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Synergistic Interactions of a Potyvirus and a Phloem-Limited Crinivirus in Sweet Potato Plants

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When infecting alone, *Sweet potato feathery mottle virus* (SPFMV, genus *Potyvirus*) and *Sweet potato chlorotic stunt virus* (SPCSV, genus *Crinivirus*) cause no or only mild symptoms (slight stunting and purpling), respectively, in the sweet potato (*Ipomoea batatas* L.). In the SPFMV-resistant cv. Tanzania, SPFMV is also present at extremely low titers, though plants are systemically infected. However, infection with both viruses results in the development of sweet potato virus disease (SPVD) characterized by severe symptoms in leaves and stunting of the plants. Data from this study showed that SPCSV remains confined to phloem and at a similar or slightly lower titer in the SPVD-affected plants, whereas the amounts of SPFMV RNA and CP antigen increase 600-fold. SPFMV was not confined to phloem, and the movement from the inoculated leaf to the upper leaves occurred at a similar rate, regardless of whether or not the plants were infected with SPCSV. Hence, resistance to SPFMV in cv. Tanzania was not based on restricted virus movement, neither did SPCSV significantly enhance the phloem loading or unloading of SPFMV. It is also noteworthy that SPVD is an unusual synergistic interaction in that the potyvirus component is not the cause of synergism but is the beneficiary. It is hypothesized that SPCSV is able to enhance the multiplication of SPFMV in tissues other than where it occurs itself, perhaps by interfering with systemic phloem-dependent signaling required in a resistance mechanism directed against SPFMV.

INTRODUCTION

Synergism between viruses refers to situations where coinfection with two viruses causes more severe symptoms than infection with either alone and in which multiplication of at least one of the viruses is enhanced by the other (Rochow and Ross, 1955; Goodman and Ross, 1974a,b; Calvert and Ghabrial, 1983; Goldberg and Brakke, 1987; Vance, 1991; Vance *et al.,* 1995; Pruss *et al.,* 1997; Scheets, 1998). Synergism can occur between plant viruses in different families as well as between viruses and their satellite viruses (Rodriguez-Alvarado *et al.,* 1994; Scholthof 1999) or associated RNA (Sanger *et al.,* 1994).

Several synergistic interactions have been described of which the best characterized are those in which a potyvirus causes an increase in titer of a second, unrelated virus. The classical example is the coinfection of tobacco plants with *Potato virus Y* (PVY, type member of the genus *Potyvirus*, family Potyviridae) and *Potato virus X* (PVX, type member of the genus *Potexvirus*): the titers of PVX RNA and coat protein increase and more severe symptoms are induced, but the titer of PVY is not affected (Rochow and Ross, 1955; Vance, 1991). Indeed, it is typical of synergistic interactions involving a potyvirus that titers of the potyvirus are either unaffected (Vance *et al.,* 1995; Pruss *et al.,* 1997 and references therein) or decline (Poolpol and Inouye, 1986). Only in three reported cases has coinfection with another virus (Lee and Ross, 1972; Valkonen, 1992; Scheets, 1998), and in one case with a viroid (Valkonen, 1992), led to enhancement of the potyvirus titers. A certain threshold titer of the enhancer virus may be required before synergism is induced as shown in the coinfection of maize with *Wheat streak mosaic virus* (WSMV, genus *Tritimovirus*, family Potyviridae) and *Maize chlorotic mottle virus* (MCMV, genus *Machlomovirus*, family Tombusviridae) (Scheets, 1998).

The molecular mechanisms of most examples of synergism are poorly understood. The multifunctional helper component-proteinase (HC-Pro) of potyviruses (reviewed by Maia *et al.,* 1996) has recently been identified as a cause of synergism (Vance *et al.,* 1995; Pruss *et al.,* 1997). The central region of HC-Pro mediates both the synergistic effect and the suppression of host posttranscriptional gene silencing (PTGS) (Shi *et al.,* 1997, Anandalakshmi *et al.,* 1998; Brigneti *et al.,* 1998; Kasschau and Carrington, 1998), suggesting that the two phenomena may be linked.

The aphid-transmitted *Sweet potato feathery mottle virus* (SPFMV, genus *Potyvirus*, family Potyviridae) and the whitely-transmitted *Sweet potato chlorotic stunt virus* (SPCSV, genus *Crinivirus*, family Closteroviridae) have positive-stranded RNA genomes and infect sweet potato (*Ipomoea batatas* L.) (reviewed by Karyeija *et al.,* 1998; Wisler *et al.,* 1998). In East African sweet potato cultivars, SPFMV alone causes no symptoms (Gibson *et al.,* 1997; Karyeija *et al.,* 1998), whereas SPCSV causes mild symptoms such as slight stunting and purpling of lower leaves and mild chlorotic mottle in the middle leaves under

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appropriate conditions (Winter *et al.,* 1992; Gibson *et al.,* 1998). However, when infected with SPFMV and SPCSV, the cultivars develop sweet potato virus disease (SPVD) (Schaefers and Terry, 1976; Gibson *et al.,* 1998), the economically most important disease of sweet potato in Africa (Geddes, 1990). This severe condition is characterized by pale green chlorotic leaves or chlorosis along main leaf veins, small crinkled, straplike or puckered leaves, and severe stunting of the plants. Yields of the SPVD-affected sweet potato are commonly reduced to less than half of the yield of the symptomless sweet potato plants (Hahn, 1976; Mukiibi, 1977; Ngeve and Bouwkamp, 1991). SPFMV is at a low titer and is difficult to detect in sweet potato plants infected by SPFMV alone (Esbenshade and Moyer, 1982; Abad and Moyer, 1992; Gibb and Padovan, 1993). However, it can be readily acquired by aphids (Schaefers and Terry, 1976), is at a high concentration (Rossel and Thottappilly, 1988) and is readily detected by ELISA in SPVD-affected sweet potato plants (Aritua *et al.,* 1998a; Gibson *et al.,* 1998). On the other hand, the data from ELISA indicate that the titers of the phloem-limited SPCSV (Cohen *et al.,* 1992; Winter *et al.,* 1992) remain the same or slightly decrease in the SPVD-affected plants as compared to plants infected with SPCSV alone (Gibson *et al.,* 1998).

The observation on increased titers of SPFMV in sweet potato plants affected by SPVD has been reported but the mechanism is unknown. The aim of this study was to examine whether it is due to a synergistic interaction between SPFMV and SPCSV, i.e., the multiplication rather than the movement of SPFMV being enhanced by SPCSV. Alternatively, the low titers of SPFMV characteristic to the resistant sweet potato plants could be associated with restricted movement of SPFMV and the enhanced titers associated with complementation of the movement of SPFMV by SPCSV, as described for many combinations of unrelated viruses (Atabekov and Taliansky, 1990). In this study, quantitative data on the amounts of SPFMV and SPCSV RNA present during the development of SPVD are provided, and the plant tissues in which each constituent virus occurs are identified. The data indicate that the great increase in the titer of the potyvirus component is not associated with enhanced virus movement but is due to a synergistic interaction with the crinivirus. While it is unusual to find the potyvirus titer to be increased in a synergistic interaction, it was even more unexpected to find it to be mediated by a phloem-limited virus. Hypotheses are proposed to explain how SPCSV may assist SPFMV to overcome resistance in sweet potato.

RESULTS

Location of SPFMV and SPCSV in infected tissues

In plants inoculated with both SPFMV and SPCSV, the symptoms of SPVD first developed in the newly emerging leaves (Fig. 1G). The time from inoculation to the appearance of symptoms varied, generally taking longer in larger and older plants, if lower leaves were inoculated, or if inoculation was by sap or aphids rather than by grafting (data not shown). The titers of SPFMV were high in all parts of the leaves in the SPVD-affected plants as examined using tissue printing (Fig. 1H) and confirmed by TAS–ELISA and NCM–ELISA (data not shown). In contrast, the plants infected with SPFMV alone remained symptomless (Fig. 1A) and SPFMV could be detected only on rare occasions by any of the three test methods in the leaves of these plants (Fig. 1B). Systemic infection with SPFMV could, however, be confirmed by grafting scions from any part of these plants to the indicator plant *I. setosa,* which developed clear symptoms of SPFMV infection. SPFMV was not confined to veins, as shown by tissue printing, regardless of whether occurring alone (Fig. 1B) or coinfecting with SPCSV (Fig. 1H).

Infection with SPCSV alone induced only mild purpling of older leaves, observed several weeks after infection under certain conditions (Fig. 1D). The fully developed leaves always tested positive for SPCSV in TAS–ELISA and NCM–ELISA (data not shown). SPCSV was confined to the leaf veins, as shown by tissue printing. The location of SPCSV in the leaves of plants infected with SPCSV only (Fig. 1F) was similar to the leaves of plants affected by SPVD (Fig. 1I).

Immunohistochemical localization using antibodies to SPCSV CP confirmed that SPCSV was confined to the phloem. Similarly, in the sweet potatoes affected by SPVD, strong staining was observed in the companion cells and no staining was observed in other tissues (Figs. 2D and 2F). SPFMV was not detected by this method in leaves or stems of plants infected by SPFMV alone due to the very low titers (Table 1). However, in some of the tissue prints (more of which could be done than was feasible for thin sections and immunohistochemical tests), SPFMV was detected and was not confined to phloem tissue (Fig. 1B) as mentioned above. In the SPVD-affected plants, SPFMV was detected in all types of leaf and stem tissues, but the highest signals were usually observed in the palisade parenchyma and guard cells of the leaves (Fig. 2C).

The amounts of viral RNA in the expanding and fully expanded leaves

RNA dot-blot analysis provided a sensitive means of quantifying the viral RNA, the lowest amounts detectable being 75 pg for SPCSV RNA and 10 pg for SPFMV RNA. The amounts of SPCSV in the mature leaves were significantly higher ($P \leq 0.001$) than in the young, expanding leaves, irrespective of whether or not plants were also infected with SPFMV. In cuttings grown from virus-infected plants, there was no significant ($P > 0.1$) difference between the amounts of SPCSV RNA (Table 1, Fig. 3) or CP antigen measured using TAS–ELISA (data not shown) in leaves of plants infected with SPCSV alone or

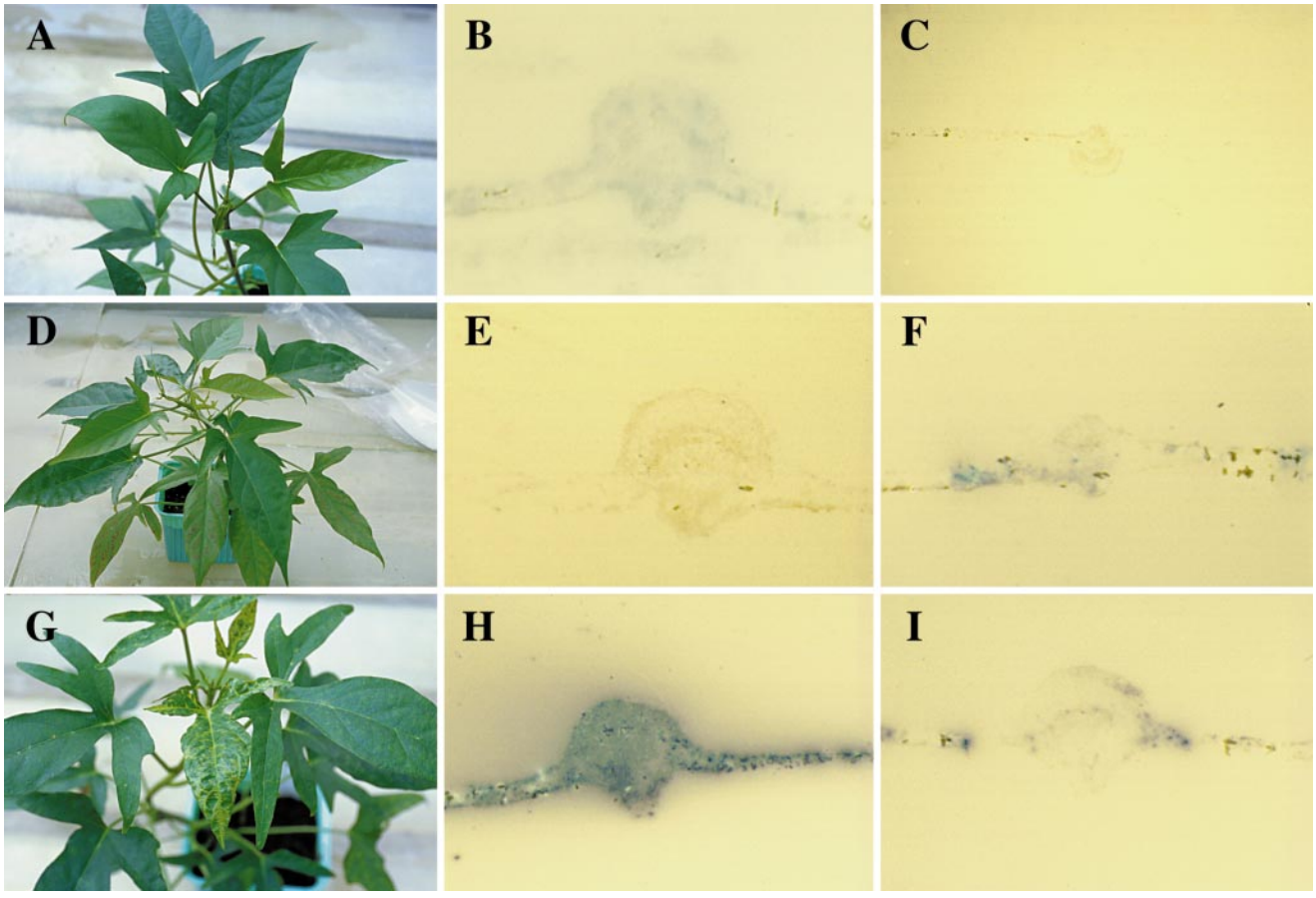

FIG. 1. Detection of SPFMV and SPCSV in tissue prints made from plants of sweet potato cv. Tanzania infected with SPFMV alone (A), SPCSV alone (D), and in a plant that was originally SPCSV-infected and was aphid-inoculated with SPFMV 3 weeks before photography (G). The tissue prints of leaf cross-sections (uppermost fully expanded leaves) prepared from each of these plants are to the right and were developed with antibodies to SPFMV (B, E, and H) or SPCSV (C, F, and I). The prints were photographed under a microscope at a magnification of ×30, except (C), which was photographed at a ×10 magnification.

of plants also infected with SPFMV. This result was consistent in the several independent experiments carried out, even though the poor growth and severe symptoms of the double-infected plants made it difficult to sample leaves at the same developmental age from the different plants of the same experiment. Sampling leaves of slightly different developmental age, in turn, was probably the reason for a relatively large variation in the measured titers of SPCSV in the SPVD-affected plants because the older leaves had higher titers (Table 1). Comparison of SPCSV titers in the expanding, systemically infected leaves between plants inoculated with SPCSV alone and plants coinoculated with both viruses at 15–33 days p.i. confirmed that the SPCSV titers were similarly low in both cases (see below; Fig. 4).

The titers of SPFMV were much greater in plants also infected with SPCSV than in plants infected with only SPFMV ($P \leq 0.001$). Very small amounts of SPFMV RNA could be detected in just a few of the plants infected with SPFMV alone, whereas large amounts of SPFMV RNA, ranging from 3750 to 80,000 pg/μg ($\leq$8%) of the total RNA (Table 1), were detected in the plants infected with both SPFMV and SPCSV. The titers of the SPFMV CP antigen similarly ranged from nondetectable levels in plants infected only with SPFMV to very high levels in plants also infected with SPCSV. In the latter case, the ELISA absorbence values were as high as, or higher than those generated by samples of SPFMV-infected plants of the indicator host *I. setosa* (data not shown).

SPFMV RNA was detected in the top leaves of plants coinoculated with SPFMV and SPCSV at 11 days p.i. (Fig. 4), and the first symptoms were observed at 12 days p.i. The data suggested that, on average, SPFMV RNA concentration of 600 pg/μg total RNA was required for the appearance of symptoms. The amount of SPFMV RNA varied between plants, correlating positively with symptom severity. The same samples were tested also for titers of SPCSV, which remained low or decreased by time (Fig. 4) similar to plants infected with SPCSV alone.

The rate of viral translocation

To compare the rate of translocation of SPFMV from inoculated leaves in the presence or absence of SPCSV, leaves of healthy or SPCSV-infected sweet potato plants were inoculated with SPFMV by sap-inoculation or aphid

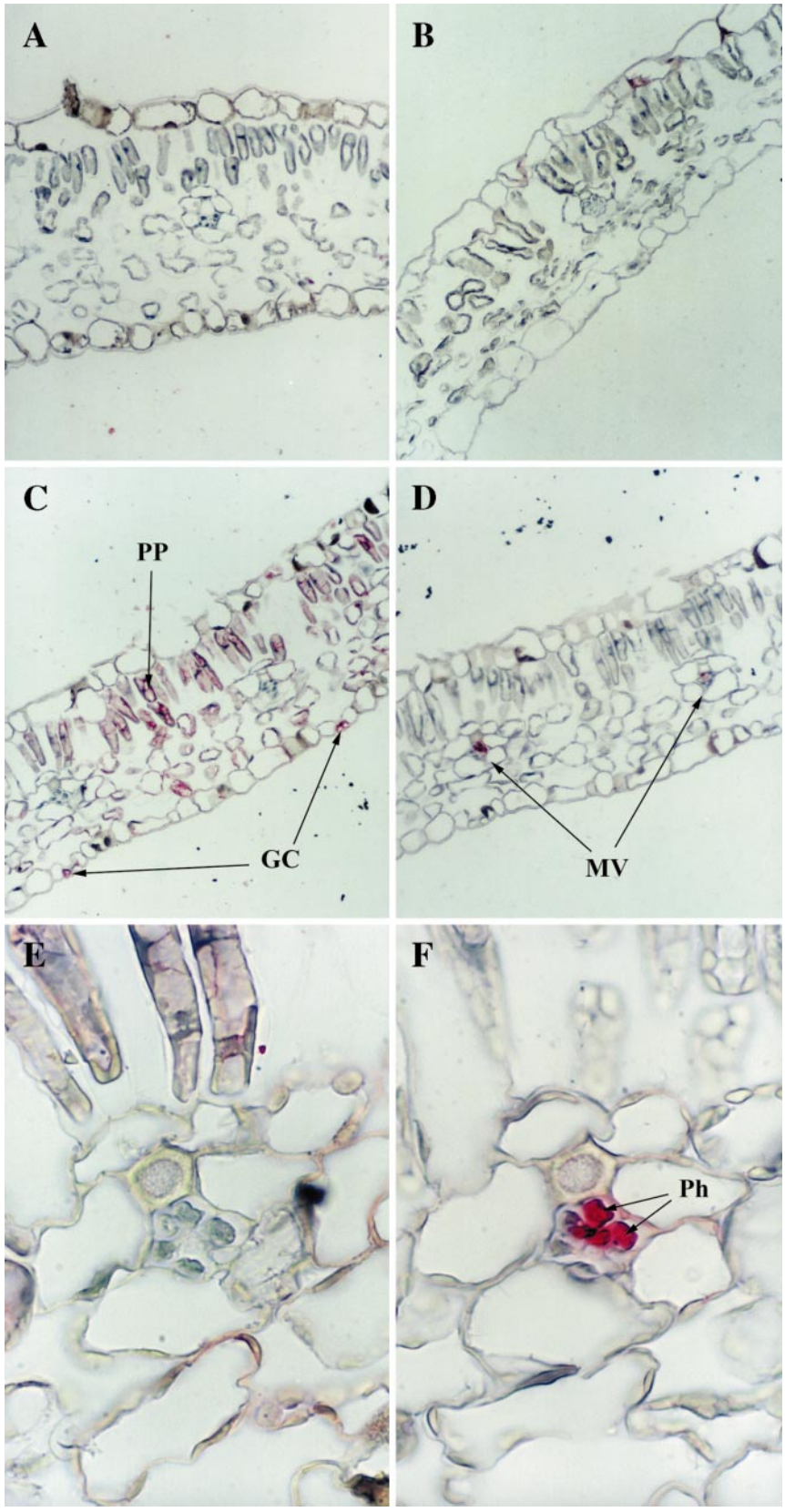

FIG. 2. Immunohistochemical localization of SPFMV and SPCSV using thin sections prepared from the fourth leaf below the uppermost fully expanded leaf on young axillary shoots of healthy (A and B) and SPVD-affected (C–F) plants of sweet potato cv. Tanzania. The sections in (A), (C), and (E) were stained using a monoclonal antibody to the SPFMV CP, and the sections in (B), (D), and (F) were stained with a monoclonal antibody to the SPCSV CP. Staining with the antibodies to SPFMV CP is visible in palisade parenchyma (PP) and guard cells (GC) (C and E), whereas the staining with antibodies to SPCSV CP is observed only in the phloem (Ph) (D and F). Healthy plants show no staining with either antibody (A and B). Magnification of the sections in (A–D) is ×20, and those in (E) and (F) are close-ups of the minor veins (MV) on the left of (C) and (D) (magnification ×100). (C) and (D) are consecutive sections (thickness of section, 5 μm).

TABLE 1

Mean Amounts of Viral RNA in Expanding and Fully Expanded Leaves of Plants of Sweet Potato cv. Tanzania Infected with SPCSV, SPFMV, or Both Viruses[a]

	SPCSV RNA				SPFMV RNA			
	Expanding leaf		Mature leaf		Expanding leaf		Mature leaf	
Virus	No.	pg	No.	pg	No.	pg	No.	pg
SPFMV	0/7	0	0/6	0	1/7	312	0/2	<10
SPCSV	4/10	309 ± 230	6/6	13330 ± 4082	0/10	0	0/6	0
SPFMV + SPCSV	3/11	5013 ± 4981	6/6	15830 ± 12810	11/11	27160 ± 25640	6/6	26670 ± 29270

[a] Means of the amounts (pg) of viral RNA per microgram of total RNA (±SE) measured from the first leaf above the uppermost fully expanded leaf (expanding leaf) and the fourth leaf below the uppermost fully expanded leaf (mature leaf). No., number of plants containing detectable amounts of viral RNA, and from which the means were calculated, per the total number of plants examined.

transmission. Plants only became systemically infected with SPFMV if the inoculated leaf was removed from the plant ≥9 days after inoculation, regardless of whether the plants were originally healthy or SPCSV-infected (Table 2). To avoid disrupting source-sink partitioning of photosynthates (the mechanism also carrying viruses in phloem over long distances in the plants; Séron and Haenni, 1996), *I. setosa* scions were also grafted to the tops of virus-free or SPCSV-infected sweet potato plants prior to inoculation with SPFMV. Scions tested positive for SPFMV at 11–15 days p.i. of the sweet potato rootstocks, again, irrespective of the presence or absence of SPCSV (Table 3).

DISCUSSION

The data from this study on sweet potato cv. Tanzania were consistent with the few previous reports showing that East African sweet potato cultivars express resistance to SPFMV, which is characterized by extremely low titers of SPFMV and a lack of symptoms (Aritua *et al.,* 1998a,b; Gibson *et al.,* 1998; Karyeija *et al.,* 1998). However, the plants are systemically infected, as shown by back-grafting to the indicator plant *I. setosa*. The resistance to SPFMV is overcome in plants infected with SPCSV. Severe symptoms of SPVD develop, and according to this study, the amounts of SPFMV RNA and CP antigen increase dramatically (600-fold on average). In contrast, the titers of SPCSV are unaffected or slightly decrease, as noted previously (Gibson *et al.,* 1998). Thus an unusual aspect of our results, in contrast to most of the previous studies (Rochow and Ross, 1955; Poolpol and Inouye, 1986; Vance, 1991; Vance *et al.,* 1995; Pruss *et al.,* 1997), is that SPVD is a synergistic viral interaction where the potyvirus component is not the cause of synergism but is the beneficiary.

Because the titers of SPCSV were not significantly increased in doubly infected plants and because the plants infected with SPCSV alone were mostly symptomless or displayed symptoms different from SPVD, the symptoms of SPVD are presumably caused solely or largely by SPFMV. This is supported by the low or undetectable titers of SPCSV in the young leaves with severe symptoms and by the correlation of the SPFMV titers with the severity of the symptoms. Furthermore the symptoms caused by SPVD resemble those typical of potyviruses (Shukla *et al.,* 1994).

Potyviruses are not limited to any particular tissues in infected leaves (Shukla *et al.,* 1994), consistent with the high titers of SPFMV present in all the leaf tissues in the SPVD-affected plants. However, there are several examples of resistant hosts restricting cell to cell (Valkonen *et al.,* 1991; Valkonen and Somersalo, 1996; Nicolas *et al.,* 1997) or vascular movement (Schaad and Carrington, 1996; Rajamäki and Valkonen, 1999) of potyviruses, and this was considered as a possible mechanism of resistance to SPFMV in sweet potato. Furthermore complementation of a defective virus transport function by an unrelated virus can overcome resistance (Atabekov and Taliansky, 1990). For example, a heat-shock protein (HSP70) homologue produced by a member of the Closteroviridae can complement the cell-to-cell movement of nonrelated, transport-defective viruses (Agranovsky *et al.,* 1998). Our data showed that in the cases where SPFMV could be detected and localized in the systemically infected leaves of the resistant sweet potato plants infected with SPFMV alone, the virus was not confined to phloem. Furthermore the movement of SPFMV from the inoculated leaf to the upper leaves occurred at a similar rate regardless whether or not the sweet potato plants were infected with SPCSV. Hence the data did not support restricted virus movement as a mechanism of resistance to SPFMV or that SPCSV may be overcoming such resistance by enhancing the phloem loading and/or unloading of SPFMV.

A surprising aspect of SPVD is that phloem-limited crinivirus is increasing the titer of an unrelated virus in nonphloem tissue. It has been observed that the phloem-limited *Potato leaf roll virus* (genus *Polerovirus*, family Luteoviridae) can exit the phloem in a mixed infection with the nonphloem-limited potyvirus, PVY (Barker, 1987).

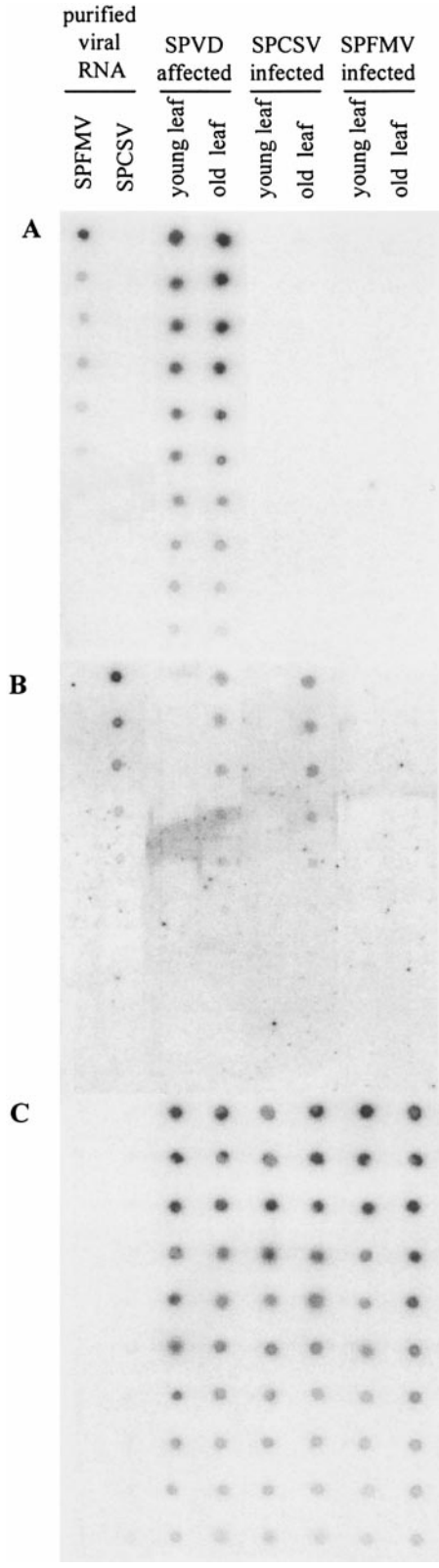

FIG. 3. RNA dot blots of purified viral RNA and total plant RNA from plants infected with SPFMV, SPCSV, or both SPFMV and SPCSV. RNA was isolated from the expanding leaf directly above the uppermost fully expanded leaf (young leaf) and the fourth leaf below the uppermost fully expanded leaf (see also Fig. 2 and Table 1). RNA was applied in a dilution series (1 μl/dot) ranging from 1000 to 2 ng of total RNA or 5 to 0.01ng of purified viral RNA, diluted twofold between steps. The same blots were hybridized with probes to the coat protein gene of SPFMV (A) or SPCSV (B) and with a ribosomal DNA probe (C).

The data of our study did not indicate that SPCSV exits the phloem when coinfecting with SPFMV or that the synergistic effects occurred in coinfected cells only. While further studies are required to reveal the molecular mechanisms of the synergism, hypotheses may be proposed. Some proteins encoded by SPCSV may be transported from the phloem to other tissues enhancing the multiplication of SPFMV and hence mediating the synergism. For example, the potyviral HC-Pro and the crinivirus and closterovirus proteinases (P-Pro and L-Pro) may be functionally analogous. They share an amino acid signature of papain-like proteinases at the C-proximal part (Klaassen *et al.,* 1995), and the central regions are shown (potyviruses; Kasschau *et al.,* 1997) or suggested (Closteroviridae; Dolja *et al.,* 1997) to mediate genome amplification. Translation of HSP70 is induced in plants while the expression of many other host genes is shut off at the initial stages of potyvirus infection, suggesting some positive role for HSP70 in potyvirus infections (Aranda *et al.,* 1996). As mentioned above, SPCSV encodes a HSP70-like protein with high homology to the HSP70 class of molecular chaperones (Alicai *et al.,* 1999).

Another possible mechanism for synergism between SPFMV and SPCSV is that sweet potato actively inhibits the multiplication of SPFMV and that SPCSV suppresses the resistance mechanism. Sweet potatoes are also resistant to *Cucumber mosaic virus* (CMV, genus *Cucumovirus*, family Bromoviridae), but, as with SPFMV, the resistance to CMV is overcome by coinfection with SPCSV (Cohen and Loebenstein, 1991; Cohen *et al.,* 1995). Therefore SPCSV infection may affect a fundamental mechanism that is normally capable of suppressing infection by some other viruses. Both cucumoviruses and potyviruses produce a suppressor of posttranscriptional gene silencing (PTGS) (Anandalakshmi *et al.,* 1998; Brigneti *et al.,* 1998; Kasschau and Carrington, 1998), and the viral suppressor of PTGS can be the target of a host resistance mechanism (Li *et al.,* 1999). PTGS involves systemic signaling that follows the same route through the phloem as used by viruses (Santa Cruz, 1999). Perhaps SPCSV either supplies a factor aiding the partially functional suppressors of PTGS produced by SPFMV or CMV or interferes with the systemic signaling of PTGS. For example, analogous functions have been suggested for the multifunctional HC-Pro, in which the central region is implicated as the suppressor of PTGS (Anandalakshmi *et al.,* 1998) and the proteinases of Closteroviridae (Dolja *et al.,* 1997). These alternative hypotheses will be addressed by future studies.

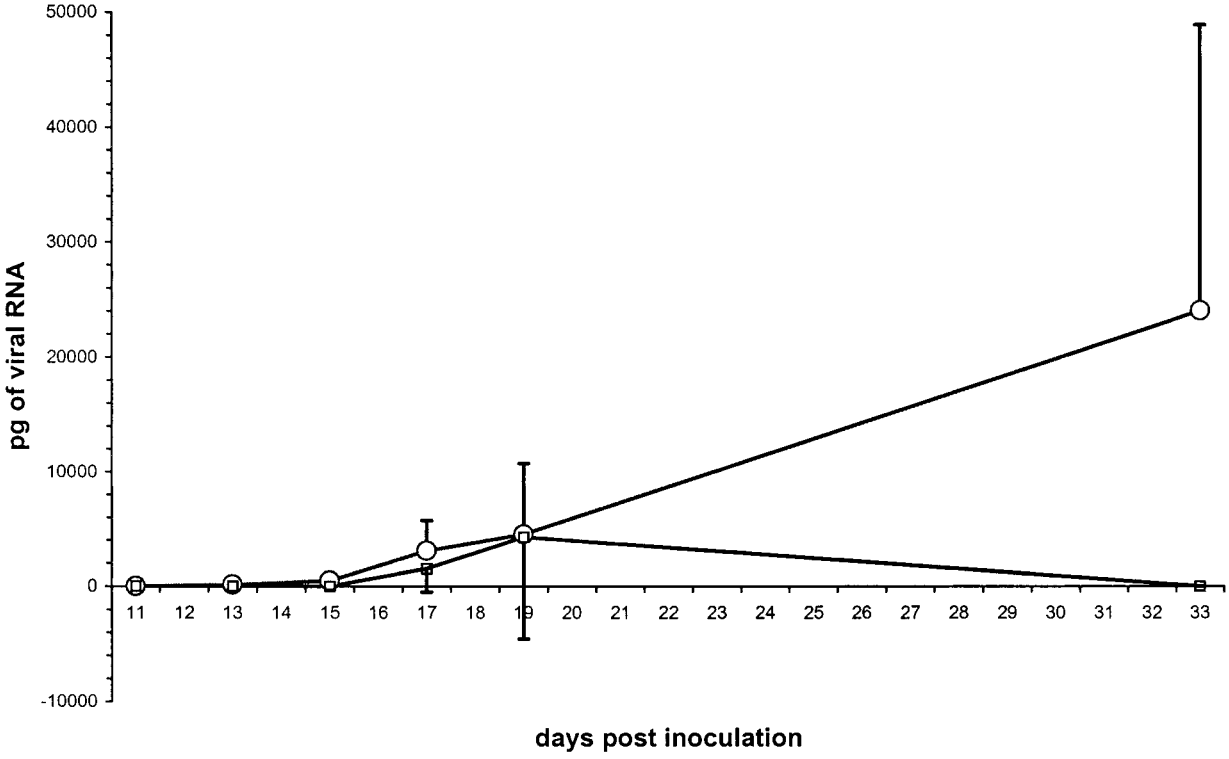

FIG. 4. Changes in the amounts of SPFMV (○) and SPCSV (□) RNAs by time in the top leaves (the first leaf above the fully expanded leaf) of plants infected with both SPFMV and SPCSV by graft inoculation. The amounts of viral RNA for both viruses were tested from the same leaf samples. Each point represents the mean from the measurement of five plants, and the bars indicate standard errors. The amounts of SPFMV in plants infected with SPFMV alone were nondetectable (not shown). The changes in amounts of SPCSV RNA in the plants infected with both SPCSV and SPFMV (shown here) were similar to the plants infected with SPCSV alone (not shown).

MATERIALS AND METHODS

Plant material and virus isolates

Healthy *in vitro* plantlets of the East African sweet potato cv. Tanzania were obtained from the International Potato Center (CIP), Lima, Peru. They were transferred to soil and multiplied by taking cuttings in a greenhouse at SLU, Uppsala, Sweden. Temperature was 25–30°C. Sodium halide lamps were used to extend day length to 16 h during winter. SPVD-affected sweet potato plants were grown in a separate chamber of the same greenhouse under similar conditions.

The SPFMV isolate (Nam1; Kreuze *et al.,* 2000), and the SPCSV isolate used (serotype East Africa 2; Alicai *et al.,* 1999) were from the Mpigi district, Uganda. The absence of S*weet potato mild mottle virus*, S*weet potato chlorotic fleck virus,* and S*weet potato latent virus* was confirmed by triple-antibody sandwich (TAS) ELISA (Gibson *et al.,* 1998). Plants were multiplied for experiments by taking stem cuttings.

SPFMV and SPCSV were purified from infected plants of *I. setosa* according to Cohen *et al.* (1988; 1992, respectively). The purified viruses and the RNA extracted from them were used as controls in ELISA and RNA dot-blot analysis, respectively.

TABLE 2

Numbers of Plants of Sweet Potato cv. Tanzania Systemically Infected with SPFMV Following Inoculation Using Aphids[a]

	Plants originally SPCSV-infected			Plants originally virus-free		
Days after inoculation	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2	Expt 3
9	0/5	0/4	0/5	0/4	0/4	0/5
11	nt	0/4	1/5	nt	0/4	2/5
13	2/5	0/4	2/5	3/4	1/4	4/5
15	5/5	3/4	3/5	4/4	4/4	4/5

Note. The numbers of plants infected with SPFMV per the number of plants inoculated are indicated. Nt, not tested.

[a] Systemic infection with SPFMV was detected from the shoots of *I. setosa* top-grafted on to the sweet potato plants prior to inoculation with SPFMV (see Materials and Methods).

TABLE 3

Numbers of Plants of Sweet Potato cv. Tanzania Which Became Systemically Infected After Removing the Sap-Inoculated Leaf at Different Times After Inoculation[a]

Days after inoculation	Plants originally SPCSV-infected		Plants originally virus-free	
	Expt 1	Expt 2	Expt 1	Expt 2
3	0/2	0/2	0/2	0/2
5	0/2	0/2	0/2	0/2
7	0/2	0/2	0/2	0/2
9	1/2	2/2	2/2	1/2
Not trimmed	2/2	1/2	2/2	2/2

[a] Systemic infection with SPFMV was detected based on symptom development and/or NCM–ELISA (in plants originally SPCSV-infected) or from shoots of *I. setosa* top-grafted on to the sweet potato plants (see Materials and Methods).

Detection of SPFMV and SPCSV

For detection of SPFMV by ELISA, polyclonal antisera and the monoclonal antibody 7H8G2 (dilution 1:500) were provided by CIP. For detecting SPCSV, the polyclonal antiserum SPCSV-KYCP (dilution 1:500) and the monoclonal antibody SPCSV-1–2G8 (dilution 1:500) were provided by Dr. H. J. Vetten (Federal Biological Research Center for Agriculture and Forestry, Braunschweig, Germany). Anti-rabbit and anti-mouse alkaline phosphatase conjugated antisera were obtained from Sigma Chemical Co. (St. Louis, MO; dilution 1:25000).

Nitrocellulose membrane ELISA (NCM–ELISA) was carried out as described by Abad and Moyer (1992). TAS–ELISA was carried out according to Gibson *et al.* (1998). Leaf samples were collected by excising discs with a cork borer, weighing them, and grinding the discs in sample buffer at 1 g/10ml. The sap was allowed to clarify for 30 min after which aliquots of 10 or 100 μl were dotted on NCM membrane (Bio-Rad Laboratories) or transferred to a microtitre plate (Greiner Laborteknik, Germany), respectively. In TAS–ELISA, absorbences were recorded at 405 nm (A_{405}) using a microplate reader (Benchmark, Bio-Rad). A_{405} values two times the values of the uninoculated plants plus the standard error were considered as virus-infected.

Prints of cross-sections of leaves from plants infected with SPCSV, SPFMV, and both viruses were made on NCM. The leaf was placed between two pieces of sterox and cut with a razor blade, and the freshly cut surface was then pressed onto the membrane. The tissue prints were tested for SPCSV and SPFMV as described for NCM–ELISA.

Immunohistochemical staining of thin sections

Leaf and stem tissues (0.5 cm^2) of healthy, SPFMV-infected, SPCSV-infected, or SPVD-affected sweet potato plants were fixed in 4% formaldehyde for 24 h immediately after harvest. The samples were dehydrated, treated in Histoclear (National Diagnostics, New Jersey), and embedded in paraffin (Paraplast Embedding Media, Sigma), according to Jackson (1989). Thin sections (5 μm) were cut from the embedded samples with a microtome (Microm, Heidelberg), transferred to microslides covered by poly-L-lysine (Polysin, Menzel-Gläser, Germany) and incubated overnight at 37°C. Paraffin was removed by washing twice in xylene, after which the samples were rehydrated and washed in PBS. Preincubation in PBS containing 4% BSA for 30 min was followed by incubation at 4°C overnight with the monoclonal antibodies to SPFMV (MAb SPFMV-7H8G2, dilution 1:500) or SPCSV (MAb SPCSV-1–2G8, dilution 1:25). After washing in PBS three times, the incubation with the secondary antibody (anti-mouse-AP conjugate, dilution 1:50) was carried out for 30 min, followed by washing in PBS three times. The samples were stained using a fresh Fuchsin substrate solution, and the plant tissue was counterstained using Mayers reagent (Hematoxylin sol., Apoteksbolaget, Sweden), according to Naish *et al.* (1989).

RNA isolation and analysis

RNA was extracted from purified virus as described by Klaassen *et al.* (1994), and the concentrations of RNA determined at A_{260} with an UV-spectrophotometer. Total RNA was isolated from virus-infected and healthy sweet potato plants by the hot-phenol method (Verwoerd *et al.,*1989). Quality of the extracted RNA was checked under UV light after electrophoresis in a standard formaldehyde gel and staining with ethidium bromide (Sambrook *et al.,* 1989).

Coat protein gene fragments from SPFMV or SPCSV were amplified by the reverse transcription polymerase chain reaction (RT–PCR) from the total RNA extracted from SPVD-affected sweet potato using the following primer pairs: 3′NTR34– (TTA AAG GCA TAC TAA AGA TAA) and Nib1536+ (GAG AAT GAG TTA GAA GTA TAT) for SPFMV (Kreuze *et al.,* 2000) and CP1 (CTG CTA GAT TGT TAG AAA) and CP2 (TAT ATG AAA ATA TAG TTC) for SPCSV (Alicai *et al.,* 1999). The amplified fragments were then ligated into the pCRII vector (Invitrogen, Carlsbad, CA) and transformed into *Escherichia coli* strain INVαF′ (TA Cloning Kit; Invitrogen) according to the manufacturer's instructions. A plasmid containing a ribosomal DNA (rDNA) fragment from tobacco was kindly provided by Sabina Vidal (Department of Plant Biology, SLU, Uppsala).

For the preparation of probes, the viral cDNA or tobacco rDNA gene fragments were cut from the plasmids with *Eco*RV, separated by electrophoresis in a 1% agarose gel, and purified from the gel by a QIAquick purification kit (QIAGEN Inc.). Probes labeled with [^{32}P]dATP were synthesized from the CP gene fragments with the *redi*prime II random prime labeling system (Amersham Pharmacia Biotech Ltd., UK) according to the manufacturer's instructions.

A dilution series was prepared from each RNA sample, starting from 1000 ng/μl and diluting by a factor of two until 2 ng/μl (10 dilutions). One microliter from each RNA dilution was dotted directly on to a nylon membrane (Hybond-N; Amersham Pharmacia Biotech Ltd., UK). Dilution series from the purified viral RNAs of SPCSV and SPFMV were prepared and applied to every membrane as standards in the same manner, except that the viral RNA amounts in the dots ranged from 5 to 0.01 ng.

The blots were UV crosslinked, prehybridized, hybridized, and washed in hybridization tubes according to Sambrook *et al.* (1989). After washing, the blots were wrapped in polyethylene plastic and exposed to a phosphor exposure cassette (Molecular Dynamics) overnight. The cassette was then scanned with a PhosphorImager and the results were analyzed using the ImageQuant software. The probe was removed from the membranes, and the same blot was used for hybridization with the next probe. Each blot was hybridized with the probes for the SPFMV CP gene, the SPCSV CP gene, and rDNA (used as an internal control for the amounts of total RNA applied).

Translocation of viruses

Five virus-free and five SPCSV-infected cv. Tanzania plants were top-grafted with *I. setosa* and allowed to unite. Leaves at the same position in each plant were inoculated with SPFMV by aphids as described previously (Gibson *et al.,* 1998). A culture of *Myzus persicae* Sulzer supplied by Britt-Louise Lennefors (Novartis Seeds, Landskrona, Sweden), reared on oilseed rape plants, was used. The time SPFMV took to get to the top-grafted *I. setosa* was monitored by symptom expression and by NCM–ELISA. The experiment was repeated three times (Table 2).

In another experiment (Table 3), half leaves of 10 virus-free and 10 SPCSV-infected cv. Tanzania plants were mechanically inoculated with SPFMV by using sap extracted from an SPVD-affected sweet potato plant. At 3, 5, 7, and 9 days p.i., virus-inoculated leaves were excised from two plants per batch, and the virus-inoculated leaves from two plants from each batch were not removed. Systemic infection with SPFMV was detected by the development of SPVD symptoms and using NCM–ELISA (in SPCSV-infected plants). Plants that developed no symptoms were top-grafted with *I. setosa* scions 3 weeks after inoculation. The scions were observed for symptoms and tested for SPFMV by NCM–ELISA.

Amounts of viral RNA and CP antigen in expanding and fully expanded leaves

Sample leaf discs (diameter 1 cm) were taken from full-grown leaves (the 4th leaf below the uppermost fully expanded leaf) and young, expanding leaves (first leaf above the uppermost fully expanded leaf) of sweet potato plants grown from cuttings taken from plants infected with SPFMV, SPCSV, or both viruses, or (in the case of expanding leaves) also from plants inoculated with SPFMV, SPCSV, or both viruses 33 days earlier. At least six plants from each plant–virus combination were sampled. The total RNA was extracted from the samples and the amounts of SPCSV and SPFMV RNA compared using virus-specific RNA probes or the amounts of the CP antigen were compared using TAS–ELISA.

Testing the viral RNA amounts during the development of disease

Three batches of five plants of cv. Tanzania were inoculated by side grafting. In one batch, the plants were grafted with scions from *I. setosa* infected with SPFMV. In a second batch, the plants were grafted with scions from *I. setosa* infected with SPCSV. In a third batch, the plants were grafted with a pair of scions, one of which was from *I. setosa* infected with SPFMV and the other from an SPCSV-infected *I. setosa*. Sampling started 11 days after grafting. Leaf disks (diameter 1 cm) were taken from the first leaf above the uppermost fully expanded leaf every second day. The samples were frozen in liquid nitrogen and stored at −80°C until RNA extraction.

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