Effect of Cultivation Temperature on the Spontaneous Development of Deletions in Soilborne Wheat Mosaic Furovirus RNA 2

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ABSTRACT

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Intraplant movement of soilborne wheat mosaic virus (SBWMV) and the spontaneous development of sequence deletions in SBWMV RNA 2 were monitored in individual plants of *Triticum aestivum* cultivars Galahad and Vona inoculated mechanically or with viruliferous zoospores of *Polymyxa graminis* and grown at different temperatures. Movement of SBWMV RNAs 1 and 2 (either full-length or deleted forms) from foliage to roots was detected 2 days after mechanical inoculation. Plants infected by *P. graminis* and maintained at high temperatures (25–30 C) showed extensive deletions in the readthrough (RT) domain of the coat protein-RT gene within 4–12 wk. In contrast, plants kept at 17 C over the same period contained only full-length RNA 2 molecules (3,593 nucleotides). Thus, in addition to prolonged cultivation at 15–17 C and/or serial mechanical transfers to healthy plants, higher temperatures resulted in truncated forms of SBWMV RNA 2. Our observations confirm that only full-length SBWMV RNA 2 is transmitted to wheat roots by viruliferous *P. graminis* from field soil and that there is no intraplant barrier to the movement of deleted forms of RNA 2 between roots and leaves. Deleted forms of SBWMV RNA 2 appear to cause more severe symptoms only after mechanical inoculation to young, healthy plants. The significance of these observations for events in fields of winter wheat or barley, especially those expressing a temperature-sensitive host resistance gene against SBWMV, are discussed. These studies may also help elucidate the mechanism of spontaneous RNA deletion.

Additional keywords: enzyme-linked immunosorbent assay, Northern blotting, reverse transcriptase-polymerase chain reaction, Southern blotting.

Soilborne wheat mosaic virus (SBWMV) is one of the most prevalent viruses of winter wheat (*Triticum aestivum* L.) in North America, Japan, China, Italy, France, Egypt, and Brazil (5,6,23) and can cause severe yellowing and stunting of susceptible cultivars. Typical crop losses are between 10 and 30%, but losses of up to 80% have been reported (6,12,14). SBWMV is transmitted by motile zoospores of the Plasmodiophoromycete fungus *Polymyxa graminis* Ledingham (4,9,16), an obligate parasite of plant roots that has extremely thick-walled resting spores. SBWMV is believed to exist and persist within the resting spores (2), which contaminate soils indefinitely (1).

SBWMV genomic RNA is bipartite, giving long (280–300 × 20 nm) and short (140–160 × 20 nm) rigid helical particles (23). The genome of SBWMV type strain (Nebraska isolate) has been sequenced completely and comprises positive-sense, single-stranded RNAs of 7,099 and 3,593 nucleotides (nt), respectively (22). SBWMV RNA 1 (7,099 nt) appears to encode RNA replication and cell-to-cell movement functions. SBWMV RNA 2 (3,593 nt) encodes the coat protein (CP) (19 kDa), a CP-readthrough fusion protein with an N terminal CP domain and a C terminal readthrough domain (CP-RT) (84 kDa), and a cysteine-rich polypeptide (Cys) (19 kDa) of unknown function (Fig. 1).

In 1984, field-infected plants were transferred to a glasshouse at 17 C for 4–8 mo, after which a number of spontaneous deletions in the SBWMV RNA 2 molecule were detected. Serial passages of the Nebraska field isolate at bimonthly intervals over 2 yr also caused extensive deletion of RNA 2 and more severe symptoms on wheat (19,20). The sequence of RNA 2 from a stable deleted form of SBWMV (Nebraska), the Lab1 mutant, was determined (18) to confirm that the CP-RT gene was truncated (21) by at least 1,058 nt (7,18).

In 1993, we obtained a new field isolate of SBWMV from Oklahoma (12) that was collected in 1992 and stored frozen in leaf tissue and contained particles with only full-length RNAs 1 and 2 (7). Over 32 wk at low ambient temperatures (15–20 C), the virus (Okl-0) was serially passaged seven times by mechanical inoculation through *T. aestivum* cv. Galahad. After 11 wk, progeny virus in the first inoculated plants (Okl-1) contained a population of deleted forms of SBWMV RNA 2 in addition to the full-length molecule. During subsequent passages, the ratio of deleted to full-length RNA 2 molecules increased, and one species of approximately 2.8 kb predominated. By the fifth passage (20 wk from Okl-0), no full-length RNA 2 remained. Both Nebraska Lab1 and the Okl-7 deleted forms of SBWMV elicited more severe symptoms in susceptible wheat plants than the respective parent virus. Reverse transcriptase-polymerase chain reaction (RT-PCR), restriction enzyme mapping, and direct RNA sequencing showed that a 759-nt deletion had occurred within the RT domain of the CP-RT gene (fusing Oklahoma SBWMV RNA 2 genome coordinates 1,420 and 2,180) in a location similar to the 1,058-nt deletion confirmed for Nebraska Lab1 RNA 2 (7,18).

The 84-kDa CP-RT protein of beet necrotic yellow vein furovirus (BNYVV) (24) is required for acquisition and transmission by *P. graminis*. As is the case with BNYVV and other furoviruses, the absence of selection to maintain transmission by the vector fungus results in the rapid loss, probably during RNA replication, of sequences in the CP-RT region of SBWMV. To establish a *P. graminis*-SBWMV transmission system and to test the vector-mediated spread of deleted forms of RNA 2, rapid and sensitive

methods to detect SBWMV must be developed; these methods must also be capable of distinguishing full-length and truncated virus RNAs. RT-PCR of full-length SBWMV RNAs 1 or 2 has been reported (15), but similar methods to detect truncated RNA molecules have not been described. To begin to understand the mechanism of spontaneous RNA deletion in vivo, we have also examined when SBWMV RNA 2 deletions arise after infection at high (25–30 C) or low (17–20 C) temperatures.

MATERIALS AND METHODS

Wheat cultivars, virus isolates, and inoculation conditions. Leaves of *T. aestivum* cv. Galahad, which contained the first passage of SBWMV (Oklahoma isolate; Okl-1) described by Chen et al (7) were stored at −20 C before use. Infected leaf material was homogenized in a precooled mortar with 0.2 g of leaf material per milliliter of 0.1 M K_2HPO_4, mixed with Carborundum, and inoculated to plants. For each experiment, six to 10 wheat plants (cultivar Galahad) were grown together in 4-in.-diameter pots, and the plants were inoculated at the two- to three-leaf stage. Five to 10 min after inoculation, the plants were rinsed thoroughly with tap water and then kept shaded for 2 days at 17 C before propagation in the greenhouse with supplemented light for 12–16 h each day. Fungus-inoculation experiments used *T. aestivum* cv. Vona. Okl-1 comprises both full-length and at least five truncated forms of SBWMV RNA 2. Plants were grown in a controlled environment chamber or glasshouse at 17 or 25–30 C. Leaves and roots were collected separately from plants at various times after inoculation as described in the text. For the initial detection experiments (Table 1), leaves or roots from two plants showing symptoms or six symptomless plants were pooled for each sample. Half the material was extracted immediately to prepare total RNA as outlined below, and the remainder was stored at −20 C for enzyme-linked immunosorbent assay (ELISA).

SBWMV infection via viruliferous fungal spores. Caryopses of *T. aestivum* cv. Vona (a gift from J. L. Sherwood, Oklahoma State University, Stillwater) were germinated in sand at 17–20 C and 3 days later transferred to a 1:100 mixture of viruliferous *P. graminis*-infested field soil from Oklahoma (also from J. L. Sherwood) and soil-free compost (10 seeds per 4-in. pot). The pots were kept dry for 14 days and then watered excessively for 14 days to encourage zoospore production and SBWMV infection of roots of the young seedlings. Four weeks from sowing, plants were coded and a small sample of leaf tissue was taken from each for triple antibody sandwich (TAS)-ELISA.

RNA extraction. Samples of wheat leaf or root tissue (0.2 g) were frozen in liquid nitrogen in polypropylene microcentrifuge tubes (2 ml) and ground to a powder. RNA extraction was based on the method of Hall et al (11). The powdered tissue was thawed to 0 C; 0.8 ml of extraction buffer (0.2 M sodium borate, pH 9.0, 30 mM EDTA, 1% sodium dodecyl sulfate [SDS], and 5 mM dithiothreitol) was added; and the mixture was shaken vigorously for 1 min. Proteinase K (50 μl of 50 mg/ml) (Sigma, St. Louis, MO) was added, and samples were incubated at 37 C for 30 min before 80 μl of 2 M KCl was added. The mixtures were left on ice for 30 min before centrifugation at 12,000 *g* for 15 min at 4 C. The supernatant was made up to 2 M KCl, and the RNA was precipitated overnight on ice, collected by centrifugation, redissolved in 0.2 ml of sterile distilled water, and extracted with phenol-chloroform-isoamyl alcohol (25:24:1). RNA was precipitated with 2 vol of ethanol at −20 C, recovered by centrifugation, dried, and dissolved in 30 μl of water for storage at −70 C.

First-strand cDNA synthesis. Total RNA samples (5 μl from 30 μl) extracted from leaf or root tissue (approximately 33 mg) were used as templates for first-strand cDNA synthesis with a 33-nt primer (5′-GGGCATGCCCCGGGCCGGATAACCCTCC GGATG-3′), which contained *Sph*I and *Sma*I restriction sites

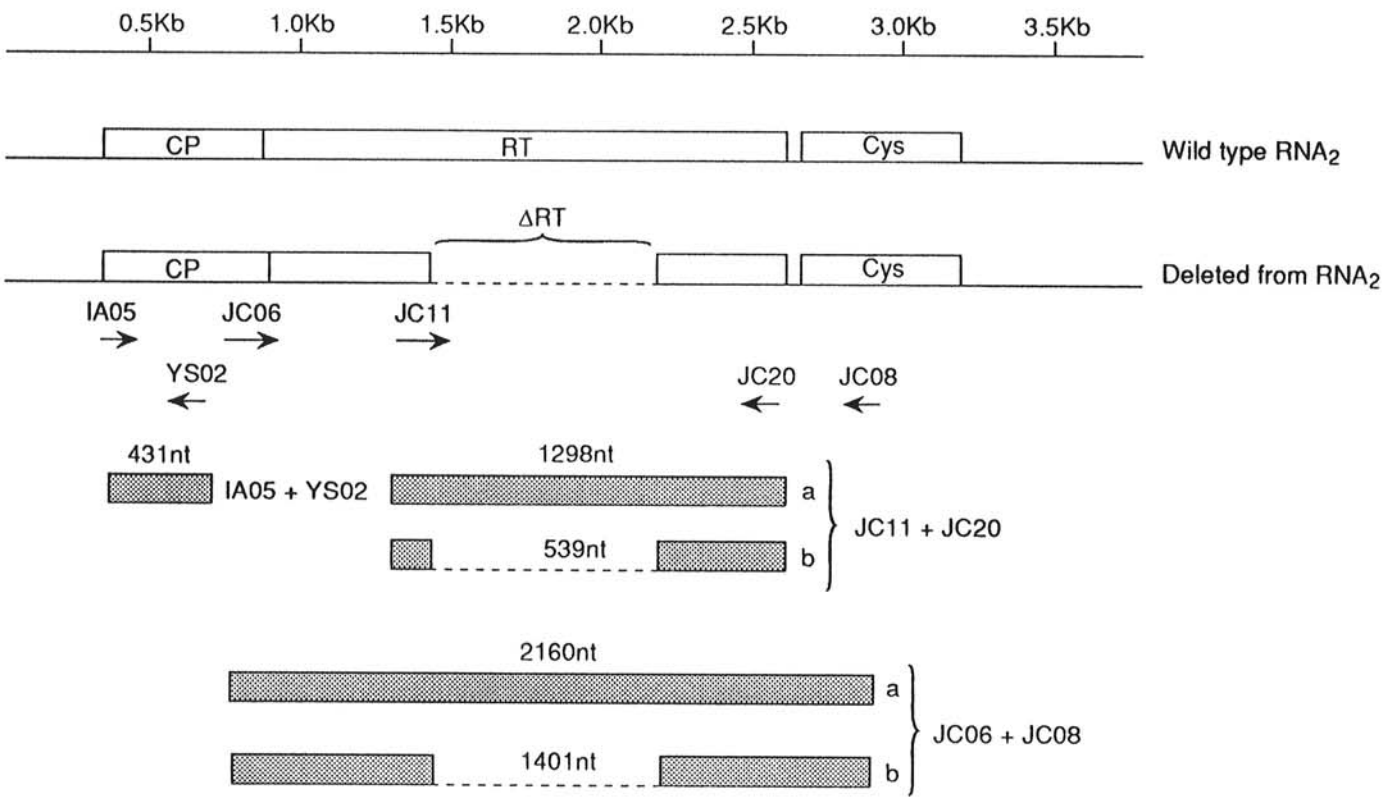

Fig. 1. Genome organization and location of polymerase chain reaction (PCR) primers for amplification of wild-type and deleted forms of soilborne wheat mosaic virus RNA 2. RNA 2-encoded polypeptides are boxed: CP (19-kDa coat protein), RT (65-kDa readthrough domain of the 84-kDa CP-RT fusion protein), Cys (19-kDa cysteine-rich protein), and ΔRT (56-kDa deleted readthrough domain of Okl-7). The deleted sequence (759 nt) is shown as a dotted line. Primer binding positions are indicated by arrows (arrowhead = 3′ end). Rightward-facing arrows are virus-sense primers, and leftward arrows are complementary (minus-sense) primers. Stippled bars below show the positions and predicted sizes (in base pairs) of the reverse transcriptase-PCR amplified fragments for different primer pair combinations derived from full-length RNA 2 (a) and deleted RNA 2 (b).

(underlined) and 22 nt (bold) complementary to the 3′ terminal sequence of SBWMV RNA; the last A is omitted (22).

First-strand SBWMV cDNA 2 synthesis used Superscript reverse transcriptase (Gibco/BRL, Gaithersburg, MD) in a 20-μl reaction, according to the manufacturer's instructions. After incubation, the volume was raised to 100 μl with water and stored at −20 C.

PCR amplification. Three pairs of primers were used to prime the amplification of DNA fragments from cDNA 2 templates (Fig. 1). Plasmid p42 (clone 35) contained a PCR fragment (2,160 bp) of full-length RNA 2, which had been sequenced (7) and was included as a positive control. PCR reactions (50 μl) contained 1 μl of the cDNA reaction or 5 ng of p42 DNA as template and 20 pmoles of each primer in the presence of 200 μM each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl, pH 8.3. Template DNA was denatured at 94 C for 3 min, and then 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN) was added. All PCR amplifications were for 30 cycles of 94 C for 1 min (denaturation), 45–47 C for 1 min (annealing), and 72 C for 1–3 min (synthesis). Details are described below.

Primers IA05(+) and YS02(−). IA05 (21 nt) corresponds to SBWMV RNA 2 genome coordinates 334–351, with an additional 5′-3 nt (underlined) (5′-GCCATGGCGGTAAATAAAGGT-3′. YS02 (17 nt) is complementary to positions 749–765 of RNA 2 (5′-TAACTCAGCAACTGCAT-3′). This PCR primer pair required a 1-min synthesis time and allowed the amplification of a 432-bp region of the CP gene (amino acids 1–144).

Primers JC06(+) and JC08(−). JC06 (17 nt) corresponds to SBWMV RNA 2 genome coordinates 721–737 (5′-CAGACCGAT CAGACTCT-3′). JC08 (17 nt) is complementary to positions 2,864–2,880 of RNA 2 (5′-TCCACAGTAACCGTCCA-3′). This PCR primer pair required a 2.5-min synthesis time and primed the amplification of fragments of the CP-RT gene of 2,160 bp (wild-type) or 1,401 bp (Okl-7 deletion).

Primers JC11(+) and JC20(−). JC11 (17 nt) corresponds to SBWMV RNA 2 genome coordinates 1,300–1,316 (5′-TGTAGAC GCTTTGCTAC-3′). JC20 (26 nt) is complementary to positions 2,580–2,598 (the 3′ end of the CP-RT domain) with an additional *Xba*I site (underlined) at the 5′ end (5′-GTTCTAGA GGACGCCATAGGCTTA-3′). This PCR primer pair required a 1.5-min synthesis time and primed the amplification of fragments of the CP-RT gene of 1,298 bp (wild-type) or 539 bp (Okl-7 deletion).

Samples (5 μl) of PCR reaction mixtures were separated by electrophoresis in 1.0 or 1.5% (w/v) agarose gels and stained with ethidium bromide. RT-PCR products were sized by comparison with the 1-kb DNA ladder (Gibco/BRL) run in the same gel.

Southern hybridization. For Southern blotting of PCR products, agarose gels were depurinated in 0.25 M HCl for 30 min, denatured in 0.5 M NaOH plus 1.5 M NaCl for two 30-min washes, and neutralized in 1 M Tris-HCl plus 1.5 M NaCl, pH 7.5, for two 30-min washes. DNA was then transferred to a nylon membrane and probed with ^{32}P-labeled DNA made from p309 (22), a near full-length cDNA clone of SBWMV RNA 2 (Nebraska strain), as described (7). After hybridization, the membrane was washed twice for 30 min each in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS and once for 30 min in 0.1× SSC plus 0.5% SDS at 65 C, exposed to X-ray film for 30 min, and developed.

Northern hybridization. Total RNAs extracted from leaves or roots (5 μl from 30 μl of stock) were heated to 65 C for 5 min, chilled on ice, denatured with formamide plus formaldehyde, separated by electrophoresis in formaldehyde-agarose gels, and transferred to nitrocellulose (17). Membranes were probed with SBWMV RNA 2-specific DNA from p309, labeled with [α-^{32}P]-dATP (10), and exposed to X-ray film at −70 C.

Antibodies and ELISA procedures. Rabbit polyclonal antiserum to SBWMV (Oklahoma isolate) was prepared with a titer of 1/256 in microprecipitin tests. A monoclonal antibody (MAb) (3) to the same virus isolate was supplied by J. L. Sherwood. All ELISA tests were performed in polystyrene microtiter plates (NUNC Immunological, Roskilde, Denmark), but the outer wells were not used. Three replicate wells were used for each sample, and the A_{405} results were averaged. Sap from leaves or roots was diluted 100-fold, and viral antigen was trapped on plates precoated with polyclonal SBWMV antiserum diluted 1:4,000. After thorough washing, MAb culture fluid (diluted 500-fold) was added to detect and quantify the virus. All dilutions, including the antibody-conjugate used for detection, rabbit anti-mouse immunoglobulin G-alkaline phosphatase (Sigma), were prepared with buffer containing 10 g of milk powder per liter (1). A_{405} values were recorded at intervals up to 18 h in a Titertek Multiskan Plus photometer (Flow Laboratories Inc., McLean, VA) with air blanks. Average absorbance values greater than twice those of the appropriate healthy control sap were considered positive and significant. To assess the efficiency of TAS-ELISA for detecting SBWMV CP, sap extracted from infected wheat leaves showing symptoms 3 mo after mechanical inoculation was serially diluted from four- to 2,056-fold and tested as described. All samples (1/4, 1/8 . . . 1/2,056) were positive, ranging from A_{405} = 1.525 (fourfold dilution) to A_{405} = 1.144 (2,056-fold), while all healthy sap controls had A_{405} values of <0.08. For further experiments, leaf or root extracts were therefore diluted 100-fold.

RESULTS

Comparison of methods to detect SBWMV in infected wheat leaves and roots. Primers IA05 and YS02 were used in RT-PCR amplification reactions to detect the SBWMV CP gene in wheat leaf and root RNA samples collected in four independent experiments at various times up to 35 days after mechanical inoculation. In all cases examined (Table 1), the predicted 432-bp DNA fragment was detected. No product was obtained by RT-PCR of healthy wheat samples (Fig. 2). Plasmid p42 (clone 35) and Okl-1 sap inoculum were included as positive controls.

The samples tested by RT-PCR were also examined by TAS-ELISA. In these experiments, all uninoculated, healthy wheat leaf or root samples resulted in A_{405} values of <0.07. SBWMV antigen was not reliably detected by TAS-ELISA in either root or leaf tissue prior to 7–14 days postinoculation (dpi). When RNA 2-specific primers IA05 and YS02 were used, RT-PCR signals

TABLE 1. Detection of soilborne wheat mosaic virus (SBWMV) by reverse transcriptase-polymerase chain reaction (PCR) and triple antibody sandwich-enzyme-linked immunosorbent assay at different days after mechanical inoculation[a]

Days after inoculation	Experiment 1				Experiment 2				Experiment 3				Experiment 4			
	Leaf		Root		Leaf		Root		Leaf		Root		Leaf		Root	
	PCR	A_{405}	PCR	A_{405}	PCR	A_{405}	PCR	A_{405}	PCR	A_{405}	PCR	A_{405}	PCR	A_{405}	PCR	A_{405}
2	NS	...	NS	...	NS	...	NS	...	++	0.071	+	0.070	+	0.072	+	0.100
7	NS	...	NS	...	NS	...	NS	...	++	0.193	++	0.096	++	0.082	++	0.086
14	++	0.478	++	0.540	++	0.561	++	0.479	++	0.609	++	0.544	++	0.571	++	0.501
21	++	0.612	++	0.582	NS	...	NS	...	NS	...	NS	...	NS	...	NS	...
28	++	0.628	++	0.638	++	0.587	++	0.599	NS	...	NS	...	NS	...	NS	...
35	++	0.599	++	0.444	NS	...	NS	...	NS	...	NS	...	NS	...	NS	...

[a]NS = not sampled; + = low and ++ = high yield of PCR products. Primers IA05 and YS02 were used to amplify a 432-bp region of the coat protein gene from SBWMV RNA 2.

were generated from samples taken at 2 dpi, indicating that viral RNA or particles moved rapidly to root tissue after leaf inoculation.

In Northern blots, SBWMV RNA 2 was detected only in RNA samples taken from leaves, and all leaf samples collected more than 14 dpi contained both full-length and deleted RNA 2 species (Fig. 3). Moreover, in plants grown at 25 C and sampled at 21 dpi, only the deleted form of SBWMV RNA 2 was detected. Similar results were obtained from three separately inoculated batches of wheat.

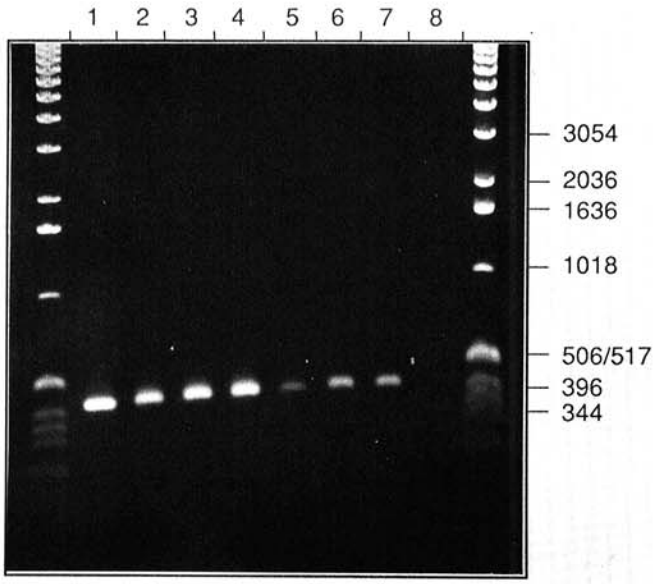

Fig. 2. Detection of the soilborne wheat mosaic virus coat protein gene by reverse transcriptase-polymerase chain reaction (RT-PCR) with primers IA05 and YS02 (432-bp fragment) in total RNA from wheat leaves or roots. PCR products were separated in an agarose gel and stained with ethidium bromide. Flanking lanes contain 1-kb DNA size markers (sizes on the right are in base pairs). Lane 1 represents the Okl-1 leaf sap used as inoculum. Lanes 2–4 are wheat leaf samples 2, 7, or 14 days postinoculation (dpi), respectively. Lanes 5–7 are root samples from the same wheat plants 2, 7, or 14 dpi, respectively. Lane 8 is leaf sap from a healthy wheat plant.

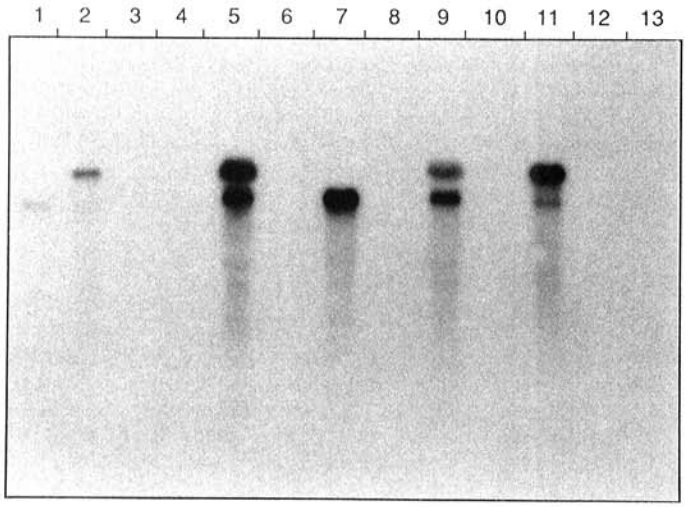

Fig. 3. Detection of soilborne wheat mosaic virus (SBWMV) RNA 2-specific sequences by Northern blot hybridization in wheat leaf or root RNA samples taken 7, 14, 21, or 28 days postinoculation (lanes 3 and 4; 5 and 6; 7–10; or 11 and 12, respectively). Lanes 7 and 8 are leaf and root samples, respectively, derived from plants grown at 25 C. All other plants were cultivated at 17 C. As markers, lane 1 contains the 759-nt deletion mutant Okl-7, and lane 2 contains the Okl-1 inoculum sap (3,593 nt). Lane 13 contains sap from a healthy wheat leaf. Lanes 4, 6, 8, 10, and 12 are root samples, and lanes 3, 5, 7, 9, and 11 are leaf samples. Samples in lanes 3–6 were derived from experiment 1, lanes 7 and 8 from experiment 2, lanes 9 and 10 from experiment 3, and lanes 11 and 12 from experiment 4. All RNA samples from root tissue routinely failed to give signals by Northern blot hybridization, although reverse transcriptase-polymerase chain reaction later confirmed the presence of SBWMV RNA 2 sequences.

RT-PCR detection of a SBWMV RNA 2 deletion mutant in leaves and roots. RT-PCR primers JC11 and JC20 were used to detect and distinguish full-length, wild-type, and a stable deleted form (Okl-7) of SBWMV RNA 2 in leaf or root RNA sampled 2 or 14 dpi when wheat plants were grown at 17 C (Fig. 4A and B). Both forms of RNA 2 were detected in the original inoculum (Okl-1) and were maintained in leaf and root sap up to 14 dpi in two independent experiments (Fig. 4A and B, lanes 4–9). In one experiment, there was a tendency for the deleted form of RNA 2 to predominate in root samples (lanes 5 and 7). Thus, these data confirm that SBWMV RNA 2 (full-length and deleted form) moves rapidly to roots after mechanical inoculation of leaf tissue.

Effect of growth temperature on the accumulation of an SBWMV RNA 2 deletion mutant after mechanical inoculation. During the late spring and early summer, glasshouse temperatures rose to 25–30 C. Under these conditions, mechanical inoculation experiments with SBWMV Okl-1 on Galahad wheat were repeated, and inoculated plants were sampled at 7, 12, and 28 dpi. SBWMV RNA 2 species were detected in leaf and root sap by RT-PCR with primers JC06 and JC08. Unexpectedly, all leaf sap samples contained only the 1.4-kbp PCR fragment derived from the deleted

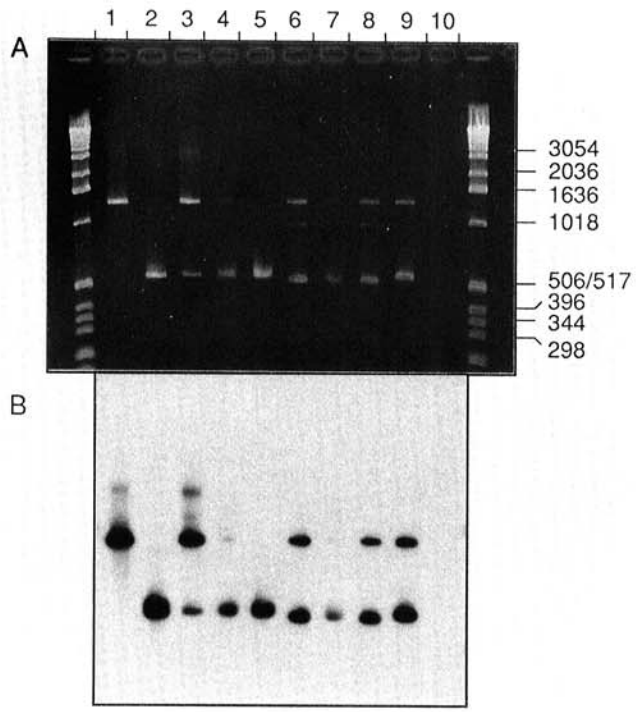

Fig. 4. Detection of full-length and/or deleted soilborne wheat mosaic virus (SBWMV) RNA 2 molecules by reverse transcriptase-polymerase chain reaction (RT-PCR) of wheat leaf or root RNA extracts with primers JC11 and JC20 via diagnostic fragments of 1,298 and 539 bp, respectively. **A,** Ethidium bromide-stained gel; **B,** autoradiograph of a Southern blot of the gel in **A** with an SBWMV RNA 2-specific probe. As reference markers, lane 1 template is sequenced plasmid p42 (clone 35) containing the full-length RT-PCR fragment of the coat protein-readthrough gene; lane 2 template is leaf RNA containing Okl-7 deleted SBWMV RNA 2; and lane 3 template is RNA of the leaf sap inoculum Okl-1. Plants grown at 17 C from two separate inoculation experiments are shown in lanes 4–7 and lanes 8 and 9. Lanes 4 and 5 are leaf and root samples, respectively, at 2 days postinoculation (dpi); lanes 6 and 8 are 14-dpi leaf samples; and lanes 7 and 9 are 14-dpi root samples. Lane 10 is a healthy leaf control. Flanking lanes are the 1-kb DNA markers, with appropriate sizes (in base pairs) shown on the right of **A.** The significant RT-PCR products are confirmed to be derived from SBWMV RNA 2 in **B.**

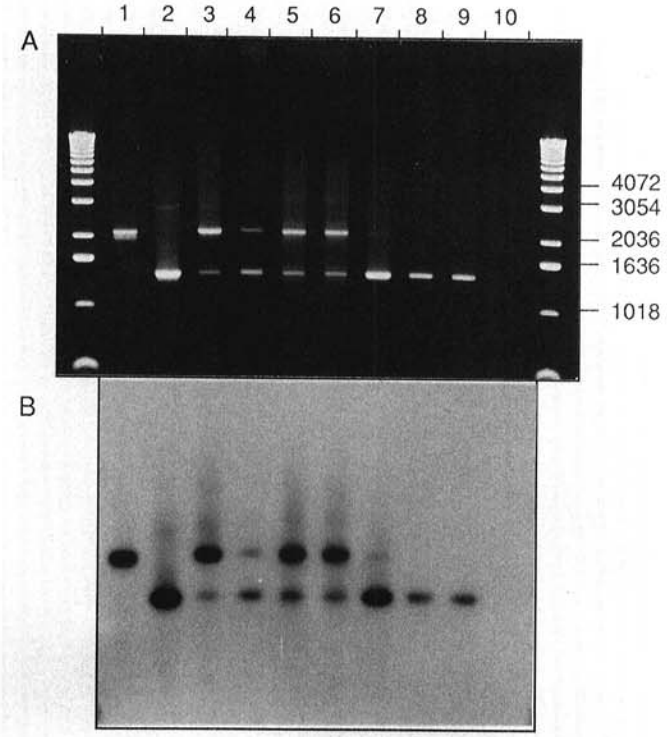

Fig. 5. The effect of temperature on the process of soilborne wheat mosaic virus (SBWMV) RNA 2 deletion in wheat leaves. Reverse transcriptase-polymerase chain reaction (RT-PCR) products made with primers JC06 and JC08 were **A,** stained with ethidium bromide or **B,** Southern blotted with an RNA 2-specific probe and autoradiographed. Marker RT-PCR products are lane 1, full-length coat protein-readthrough fragment (2.1 kbp) from sequenced plasmid p42 (clone 35); lane 2, deleted fragment (1.4 kbp) from Okl-7-infected sap; and lane 3, the mixture of Okl-1-infected sap used as primary inoculum. Lanes 4–6 are leaf sap samples collected at 2, 14, or 28 days postinoculation, respectively, from wheat plants grown at 17 C. Lanes 7–9 are the same but are samples from plants grown at 25–30 C. Lane 10 is sap from a healthy wheat leaf. The significant RT-PCR products are confirmed to be derived from SBWMV RNA 2 in **B.**

form of RNA 2 (7), and no SBWMV RNA 2 was detected in any root sample (data not shown). To confirm this temperature-dependent enhancement in the accumulation of the deleted form of SBWMV RNA 2, an additional 48 pots of Galahad wheat (10 seedlings per pot) were manually inoculated with SBWMV Okl-1. Twenty-four pots were then grown at 17 C and 24 pots at 25 C. Leaf samples were collected at 2, 14, or 28 dpi at both temperatures, and total RNA was probed by RT-PCR with primers JC06 and JC08 (Fig. 5A and B). As observed earlier at 25 C, between 2 and 14 dpi, full-length SBWMV RNA 2 was not detected. This contrasts with the detection of full-length SBWMV RNA 2 in plants grown at 17 C. The deletion corresponded to that mapped and sequenced in Okl-7 (7), i.e., 759 nt between genome coordinates 1,420 and 2,180. This result was confirmed, independently, by Northern blotting of total RNA from mechanically inoculated wheat leaves grown at 25 C for 21 dpi (Fig. 3, lane 7).

Effect of growth temperature on the accumulation of an SBWMV RNA 2 deletion mutant after fungus-mediated infection. Oklahoma field-infested soil carrying viruliferous (SBWMV) resting spores of *P. graminis* was used to infect 3-day-old susceptible seedlings of wheat cultivar Vona at 17 C. TAS-ELISA confirmed the presence of SBWMV in small leaf samples of 20 of 50 seedlings 4 wk after germination. Northern blotting with RNA 2-specific probes (Fig. 6A) or a mixture of RNA 1- and RNA 2-specific probes (Fig. 6B) confirmed the presence of only full-length SBWMV RNAs 1 and 2 in the roots (lane 1) and leaves (lane 2) of these 4-wk-old infected but symptomless wheat plants maintained at 17–20 C. As before, the signals observed for SBWMV RNA 2 (and RNA 1) by Northern hybridization of total root RNA samples were extremely weak (Fig. 6A and B, lane 1). RT-PCR with primers JC06 and JC08 also confirmed that only full-length SBWMV RNA 2 was present in leaves and roots (Fig. 7A and B, lanes 1 and 2). No deleted form of RNA 2 was detected by either method.

Half of the fungus-infected plants were kept in a growth chamber at 17 C (16-h photoperiod), and the others were transferred to a glasshouse that varied from 25 to 30 C. After 4 wk at the higher temperature (8 wk from sowing), total leaf RNA was prepared from four plants and analyzed by Northern blotting with RNA 2- or RNA 1- and RNA 2-specific probes (Fig. 6A and B, lanes 3–6). In no case was RNA 1 altered. However, only one plant (plant X) contained full-length RNA 2 alone (Fig. 6A

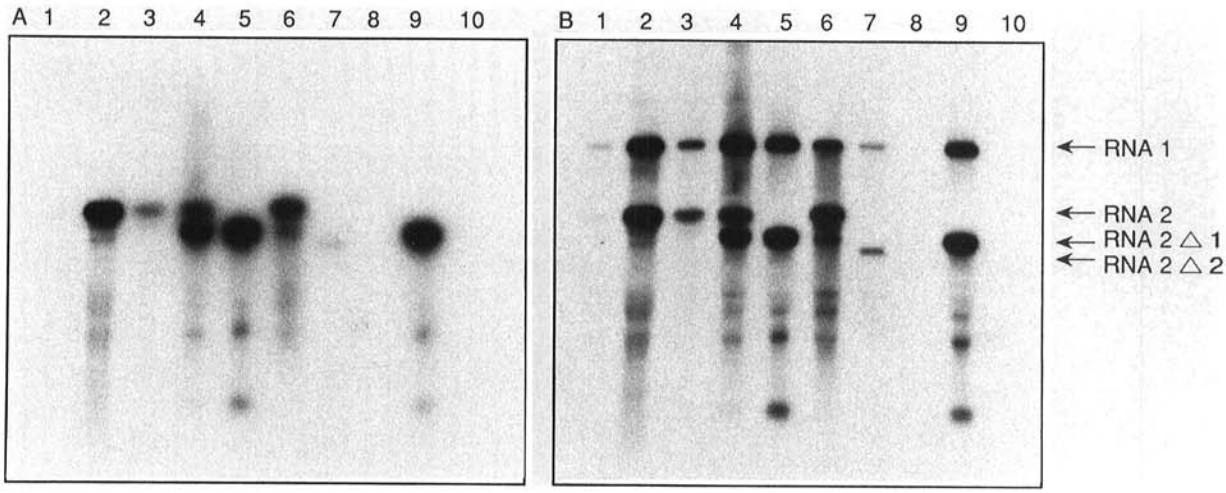

Fig. 6. Northern blot hybridization analysis of soilborne wheat mosaic virus (SBWMV) **A,** RNA 2- or **B,** RNAs 1 and 2-related species in leaf or root RNA samples of fungus-infected wheat seedlings maintained at 25–30 C. Total RNA is from root (lane 1) and leaf (lane 2) tissue of a wheat seedling infected with viruliferous *Polymyxa graminis* zoospores and grown at 17 C for 4 wk. Leaf RNA samples are from four separate plants 8 wk after infection (lanes 3–6) showing only the full-length form (lane 3), only the 759-nt deleted form (lane 5), or a mixture of both forms (lanes 4 and 6) of SBWMV RNA 2. After 12 wk, total leaf (lane 7) and root (lane 8) RNAs were extracted from another wheat plant that showed an even more extensively (1,028-nt) deleted form of SBWMV RNA 2. Leaf sap from all infected plants was then pooled and used to mechanically inoculate healthy young Vona wheat seedlings. After 2 wk, total leaf RNA from these test plants showed only the 759-nt deletion in RNA 2 (lane 9). Healthy wheat leaf RNAs gave no SBWMV-specific signal (lane 10). The identities of the SBWMV RNA species are shown on the right. RNA 2 △1 is the 759-nt deletion Okl-7. RNA 2 △2 is the 1,028-nt deleted form.

and B, lane 3). All the fungus-inoculated plants, including plant X, were asymptomatic. Two plants had a mixture of approximately 3.6- and 2.8-kb RNA 2 molecules (lanes 4 and 6), and one contained only the deleted form of RNA 2 (lane 5). These conclusions were confirmed by RT-PCR (Fig. 7A and B, lanes 3–6) with primers JC06 and JC08. The stability of SBWMV RNAs 1 and 2 in plant X was monitored further by Northern hybridization. Small amounts of leaf RNA were extracted at weekly intervals 9–13 wk after fungus infection of plant X, which was maintained at 25 C. During that period, the deleted form of SBWMV RNA 2 became dominant within plant X, but as with the other fungus-inoculated plants, plant X still showed no symptoms of virus infection. RT-PCR was used to amplify a genome fragment carrying the deletion, which was then cloned, sequenced, and shown to be identical to the deleted form of SBWMV RNA 2 found after serial mechanical transmission of the virus (7). The deletion occurred between SBWMV RNA 2 genome coordinates 1,420 and 2,180.

After 13 wk at 17 C, a sample of total leaf RNA pooled from all 10 infected plants showed only full-length RNA 2 (and RNA 1)

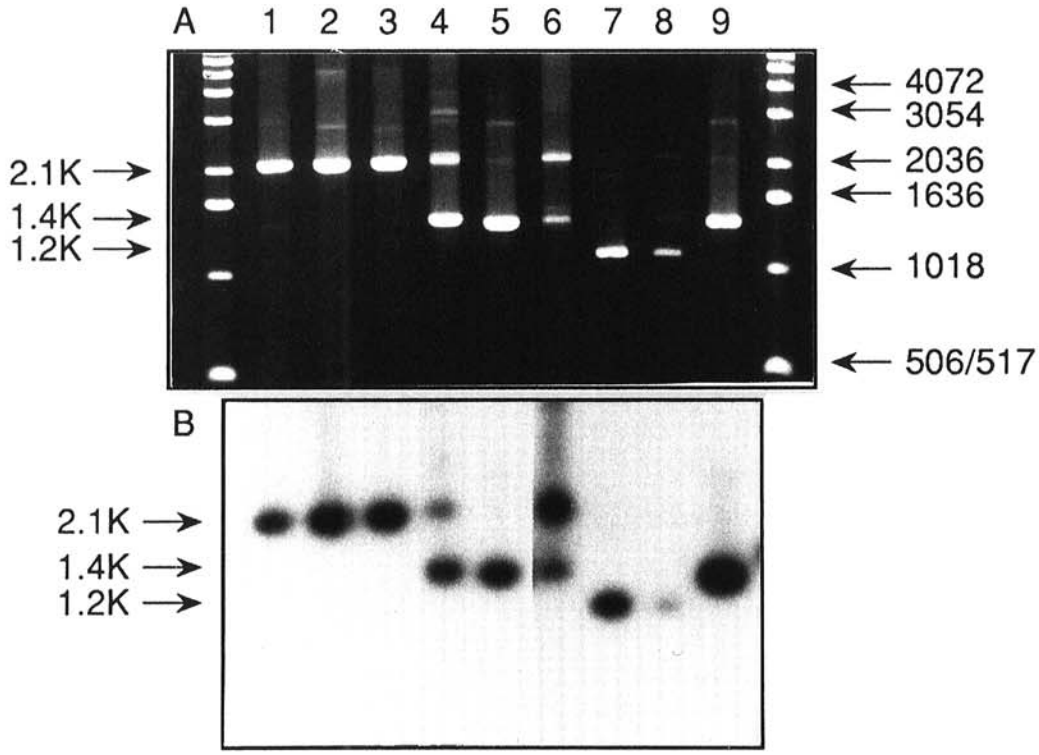

Fig. 7. Reverse transcriptase-polymerase chain reaction (RT-PCR) detection and analysis of soilborne wheat mosaic virus (SBWMV) RNA 2-derived fragments (with primers JC06 and JC08) in total leaf or root RNA samples from fungus-inoculated wheat plants grown at 25–30 C. RT-PCR products were **A,** stained with ethidium bromide and **B,** confirmed to be SBWMV related by Southern hybridization with an RNA 2-specific probe. Template RNAs used for the RT-PCR reactions shown in lanes 1–9 are described in Figure 6. Flanking lanes **(A)** contain DNA size markers, and their sizes (in base pairs) are given on the right. The significant RT-PCR products (2.1, 1.4, and 1.2 kbp) are indicated by arrows on the left.

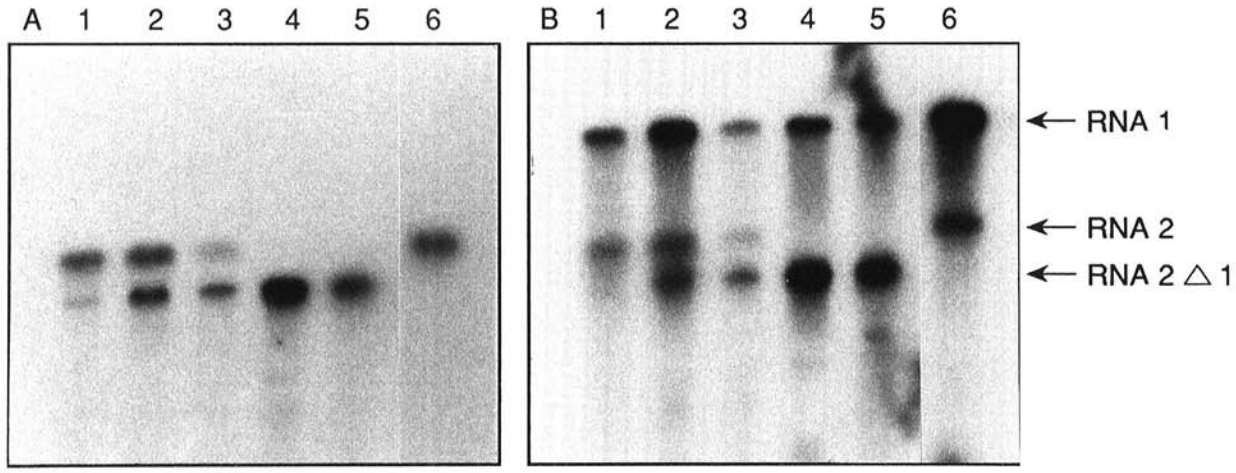

Fig. 8. Northern blot hybridization analysis of soilborne wheat mosaic virus (SBWMV) **A,** RNA 2- or **B,** RNAs 1- and 2-related species in leaves of a fungus-infected wheat seedling (plant X; lane 3 in Figs. 6A and B and 7A and B) propagated at 25–30 C for 9-13 wk. Total leaf RNA samples were taken at weekly intervals and probed as in Figure 6. Lanes 1–5 represent 9- to 13-wk samples, respectively. Pooled leaf RNA samples from plants grown at 17 C for 13 wk retained only full-length SBWMV RNAs 1 and 2 (lane 6). The identities of the SBWMV RNA species are shown on the right. RNA 2 △1 is the 759-nt deletion Okl-7.

by Northern blotting (Fig. 8A and B, lane 6).

More extensive but unstable or poorly replicated deletions arise in RNA 2 in some SBWMV-infected plants at higher temperatures. Twelve weeks from sowing, leaf and root RNAs were prepared from another of the 10 plants transferred to the glasshouse (25–30 C) 8 wk previously. This plant was symptomless and contained intact SBWMV RNA 1 (Fig. 6B, lane 7) but had a more extensively deleted form of RNA 2 (approximately 2.6 kb) (Fig. 6A and B, lane 7). No signal was obtained in Northern blots with RNA from roots (Fig. 6A and B, lane 8), but RT-PCR products were obtained that were identical to those amplified from leaf samples (Fig. 7A and B, lanes 7 and 8). This approximately 1-kb deletion in SBWMV RNA 2 resulted in an RT-PCR fragment of 1.2 kbp (primers JC06 and JC08) (Fig. 7A and B, lanes 7 and 8). Cloning and sequencing showed the deletion to be between genome coordinates 1,460 and 2,489.

When sap from this plant and other wheat plants propagated at 25–30 C for 8 wk (12 wk from sowing) was combined and used to mechanically inoculate wheat seedlings (cultivar Vona) at the two-thirds-leaf stage, only the 759-nt deleted form of SBWMV RNA 2 was detected after an additional growth period of 2 wk at the higher temperature (Figs. 6A and B and 7A and B, lane 9). After this single mechanical passage, these virus-inoculated plants showed stronger symptoms than the plants kept at 25 C for 12 wk after infection by viruliferous zoospores. Thus, both the full-length (3.6-kb) and the more extensively deleted (2.6-kb) forms of SBWMV RNA 2 were no longer detected in plants after propagation at 25–30 C for between 4 and 12 wk followed by a single mechanical transfer and additional growth for 2 wk at the higher temperature. In individual wheat plants, we have seen that the loss of extensively deleted RNA 2 species can occur even more rapidly (Figs. 6A and B and 7A and B, lane 5).

DISCUSSION

In this paper, we report that RT-PCR methods are the most sensitive for detecting SBWMV in root or leaf RNA extracts and that Northern blotting is sufficiently sensitive for detecting SBWMV RNA only in leaf samples. TAS-ELISA is also comparatively insensitive (Table 1).

After mechanical inoculation, SBWMV RNA 1 and full-length or deleted RNA 2 moves rapidly from the leaves to the roots. After inoculation by fungus, the spread of full-length and deleted virus from roots to leaves is equally rapid. The process of spontaneous deletion in SBWMV RNA 2 is accelerated at higher growth temperatures (25–30 vs. 15–17 C), but the deleted form seems to cause more severe symptoms only after one or more mechanical transfers to younger, healthy test plants. Full-length SBWMV, introduced as virions by viruliferous zoospores of *P. graminis,* also undergoes spontaneous RNA 2 deletion, especially at 25–30 C, but does not cause symptoms. Thus, the presence of deleted RNA 2 or a truncated CP-RT polypeptide is not responsible per se for the severe symptoms observed previously (7,19). It may be that symptom severity is determined by the route of virus entry into the foliage (direct infection after mechanical abrasion as opposed to vascular transport from fungus-infected roots) or, more likely, by the developmental stage of leaf tissues first exposed to the deleted form of SBWMV RNA 2. Thus, in field-grown plants in late spring, although higher temperatures and prolonged infection since the previous autumn may favor the accumulation of deleted forms of SBWMV RNA 2, the new growth of the host plant displays reduced symptoms of virus infection. If the spontaneously deleted forms of SBWMV are then mechanically transferred to healthy cereal plants, however, severe symptoms occur.

We have shown that in mechanically inoculated plants grown in the greenhouse, deletions greater than 759 nt occur in RNA 2 of the Oklahoma isolate of SBWMV but that during further passaging, the 759-nt deletion becomes the dominant form whereas the more extensive deletions are not maintained (7). We have sequenced several independent RT-PCR clones of each of the different truncated forms of RNA 2 found in Okl-1 and in subsequent serial passages (7) and have compared them with the sequence of full-length SBWMV RNA 2 (Oklahoma isolate). In all clones, regardless of the extent of the deletion, the 5′-deletion site mapped at or very close to genome position 1,460. The different deletion endpoints contain no obvious sequence homologies or duplications, which argues against a mechanism of deletion formation as a result of homologous recombination. However, there is some evidence that RNA secondary structure might play a role in the deletion of RNA 2 sequences (J. Chen, S. MacFarlane, and T. Wilson, *unpublished*). We hope that our observations in this study, together with those of previous studies of the 1,058-nt deletion in the Nebraska Lab1 mutant of SBWMV RNA 2 (18) and mapped deletion events in other furovirus RNAs, will lead to an understanding of the molecular mechanism for this phenomenon during RNA replication in planta.

Our findings may also be pertinent to a recent report (8) on the expression of resistance to SBWMV in resistant cultivars of hard red winter wheat (Hawk and Newton). An inhibition of virus movement from fungus-inoculated roots to foliage, which operated at 15 C, broke down at elevated temperatures (23 C) over 24 days (8). These workers used ELISA as a sensitive, economic method to detect SBWMV in both tissue types (8,13,15). However, since ELISA detects CP and not RNA, it cannot detect or distinguish the presence of full-length or truncated RNA 2 molecules in roots or shoots. Thus, it may be instructive to study RNA deletion and long-distance movement phenomena together in resistant or susceptible cultivars at high and low temperatures.

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