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Beet western yellows virus-associated RNA: An independently replicating RNA that stimulates virus accumulation

(associated RNA replication/genomic RNA/RNA encapsidation/satellite RNA)

BONI K. PASSMORE, MARGARET SANGER, LIH-SHEN CHIN, BRYCE W. FALK*, AND GEORGE BRUENING

Department of Plant Pathology, College of Agricultural and Environmental Sciences, University of California, Davis, CA 95616

Contributed by George Bruening, August 2, 1993

ABSTRACT **Infections of plants by subviral RNA agents, alone or in association with virus genomic RNA molecules, are well known. The ST9 strain of beet western yellows virus encapsidates not only the 5.6-kilobase genomic RNA that is typical of luteoviruses, but also a 2.8-kilobase-associated RNA that has a distinct nucleotide sequence. The ST9-associated RNA has been postulated to be a satellite RNA, which by definition would be capable of replicating only in coinfections with beet western yellows virus or closely related viruses. To characterize the associated RNA, we inoculated protoplasts and leaves with *in vitro* transcripts of the virus genomic RNA and the ST9-associated RNA separately and in combination. Surprisingly, the ST9-associated RNA alone replicated efficiently in both protoplasts and leaves, and it stimulated accumulation of the virus genomic RNA in protoplasts. Thus, the ST9-associated RNA is a newly discovered type of plant infectious agent, which depends on its associated virus, beet western yellows virus, for encapsidation but not for replication.**

The plant virus infection cycle requires that the virus genome, once introduced into the host cell, must replicate, move from the initially infected cell to other cells, and, ultimately, gain access to a new host plant. Virus genomes encode proteins required for replication, encapsidation, movement in the plant, and other functions. One group of subviral RNA agents, the viroids, are independently replicating and complete their infection cycle without generating a capsid or other proteins. A variety of other subviral RNA agents (Table 1) lack one or more critical functions necessary for completion of the infection cycle and apparently have evolved in association with a virus that supplies the necessary functions. Often the association results in a decrease of virus titer. We investigate here a system in which a subviral, virus-associated RNA increases the titer of the corresponding virus, at least in protoplasts and probably in infected plants as well.

The name luteovirus is derived from the Latin root for yellow, reflecting the tendency of members of the group to induce yellowing symptoms. Luteoviruses such as barley yellow dwarf virus, beet western yellows virus (BWYV), carrot red leaf virus, legume yellows virus, and soybean dwarf virus infect and cause significant losses to all of the major groups of food crops. BWYV and other luteoviruses are characterized by obligate aphid transmission to host plants, by limitation of infection to the phloem tissue, and by low titer, presumably as a consequence of phloem limitation (5–7). Like other luteoviruses, BWYV has a single genomic RNA (Fig. 1*a*) of ≈6000 nt, with 6 open reading frames (ORFs). Virions have isometric capsids, probably composed of 180 coat protein molecules as is characteristic of a $T = 3$ structure. The bulk of the coat protein is translated from ORF-3, but a few coat protein molecules result from readthrough of the ORF-3 termination codon (Fig. 1*a*) to give an ORF-3/5 fusion (6).

The ST9 strain of BWYV is unique among BWYV strains because virion RNA preparations contain not only the genomic RNA (ST9gRNA) but also an associated RNA (ST9aRNA) (11). ST9aRNA (Fig. 1*b*), at 2843 nt, is about half the size of the ST9gRNA (Fig. 1*a*) and has a distinct nucleotide sequence (9). Plants infected by ST9 BWYV exhibit a more severe symptom phenotype and contain ≈10-fold more virions per g of tissue than do plants infected by common BWYV isolates (11). The ST9aRNA nucleotide sequence contains three large ORFs, which, at least *in vitro*, can be translated to yield products of ≈85 kDa, corresponding to ORFs-1, -2, plus -3, as well as products with sizes corresponding to ORF-1, ORFs-2, plus -3, and ORF-3 but not ORF-2 alone. The deduced amino acid sequence of two regions of the 85-kDa protein contain significant homology with RNA-dependent RNA polymerases of carmoviruses (9), including the GDD sequence common to most such enzymes. ST9aRNA has some of the characteristics of a satellite RNA (Table 1). Specifically, ST9aRNA is encapsidated in ST9 BWYV coat protein and lacks sequence similarity with ST9gRNA. However, a satellite RNA is capable of replicating only in cells that are infected by any one of a few, closely related "supporting" or "helper" viruses. Results presented here suggest that ST9aRNA, although it has an intimate biological relationship with its associated virus, is not a satellite RNA. The ST9aRNA appears to be a different type of plant infectious agent that depends on BWYV for encapsidation but not for replication.

MATERIALS AND METHODS

Construction of Full-Length cDNA Clones. We constructed plasmid pBW7120-7 from pBW7120 (12), a cDNA clone encoding the entire ST9gRNA sequence except for the 5′-most 13 nt. Two micrograms of RNA extracted from ST9 BWYV virions was used as template for first-strand cDNA synthesis in 20 μl of 50 mM KCl/10 mM Tris·HCl/1.5 mM $MgCl_2$/0.5 mM each dNTP, pH 8.3. One microgram of the oligodeoxyribonucleotide BW-2, complementary to nt 82–101 of ST9gRNA, and 20 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL) were added to initiate the reaction. Incubation was for 1 hr at 45°C. The resulting cDNA was the template for a PCR (13) using primers BW-2 and BW-3, the latter having a *Pst* I restriction site followed by a bacteriophage T3 promoter and 14 nt of ST9gRNA 5′ sequence. The 130-base-pair DNA product was digested with *Pst* I and *Xba* I, the site for the latter being located at nt 64 in ST9gRNA, and was inserted

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Abbreviations: BWYV, beet western yellows virus; ORF, open reading frame.

*To whom reprint requests should be addressed.

Table 1. Subviral RNA agents that are associated with plant viruses

RNA type	Example	Independent replication*	Sequence similarity†	Protein synthesis‡	Virus titer§
Small satellite RNA	Satellite tobacco ring-spot virus RNA (1)	No	No	Generally no	Generally reduction
Large satellite RNA	Satellite tomato black-ring virus RNA (2)	No	No	Noncoat protein	Generally reduction
Defective-interfering RNA	Defective-interfering RNA of tomato bushy stunt virus (3)	No	Yes	Generally no	Generally reduction
Satellite virus genomic RNA	Genomic RNA of the satellite virus of tobacco necrosis virus (4)	No	No	Coat protein	Generally reduction
Coat-dependent RNA replicon	ST9aRNA	Yes	No	Yes	Increase

*Yes, agent replicates independently of the associated virus.
†Yes, virus-associated RNA and virus genomic RNA have extensive similarity of nucleotide sequence.
‡Indication of whether the virus-associated RNA is a mRNA.
§Indication of effect on the virus titer of coinoculation of the associated RNA with the virus, the reference point being virus inoculated alone.

into similarly cut pBW7120 to yield pBW7120-7, which has a full-length insert.

Plasmid cDNA clone pST9106 has as insert the ST9aRNA sequence except for 13 nt at the 5′ end and 10 nt at the 3′ end (9). Plasmid pST9106-8 was constructed by a PCR (13) with pST9106 used as the template. The first of the two primers has a *Sal* I restriction site, a bacteriophage T3 promoter, and 20 nt of ST9aRNA 5′ sequence. The second primer is complementary to the 3′ 20 nt of ST9aRNA and incorporates *Sma* I and *Sph* I sites. The PCR product was digested with *Sph* I and *Sal* I and was inserted into a similarly digested plasmid pSport1 (Bethesda Research Laboratories).

RNA Inocula. Virion RNA inoculum was prepared by extraction of purified ST9 BWYV virions according to the method of Falk *et al.* (11). Transcripts of restriction endonuclease *Not* I-linearized pBW7120-7 and *Sma* I-linearized pST9106-8, in the presence or absence of cap analogue $m^7G(5')ppp(5')G$ (New England Biolabs), were synthesized by the action of bacteriophage T3 RNA polymerase as described (14). To generate complementary ST9aRNA, *Sal* I-cut pST9106-8 was transcribed by bacteriophage SP6 RNA polymerase. The full-length transcript of *Not* I-linearized pBW7120-7, tST9gRNA, is expected to be flanked at the 3′ end by 6 nt of plasmid sequence. The transcript of *Sma* I-linearized pST9106-8, tST9aRNA, is expected to have the full ST9aRNA sequence, with no flanking, nonviral nucleotides. Concentrations of transcripts were estimated by comparison with standards after agarose gel electrophoresis and ethidium bromide staining. tST9a/xRNA is a tST9aRNA-like molecule with 62 nt of plasmid-derived 5′ flanking sequence and a 730-nt truncation of the 3′ end. It was transcribed by bacteriophage T7 RNA polymerase from plasmid pST9106-8 that had been linearized with *Xho* I.

Electroporation of Protoplasts. Cultured tobacco (*Nicotiana tabacum* cv. Xanthi nc) suspension cells were maintained on a rotary shaker at 125 rpm and 28°C in Murashige and Skoog minimal organics medium (GIBCO/BRL) supplemented with 0.9 μM 2,4-dichlorophenoxyacetic acid, 0.4 μM kinetin, and 1.5 μM thiamine hydrochloride and adjusted to pH 5.8. To obtain protoplasts, cells were harvested by centrifugation at ≈400 × *g* for 4 min and were dispersed in the original volume of 5 mM $CaCl_2$/12 mM sodium acetate/425 mM mannitol, pH 5.8 (wash solution), and were centrifuged again. Approximately 8 ml of packed cells from 100–150 ml of culture was suspended in 100 ml of solution containing 1% Cellulysin (Calbiochem), 0.6% cellulase RS (Yakult Honsha, Tokyo), 0.4% macerase (Yakult), 5 mM $CaCl_2$, 12 mM sodium acetate, and 362 mM mannitol adjusted to pH 5.8. The suspension was placed on a rotary shaker (40 rpm) at ≈25°C for 5–6 hr. Protoplasts were separated from debris by

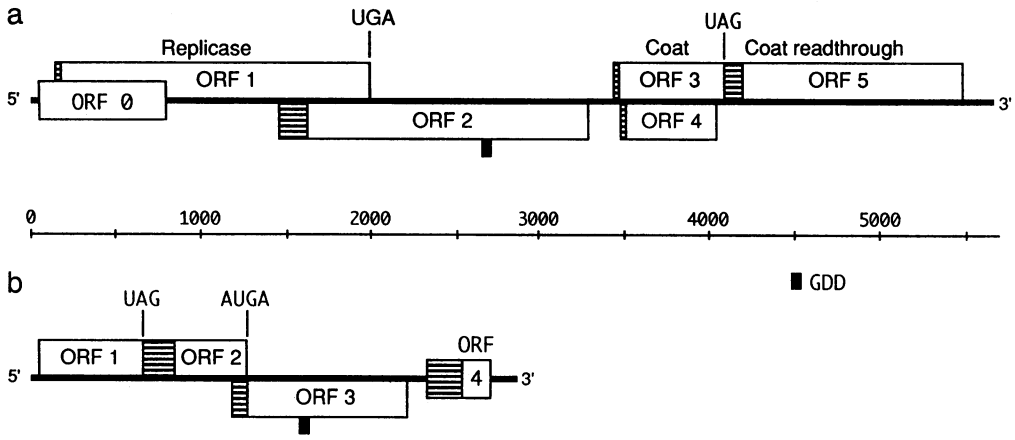

FIG. 1. Numbered ORFs of the 5641-nt genomic RNA of BWYV strain FL1 [FL1gRNA (8)] (*a*) and of the 2843-nt associated RNA of the ST9 strain of BWYV [ST9aRNA (9)] (*b*). Upper, center, and lower positionings of open rectangles relative to the central heavy line indicate the three reading frame registers of ORFs in the single-stranded, encapsidated, positive-polarity RNA. For all but the 5′-most ORF, horizontally hatched rectangles define the extent to which in-register nonstop codons are located to the 5′ side of the AUG start codon. Those stop codons at which a frameshift or a readthrough of translation is suspected (8, 9) are indicated by a stop codon designated above the diagram, including an AUGA overlapping start codon and stop codon. Small solid rectangles locate the GDD amino acid sequence that is characteristic of RNA-dependent polymerases (10), and a nucleotide scale lies between *a* and *b*.

filtration through 60-μm nylon mesh and then centrifuged and washed three times in 30 ml of wash solution.

Electroporation was by procedures modified from Fromm *et al.* (15) using 1–2 × 10^6 protoplasts suspended in 0.7 ml of electroporation buffer (10 mM NaHepes/120 mM KCl/10 mM NaCl/4 mM $CaCl_2$/200 mM mannitol, pH 7.1). The suspension was combined immediately with 100 μl of electroporation buffer containing the indicated amounts of RNA from virions or the transcription reactions and 300 μg of sheared salmon sperm DNA. Electroporation was at 4°C in cuvettes (Bio-Rad) with 4-mm-spaced aluminum electrodes, using a Hoefer Progenitor II apparatus set at 490 μF and 330 V and with a discharge pulse of 8 msec. Five minutes after electroporation, the protoplasts were diluted into 9.2 ml of protoplast culture medium in a 100-mm Petri plate and were incubated at 25°C. At the indicated times, 1-ml aliquots were removed, and cells were collected by centrifugation, quick frozen in dry ice/ethanol, and stored at −70°C. Protoplast culture medium (pH 5.8) was composed of 0.75 vol of fresh cell culture medium, 0.05 vol of coconut water (GIBCO/BRL), mannitol to a final concentration of 260 mM, and supernatant from centrifuged tobacco cell culture (conditioned culture medium) to volume.

Purification and Analysis of Cell RNA. Frozen cells derived from 1 ml of protoplast culture were suspended in 400 μl of 50 mM Tris·HCl, pH 7.4/1 mM EDTA (TE buffer) containing 10 mg of SDS per ml. Proteinase K was added to a final concentration of 100 μg/ml. The mixture was incubated at 37°C for 30 min followed by extraction with 400 μl of TE-equilibrated phenol. After centrifugation, the aqueous phase was removed, and the nucleic acids were precipitated by the addition of 40 μl of 3 M sodium acetate and 1 ml of ethanol. The precipitated nucleic acids were collected by centrifugation, washed once with 70% ethanol, dried, and suspended in 50 μl of diethylpyrocarbonate-treated water. Three-microliter aliquots were diluted to 12 μl with a solution of 60% dimethyl sulfoxide, 9.5% deionized glyoxal, 1.67 mM Na_2EDTA, 33 mM Hepes (pH 7.0) and were incubated at 55°C for 1 hr.

Samples were analyzed by electrophoresis through 1.0% agarose gels in 1× Hepes buffer (20 mM NaHepes/1 mM Na_2EDTA, pH 7.0) at 10 V/cm. The gels were blotted to Hybond N^+ (Amersham) nylon membrane with 50 mM NaOH for 3–4 hr, rinsed in 6× SSC (1× SSC is 0.15 M sodium chloride/15 mM citric acid, pH 7), and air dried. A probe complementary to the 3′ 1000 nt of ST9gRNA and another probe complementary to the full length of ST9aRNA were prepared by *in vitro* transcription with bacteriophage SP6 RNA polymerase of *Eco*RI-cut pBW7120-7 and *Sal* I-cut pST9106-8, respectively. Nylon membranes were subject to hybridization conditions at 55°C, washed according to the methods of Amasino (16), air dried, sealed in plastic bags, and exposed to Kodak XAR film.

Inoculation of Shepherd's Purse Seedlings and Extraction of Leaf RNA. For inocula applied to shepherd's purse (*Capsella bursa-pastoris*) seedlings, all transcription reaction mixtures were treated with RQ1 DNase (Promega) at ≈1 unit per μg of DNA and were extracted with phenol and chloroform. Nucleic acids were precipitated by the addition of 0.1 vol of 3 M sodium acetate and ethanol to 70%. Dried RNA was resuspended in 12 mM Na_2HPO_4/8 mM KH_2PO_4/0.1 mM Na_2EDTA, pH 7, to a final concentration of 0.1 mg/ml. Carborundum-dusted leaves were inoculated at the four- or five-leaf stage with 10 μl of RNA solution. Two weeks after inoculation, nucleic acids were purified (17) from selected leaves and analyzed as described above.

RESULTS AND DISCUSSION

Infectious Transcripts of Plasmids Encoding Genomic and Associated RNAs. BWYV ST9 genomic RNA transcript tST9gRNA (from plasmid pBW7120-7) and associated RNA transcript tST9aRNA (from plasmid pST9106-8), when electroporated into protoplasts, each engendered an increase with time in the corresponding RNA (Fig. 2, lanes 1–8). A coelectroporated mixture of tST9gRNA and tST9aRNA (lanes 9–12) initiated an infection qualitatively similar to infection by ST9 BWYV virion RNA (lanes 13–16), which contains principally a mixture of ST9gRNA and ST9aRNA. The intensities of bands in this experiment suggest that coelectroporation of tST9aRNA with tST9gRNA stimulated a more rapid appearance of ST9gRNA (e.g., lane 11 compared to lane 3). An increased accumulation of ST9gRNA also is indicated by results observed after coinoculation of tST9gRNA with increasing amounts of tST9aRNA (Fig. 3, lanes 1–4). The cell culture-derived protoplasts used in these experiments proceed to divide in culture medium for at least 5 days postelectroporation under the conditions described. Results similar to those obtained at 3 days (Fig. 3) also were obtained at 5 days (data not shown), suggesting that the stimulation of ST9gRNA accumulation by ST9aRNA was not a transient effect. Preparation and electroporation of transcripts complementary to ST9aRNA did not elicit detected ST9aRNA accumulation (lane 9) and failed to stimulate ST9gRNA accumulation (lanes 5–7). This last result suggests that the added tST9aRNA (lanes 2–4) did not act merely by protecting the tST9gRNA inoculum from degradation. The presence of ST9gRNA seems not to have an effect on level

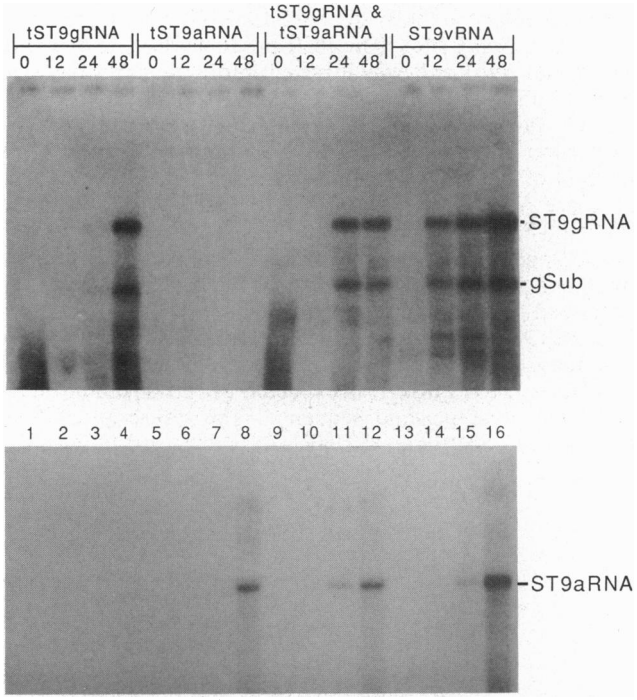

FIG. 2. Infectious transcripts of cDNA clones of strain ST9 BWYV genomic RNA (ST9gRNA) and the associated ST9aRNA. Protoplasts from tobacco (*N. tabacum* cv. Xanthi nc) suspension cell culture were electroporated with 5 μg of capped transcript of plasmid clone pBW7120-7 (tST9gRNA; lanes 1–4), 0.5 μg of noncapped transcript of clone pST9106-8 (tST9aRNA; lanes 5–8), a mixture of 5 μg of tST9gRNA and 0.5 μg of tST9aRNA (lanes 9–12), and 0.5 μg of virion RNA (mixture of ST9gRNA and ST9aRNA) from BWYV strain ST9 (ST9vRNA; lanes 13–16). Time of incubation of protoplasts (hr) is indicated above lanes. Nucleic acids extracted from protoplasts were denatured and analyzed by electrophoresis through a 1% agarose gel. After blotting, ^{32}P-labeled probes were applied to detect accumulated positive polarity ST9gRNA (*Upper*) or ST9aRNA (*Lower*). Migration positions are indicated for ST9gRNA, the corresponding subgenomic (gSub) RNA, and ST9aRNA, which migrates just behind gSub.

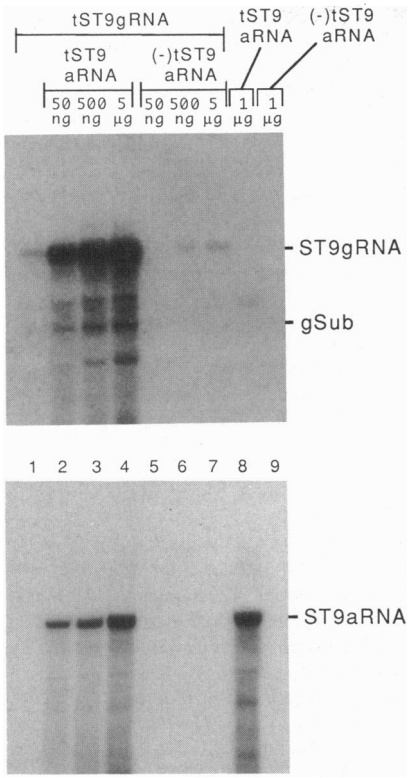

FIG. 3. Increased accumulation of ST9gRNA in protoplasts by coelectroporation of tST9aRNA with tST9gRNA. Protoplasts were electroporated with 2 µg of tST9gRNA without (lane 1) or with the indicated amounts of tST9aRNA (lanes 2–4) or its complement [(−)tST9aRNA; lanes 5–7]. The tST9aRNAs also were inoculated alone (lanes 8 and 9). Protoplasts were incubated for 72 hr after electroporation, and nucleic acids were extracted and analyzed as described in Fig. 2.

of replication of ST9aRNA (compare lanes 3, 4, and 8), and the slight difference seen in Fig. 2 between lanes 7 and 11 probably was due to sample variability.

Replication of ST9aRNA in Inoculated Leaves. We inoculated leaves of shepherd's purse seedlings with tST9gRNA with tST9aRNA, alone or combined with tST9gRNA, and with tST9a/xRNA. No ST9gRNA was detected in the inoculated or upper leaves of these plants (data not shown), supporting previous observations of the inability of BWYV to be propagated by mechanical inoculation. Surprisingly, tST9aRNA inoculated alone resulted in accumulation of ST9aRNA in the inoculated leaves (Fig. 4, lane 2). Comparisons with standards indicate that ST9aRNA accumulated to ≈100 ng per g of tissue (results not shown), which is similar to levels achieved by the virion RNA of a typical luteovirus. However, ST9aRNA was not detected in upper (lane 3) or other (data not shown) uninoculated leaves. When tST9a/xRNA, expected not to be infectious, was inoculated to leaves as a control, it was not detected in leaf extracts (lane 1). This observation and the appearance of a characteristic subgenomic RNA derived from ST9aRNA (lane 2) strongly support our contention that ST9aRNA is capable of independent replication in inoculated shepherd's purse leaves. In these experiments, the age of the shepherd's purse seedlings was critical. Plants with fewer than four or five leaves died after inoculation, and older plants did not support a detected increase in ST9aRNA. When tST9gRNA was coinoculated with tST9aRNA, no increase in tST9aRNA accumulation was noted (data not shown).

ST9aRNA, satellite RNAs, and the genomic RNAs of satellite viruses all have a nucleotide sequence distinct from that of the genomic RNA of the associated virus (Table 1, column 4). Satellite RNAs [including a documented luteovirus satellite RNA, the satellite RNA of barley yellow dwarf virus (18)], satellite viruses, and defective interfering RNAs all are dependent on the helper virus for replication (Table 1, column 3). The ST9aRNA is clearly distinct from the replication-dependent satellite agents because of its ability to replicate independently of the associated virus BWYV. ST9aRNA also is distinctive because of its the ability to stimulate the increased accumulation of ST9gRNA in protoplasts. In infected plants, virions of the ST9 strain of BWYV accumulate to ≈10 times the titer of BWYV strains that lack an associated RNA (11), suggesting, but not proving, that an interaction of ST9aRNA and ST9gRNA favors increased accumulation of the latter in leaves as well as in protoplasts.

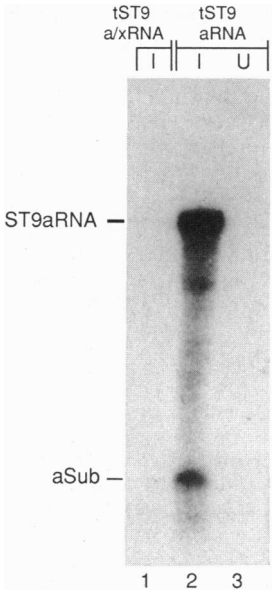

FIG. 4. Accumulation of ST9aRNA in inoculated leaves of shepherd's purse. Nucleic acids were purified from DNase-treated transcription reactions for inoculation to shepherd's purse seedlings. After 2 weeks, nucleic acids, predominantly RNA, were extracted, and 5 µg was applied to each lane of a 1% agarose gel. Lane 1, nucleic acids from leaves inoculated (I) with tST9a/xRNA, which served as a control for the survival of inoculated but presumably not replicating RNA. Lane 2, nucleic acids from leaves inoculated with tST9aRNA. Lane 3, nucleic acids from upper (U) leaves just above the inoculated leaves. Migration positions of ST9aRNA and its presumed subgenomic RNA (aSub) are indicated.

Properties of the ungrouped pea enation mosaic virus (PEMV) suggest certain similarities with the luteovirus/carmovirus-related BWYV strain ST9 system. All isolates of PEMV have two encapsidated RNAs, both of which are able to independently replicate in protoplasts but not in whole plants (19). For other plant viruses with two genomic RNAs, only one is infectious. PEMV RNA 2, like ST9aRNA, does not encode a coat protein (19). Unlike ST9aRNA, PEMV RNA 2 failed to accumulate in inoculated plants to a detected level.

The ease with which we detected ST9aRNA in inoculated leaves of shepherd's purse (Fig. 4, lane 2) and our inability to detect ST9aRNA elsewhere in the inoculated plants suggest that ST9aRNA replicates and probably spreads from cell to cell within the inoculated leaf. The aphid vector of BWYV is the green peach aphid, *Myzus persicae*. It is unlikely that an aphid could transmit unencapsidated ST9aRNA from plant to plant, as the aphid vector transmission specificity of plant viruses, which are transmitted in a circulative nonpropagative manner (as is BWYV), is correlated with the protein capsid (20). Thus, in nature both the spread of ST9aRNA from the inoculated leaf to other parts of the plant and

ST9aRNA transmission from plant to plant presumably rely on coreplication of ST9aRNA with ST9gRNA and encapsidation of ST9aRNA in ST9gRNA-encoded coat protein.

We postulate that the stimulation of ST9gRNA increase by ST9aRNA (11) has a survival benefit for ST9aRNA. High ST9gRNA titer would assure ST9aRNA, which is relatively high titer and possibly efficiently encapsidated, of a more abundant supply of capsids than might be provided by the typically low titer BWYV alone. An additional possible benefit of high ST9gRNA titers is an increased accumulation of encapsidated ST9gRNA, which should enhance the efficiency of spread of the ST9aRNA from the primary infection foci introduced by aphid transmission of encapsidated ST9aRNA and ST9gRNA.

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