Research Note

A Synergism Induced by Satellite Panicum Mosaic Virus

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Panicum mosaic virus (PMV) induces a mild mottle on millet plants, but the addition of its satellite virus (SPMV) causes a severe chlorosis and stunting. Immunoblots, RNA blots, and sucrose density gradient analyses revealed increases of PMV RNA and its p8 and capsid proteins when plants were co-infected with SPMV. A unique feature associated with this satellite virus interaction was an increased rate of systemic infection of PMV.

Virus interactions resulting in a synergism have been described in association with many mixed virus infections, but particularly with potyviruses. A synergism is generally recognized as one virus acting as a catalyst to up-regulate the replication of a second, unrelated virus, resulting in more severe symptoms on the host (Goldberg and Brakke 1987; Pruss et al. 1997; Rochow and Ross 1955; Scheets 1998; Vance 1991; Vance et al. 1995). A synergism has also been reported with mixed infections of beet western yellows luteovirus and its ST9-associated RNA, which encodes a carmovirus-like replicase (Sanger et al. 1994). Although a satellite virus-associated synergism per se has not been reported, co-infections of tobacco mosaic virus (TMV) and its satellite virus, STMV, result in more severe symptoms on pepper plants than observed for TMV alone (Rodríguez-Alvarado et al. 1994).

Satellite panicum mosaic virus (SPMV) depends on its helper virus, panicum mosaic virus (PMV), for replication and movement. SPMV expresses a 17-kDa capsid protein from its 826 nucleotide (nt) genome to form 16-nm icosahedral particles (Buzen et al. 1984; Masuta et al. 1987; Scholthof et al., *in press*). PMV, a single-stranded, positive-sense RNA virus of 4,326 nt is the type member of the provisional genus *Panicovirus* within the Tombusviridae (Turina et al. 1998). A mixed infection of PMV and SPMV on St. Augustinegrass results in “St. Augustine decline,” an economically important disease of St. Augustinegrass in the southern United States (Buzen et al. 1984; Holcomb et al. 1989; Scholthof et al. 1999). To investigate the biological complexity of the helper-satellite-host interactions in the *Gramineae* and the role of satellites as “molecular parasites,” disease progression in pearl millet plants was monitored following inoculation with PMV or PMV+SPMV.

PMV-infected pearl millet plants generally are slightly stunted and the leaves display a mild chlorotic mottle (Fig. 1A). In contrast, PMV+SPMV infected plants develop chlorotic streaks on the emerging uninoculated leaves 3 to 4 days after the virus is first detected in the inoculated leaves; the symptoms rapidly progress to a severe chlorotic mottle with occasional regions of bleaching and/or necrosis. Mature plants and their panicles are severely stunted and rarely produce viable seed (Fig. 1B). To characterize the role of SPMV in this interaction, plants were monitored daily by immunoblot analyses to detect the capsid protein of PMV and SPMV. The PMV p8 protein, which is likely associated with cell-to-cell movement based on conserved similarity with the carmo- and necrovirus homologues (Turina et al. 1998), also was monitored by immunoblots.

A polyclonal antibody was derived to analyze PMV p8 protein in infected plants. To amplify the p8 open reading frame by PCR (polymerase chain reaction) from the full-length infectious PMV cDNA clone, pPMV85 (Turina et al. 1998), a 5' primer (5'-ATACGGATCCAATGTCTACTGTT-3') was used to introduce a *Bam*HI site (underline) just upstream of the predicted p8 start codon (nucleotides 2875 to 2877; double underline). The 3' primer (5'-GTTGAGAACC CAGGAGCC-3') extended to a region downstream of the putative p8 stop codon. This PCR-derived DNA fragment was digested with the restriction enzymes *Bam*HI and *Sca*I (PMV nucleotide 3149) and ligated in frame into the *Bam*HI:*Sma*I-digested pGEX-1 vector (Pharmacia, Piscataway, NJ) to create pKB137. Following transformation of *Escherichia coli* XL1 Blue (Stratagene, La Jolla, CA) with this construct, 1 mM isopropyl- β -thiogalactopyranoside was added to induce the overexpression of a 34-kDa glutathione S-transferase and p8 chimera (GST-p8). This GST-p8 protein was purified from sodium dodecyl sulfate (SDS)-polyacrylamide gels as described previously (Scholthof et al. 1994, 1995c) and a polyclonal antibody was prepared in New Zealand white rabbits (HTI BioProducts, San Ramon, CA).

PMV or PMV+SPMV RNA transcripts from cDNA were inoculated to pearl millet (*Pennisetum glaucum*) plants as described previously (Turina et al. 1998) and these primary infected plants were used as inoculum for subsequent experiments. Infected leaves were ground with a mortar and pestle in water (approximately 3:1 wt/vol) and inoculated onto two fully expanded leaves of young millet plants. Inoculated and upper uninoculated leaves pooled from three inoculated plants

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were collected at 3, 4, 5, 6, 7, and 12 days post-inoculation (dpi) for protein assays.

To detect PMV and SPMV capsid proteins and PMV p8 protein, approximately 0.1 g of inoculated and 0.1 g of upper uninoculated leaves were pulverized separately in 1 ml of TE (10 mM Tris, 1 mM EDTA, pH 7.4) with 1 mM dithiothreitol and disrupted in 2% SDS prior to boiling for 3 min. The denatured samples were centrifuged for 5 min at $12,000 \times g$ and equal volumes of supernatant were electrophoresed on 12.5% SDS-polyacrylamide gels. After electrophoretic transfer of the proteins to nitrocellulose membrane, antibodies specific to PMV and SPMV capsid proteins were used at dilutions of 1:20,000 and 1:10,000, respectively (Turina et al. 1998). An IgG-enriched fraction of the GST-p8 polyclonal antiserum was used at a dilution of 1:100. The details of protein preparation, immunoblots, and use of chemiluminescent substrate were described previously (Scholthof et al. 1994, 1995c; Turina et al. 1998).

At 3 dpi, very small amounts of the PMV and SPMV capsid proteins were detected in the inoculated leaves (Fig. 2). At 4 dpi both the PMV capsid and p8 proteins were detected at a higher titer in the mixed PMV+SPMV infection than for an infection with PMV alone. The most significant effect at 4 dpi was the clear establishment of PMV+SPMV in upper uninoculated leaves, compared with the absence of detectable protein in PMV infection alone (Fig. 2). By 5 dpi, the PMV p8 and capsid proteins were readily detected in both single and mixed infections in the upper uninoculated leaves.

Later in the timecourse assay, at 7 and 12 dpi, the titer of p8 increased and was represented either as a dimer of p8 or perhaps as p8-FS (Fig. 2), a putative 14.6-kDa frameshift protein of p8 (Turina et al. 1998). On foxtail millet (*Setaria italica* cv. German R), both single and mixed infections showed a pattern of capsid and p8 protein accumulation similar to that on pearl millet (not shown).

On pearl millet, these immunoblot results were supported by total RNA extracts analyzed on 7.5 to 30% sucrose density gradients and RNA blots (Fig. 3). Sucrose density gradients, with an equivalent of 9.5 mg of tissue per sample, were used to evaluate the chloroplast and cytoplasmic rRNA profiles as well as PMV and SPMV RNAs at 5, 7, and 10 dpi by a method previously described (Goldberg and Brakke 1987). Plants infected with PMV and PMV+SPMV consistently sustained a slight, but similar, reduction of rRNAs at 5 and 7 dpi (not shown), compared with healthy plant extracts. A small amount of PMV and SPMV was detected on the sucrose density gradients at 7 dpi. By 10 dpi, both PMV and PMV+SPMV infections resulted in similarly diminished levels of host rRNAs on systemically infected leaves when compared with the healthy rRNA profiles (Fig. 3A). The sucrose density gradient profiles also clearly show a small, yet consistent increase of PMV RNA, approximately two- to three-fold, in association with SPMV vs the single infection (Fig. 3A). Total RNA was also extracted from plants for RNA blot analyses (Turina et al. 1998). The RNA blots confirmed that the PMV and SPMV RNAs defined in the sucrose density gradients are found in the upper uninoculated leaves (Fig. 3B).

These experiments suggest that the effect of SPMV shares classic features associated with a plant virus synergism, including increased virus titer, exacerbated symptoms, and more rapid systemic spread of the virus complex (Goldberg and

Brakke 1987; Pruss et al. 1997; Rochow and Ross 1955; Scheets 1998; Vance 1991; Vance et al. 1995). The potyvirus-induced synergism is associated with the HC-Pro domain, which perturbs the normal regulation of minus-strand RNA accumulation of the second virus (Pruss et al. 1997; Vance 1991). The unveiling of mechanisms associated with the up-regulation of PMV by SPMV will be necessary to determine if it mimics the well-characterized, potyvirus-associated synergism. However, SPMV has some obvious differences from potyviruses and the ST9-associated RNA in that the satellite virus is completely dependent on PMV for both its replication and movement. In fact, one might expect SPMV to suppress

Fig. 1. Pearl millet plants infected with panicum mosaic virus (PMV) or both PMV and satellite PMV (SPMV) at 30 days post inoculation (dpi). Millet plants (A) and panicles (B) infected with PMV (center) or PMV+SPMV (right) at 30 dpi. Uninoculated controls are leftmost plant (A) and panicles (B).

the helper virus titer and symptoms, as is observed with defective interfering RNAs and some satellite viruses (Roossinck et al. 1992; Scholthof et al. 1995b). Instead, a slight increase of at least two helper virus proteins, p8 and capsid protein, and the PMV RNA (Figs. 2 and 3) as well as more rapidly progressing disease are observed in PMV+SPMV infected millet plants (Fig. 1). Genetic and mutational analyses of PMV and SPMV are needed to elucidate

the mechanisms that control this unique, satellite virus-induced, synergistic interaction. Recently, it has been shown that 34 nt from RNA-2 of red clover necrotic mosaic dianthovirus (RCNMV) can transactivate the accumulation of the RNA-1 subgenomic RNA (Sit et al. 1998). It is possible that SPMV RNA has a role in regulating the accumulation of the proteins encoded from the PMV subgenomic RNA, particularly in consideration of the early and more rapid movement in

Fig. 2. Detection of panicum mosaic virus (PMV) capsid protein (CP) and p8 protein and satellite PMV (SPMV) CP in pearl millet plants. Leaves inoculated with PMV or PMV+SPMV were assayed at 3 to 5 days post inoculation (dpi) and upper uninoculated leaves were collected at 3 to 7 and 12 dpi. Total proteins were extracted, disrupted in 2% sodium dodecyl sulfate (SDS), and electrophoresed through 12.5% SDS-polyacrylamide gels with either Tris-glycine (for CP) or Tris-tricine (for p8) buffer systems as described previously (Turina et al. 1998). Proteins were transferred to nitrocellulose membranes, blocked with nonfat dry milk, and incubated with the primary antibody overnight. Secondary antibody (goat anti-rabbit HRP) was added for 2 to 4 h followed by addition of a chemiluminescent substrate and visualization on X-ray film. Indicated above the panels: the dpi, inoculated (I) and upper uninoculated (U) leaves, and inoculation with PMV (P) or PMV+SPMV (S). Antibodies used for specific detection of PMV CP, PMV p8, and SPMV CP are noted to the right of each panel. Asterisk (*) above p8 protein reflects either a protein dimer of p8 or the putative PMV p8-FS product of approximately 14.6 kDa (Turina et al. 1998).

Fig. 3. Sucrose density gradients and RNA blot analyses of total RNA extracts from pearl millet plants 10 days post inoculation (dpi) with panicum mosaic virus (PMV) or both PMV and satellite PMV (SPMV). **A**, Absorbance scans (254 nm) of 7.5 to 30% sucrose density gradients in NaGPS buffer (0.05 M Na_2HPO_4 , 0.3 M NaCl, 0.1 M glycine, and 1 mM EDTA, pH 9.4) following centrifugation at 54,000 rpm for 3 h at 15 C in an SW60 rotor (Beckman, Fullerton, CA) (Goldberg and Brakke 1987). Total RNA from systemically infected and healthy millet leaves was extracted in 0.1 M $(\text{NH}_4)_2\text{CO}_3$, 0.3 M NH_4Cl , 1 mM EDTA, and 1% sodium dodecyl sulfate, pH 9.4 (Goldberg and Brakke 1987). Peak 1 represents 16 S and 18 S rRNAs; peak 2, the 23 S rRNA; peak 3, the 28 S rRNA. Peaks P and S represent PMV and SPMV RNAs, respectively. **B**, RNA blots of inoculated (I) and upper uninoculated (U) millet leaves infected with PMV (P) or PMV+SPMV (S). Total RNA was extracted from plants at 10 dpi and electrophoresed through duplicate 1% agarose gels prior to transfer to nylon membrane. The membranes were incubated overnight with ^{32}P -labeled probes specific to the pPMV85 or pSPMV1 full-length cDNA clones and autoradiographed at -80°C for 4 h. The origin of the RNA band (denoted by asterisk) running slightly ahead of the 826 nucleotide SPMV RNA is unknown. Location of PMV and SPMV RNA is indicated on right side of panel.

association with the accumulation of p8, a putative PMV movement protein (Fig. 2). Alternatively, or additionally, the SPMV capsid protein may be directly involved in exacerbation of symptom development, possibly akin to the effects of the gene VI protein of caulimoviruses (Goldberg et al. 1991) or the p19 protein of tomato bushy stunt virus (Scholthof et al. 1995a).

Two possible mechanisms have been proposed to understand plant virus synergisms (Goldberg and Brakke 1987; Rochow and Ross 1955). A synergism may induce a higher concentration of virus in each cell, possibly due to a perturbation of normal cellular regulation. Within this framework, it is tempting to speculate that SPMV may suppress or interfere with host defense responses, as recently described for the potyvirus system (Anandalakshmi et al. 1998). Alternatively, more cells may be infected, which suggests that the synergism facilitates cell-to-cell movement or perhaps "relaxes" the cell-type specificity. Evaluation of the data associated with the accumulation of the helper and satellite virus capsid proteins and PMV p8 suggests that more rapid cell-to-cell spread of PMV may result in a greater percentage of cells infected in the upper uninoculated leaves, and an increase in titer as the infection progresses (Figs. 2 and 3). The rRNA profiles indicate that the PMV and PMV+SPMV infections have similar effects on the host chloroplast and cytoplasmic ribosomes. Therefore, the severe systemic symptoms associated with SPMV appear to be due to yet undetermined effects on the host cell that apparently do not differentially modify the rRNA profiles. Elucidation of the means by which SPMV affects both the helper virus and the host plant may provide us with new strategies to control an economically important disease complex associated with *St. Augustinegrass* and other monocotyledonous host plants.

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