A PCR-Based Assay for Wheat Soilborne Mosaic Virus in Hard Red Winter Wheat

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ABSTRACT

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A reverse transcription (RT)–polymerase chain reaction (PCR)–based assay that allows individual detection of RNA1 and RNA2 of wheat soilborne mosaic virus (WSBMV) was developed and utilized in conjunction with ELISA to detect WSBMV in *Triticum aestivum*. Resistant cultivars Newton and Hawk and susceptible cultivars Vona and Sage were planted 30 September 1991 in a field with a history of severe wheat soilborne mosaic. In ELISA, WSBMV was detected only rarely in root samples from either susceptible or resistant cultivars taken on 16 October or 2 November but in nearly all root samples taken on or after 4 December. RNA2 was detected in all ELISA-positive root samples and in most ELISA-negative root samples from either susceptible or resistant cultivars. Thus, RNA2 could be found in root samples of susceptible or resistant cultivars up to 7 wk before virus could be detected by ELISA. RNA1 was detected in most samples in which RNA2 was detected but was detected only rarely in samples in which RNA2 was not detected. In ELISA and/or RT-PCR, WSBMV was detected in most foliar samples from susceptible cultivars taken on or after 4 December. In ELISA and/or RT-PCR of foliar samples from resistant cultivars, however, detection of WSBMV was limited until the 1 March sampling. These results support the use of ELISA for evaluating cultivars for resistance to WSBMV and support the hypothesis that resistance is expressed as an inhibition of virus movement. The results do not support the possibility that resistance is expressed differentially against the two WSBMV RNAs.

Wheat soilborne mosaic virus (WSBMV) causes wheat soilborne mosaic (WSBM), a major disease of hard red winter wheat (*Triticum aestivum* L.) in the Great Plains states (2). Long (281 × 20 nm) and short (142 × 20 nm) particles of the virus contain, respectively, 7,090 base (RNA1) and 3,593 base (RNA2) components of a bipartite RNA genome (11,12; Y. Shirako and T. M. A. Wilson, *personal communication*). The short particle is approximately 20 times more prevalent in infected tissue than is the long particle, but both particles, or their RNAs, are required for infection (12). The virus is transmitted by the soil fungus *Polymyxa graminis* Ledingham (3,4). Viruliferous resting spores of *P. graminis* survive many years in the soil and cannot be eliminated by economically feasible methods.

Efforts to control WSBM have focused on development and planting of resistant cultivars, but the mechanism of resistance of resistant cultivars is unclear (2). *P. graminis* can colonize the roots of both susceptible and resistant cultivars (6,8), suggesting that resistance is directed at the virus rather than the vector. Although more virus and viral antigen can be detected in susceptible plants (1), the detection of virus and viral antigen in foliage of both resistant and susceptible plants following spring growth suggests that resistance involves more than inhibition of viral replication. In a growth chamber study (10), viral antigen was found in the roots of both susceptible and resistant plants following growth in soil containing viruliferous *P. graminis*, but in the foliage of only the susceptible plants. These results, obtained using ELISA, indicated resistance may be due to inhibition of virus movement. To test and refine this hypothesis, alternative methods are needed to detect virus and viral components following infection by viruliferous zoospores.

Reverse transcription (RT)–polymerase chain reaction (PCR)–based assays have been increasingly used for detection of plant viruses (7,13), but the utility of RT-PCR as a supplement to ELISA in the study of the early host-virus interaction has not been explored. Here we report development of an RT-PCR assay to assess the spread of WSBMV in field-planted hard red winter wheat. Detection of viral antigen and RNA were by ELISA and RT-PCR, respectively, in root and foliar samples from plants of susceptible and resistant cultivars.

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MATERIALS AND METHODS

Cultivars. The WSBM-susceptible cultivars Vona (CI 17441) and Sage (CI 17277) and the WSBM-resistant cultivars Newton (CI 17715) and Hawk (CI 17952) were used. The reaction to WSBM of these cultivars has been previously characterized (4).

Field study. Pots 25 cm in diameter and 45 cm deep were filled with soil from a WSBMV-infested field west of Stillwater, Oklahoma, and buried to the rim in this field. Pots were used to prevent the intermingling of the root systems of separate samples. For each selected cultivar, five to 10 seeds were sown in each of 20 pots (80 pots total). Pots were arrayed randomly in the field, and seed was planted on 30 September 1991. Plants were thinned to three to five per pot after 4 wk.

Sampling was performed five times over the growing season (16 October, 2 November, and 4 December 1991 and 1 March and 27 April 1992). Plants from two or three randomly selected pots of each cultivar were removed at each sampling date. Plants within each pot were pooled, cleansed of soil, and separated into root (portion from the crown down) and foliar (portion above the crown) subsamples. Within 24 hr, root and foliar subsamples were ground separately in liquid nitrogen and further subdivided for analysis by ELISA and isolation of viral RNA.

ELISA. Virus was detected by ELISA as previously described (5). A_{405nm} values ≥ 0.10 were considered positive for WSBMV (5). Values reported are mean values of three values taken.

Viral RNA isolation and RT-PCR assay. Viral RNA was isolated as described by Langeveld et al (7). Frozen plant tissue samples (approximately 150 mg) were ground in 0.4–1 ml of extraction buffer containing 1 mM Tris (pH 8), 100 mM LiCl, 1% SDS, and 10 mM EDTA. Homogenates were extracted with water-saturated phenol at 65 C for 5 min. Aqueous phases were then extracted three times at room temperature with phenol:chloroform:isoamyl alcohol (25:24:1). The RNA in the final aqueous phases was precipitated overnight at −20 C with 0.1 volume 3 M sodium acetate and 2.5 volumes 95% EtOH. Precipitated RNA was pelleted by centrifugation, washed with 75% ethanol, dried under vacuum, and resuspended in 50 µl of sterile H_2O. The RNA isolation procedure was also performed on 10- to 20-mg samples of soil collected at each sampling date from the pots containing the plants.

Production of cDNA from viral RNA templates was performed using Superscript reverse transcriptase (Gibco BRL, Gaithersburg, MD) in 40-μl reaction volumes containing a 1-μl sample. Reaction conditions were those suggested by the supplier. Priming was with downstream PCR primers specific for WSBMV RNA1 (1-1020−) and RNA2 (2-860−) (Fig. 1).

PCR amplification was performed in 0.5-ml GeneAmp tubes (Perkin Elmer Cetus, Norwalk, CT) using 1 μl of cDNA reaction mixture, 25 pmol each of a selected upstream and a selected downstream primer (Fig. 1) (Oligos Etc. Inc., Wilsonville, OR), and 0.25 units of *Taq* polymerase (Perkin Elmer Cetus) in 50-μl reaction mixtures prepared as suggested by the supplier. For PCR amplification of WSBMV RNA1, primer 1-1020− was used in conjunction with either primer 1-361+ or primer 1-501+. For amplification of WSBMV RNA2, primer 2-860− was used in conjunction with either primer 2-341+ or primer 2-481+. The 5′ to 3′ sequences of primers used were as follows: AACGCGGCACACATAGTTTT (1-361+), GTGCATTGTTGCTGTCCCAC (1-501+), CGAAAGTCTTAGTAAGATAT (1-1020−), TAAATAAAGGTTACACTGGT (2-341+), ATGCTTAATGGCGTGAGTAA (2-481+), and CTCGAACCTTCCCATTTCAA (2-860−). Thermocycling was performed in a PTC-100 model 60 (MJ Research, Inc., Watertown, MA) programmed as follows: 94 C for 1 min; followed by 40 cycles of 90 C for 30 sec, 55 C for 45 sec, and 70 C for 45 sec; and 70 C for 3 min.

PCR products were loaded (20 μl) on 2% agarose gels prepared in Tris-acetate-EDTA buffer and analyzed by electrophoresis and ethidium bromide staining (Fig. 2). Stained gels were examined for the presence of bands whose apparent size matched that predicted, considering the primer pair used for PCR amplification (Fig. 1). Selected PCR products were subjected to restriction analysis to corroborate their identity. Faint bands of the predicted size were noted. Positive controls were cDNAs prepared from samples with A_{405nm} values ≥0.50. Negative controls consisted of PCR reactions performed with no cDNA or RT-PCR performed on RNA isolated from healthy tissue.

RESULTS

Nine of 12 root subsamples from susceptible cultivars and 10 of 11 root subsamples from resistant cultivars taken on the first two sampling dates (16 October and 2 November) were negative in ELISA for WSBMV (Table 1). In contrast, on the 4 December and subsequent samplings, all root subsamples were positive in ELISA for WSBMV. All ELISA-positive root subsamples were also positive in RT-PCR when primers specific for the coat protein region of RNA2 were used (Table 1). Additionally, RNA2 was detected in all ELISA-negative root subsamples taken 2 November and in three of four ELISA-negative subsamples of susceptible cultivars and two of five ELISA-negative subsamples of resistant cultivars taken on 16 October. Attempts to detect by RT-PCR viral RNA in soil infested with *P. graminis* were unsuccessful (*data not shown*; T. M. A. Wilson, *personal communication*).

Using ELISA, WSBMV was first detected in shoots in three of six samples of susceptible cultivars taken on 4 December. All shoot samples from resistant cultivars taken on or before 4 December were negative in ELISA. Six shoot subsamples of susceptible cultivars taken on or before 4 December were negative in ELISA, whereas their respective root subsamples were positive. Shoot subsamples of four of these six were strongly positive by RT-PCR assay for RNA2, one was weakly positive for RNA2, and one was negative for RNA2. All seven samples of resistant cultivars that were taken on or before 4 December and that had root subsamples positive for WSBMV in ELISA had shoot subsamples that were negative for WSBMV in ELISA. Four shoot subsamples of these seven were negative in RT-PCR for RNA2 and three were weakly positive. All shoot subsamples that tested positive for WSBMV in ELISA were positive for RNA2 in RT-PCR. With one exception (a cv. Newton sample taken at the 16 October sampling), RNA2 was not detected in shoots unless the corresponding roots were positive by ELISA (18 samples total).

In both resistant and susceptible cultivars, fewer positive results were obtained in RT-PCR when RNA1-specific primers were used instead of RNA2-specific primers. Whereas RNA2 was detected in each of 25 samples with ELISA values >0.03, six samples with ELISA values between 0.03 and 0.22 tested negative for RNA1 (two of four in susceptible cultivars and four of seven in resistant cultivars). Sixteen samples (nine in resistant and seven in susceptible cultivars) with ELISA values <0.04 contained RNA2, whereas RNA1 was detected in only five of these (two from resistant and three from susceptible

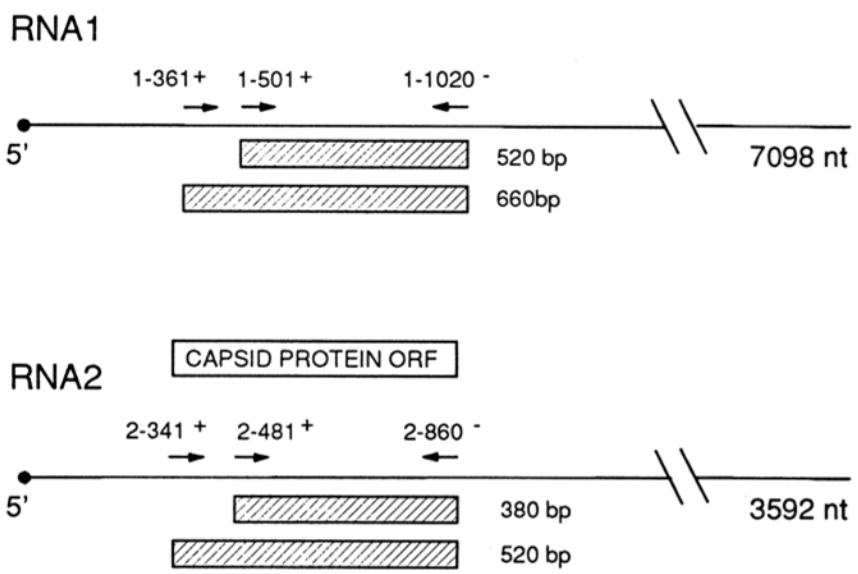

Fig. 1. Wheat soilborne mosaic virus (WSBMV) RNA sequence (11; Y. Shirako and T. M. A. Wilson, *personal communication*) coordinates of primers (arrows) used for reverse transcription–polymerase chain reaction–based amplification of WSBMV RNA1 and RNA2. Location of capsid protein ORF is indicated in open bar and sizes in base pairs (bp) of amplification targets are indicated by hatched bars.

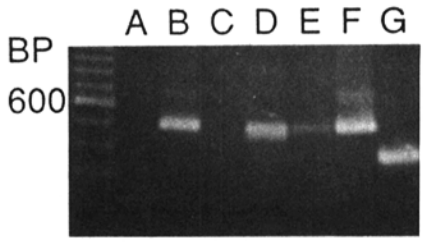

Fig. 2. Detection by reverse transcription–polymerase chain reaction (PCR) assay of wheat soilborne mosaic virus RNA1 and RNA2 in selected samples of hard red winter wheat. A 20-μl aliquot of the 50-μl PCR reaction mixture was analyzed in a 2.0% agarose gel. Lanes A, B, E, and F = amplification of 520-bp fragment using RNA2-specific primers (2-341+ and 2-860−); lanes C and D = amplification of 520-bp fragment using RNA1-specific primers (1-501+ and 1-1020−); and lane G = amplification of 380-bp fragment using RNA2-specific primers (2-481+ and 2-860−). Samples are A = negative control, B = positive control, C and E = cv. Vona root collected 16 October, and D, F, and G = cv. Vona root collected 4 December. Left lane = 100-bp ladder size marker.

Table 1. Detection of wheat soilborne mosaic virus (WSBMV) RNA1 or RNA2 by reverse transcription–polymerase chain reaction (RT-PCR) and WSBMV coat protein by enzyme-linked immunosorbent assay (ELISA) in shoot and root samples of two susceptible cultivars (Vona and Sage) and two resistant cultivars (Newton and Hawk) of hard red winter wheat planted 30 September 1991 in a field with a history of severe wheat soilborne mosaic[a]

Sampling date	Vona						Sage						Newton						Hawk					
	Root			Shoot			Root			Shoot			Root			Shoot			Root			Shoot		
	ELISA	PCR		ELISA	PCR		ELISA	PCR		ELISA	PCR		ELISA	PCR		ELISA	PCR		ELISA	PCR		ELISA	PCR	
	A_{405}	RNA1	RNA2	A_{405}	RNA1	RNA2	A_{405}	RNA1	RNA2	A_{405}	RNA1	RNA2	A_{405}	RNA1	RNA2	A_{405}	RNA1	RNA2	A_{405}	RNA1	RNA2	A_{405}	RNA1	RNA2
16 Oct.	0.02	−	++	0.01	−	−	0.29	++	++	0.01	−	−	0.04	+	++	0.04	−	++	0.01	−	−	0.01	−	−
	0.23	++	++	0.01	−	++	0.02	+	+	0.01	−	−	0.02	+	++	0.02	−	−	0.02	−	−	0.00	−	−
	0.04	−	+	0.02	−	−	0.02	+	−	0.01	−	−							0.02	−	−	0.01	−	−
2 Nov.	0.06	−	++	0.01	−	−	0.09	++	++	0.01	−	−	0.05	++	++	0.02	NT[b]	NT	0.03	NT	NT	0.01	−	−
	0.01	+	++	0.01	−	−	0.07	++	++	0.01	−	−	0.02	+	++	0.02	−	−	0.12	−	++	0.01	−	−
	0.48	++	++	0.02	−	++	0.02	++	++	0.01	−	−	0.01	−	+	0.01	−	−	0.02	−	++	0.01	−	−
4 Dec.	0.74	++	++	0.72	NT	++	0.77	++	++	0.02	−	+	0.18	++	++	0.02	−	+	0.10	NT	NT	0.03	−	−
	0.88	++	++	0.01	−	++	0.60	++	++	0.98	++	++	0.21	−	++	0.02	−	+	0.22	+	++	0.02	−	−
	0.78	++	++	0.80	++	++	0.43	++	++	0.02	−	++	0.27	++	++	0.02	−	+	0.16	−	++	0.02	−	−
1 Mar.	0.73			0.74			0.93			0.75			0.12			0.02			0.81			0.66		
	0.76			0.84			0.69			0.86			0.55			0.72			0.17			0.91		
	0.65			0.86			NT			0.94			0.34			0.57			0.38			0.94		
27 Apr.	NT			0.77			NT			0.83			NT			0.02			NT			0.59		
	NT			0.81			NT			0.76			NT			0.79			NT			0.51		
	NT			0.84									NT			0.86			NT			0.02		

[a]Three to five plants were divided into root and shoot subsamples, which were homogenized by grinding in liquid nitrogen, then divided into portions for analysis by ELISA and for isolation of viral RNA. Samples for ELISA were stored at −20 C until assayed. Assays were performed at two different times. Positive controls consisting of previously harvested infected wheat leaves ($A_{405nm} = 0.40 \pm 0.05$) and uninfected negative controls ($A_{405nm} = 0.01 \pm 0.01$) were run at both assay times; some samples were assayed at both times to ensure consistency of results between the two times. Values are means of three ELISA absorbances. ELISA values ≥0.10 were judged positive for WSBMV. The RT-PCR assay was performed on viral RNA samples using primers specific for either WSBMV RNA1 or RNA2; 20 µl of 50-µl total PCR products was subjected to electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. − = No PCR product visible, + = PCR product faintly visible, ++ = PCR product clearly visible. Positive controls for RT-PCR were cDNAs prepared from samples with A_{405nm} values ≥0.50. Negative controls consisted of PCR reactions performed with no cDNA or RT-PCR performed on RNA isolated from healthy tissue. Positive and negative controls were run with each set of RT-PCR reactions and invariably yielded bright and undetectable product bands, respectively. The RT-PCR assay was performed on samples from the first three sampling dates.

[b]NT = not tested.

cultivars) and in only one (a root subsample of cv. Sage taken 16 October) of 26 samples that tested negative for RNA2. Positive and negative controls run with each set of RT-PCR reactions invariably yielded bright and undetectable product bands, respectively.

DISCUSSION

WSBMV was detected in the roots of resistant and susceptible cultivars at approximately the same time after exposure to viruliferous zoospores in the present field experiment (Table 1) as in previous growth chamber experiments (10). In the present experiment, WSBMV RNA2 was detected by RT-PCR assay in all ELISA-positive samples and in most root subsamples taken prior to 4 December when the majority of ELISA results were negative. As we, and others, have been unsuccessful in detecting viral RNA in samples of *P. graminis*-infested soil, the RT-PCR assay results indicate that virus or viral RNA was present in the roots of all cultivars up to 7 wk before WSBMV could be detected by ELISA.

In RT-PCR assays of both resistant and susceptible cultivars, fewer samples tested positive with RNA1-specific primers than with RNA2-specific primers. These results are consistent with the report (12) that RNA2 is 20 times more prevalent than RNA1 in infected tissue.

In the present field study, spread of virus to the foliage was delayed in resistant cultivars compared with susceptible cultivars. Virus was detected by ELISA in shoots of the susceptible cultivars by 4 December but was not detected by ELISA in shoots of resistant cultivars until 1 March, at which time spring warming had begun. The time at which virus became detectable in the roots using ELISA or RT-PCR assay did not differ between cultivars. Thus, the delay in the appearance of virus in shoots of resistant cultivars suggests that inhibition of upward movement of virus is important for resistance. Alternatively, replication of virus may be inhibited in the shoots of resistant cultivars.

Viral RNA was detected in several initial shoot samples of cv. Newton and virus was detected by ELISA in most foliar samples of cvs. Newton and Hawk taken on or after 1 March. This is consistent with a previous report (1) that resistance to WSBMV is not absolute and that virus can be detected in the foliage of resistant cultivars in spring as the temperature rises. Additional support for this view was obtained in a recent growth chamber study (9) in which inhibition of upward spread of WSBMV in resistant cultivars grown in *P. graminis*-infested soil could be overcome by growing the plants at 23 C following an initial period of growth at 15 C. The frequencies of occurrence of RNA1 and RNA2 in samples positive and negative by ELISA did not differ between cultivars. Thus, resistance appears to be temperature-dependent and not differentially targeted at either the long or short WSBMV particles or their RNAs.

We have shown that RT-PCR assay is useful for detection of WSBMV and provides important information not obtainable using ELISA alone on the WSBMV-wheat interaction. In addition, the results support the ELISA-based approach, used over several years, to evaluate resistance to WSBMV in hard red winter wheats (5).

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