Techniques

Differentiation of Two Closely Related Furoviruses Using the Polymerase Chain Reaction

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Accepted for publication 30 August 1994.

ABSTRACT

Rush, C. M., French, R., and Heidel, G. B. 1994. Differentiation of two closely related furoviruses using the polymerase chain reaction. Phytopathology 84:1366-1369.

Oligonucleotide primers based on published sequence data for beet necrotic yellow vein virus (BNYVV) were synthesized for use in the reverse transcriptase polymerase chain reaction (RT-PCR) to differentiate beet soilborne mosaic virus (BSBMV) from BNYVV. Primers designed for the 3′ end of BNYVV RNA 1 were effective in PCR amplification of a product of the predicted size, approximately 1,056 bp, from extracts of plants infected by BNYVV. The same primer pair also directed the amplification of a PCR product of approximately 1,000 bp from extracts of plants infected by BSBMV. If extracts from plants infected with BNYVV were mixed with those from plants infected with BSBMV, the primer pair allowed the amplification of only BNYVV. In addition to the slight size difference, the BSBMV product could be distinguished from the BNYVV product by digestion with *Tha*I, which cleaved the BSBMV product but not the BNYVV product. The BSBMV RT-PCR product was partially sequenced, and primers specific for BSBMV were synthesized. The primers directed the amplification of a PCR product of the predicted size, approximately 691 bp, only with extracts from plants infected by BSBMV. Only one PCR product of the size expected for BSBMV was produced from extracts containing both BSBMV and BNYVV. The BSBMV PCR product obtained with the BSBMV-specific primers could be digested by *Tha*I. PCR products of similar size were amplified using the BSBMV primers and extracts of several isolates of BSBMV differing in geographic origin and symptom phenotype.

Rhizomania, caused by beet necrotic yellow vein virus (BNYVV), was first reported in the United States in California in 1984 (3). It was next identified in Texas in 1987 (2). The disease was thought to be restricted to these two states, but during 1992–1993, rhizomania was found in Colorado, Idaho, Nebraska, and Wyoming (4,5). Because of the importance of the sugar beet industry in these states, intensive programs to determine the distribution of BNYVV were established. However, the presence of another soilborne virus infecting sugar beet, initially designated as Tx7, complicated and confused detection and identification of BNYVV (10).

Tx7 was originally identified in infected sugar beets from Texas in 1988 (10), but serologically identical viruses have since been found in California, Colorado, Idaho, Nebraska, and Wyoming. Tx7, presently designated beet soilborne mosaic virus (BSBMV), has been partially characterized (8) and is similar to BNYVV. BSBMV, a multiparticulate virus, is vectored by *Polymyxa betae* Keskin. It has a capsid protein of approximately 22.5 kDa, and the RNA species are polyadenylated. Some reports have indicated serological cross-reactivity between BNYVV and BSBMV (14). Because of the morphological similarities between BNYVV and BSBMV and the potential for serological cross-reactivity, a dependable method for differentiating these two viruses was needed. This report describes the use of the reverse transcriptase polymerase chain reaction (RT-PCR) to detect and differentiate BSBMV and BNYVV.

MATERIALS AND METHODS

Virus maintenance. All BNYVV and BSBMV isolates were obtained from infected sugar beets from Texas, California, and Nebraska (Table 1). Initial isolate selection was based on serology and diagnostic root and foliar symptoms for BNYVV and BSBMV, respectively (7). Isolates were maintained in the greenhouse in *Chenopodium quinoa* Willd. by repeated mechanical inoculation or in sugar beet root cultures (6).

PCR. A primer pair was synthesized for BNYVV RNA 1 based on published nucleotide sequence data from European isolates (1). The downstream primer BNYVV 1 (5′ TTCACACCCAG-TCAGTA 3′) is complementary to bases 6,688–6,704 of RNA 1, but the sequence is common to all four RNA species of BNYVV. The upstream primer BNYVV 3 (5′ AGATAGTGCTATAAA-CGG 3′) is identical to bases 5,649–5,666 and is specific for RNA 1.

Crude nucleic acid extracts (11) or purified BNYVV and BSBMV RNA (8) were used as templates for first-strand cDNA synthesis in reverse transcriptase reactions. Extracts from noninfected plants were used as controls. Two microliters of sample was mixed with 0.2 M 2-mercaptoethanol, 10 pmol BNYVV 1, 10 mM dNTPs, 3.5 units of AMV reverse transcriptase (Boehringer Mannheim, Indianapolis, IN), 5 μl of 5× reaction buffer, supplied with the enzyme, and H_2O to a final volume of 20 μl. The solution was incubated at 40 C for 1 h, diluted to 40 μl H_2O, and boiled 5 min to stop the reaction.

PCR amplification was carried out in 50-μl reactions using 2 μl of cDNA, 10 pmol of each primer, 10 mM dNTPs, 2.5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), 5 μl of 10× reaction buffer, supplied with the enzyme, and H_2O to volume. The mixture was overlaid with 100 μl of mineral oil and subjected to 35 cycles consisting of 1 min at 94 C, 1 min at 41 C, and 2 min at 72 C. During the first and last cycle, the extension step was held at 72 C for 10 min, and the reaction mixture was held at 4 C after the final cycle. PCR products were analyzed by electrophoresis in 1% agarose gels and visualized by staining with ethidium bromide. In some experiments, BNYVV and BSBMV RNA were mixed and used in the cDNA reaction. Alternatively, cDNAs made from the different extracts were mixed and used in PCR.

To verify the identity of amplified products, bands of the expected size were cut from gels, and DNA was extracted using the Gene Clean kit (Biosis 101, La Jolla, CA). Purified DNA was digested with restriction enzymes *Dra*I, *Tha*I, *Nhe*I, and *Spe*I following the manufacturer's instructions. Based on the published nucleotide sequence of BNYVV (1), each of these enzymes was expected to digest the BNYVV PCR product at only one site, with the exception of *Dra*I, with three predicted restriction sites (Fig. 1). Digestion products were analyzed by electrophoresis in 1% agarose gels and staining with ethidium bromide.

RESULTS

A PCR product of the expected size was produced by PCR amplification of BNYVV cDNA using the two primers BNYVV 1 and BNYVV 3. The same product was obtained using either crude nucleic acid extracts or purified virion RNA preparations. Somewhat unexpectedly, a PCR product also was produced when the BNYVV primers and BSBMV cDNA template were used (Fig. 2, lane 3). This product, approximately 1,000 bp compared to 1,056 bp for the BNYVV product, was produced with all BSBMV isolates tested (Table 1). However, when BNYVV was mixed with BSBMV, either as RNA samples for first-strand cDNA synthesis or as cDNA in PCR, only the BNYVV product was amplified (Fig. 3, lane 7).

When exposed to restriction enzyme digestion, both BNYVV and BSBMV PCR products were digested by *Dra*I, *Nhe*I, and *Spe*I, as predicted from BNYVV nucleotide sequence data (Fig. 1). However, *Tha*I digested only the BSBMV product (Fig. 2, lanes 6 and 7). When BSBMV cDNA was mixed with BNYVV cDNA and amplified by PCR, the single resulting product was not digested by *Tha*I, further indicating that only BNYVV cDNA was amplified in mixed samples. In all cases, restriction fragments corresponding to the 5′ ends of BNYVV and BSBMV were similar in size, whereas those corresponding to the 3′ end of fragments of BSBMV were smaller than those of BNYVV. These results suggest that the nucleotide sequence of the BSBMV product is quite similar to that of the BNYVV product and that the size difference between the two products is likely due to a small deletion relative to the BNYVV RNA 1 sequence, near the 3′ end of BSBMV RNA 1.

After determining that primers BNYVV 1 and 3 directed amplification of a cDNA product unique for BSBMV, the BSBMV PCR product was cloned into *Hinc*II-digested pGEM3z and partially sequenced from each end by the dideoxy method of Sanger et al (13). Based on the preliminary nucleotide sequence data, two primers specific for BSBMV were synthesized. The upstream primer, Tx7-1 (5′-TACGCAACTCATTGAAAGGTA-3′), is identical to bases 66–86 of the BSBMV PCR product, and the downstream primer, Tx7-2 (5′-AGATAACACTTGTAA-CTCGTC-3′), is complementary to bases 737–756. Using these primers and BSBMV cDNA from several isolates (Table 1), the

TABLE 1. Geographic origin, symptom phenotype, and reverse transcriptase polymerase chain reaction (RT-PCR) products of beet necrotic yellow vein virus (BNYVV) and beet soilborne mosaic virus (BSBMV) isolates

Isolate	Origin	Symptoms[a]		RT-PCR products[b]		
		Sugar beet	*C. quinoa*	BNYVV primers	BSBMV primers	*Tha*I cleavage
BNYVV	California	NFS, RB	YS	1,056	None	No
BSBMV-Neb gfs	Nebraska	NRS, SDYB	DCS	1,000	700	Yes
BSBMV-Neb gh	Nebraska	NRS, SDYB	YS	1,000	700	Yes
BSBMV-Neb Morrill	Nebraska	NRS, SDYB	YS	1,000	700	Yes
BSBMV-Harken	Texas	NRS, SDYB	DCS	1,000	700	Yes

[a]Symptoms expressed on the sugar beet from which isolates were originally obtained or on mechanically infected *Chenopodium quinoa*: NFS = no systemic foliar symptoms; NRS = no obvius root symptoms; RB = root bearding; SDYB = systemic diffuse yellow banding on leaves; YS = yellow spots; and DCS = diffuse chlorotic spots.

[b]Approximate size in base pairs.

expected PCR product of 691 bp was obtained (Fig. 4). Tx7-1 and -2 did not allow the amplification of BNYVV cDNA, and when BNYVV and BSBMV cDNA were mixed and used in PCR, only the 691-bp product of BSBMV was amplified (Fig. 3, lanes 3 and 4, respectively). This product was digested with *Tha*I, verifying its origin from BSBMV (Fig. 2, lane 7).

DISCUSSION

The results of this study support conclusions from previous studies (8,12) that BSBMV is very closely related to BNYVV. Primers specifically designed for BNYVV RNA 1 matched BSBMV RNA well enough to allow amplification of a PCR product. However, with mixtures of both viruses, the precise match of BNYVV primers with BNYVV templates likely allowed more efficient amplification of the BNYVV template. Thus, the homologous BNYVV PCR products predominated in PCR reactions with mixtures of BNYVV and BSBMV cDNAs. Furthermore, the BSBMV product had *Dra*I, *Nhe*I, and *Spe*I restriction sites in common with the BNYVV PCR product. The BSBMV product also was digested by *Tha*I as predicted, whereas the BNYVV product was not. This indicates the nucleotide sequence of the BNYVV isolate used in this study differs from the published sequence of the European isolate in at least one restriction site near the 3′ end of RNA 1.

Using restriction mapping, researchers in Europe have identified differences between isolates of BNYVV (9). Two specific genotypes or strains were identified, but the biological significance of the differences, if any, has not been determined. It would be interesting to determine the nucleotide sequence differences between isolates of BSBMV and the different genotypes of BNYVV, especially considering the fact that some BSBMV isolates superficially resemble BNYVV RNA 3 deletion mutants with regard to symptom expression on *C. quinoa* and size of RNA 3 (8,12).

The degree of genetic variation among isolates of BSBMV is unknown. However, symptoms on infected plants can be minimal or mimic those of rhizomania, indicating probable genetic diversity. Indeed, some researchers have observed sufficient symptom variation to suspect and report the existence of two separate viruses: BSBMV 1 and 2 (14). We believe the observed variation in symptom expression does not warrant naming a second new virus. Furthermore, considering the present state of virus taxonomy and the confusion about what constitutes a new virus as opposed to a viral strain, we may be premature in accepting the name BSBMV for these viruses. Until BSBMV has been completely sequenced, we will be unable to verify whether BSBMV

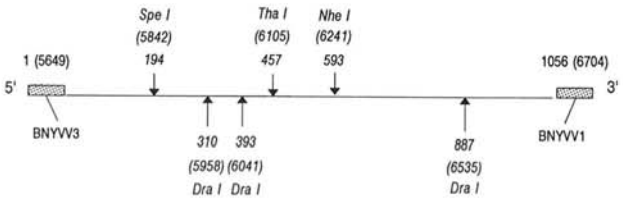

Fig. 1. Restriction map of beet necrotic yellow vein virus (BNYVV) RNA 1 reverse transcriptase polymerase chain reaction (RT-PCR) product based on published sequence data (1). Arrows indicate predicted restriction sites and numbers in parenthesis the corresponding location in the BNYVV RNA 1 sequence. The other numbers represent predicted restriction sites on the BNYVV RT-PCR product. Boxes at either end of the horizontal line indicate locations of sense (BNYVV 3) and anti-sense (BNYVV 1) primers used in amplification of the RT-PCR product.

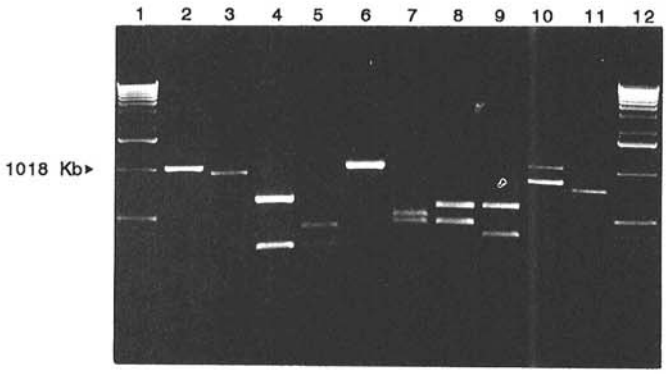

Fig. 2. Restriction endonuclease analysis of beet necrotic yellow vein virus (BNYVV) and beet soilborne mosaic virus (BSBMV) reverse transcriptase polymerase chain reaction (RT-PCR) products. RT-PCR products were analyzed after electrophoresis in a 1% agarose gel and staining with ethidium bromide. Lanes 1 and 12, 1-kb DNA ladder; lanes 2 and 3, BNYVV and BSBMV controls; lanes 4 and 5, BNYVV and BSBMV digested with *Dra*I; lanes 6 and 7, BNYVV and BSBMV digested with *Tha*I, the BNYVV band was not cleaved; lanes 8 and 9, BNYVV and BSBMV digested with *Nhe*I; lanes 10 and 11, BNYVV and BSBMV digested with *Spe*I.

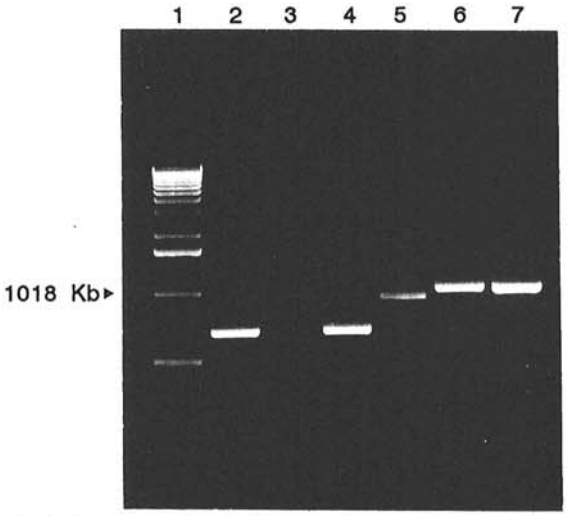

Fig. 3. Analysis of reverse transcriptase polymerase chain reaction (RT-PCR) products from single or mixed cDNA samples. Products were analyzed after electrophoresis in a 1% agarose gel and staining with ethidium bromide. Lane 1, 1-kb DNA ladder; lanes 2–4, beet soilborne mosaic virus (BSBMV), beet necrotic yellow vein virus (BNYVV), and BSBMV + BNYVV cDNA amplified by RT-PCR using primers Tx7-1 and -2; lanes 5–7, BSBMV, BNYVV, and BSBMV + BNYVV cDNA amplified by RT-PCR using primers BNYVV 1 and 3.

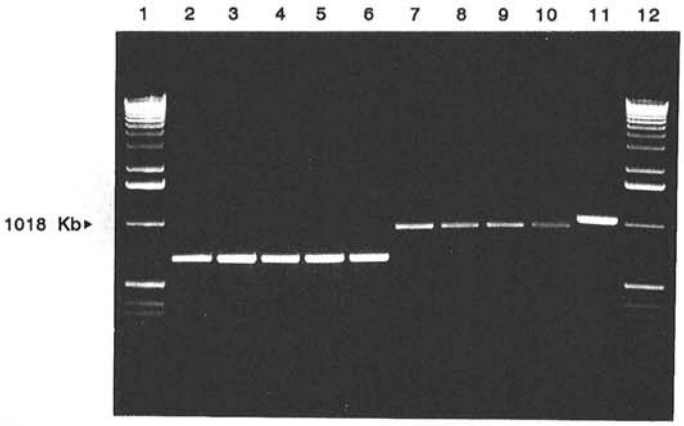

Fig. 4. Reverse transcriptase polymerase chain reaction (RT-PCR) products amplified from beet soilborne mosaic virus (BSBMV) or beet necrotic yellow vein virus (BNYVV) nucleic acid extracts using BSBMV primers Tx7-1 and -2 and BNYVV primers BNYVV 1 and 3. Products were analyzed after electrophoresis in a 1% agarose gel and staining with ethidium bromide. Lanes 1 and 12, 1-kb DNA ladder; lanes 2–5 and 7–10, BSBMV isolates from Texas and Nebraska amplified using BSBMV primers Tx7-1 and -2 and BNYVV primers BNYVV 1 and 3, respectively; lanes 6 and 11, BSBMV cDNA mixed with BNYVV cDNA amplified using primers Tx7-1 and -2 and BNYVV 1 and 3, respectively. With mixed cDNA, only RT-PCR products homologous to the primers were amplified.

is truly a distinct virus or whether isolates of the BSBMV serotype are actually biological variants or strains of BNYVV.

Although genetic variation is likely among isolates of BSBMV, RT-PCR products from different isolates appeared identical. Whether products were amplified using the BNYVV primer pair or Tx7-1 and -2, the PCR products produced with a given primer pair were apparently identical in size and restriction digestion profile. More importantly, BNYVV and BSBMV were easily detected and differentiated in infected plant tissue. If cDNAs made from BSBMV or BNYVV extracts were mixed, the two primer pairs only directed the amplification of the homologous cDNA. This suggests that if a plant is infected with both viruses, only one will be detected with a given set of primers. However, it also indicates that if the 1,000-bp BSBMV product is amplified when using the BNYVV primer pair, no BNYVV is present in the sample. This can be verified by restriction analysis with *Tha*I.

The development of RT-PCR assay for accurate and sensitive detection and differentiation of BSBMV and BNYVV is timely. In the last 3 yr, BNYVV and BSBMV have been identified in most of the western U.S. sugar beet-producing states (3–5). Incidence of BSBMV may have been overlooked in some of the initial reports of BNYVV in these states due to serological cross-reactivity between BNYVV and BSBMV. In a survey of BNYVV in Texas (7), several samples that tested positive for BSBMV in enzyme-linked immunosorbent assays also gave borderline positive readings for BNYVV. Similar results have been obtained in tests with BSBMV isolates from Colorado and Nebraska. However, subsequent RT-PCR analysis of some of these isolates indicated the presence of only BSBMV and ruled out the presence of a low level of BNYVV, as previously suspected. Although RT-PCR will not replace serology for routine testing, its specificity, as shown in this study, should be useful, especially when the possibility of dual infection by BNYVV and BSBMV exists.

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