed at harvest 11 MAP. Storage roots from the 16 innermost plants of each plot were uprooted, sliced transversely into five pieces and sections scored visually for presence and severity of CBSD using the 1–5 scale described previously by Ogwok et al.[8] No CBSD necrotic symptoms were observed within 290 root slices cut from 58 storage roots obtained from 38 different transgenic plants of line p718–001 (**Table 2**; **Fig. 1**). In contrast, all (100%) roots from TME204 and transgenic event p718–005 showed CBSD symptoms, with incidence of necrosis at 61.4%, 28.7%, and 1.6% of roots harvested from plants of 60444, p718–002 and TMS30572 respectively (**Fig. 1**). Severity of CBSD root damage remained mild to moderate in roots of p718–002, p718–005, and TMS30572 (average score 2.5–3.5)[8] compared with severe to very severe root symptoms observed in roots of non-transgenic 60444 and TME204 plants (average score 3.9–4.8) (**Table 2**).

Molecular diagnosis was performed on total RNA extracted from the youngest leaves showing CBSD symptoms, or on equivalent non-symptomatic tissue, RNA was subjected to RT-PCR, as described previously by Ogwok et al.,[8] using primers CBSVDF2 (5′-GCTMGAAATGCYGGRTAYACAA-3′) and CBSVDR (5′-GGATATGGAGAAAGRKCTCC-3′), which amplify 437 and 343 nucleotides of the 3′-terminal sequences of the UCBSV and CBSV genomes, respectively. **Table 3** summarizes diagnostic analysis of plants at 11 MAP. Virus was detected in the vast majority of plants of the susceptible cultivar TME204 and non-transgenic 60444, with most plants found to be infected with both CBSV and UCBSV. As reported in the first CFT,[8] all transgenic lines remained free of detectable UCBSV. While plants of p718–002 and p718–005 were confirmed to be infected with CBSV, all plants of p718–001 remained free of RT-PCR detectable levels of both CBSV and UCBSV (**Table 3**).

To determine presence of CBSV and UCBSV in storage roots, 1–2 cm thick root discs were obtained at harvest 11 MAP. Total RNA was isolated and subjected to RT-PCR.[8] As in the case of leaf tissues, root tissues from plants of p718–001 were found free of UCBSV and CBSV (n = 10) (**Table 4**). No UCBSV was detected in lines p718–002 or p718–005, but roots from these events were found to be positive for presence of CBSV at 60–80%.

This study has shown that plants derived from stem cuttings of cassava cultivar 60444 transgenic for the RNAi construct p718 (carrying an inverted repeat sequence of a truncated full-length UCBSV CP) remained highly resistant to CBSD across a second planting cycle of 11-month duration. Molecular analysis revealed that all RNAi-derived transgenic plants remained resistant to the homologous virus (UCBSV), and that plant line p718–001 continued to be protected against both CBSV and UCBSV. Such performance demonstrates that siRNA-imparted resistance to CBSD is durable over time and across a vegetative

cropping cycle. Importantly, CMD incidence and severity was high across all the transgenic plants in this field trial, indicating that presence of replicating African Cassava mosaic virus and East African Cassava mosaic virus, known to encode the gene silencing suppressor proteins AC4 and AC2 respectively, did not cause measurable suppression of transgenic RNAi-mediated resistance to CBSD.

The observed field resistance of transgenic cassava to CBSD through a vegetative cropping cycle as described here confirms previous greenhouse studies[6,7] and an earlier field experiment,[8] and provides proof of concept that RNAi technology can provide effective resistance to CBSD under conditions similar to that encountered in farmers' fields. Combined with a recent, independent report of RNAi-mediated control of CBSD,[10] the present study provides strong encouragement to further develop this technology to improve CBSD resistance in cultivars preferred by cassava farming communities in East Africa.[11]

Disclosure of Potential Conflicts of Interest

The authors have no conflict of interest to declare.

Acknowledgments

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