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Reaction of transgenic sweet potato (*Ipomoea batatas* L.) lines to virus challenge in the glasshouse

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ABSTRACT
Objective: Sweet potato virus disease (SPVD) is highly devastating and diseased plants produce little or no yield. Efficient methods to control the disease are not available and conventional breeding for resistance has had limited success. Breeding for resistance through genetic engineering offers an alternative solution for the control of SPVD. The objective of this study was to select transformed sweet potato lines and evaluate their reaction to virus inoculation under controlled conditions.
Methodology and results: Seven hundred and eight sweet potato lines that were putatively transformed with the coat protein (CP), replicase and inverted repeat of the CP genes of sweet potato feathery mottle virus (SPFMV) were characterized. Leaves of 597 (84.3%) were unbleached following treatment with 1% (w/v) kanamycin solution whereas those of 111 (15.7%) lines turned yellow. Kanamycin-resistant lines were graft-inoculated with sweet potato scions infected with SPVD and of the 597 lines, only 20 did not display symptoms. In PCR, amplified DNA fragments of 450 bp were realised in 7 out of the 20 transgenic lines tested using specific primers to the CP, replicase and inverted repeat of the CP genes. The confirmed transgenic lines were evaluated after inoculation with SPFMV, sweet potato chlorotic stunt virus (SPCSV) and a combination of the two under screen house conditions. Ten transgenic sweet potato lines remained symptomless and were virus-free when serologically tested by nitro-cellulose membrane (NCM) -ELISA. Results from triple antibody sandwich (TAS)-ELISA demonstrated that virus accumulation was suppressed in 7 transgenic lines as compared to the non-transgenic control plants two months after inoculation, indicating that the plants were relatively protected.
Conclusion and application of findings: This study indicates some form of protection exists against SPVD in plants that were transformed with SPFMV-derived genes. Further experimentation in the field is needed to fully determine the efficacy of the transgenes in conferring resistance to SPVD.

Key words: Transgenic sweet potato, sweet potato virus disease, virus resistance, ELISA.

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INTRODUCTION

Sweet potato (*Ipomoea batatas* L.) is a versatile crop with importance as a human food worldwide, particularly in developing countries (CIP, 1996). It is one of the highest yielding crops with higher food value and total production per unit area than other staples such as maize, rice, sorghum and millet (FAO, 2004). However, sweet potato virus disease (SPVD) caused by a dual infection with sweet potato feathery mottle virus (SPFMV) and sweet potato chlorotic stunt virus (SPCSV) is one of the most serious constraints to production (Gibson *et al*., 1998b). Although SPVD can be controlled by use of healthy planting materials, phytosanitation and cultural measures, these strategies are difficult to integrate within subsistence production systems practiced by resource-poor farmers (Gibson *et al*., 2004).

Selection by farmers of more resistant landraces has to some extent reduced SPVD incidence in the field, resulting in improved yields (Karyeija *et al*., 2000; Mwanga *et al*., 2002) but no cultivar is known that is immune to SPVD. There have been efforts to produce virus-free plants through shoot-tip culture (Nagata, 1984). However, this method is costly and plants that are initially virus-free often become re-infected after cultivation. Therefore, it is worthwhile to evaluate the use of transgenic resistance for obtaining SPVD resistance through the production of transgenic sweet potato plants expressing recombinant virus proteins, which interfere with virus replication.

Using genetic transformation, varying levels of resistance have been engineered into crops with the expression of virus genes or sequences (Berger, 2001). This form of resistance is known as pathogen-derived resistance (PDR) (Baulicombe, 1996). PDR is manifested at either the protein or the RNA level. In protein-mediated resistance, there is usually a direct correlation between the level of protein accumulation and virus resistance. On the other hand, RNA mediated resistance is typically associated with transgenic plants with low or undetectable transgene expression. This type of resistance is referred to as homology-dependent virus resistance (HDR) (Baulicombe, 1996).

There have been several studies on the transformation of sweet potato although only four succeeded and produced transgenic sweet potato plants using an *Agrobacterium*-mediated system (Otani *et al*., 1993; Newell *et al*., 1995; Gama *et al*., 1996; Otani *et al*., 1998), and one using an electroporation system (Okada *et al*., 2001). In Kenya, transgenic sweet potato lines expressing the coat protein (CP), replicase and CP inverted repeat genes of SPFMV have been produced (Njagi, 2004). However, these putatively transformed sweet potato lines have not been characterized to confirm the presence of the transgenes. The reaction of the transgenic lines to virus inoculation also needs to be determined so as to evaluate the effectiveness of the genes in conferring resistance. The objective of this study therefore was to select transformed sweet potato lines and evaluate their reaction to virus inoculation under controlled conditions.

MATERIALS AND METHODS

Kanamycin painting and graft-inoculation: Seven hundred and eight (708) sweet potato lines belonging to two varieties, i.e. CPT 560 and KSP 36, and that were putatively transformed with the coat protein (CP), replicase and inverted repeat of the CP genes of sweet potato feathery mottle virus (SPFMV) were characterized by kanamycin painting. To evaluate the lines for kanamycin resistance, the surfaces of fully expanded leaves of each plant were painted with a 1% (w/v) kanamycin solution. Observations for bleaching were recorded on a daily basis for a period of one week. The non-transformed CPT 560 and KSP 36 were expected to bleach, whereas the transgenic lines were not

expected to bleach as they express the selectable marker gene, npt II. The kanamycin-resistant sweet potato lines were graft-inoculated using the method described by Beetham and Mason (1992) with scions from sweet potato plants that were pre-infected with SPVD in an insect-proof biosafety level II glasshouse. The graft-inoculated plants were observed for development of SPVD for 8 weeks post-inoculation. Symptom severity was scored following a scale of 1-5; where 1= no symptoms and 5= very severe symptoms (Njeru *et al*, 2004). Lines that did not exhibit any symptoms of virus infection were selected for further molecular analyses.

DNA extraction and PCR amplification: Total genomic DNA was extracted from young leaves following the CTAB method (Doyle & Doyle, 1990). DNA quality and concentration were evaluated by electrophoresis in 0.8% agarose gel stained with ethidium bromide. PCR reactions were performed in 25 µl reaction mixes consisting of 10 ng of the genomic DNA template, 5 µl reverse and forward primers (5 picomoles/µl) (Table 1), 2.5 µl of l0X PCR buffer (50 mm KCl, 10 mM Tris-HCl pH 8.4, 1 mg gelatin / ml), 2 µl of 2.5 mM $MgCl_2$, 1 µl of 5 mM dNTPs, 0.1 µl Taq DNA polymerase (2 units) (Amplitaq Gold) in a 0.5 ml sterile microfuge tube. The final volume was made up with sterile water. Positive and negative control tubes were included in the reaction and contained the reaction mix with DNA from *Agrobacterium* containing the transgene and with DNA templates from non-transformed plants, respectively. A thermal cycler (GeneAmp PCR system 9700, Applied Biosystems) was programmed for 1 min at 94°C followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 sec and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. Amplification products were resolved on a 2 % agarose gel in 1X TBE buffer. Gels were run at 70 V for 45 min and were photographed under UV light after ethidium bromide staining.

Table 1: Primers used to detect the transgenes in DNA samples from transgenic sweet potato lines.

Gene targeted	Sequence	Annealing temperature	Expected product size
CP	5'GGATGGTGATGAGCAAGTGACATA3' 3'ATTGGAGAATCCTTCATCCCCA5'	55°C	450 bp
Replicase	5'CGTGGCAGACAAACGTA3' 3'TTCACTGTCTATCGACCC5'	55°C	450 bp
Inverted repeat of CP	5'GCCTGCACAGTTAGTTGACTAA3' 3'TTCCAGACCTTACCTAGAGACT5'	55°C	450 bp

Challenging confirmed transgenic lines with virus: To determine the efficacy of the transgenes in controlling the virus, transgenic sweet potato lines were graft-inoculated with SPFMV, SPCSV and a combination of SPFMV and SPCSV (SPVD) in a biosafety level II glasshouse. The treatments were replicated 3 times. The development of visual symptoms due to SPFMV, SPCSV and SPVD infections was monitored weekly for 8 weeks following inoculation. Disease severity was scored at weekly intervals, starting 4 weeks after inoculation for a period of 4 weeks using the scale described above. Inoculated sweet potato plants were serologically assayed for SPFMV and SPCSV by nitrocellulose membrane (NCM)-ELIAS (Gibb & Padovan, 1993) and triple antibody sandwich (TAS)-ELISA (Gibson *et al*., 1998), 8 weeks post-inoculation utilizing monoclonal and polyclonal antibodies.

In NCM-ELISA, two leaf discs (1-cm diameter) excised from a composite sample of two leaves taken from different points (middle and top) of a sweet potato plant were tested. Positive and negative reactions were determined by visual assessments with different grades of purple colour indicating positive reactions. To determine the level of virus accumulation, triple antibody sandwich, enzyme-linked immunosorbent assay (TAS-ELISA)

was carried out as described by Gibson *et al*. (1998b) utilizing monoclonal and polyclonal antibodies. A positive result was obtained when the absorbance value of a sample was more than twice the value for the positive control. The disease severity scores and absorbance readings from the TAS-ELISA were subjected to analysis of variance.

RESULTS

Following kanamycin painting, leaves of true transgenic sweet potato lines did not bleach, whereas those of the non-transformed lines and non-transformed control plants bleached. Out of the 708 lines screened, 111 turned yellow (bleached), whereas 597 lines remained green (not bleached). The non-transformed lines started to bleach on the third day and were fully bleached on the seventh day. All the 10 non-transformed plants used as checks were bleached. Of the 597 kanamycin-resistant lines that were graft-inoculated with SPVD, only 20 lines were not infected by the 8th week post-inoculation. Of the 145 kanamycin-resistant lines transformed with the replicase gene, 160 lines with the CP gene and 158 lines with the inverted repeat of the CP gene, 7, 9 and 4 lines were not infected, respectively (Table 2).

Table 2: Reaction of kanamycin-resistant sweet potato lines to SPVD-inoculation.

Gene construct	Kanamycin-resistant lines	Lines infected	Lines not infected
Replicase	145	138	7
CP	160	151	9
Inverted repeat of CP	158	154	4
Non-transgenic control	10	10	0
Total	607	587	20

The presence of the transgene in putatively transformed lines was confirmed by PCR amplification of a 450 bp fragment of the coat protein, replicase and inverted repeat of the coat protein in the extracted DNA. As expected, DNA from non-transformed control plants (CPT 560 and KSP 36) did not posses this fragment. A single sharp band was obtained at 450 bp positions from 7 transgenic lines and the *Agrobacterium tumefaciens* used as positive control. No amplification was observed for the DNA extracted from non-transformed control plants (KSP 36 and CPT 560) and from lines CPT 560/29/69, CPT 560/29/110, CPT 560/29/130, CPT 560/29/17, KSP 36/28/1, CPT 560/28/71, CPT 560/29/3, CPT 560/36/93 and CPT 560/36/21.

Upon challenge, all transgenic lines that were graft-inoculated with SPFMV or SPCSV alone did not develop any symptoms. Transgenic lines that were graft-inoculated with a combination of SPFMV and SPCSV exhibited delayed or no symptoms at all. At 8 weeks post-inoculation with SPVD, plants of 10 transgenic lines did not develop symptoms. These comprised 5 expressed CP, 3 lines replicase and 2 lines inverted repeat of the CP gene. Generally, the susceptible transgenic lines showed symptoms after 21-28 days, compared to the control plants that expressed symptoms within 12 days post-inoculation. The time from inoculation to the appearance of symptoms varied in the susceptible transgenic lines, generally taking longer in plants that were transformed with the CP gene. Such plants showed a 3 to 6-day delay in symptom appearance compared to the plants that were transformed with replicase gene. In the susceptible transgenic lines symptoms due to SPVD infection included vein clearing, leaf feathering, chlorotic spots on leaves, leaf deformation or decrease in leaf size and plant stunting (Plate 1).

Transgenic lines inoculated with SPFMV alone tested negative for SPFMV in both NCM-ELISA and TAS-ELISA. Similar to SPFMV, transgenic lines inoculated with SPCSV alone tested negative for SPCSV in ELISA. Among transgenic lines inoculated with SPVD-infected scions, NCM-

ELISA assays were positive only in plants that exhibited virus symptoms.

Transgenic lines CPT 560/28/71 and CPT 560/28/21 expressing the replicase gene were significantly ($P \leq 0.05$) more severely diseased than other susceptible transgenic lines at 6, 7 and 8 weeks post-inoculation (Table 3). These lines also had significantly ($p \leq 0.05$) higher disease severity scores than the susceptible transgenic lines expressing the CP and the inverted repeat of the CP. Transgenic lines CPT 560/36/137 and CPT 560/36/93 expressing the inverted repeat of the CP were significantly ($p \leq 0.05$) more diseased than susceptible transgenic lines expressing the CP gene at 8 weeks post-inoculation. Similar disease severity was observed on CPT 560/28/201, CPT 560/28/155, CPT 560/28/76, CPT 560/29/247, CPT 560/29/83, CPT 560/29/121, KSP 36/29/61, CPT 560/36/53 and CPT 560/36/19, which were significantly lower than the scores on the other lines. The non-transgenic control plants were significantly ($p \leq 0.05$) more diseased than all the transgenic sweet potato lines tested. Generally, symptom severity increased between 6 and 8 weeks post-inoculation on all the susceptible transgenic sweet potato lines.

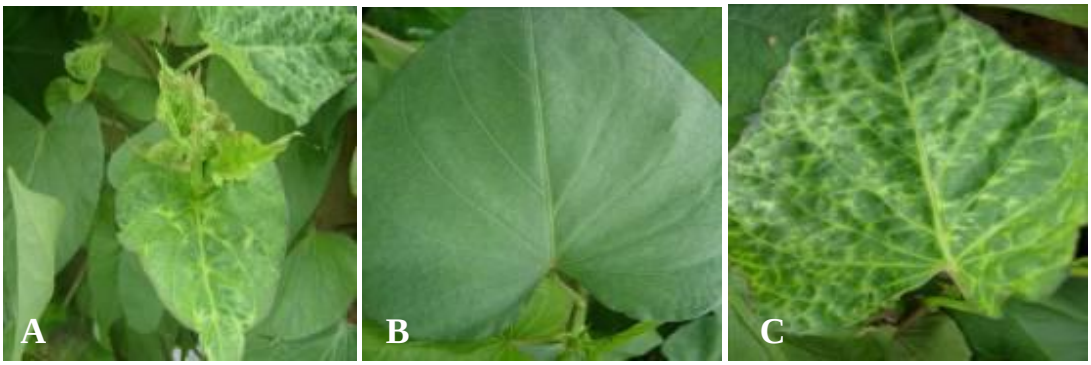

Plate 1: Plants inoculated with a combination of SPFMV and SPCSV: (A) susceptible transgenic line CPT 560/29/69 expressing vein clearing and chlorotic spots on leaves, (B) symptomless resistant line CPT 560/29/83 and (C) non-transformed control plant CPT 560 expressing severe vein clearing and chlorotic spots on leaves. Both plants A and B contain the coat protein gene of the sweet potato feathery mottle virus.

Virus accumulation in transgenic plants: Virus accumulation in transgenic plants was determined using TAS-ELISA following graft-inoculation with virus-infected sweet potato scions. The plants dually infected with SPFMV and SPCSV had higher titers of both viruses than plants that were infected with the individual viruses (Table 4). The titers of SPFMV were much greater in plants co-infected with SPCSV than in plants infected with only SPFMV in transgenic as well as in the non-transgenic plants used as control. The titers of SPCSV were significantly low in plants inoculated with SPCSV alone and plants dually infected with SPFMV and SPCSV at 8 weeks post-inoculation. In transgenic lines inoculated with SPFMV alone, the lowest virus concentration was recorded in lines expressing the CP gene as compared to lines expressing the replicase and inverted repeat of the CP genes.

In a combined inoculation of SPFMV and SPCSV, the highest titers of SPFMV were recorded in CPT 560/36/137 and CPT 560/36/53 expressing the inverted repeat of the CP whereas lowest titers were recorded in CPT 560/28/76 and CPT 560/28/71 expressing the replicase gene. The lowest titers of SPCSV were recorded in transgenic lines expressing the CP gene except CPT 560/29/247, CPT 560/29/121 and KSP 36/29/61, whereas the highest titers were recorded in lines expressing the replicase gene at 8 weeks post-inoculation in a combined inoculation of SPFMV and SPCSV. The transgenic lines CPT 560/36/137 and CPT 560/36/53 expressing the inverted repeat of the CP gene had similar titers of SPFMV and were significantly ($P \leq 0.05$) higher than the titers in the transgenic lines expressing the CP and the replicase genes in a combined infection with

SPFMV and SPCSV (Table 2). Low titers of SPFMV were recorded in CPT 560/28/155, CPT 560/28/76 and CPT 560/28/71 expressing the replicase gene and was significantly (P ≤ 0.05) different from the titers in other transgenic lines except CPT 560/29/247 and CPT 560/36/19 expressing the CP and inverted repeat of the CP genes, respectively, in a combined inoculation of SPFMV and SPCSV.

Table 1: Disease severity on transgenic sweet potato lines inoculated with combined SPFMV and SPCSV.

Transgenic line	Gene construct	Weeks post-inoculation		
		6	7	8
CPT 560/28/201	Replicase	1.0	1.0	1.0
CPT 560/28/155	Replicase	1.0	1.0	1.0
CPT 560/28/71	Replicase	3.3	3.7	3.7
CPT 560/28/76	Replicase	1.0	1.0	1.0
CPT 560/28/21	Replicase	2.9	3.0	3.3
CPT 560/29/247	CP	1.0	1.0	1.0
CPT 560/29/110	CP	2.1	2.3	2.3
CPT 560/29/69	CP	1.9	2.2	2.3
CPT 560/29/83	CP	1.0	1.0	1.0
CPT 560/29/121	CP	1.0	1.0	1.0
KSP 36/29/61	CP	1.0	1.0	1.0
CPT 560/36/53	CP inverted repeat	1.0	1.0	1.0
CPT 560/36/19	CP inverted repeat	1.0	1.0	1.0
CPT 560/36/21	CP inverted repeat	1.0	1.0	1.0
CPT 560/36/137	CP inverted repeat	2.5	2.6	2.9
CPT 560/36/93	CP inverted repeat	2.6	2.8	2.9
CPT 560	Non-transgenic	3.8	4.6	5.0
KSP 36	Non-transgenic	3.7	4.7	5.0
LSD (P=0.05)		0.21	0.23	0.26

Values are means of disease severity determined on a scale of 1-5 where 1 = no symptoms and 5 = very severe symptoms (Njeru *et al*., 2004). Disease severity was taken from week 6 to week 8 after inoculation. Replicase, CP and inverted repeat of CP were obtained from the SPFMV under the control of an enhanced 35S cauliflower mosaic virus promoter (Pe35S), neomycin phosphotransferase (npt II) selectable marker gene and a leader sequence (heat shock protein from soybean).

Transgenic lines expressing the replicase gene were significantly (p≤ 0.05) more severely diseased than lines expressing the CP and inverted repeat of the CP genes. These lines also had significantly (p≤ 0.05) higher disease severity scores and virus accumulation than transgenic lines expressing the CP and inverted repeat of the CP. As expected the non-transgenic control plants were more severely diseased and had significantly (p≤ 0.05) higher severity and virus accumulation than the transgenic lines expressing the CP, the inverted repeat of CP and the replicase genes (Table 5).

Table 4: Concentration[a] of SPFMV and SPCSV in transgenic sweet potato lines infected individually with SPFMV or SPCSV and their combination.

Transgenic line	TAS-ELISA readings for SPFMV in plants infected with		TAS-ELISA readings for SPCSV in plants infected with	
	SPFMV	SPFMV + SPCSV	SPCSV	SPFMV + SPCSV
CPT 560/28/155	0.59	0.89	0.58	0.98
CPT 560/28/76	0.66	0.70	0.57	0.59
CPT 560/28/201	0.58	0.85	0.90	1.08
CPT 560/28/71	0.60	0.79	0.58	1.00
CPT 560/28/21	0.58	1.24	0.60	1.28
CPT 560/29/121	0.55	1.10	0.58	0.88
CPT 560/29/83	0.56	1.40	0.58	1.38
KSP 36/29/61	0.59	1.28	0.59	0.95
CPT 560/29/110	0.56	1.18	0.57	1.18
CPT 560/29/69	0.56	1.29	0.59	0.98
CPT 560/29/247	0.57	0.84	0.62	0.69
CPT 560/36/19	0.61	0.82	0.57	0.58
CPT 560/36/53	0.57	1.44	0.60	1.42
CPT 560/36/93	0.67	1.32	0.59	1.32
CPT 560/36/137	0.64	1.45	0.64	1.41
CPT 560 healthy	0.54	0.57	0.56	0.55
KSP 36 healthy	0.55	0.57	0.43	0.57
Non-transgenic	1.49	1.82	1.33	1.73
LSD (P=0.05)	0.207	0.238	0.207	0.209

[a]Values are absorbance readings at 405 nm. Readings were taken after 1 hr. substrate incubation.

Table 5: Effect of gene construct on disease severity and virus accumulation in sweet potato inoculated with SPFMV and SPCSV.

Gene construct	Disease severity*	Virus accumulation**
CP	1.3	0.89
Inverted repeat of CP	1.4	0.92
Replicase	2.0	1.19
Non-transgenic	3.5	1.71
LSD (P=0.05)	0.23	0.29

* Sweet potato virus disease severity determined on a scale of 1-5 where; 1 = no symptoms and 5 = very severe symptoms (Njeru *et al*., 2004). ** TAS-ELISA readings at 405 nm.

DISCUSSIONS

Simple, fast and cost effective protocols were used to confirm the presence of the transgene in putatively transformed sweet potato lines. The gene constructs that were used in the transformation process comprised the selectable marker npt II that breaks down aminoglycoside antibiotic kanamycin, gentamycin and paramomycin (Fraley *et al*., 1986). The npt II gene acts by inactivating kanamycin through a phosphorylation reaction, thereby permitting transformed cells to grow and differentiate. Following kanamycin painting, leaves of transgenic lines were not bleached suggesting that they were expressing the selectable marker gene (npt II). However, not all the plants that were not bleached were transgenic because during transformation, the selectable marker gene might

detach from the transgene and be incorporated into the cells of the explants without the transgene. Such plants were not bleached following treatment with kanamycin due to the presence of the selectable marker gene and not the virus derived gene.

PCR has been used effectively in numerous studies for screening transgenic plants (Sonoda *et al*., 1999; Okada *et al*., 2001). The PCR results in this study provided preliminary evidence of the presence of the CP, replicase and inverted repeat of the CP genes of SPFMV in transgenic sweet potato plants. Amplified DNA fragments of expected size (450 bp) were obtained from 7 transgenic lines. Some of the reasons for negative results in PCR even after plants have been transformed are (i) the transgenes may have been silenced, or (ii) the gene may have undergone some positional effects resulting in random integration and were hence not expressed in the tissues tested, or (iii) the genes may have been integrated in a promoter and therefore became suppressed. Variable expression of transgenes or gene silencing is a common phenomenon in transgenic plants whether produced by direct DNA uptake or by *Agrobacterium*-mediated transformation (Sonoda *et al*., 1999).

Transgenic sweet potato plants expressing the SPFMV CP, replicase and inverted repeat of the CP genes were challenged by graft-inoculation with SPFMV and SPCSV individually and in combination. The low virus concentration recorded in the resistant lines could be an indication of restricted virus multiplication. Furthermore, the resistant lines did not develop symptoms, perhaps due to the ability of the transgene to limit infection. The lack of symptoms and the low virus accumulation in transgenic sweet potato plants inoculated with SPFMV were consistent with the few previous reports which suggest that transgenic sweet potato plants are protected against SPFMV, with the resistance being characterized by extremely low virus titers and a lack of symptoms (Cipriani *et al*., 2001; Okada *et al*., 2001). Okada *et al*. (2001) has previously reported that sweet potato plants transformed with the CP gene of SPFMV were highly protected against SPFMV with virus accumulation being suppressed in the transgenic lines. However, SPFMV itself is not the problem, but rather the SPVD caused by the synergistic co-infection with SPCSV. It is still unknown whether transgenic resistance to SPFMV would be sufficient to prevent the development of SPVD.

Transgenic plants inoculated with a combination of SPFMV and SPCSV showed increased resistance with less severe symptoms than the non-transformed lines. Transgenic lines CPT 560/28/201, CPT 560/29/83 and CPT 560/36/53 seemed to support virus multiplication but the accumulation was not high enough to elicit the development of symptoms. This observation is an exception to the positive correlation noted between disease severity and virus accumulation among transgenic plants (Lawson *et al*., 1990; Baulicombe, 1996). Previously, it was reported that virus resistance in transgenic plants with viral sequences was based on post-transcriptional gene silencing (Baulicombe, 1996). However, from this study, 7 transgenic sweet potato lines of which 4 expressed the CP gene and 3 expressed the replicase gene showed significant resistance to the virus suggesting that this mechanism of resistance is CP- or replicase mediated.

Although the results of this study indicate some form of protection against SPVD in plants that were transformed with SPFMV-derived genes, it is not known whether this would be sufficient to control SPVD. Since aphid and whitefly inoculation is the natural route to infection, further experimentation in the field is needed to fully determine the efficacy of the transgenes.

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