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Research Note

Satellite RNA of Barley Yellow Dwarf-RPV Virus Reduces Accumulation of RPV Helper Virus RNA and Attenuates RPV Symptoms in Oats

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Infectious transcripts of satellite (satRPV) RNA of RPV barley yellow dwarf luteovirus were transmitted to oat plants by aphids (*Rhopalosiphum padi*) that acquired virus from satellite-infected oat protoplasts. Plants infected with RPV and satRPV RNA had milder symptoms than those infected with RPV helper virus alone. Presence of satRPV RNA reduced RPV helper RNA accumulation in plants and protoplasts. SatRPV RNA did not affect symptoms caused by the severe mixed infection of RPV and PAV barley yellow dwarf viruses and had no effect on PAV RNA accumulation.

Additional keywords: encapsidation, RNA replication.

Barley yellow dwarf luteovirus (BYDV) infection results in stunting, yellowing, or reddening of leaves, delayed or no heading and marked increase in sterile florets in its hosts (Gramineae). BYDVs are transmitted to plants only by aphid transmission of virions (Power and Gray 1995). BYDVs have been divided into two subgroups (D'Arcy 1986; Mayo and Ziegler-Graff 1996; Miller et al. 1995). Subgroup I includes the PAV and MAV serotypes; subgroup II includes RPV, SGV, and RMV. RPV and PAV, subjects of this report, are so different from each other that they are considered to be separate viruses (D'Arcy 1986; Miller 1994) and we refer to them as such.

Satellite RNAs (satRNAs) are subviral RNAs that depend for their productive replication on coinfection of a host cell with a helper virus (Mayo et al. 1995). They are encapsidated in helper virus virions and have no sequence similarity to the helper or host. SatRNAs can attenuate or exacerbate symptoms, or produce new symptoms that are not caused by the helper virus alone (Roossinck et al. 1992; Collmer and Howell 1992). A small molecular weight RNA (satRPV RNA, formerly sBYDV RNA) associated with an Australian RPV-like isolate was shown to have all the properties of satellite RNA in oat protoplasts (Silver et al. 1994). SatRPV RNA is 322-nt long with no significant sequence homology to its helper virus (Miller et al. 1991). Circular and multimeric forms capable of self-cleavage at hammerhead ribozyme structures were identi-

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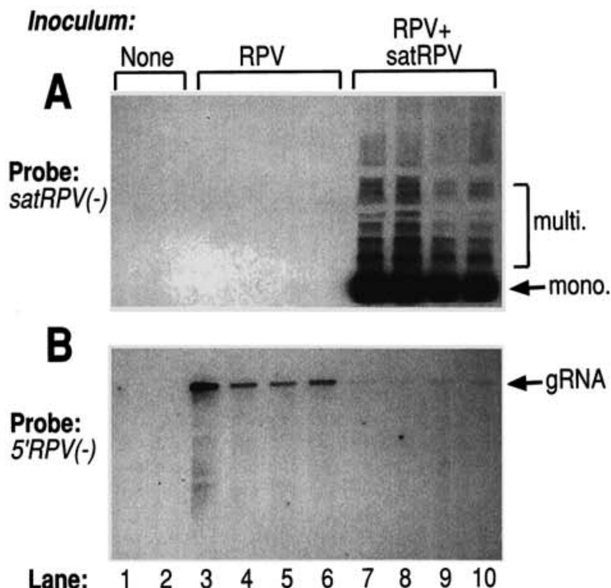

Fig. 1. Northern blot analysis of total RNA extracted from oat plants inoculated with *Rhopalosiphum padi* that had acquired virus from protoplast extracts infected with the indicated inoculum. Total RNA was isolated in quadruplicate from pooled tissue of 15 plants 2 weeks after inoculation by the procedure of Wadsworth et al. (1988) as modified by Dinesh-Kumar and Miller (1993). Equal amounts of RNA, verified by ethidium bromide staining of ribosomal RNAs, were loaded on each lane. RNA was fractionated on a 1% denaturing agarose gel, blotted to nylon membrane and hybridized with the indicated probes as described in Mohan et al. (1995). ^{32}P-labeled RNA probes were synthesized by in vitro transcription as described by Promega (Madison, WI) using [α ^{32}P]-CTP. Antisense satRPV RNA probe was transcribed with SP6 RNA polymerase from pT7Sat linearized with *Hin*dIII. Antisense 5′RPV probe, complementary to bases 809 to 1191, was synthesized by SP6 RNA polymerase transcription of *Apa*I-cut plasmid pML5 (Silver et al. 1994). Mobilities of satRPV RNA monomers (mono., 322 nt), multimers (multi.), and RPV genomic RNA (gRNA, 5,723 nt) are indicated at right. Equal amounts of probe (5×10^5 cpm/ml) were used in each hybridization. One blot was used, stripped and re-probed. Exposures were at −80°C with one intensifying screen for 1 h (panel A) and 72 h (panel B). Radioactive bands were detected using a Phosphorimager model 400E and quantified with Imagequant 3.3 software (Molecular Dynamics, Sunnyvale, CA).

fied in both strands (Miller and Silver 1991), indicating that it replicates by a symmetrical rolling circle mechanism (Silver et al. 1994). Full-length satRPV RNA transcripts from a cDNA clone were infectious in oat protoplasts in the presence of RPV helper virus (Silver et al. 1994). In contrast, the PAV BYDV is unable to support satRPV RNA replication (Silver et al. 1994). One of the most obvious unanswered questions about the satellite has been its effect on disease symptoms in plants. Here, we deliver satRPV RNA to whole plants and observe the effect on helper virus replication and disease symptoms.

Although luteoviruses are obligately aphid transmitted, it was conceivable that satRPV RNA could be mechanically transmissible because similar satRNAs such as satRNA of tobacco ringspot virus are extremely infectious in plants (Gerlach et al. 1986) and an RNA that is normally dependent on a luteovirus helper, beet western yellows ST9-associated RNA, is mechanically transmissible (Passmore et al. 1993). To attempt mechanical transmission, 1 week following aphid-inoculation of oats with an RPV+PAV mixture, 1 μg of linear, monomeric satRPV RNA transcript was rubbed on the first leaves with RNase-free abrasive. This transcript was derived from permuted dimeric cDNA clone pT7Sat that was linearized with *Eco*RI prior to transcription with T7 RNA polymerase (T7 Megascript kit, Ambion, Austin, TX). The monomeric self-cleavage product (322 nt) that arose during transcription of the permuted, dimeric RNA was gel purified, prior to inoculation (Silver et al. 1994). Plasmid pT7Sat is the same as pFL-WT (Silver et al. 1994) but has the satRPV RNA sequence inverted relative to the promoters. Satellite-free helper virus isolates used were NY-RPV (from Stewart Gray, USDA/ARS, Cornell University), and IL-PAV (from Anna Hewings, formerly USDA/ARS, University of Illinois). Despite several attempts, no replication of satRPV RNA as indicated by accumulation of abundant multimeric or circular forms, that are formed during the rolling circle replication, was detected (data not shown) in mechanically inoculated plants.

We next attempted to transmit satRPV RNA to plants by feeding aphids on partially purified virus extracts from infected protoplasts. Protoplasts were isolated from oat (*Avena sativa* cv. Stout) suspension culture (cell line S226 obtained from Howard Rines, USDA/ARS, University of Minnesota) and electroporated with 100 ng of genomic RNA extracted from RPV virions and 50 ng of gel-purified, monomeric satRPV RNA transcript as described by Silver et al. (1994). Virus was partially purified from infected protoplasts (Wang et al. 1995) with the following modifications. Infected protoplasts were pelleted 72 h after inoculation by centrifugation at $100 \times g$ for 5 min. The pellet was resuspended in 100 μl of 10 mM phosphate buffer, pH 7.0, sonicated for 15 s and centrifuged at $7,826 \times g$ for 5 min. The supernatant contained par-

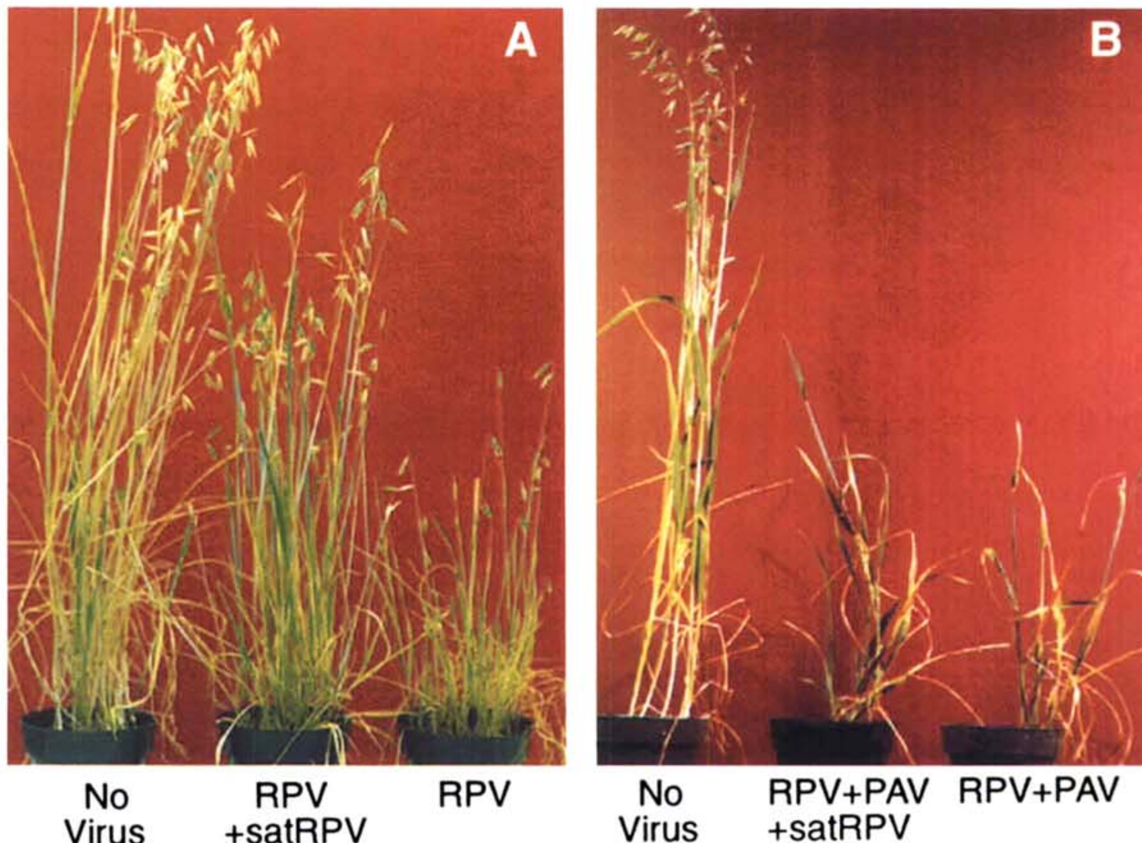

Fig. 2. Oat plants (Clintland 64) 8 weeks after aphid inoculation with the indicated combinations of virus and satRPV RNA. Plants per pot: **A,** 7-8; **B,** 4-5.

tially purified virions and was designated "protoplast extract." Aphids (*Rhopalosiphum padi*) were maintained on RPV-infected, satellite-free plants prior to feeding on extracts from protoplasts through Parafilm membranes. Aphid acquisition of virus through membranes and inoculation of plants was as described by Wang et al. (1995). Plants were then grown with 16 h days, at 21°C. Plants were assayed for viral RNA and coat protein accumulation by Northern hybridization and ELISA (Converse and Martin 1990), respectively, using RPV-specific antibodies (a gift from S. Gray, Cornell University).

Aphid transmission from protoplasts to plants was successful, as indicated by accumulation of abundant monomeric and multimeric forms of satRPV RNA in oat leaves (Fig. 1A). Plants infected with satRPV RNA accumulated much less genomic RNA than those infected with RPV alone (Fig. 1B, compare lanes 3 to 6 and 7 to 10). The amount of RPV genomic RNA was reduced by 87 ± 3% in the presence of satRPV RNA. The severity of symptoms induced by RPV in oats was also reduced strikingly (Fig. 2A). Compared to plants infected with RPV alone, those which also contained satRPV RNA (i) showed less stunting, (ii) headed 1 week earlier (but 1 week later than healthy plants), (iii) produced three times as many seeds, and (iv) yielded 34.8 g of total leaf tissue per 10 plants compared to 16.3 g from plants infected with RPV alone and 58.5 g from uninoculated plants. The differences between plants infected with RPV alone and those infected with RPV and satRPV RNA were consistent in three passages and especially apparent late in infection. SatRPV RNA reduced accumulation of RPV coat protein only marginally (17 ± 6% reduction in A_{405}). Thus satRPV RNA decreases accumulation of helper virus genomic RNA substantially more than the coat protein in oat plants and it attenuates disease symptoms caused by its helper virus.

SatRPV RNA may reduce RPV helper RNA accumulation in plants by affecting intracellular levels directly, or by reducing the ability to spread through the plant. To distinguish between these possibilities, we examined the effect of satRPV RNA on RPV helper RNA accumulation in protoplasts. In the presence of pure RPV RNA helper, satRPV RNA replicated to high levels by 72 hpi (Fig. 3A). In three independent samples, the amounts of RPV genomic RNA was reduced by 68 ± 4% in the presence of satRPV RNA (Fig. 3B, compare lanes 1 to 3 and 4 to 6). Therefore, satRPV RNA decreases the helper viral RNA accumulation at the cellular as well as the whole plant level.

Reduction of helper virus RNA accumulation in plants infected with RPV probably explains the reduced severity of symptoms caused in the presence of satRPV RNA. Concomitant reduction of helper virus RNA levels and disease symptoms has been observed for many other satellite RNAs (Collmer and Howell 1992). We propose that satRPV RNA

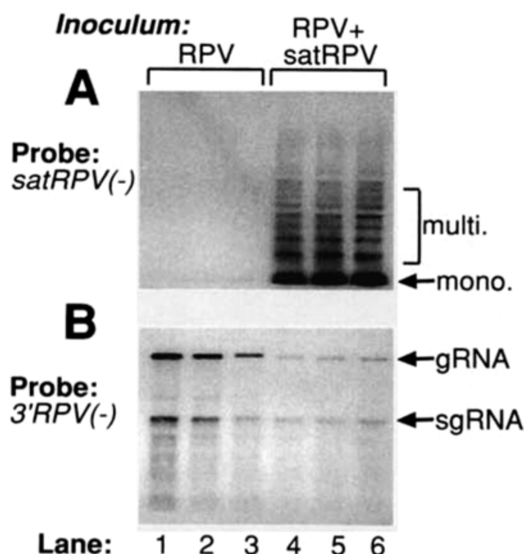

Fig. 3. Northern blot analysis of total RNA extracted from oat protoplasts 72 h postinoculation with the indicated inoculum. Each lane represents an equal amount of RNA isolated from an independent sample (~300,000 protoplasts per sample). Antisense satRPV RNA probe was the same as in Fig. 1. Antisense 3′RPV probe, comprising nucleotides 5026 to the end of the RPV genome, was transcribed with SP6 RNA polymerase from *Eco*RI-linearized pMB102. Mobilities of satRPV RNA monomers (mono., 322 nt), multimers (multi.), and RPV genomic (gRNA, 5,723 nt) and subgenomic (sgRNA, approximately 2.6 kb) RNAs are indicated at right. Equal amounts of probe were used in each hybridization. One blot was used, stripped and re-probed. Exposures were at −80°C with one intensifying screen for 2 h (**A**) and 6 h (**B**). Radioactive bands were detected using a Phosphorimager model 400E and quantified with Imagequant 3.3 software (Molecular Dynamics, Sunnyvale, CA).

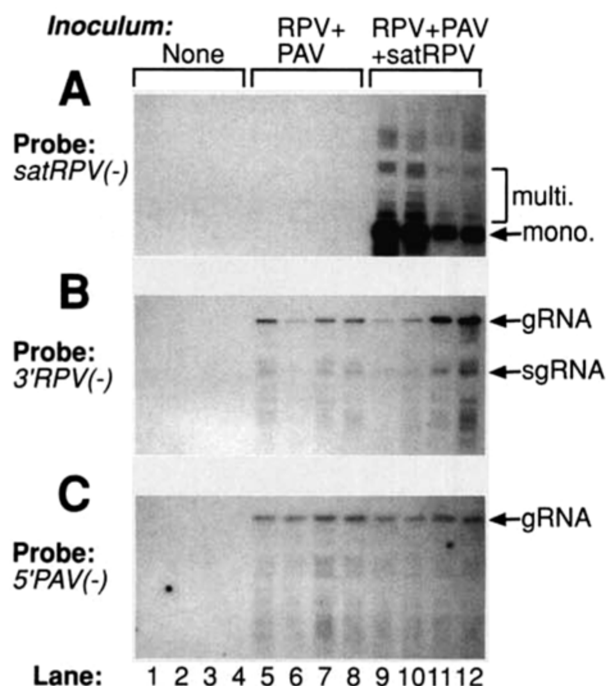

Fig. 4. Northern blot analysis of total RNA extracted from oat plants inoculated with *Rhopalosiphum padi* that had fed on protoplast extract infected with the indicated inoculum. Total RNA was isolated from second leaves of four individual plants 2 weeks after inoculation. Equal amounts of RNA were loaded per lane. RNA was fractionated on a 1% denaturing agarose gel, blotted to nylon membrane and hybridized with the indicated probes. Antisense satRPV RNA and 3′RPV probes were the same as described in Figs. 1 and 3. Antisense 5′PAV probe was transcribed with T7 RNA polymerase from plasmid pPAV1-1 (Mohan et al. 1995) linearized with *Eco*RI. Equal amounts of probe were used in each hybridization. One blot was used, stripped and reprobed. Mobilities of satRPV RNA monomers (mono., 322 nt), multimers (multi.), RPV genomic (gRNA, 5,723 nt) and subgenomic (sgRNA, approx 2.6 kb) RNAs and PAV genomic RNA (gRNA, 5,677 nt) are indicated at right. Blots were exposed at -80°C with one intensifying screen for 3 h (panel A), 18 h (panel B), and 24 h (panel C).

reduced RPV genomic RNA levels by competing for the replicase or encapsidation. RPV coat protein encapsidates PAV RNA, but not vice versa (Wen and Lister 1991), so it may be less discriminatory against non-RPV RNAs. SatRPV RNA that replicates to much higher levels than its helper RNA could thus be more likely to be encapsidated than genomic RPV RNA. The small reduction in coat protein accumulation relative to the larger reduction in RPV genomic RNA in satRPV RNA-infected plants is consistent with this mechanism. Lack of encapsidation reduces luteovirus genomic RNA accumulation considerably but subgenomic RNA only slightly (Mohan et al. 1995; Reutenaur et al. 1993). Similar preferential reduction of RPV genomic relative to subgenomic RNAs is caused by satRPV RNA (Fig. 3). The results also indicate that RPV coat protein is not the major symptom determinant because symptom amelioration in the presence of satRPV RNA was correlated with reduced RPV RNA levels but not coat protein levels as measured by ELISA. Competition for components involved in cell-to-cell or long-distance movement is possible in plants, but cannot be the sole explanation, because satRPV RNA reduces helper virus RNA accumulation in protoplasts in which no such movement is likely.

RPV often exists in a mixture with PAV both in nature and the laboratory. Because the mixed infection results in more severe symptoms than infection with RPV alone, we tested the symptom-modulating properties of satRPV RNA in oats infected with RPV in the presence of the PAV virus. Initially, satRPV RNA could not be transmitted from protoplasts infected with the mixture of RPV + PAV and satRPV RNAs to plants by aphids. Very little, if any, encapsidated RPV genomic RNA was detected (data not shown) so the amount of encapsidated helper RNA was increased by spiking the protoplast extract with satellite-free RPV virus to the final concentration of 30 μg/ml. Following aphid acquisition and transmission of this spiked extract, satRPV RNA replicated in second leaves of all four inoculated plants (Fig. 4A). This shows that helper virus titer was a limiting factor for aphid transmission, consistent with observations of Periera et al. (1989). Furthermore, the helper virus genome need not be in the same particle as satRNA. Preferential encapsidation has been observed for other satRNAs (Buzayan et al. 1986; Randles et al. 1981) but it was not reported to affect the efficiency of transmission.

Oats infected with satRPV RNA in the presence of RPV+PAV mixture showed typical BYD symptoms (Fig. 2B). We observed no significant difference in symptoms or virion concentration between plants infected with RPV + PAV alone and those infected with RPV + PAV and satRPV RNA (Fig. 2B) in three passages. There was plant-to-plant variation in levels of RPV genomic and satRPV RNAs between plant samples (each lane in Fig. 4A and B) probably due to chance in sampling. In contrast, PAV appeared to be distributed more uniformly and was unaffected by the presence of satRPV RNA. In all samples, satRPV RNA accumulated to much higher levels than either RPV or PAV RNAs (note differences in exposure times between panels in Fig. 4). Mixed infections with RPV and PAV can cause more severe symptoms than RPV alone and this PAV isolate alone causes more severe symptoms than RPV in oats (Martin and D'Arcy 1995). We surmise that the effect of satRPV RNA in the mixed infection was masked by PAV. We also conclude that symptom reduction in the absence of PAV must be attributable to diminished RPV RNA levels, rather than to high accumulation of satRPV RNA per se. Thus, satRPV RNA does not attenuate symptoms by inducing a nonspecific host defense response.

Finally, we must bear in mind that these experiments used only one set of growth conditions and one host cultivar. Variation of environmental conditions has been shown to affect symptom modulation by satCMV RNA (Kaper et al. 1995; White et al. 1995). SatRNA symptom modulation involves a complex interaction among helper virus, satellite, and host plant (Roossinck et al. 1992). The effects of satRPV RNA on different helpers, hosts and in different environmental conditions, remain to be determined.

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