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Detection and discrimination of wheat spindle streak mosaic virus and wheat yellow mosaic virus using multiplex RT-PCR

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Abstract

Wheat spindle streak mosaic virus (WSSMV) and wheat yellow mosaic virus (WYMV) are two closely related bymoviruses which cause significant yield losses in wheat. There is no molecular diagnostic protocol available for either virus nor are serological methods able to discriminate them. A multiplex reverse transcription-polymerase chain reaction (RT-PCR) protocol was developed for their detection and discrimination. Twenty-three isolates of WSSMV and WYMV were collected from a range of countries and the sequence of 834–837 nucleotides of the coat protein gene of three representative WSSMV isolates and one WYMV isolate was determined. This sequence data provided further evidence that the two viruses are distinct species and was used together with previously published sequence data to design specific oligonucleotide primers to discriminate the two viruses. Using total RNA isolated from eighteen WSSMV and five WYMV isolates in either one-step or two-step RT-PCR, these primers were shown to detect and discriminate the two viruses reliably. The primers were specific to either WSSMV or WYMV and no PCR product was obtained with either soil-borne wheat mosaic virus, which is frequently associated with both diseases, or with the closely related viruses, barley yellow mosaic virus, barley mild mosaic virus or oat mosaic virus. This new diagnostic protocol will enable more effective management of the diseases caused by these viruses by enabling correct identification of the causal pathogen and earlier detection than is possible by serological methods.

Members of the genus *Bymovirus* such as barley mild mosaic virus (BaMMV), barley yellow mosaic virus (BaYMV), oat mosaic virus (OMV), rice necrosis mosaic virus, wheat spindle streak mosaic virus (WSSMV) and wheat yellow mosaic virus (WYMV) cause economically important diseases of cereals. However, only WSSMV and WYMV in this group infect wheat. Both viruses have similar physical properties and morphology and are transmitted by *Polymyxa graminis* (Slykhuis, 1976; Haufler 1996; Inouye, 1996). They also cause similar symptoms on susceptible plants, i.e. chlorotic, spindle-shaped streaks on infected leaves, reduced tillering and decreased seed yield. Disease severity is determined by host susceptibility, virus pathogenicity and climatic factors, in particular temperature (symptoms are decreased at temperatures above 15–20 °C) (Haufler, 1996). In severe cases, yield reductions may be substantial, e.g. Slykhuis (1970) reported losses of up to 25–50%.

Both viruses have a widespread distribution; WSSMV has been reported from Canada (Slykhuis, 1976), France, Germany, India, Italy, Japan, the USA (Haufler, 1996) and Zambia (Kapooria and Ndunguru, 1998), and WYMV from China, France, Germany, Japan, North Korea and the Korean Republic (Inouye, 1996). However the taxonomic relationship between WSSMV and WYMV has been confused. On the basis of their physical, serological and pathological characteristics, Usugi and Saito (1979) concluded that WSSMV and WYMV represented strains of the same virus. This view was reiterated by Haufler (1996) who stated that WSSMV was probably a strain of WYMV.

In general viruses of this type from North America have been arbitrarily described as WSSMV, while those from China and Japan have been described as WYMV. In Europe the position has been even more confusing with similar strains being described as both WSSMV and WYMV, e.g. in France (Signoret et al., 1977; Hariri et al., 1987) and Germany (Proeseler and Stanarius, 1983; Huth and Lesemann, 1996). However, more recently the taxonomy of the group has been resolved by sequence analysis. In 1994, Sohn et al. reported the sequence of 4.6 kb representing the 3′-terminal half of RNA 1 of a French isolate of WSSMV (WSSMV-F), while Namba et al. (1998) sequenced the complete genome of a Japanese WYMV isolate (WYMV-J). By comparing the two sequences Namba et al. (1998) demonstrated that the two viruses are indeed distinct species; WSSMV and WYMV shared only 68.3% nucleotide homology in the 3′-terminal half of RNA 1. This discrimation was supported by sequencing of a 1.7 kb fragment representing the 3′-terminal sequence of RNA 1 of a Canadian WSSMV isolate (Lu et al., 1998); this sequence shared 98.0% homology with the French WSSMV isolate.

Discrimination of WSSMV and WYMV is important for practical as well as taxonomic reasons. WSSMV has a broader host range than WYMV, infecting not only *Triticum aestivum* but also *T. durum* and *Secale cereale* (Haufler, 1996; Inouye, 1996). Susceptibility of cereal cultivars to these viruses is also likely to differ which is particularly pertinent since host resistance is the only control method available. Furthermore, WSSMV but not WYMV infection has been reported to break down field resistance to the important pathogen, soil-borne wheat mosaic virus (SBWMV; genus *Furovirus*) (Lommel et al., 1986). Currently no diagnostic methods are available to discriminate between WSSMV and WYMV. No molecular protocols have been published for either virus, nor are either polyclonal antisera (Carroll et al., 1995) or monoclonal antibodies (Hariri et al., 1996) able to distinguish them. This paper describes the design of specific primers and an RT-PCR protocol to discriminate WSSMV and WYMV.

Four soils containing viruliferous spores of either WSSMV (Cornell, USA; Bay, USA; and Montpellier, France) or WYMV (Dengzhou, China) (Table 1) were used to infect plants. Two-week-old seedlings of a susceptible wheat cultivar (*Triticum aestivum* cv. Thunderbird) were incubated in a slurry of 40 g of infected soil at 20 °C for 10–14 days before transplanting into sufficient sterile sand to give a 1 : 9 dilution of the infected soil. The plants were grown for a further 3 weeks at 20 °C before decreasing the temperature to 10 °C. The plants were grown for up to a further 9–12 months and tested periodically for infection by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using antisera provided by G. Bergstrom (Carroll et al., 1995), W. Huth (BBA-Braunschweig, Germany) and P. Signoret (ENSAM/INRA, Montpellier, France). Further isolates of WSSMV, WYMV, BaYMV, BaMMV, OMV, and SBWMV were obtained in either dried leaves or roots (Table 1). Total RNA was extracted from 200 mg of either root or leaf tissue as described previously (Clover et al., 1999).

Two degenerate primers were designed from the sequence data of Sohn et al. (1994) and Namba et al. (1998) to complement the 3′ (WMVCPR) and 5′ (WMVCPF) ends of the coat protein (CP) genes of both WSSMV and WYMV (Table 2). These primers were used in RT-PCR to amplify the CP gene of three WSSMV isolates (Cornell, USA; Bay, USA; and Montpellier, France) and one WYMV isolate from Dengzhou, China (Table 1). Reverse transcription was performed using 0.5 µl of template RNA, 4 µl of WMVCPR (5 µM) and 100 units of MMLV reverse transcriptase (Promega, Southampton, UK) in a 10 µl volume at 37 °C for 1 h according to the manufacturer's instructions. The PCR reaction was carried using the Expand high fidelity PCR system (Roche Molecular Biochemicals, Lewes, UK) according to the manufacturer's protocol. Reactions were performed in a 100 µl volume containing 5 µl of cDNA, 10 µl of 10× Expand HF buffer, 2 µl each of dATP, dCTP, dGTP and dTTP (10 mM), 4 µl each of WMVCPR (5 µM) and WMVCPF (5 µM) and 2.6 units of Expand enzyme mix. The PCR thermal cycling conditions were 94 °C for 2 min, then 10 cycles of 94 °C for 45 s, 50 °C for 30 s and 72 °C for 1 min, then 20 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 3 min, followed by 72 °C for 7 min. Amplified products were observed after electrophoresis in ethidium bromide-stained 1.2% agarose gels. The amplified products representing the CP genes of these isolates were of the expected size (i.e. 879–882 nucleotides) and were ligated into the pSCREEN-T vector (Novagen, Madison, USA) prior to transformation and sequencing as decribed previously (Clover et al., 1999).

Multiple sequence analysis of the CP genes of these isolates together with those previously published, that

Table 1. Isolates of soil-borne cereal viruses used during this study

Virus	Origin	Supplier
WSSMV	Bay, Arkansas, USA	Professor E. Milus
	Urbana, Illinois, USA	Professor F. Kolb
	Boyd, Indiana, USA	Professor K. Perry
	West Lafayette, Indiana, USA	Professor K. Perry
	Hesston, Kansas, USA	Professor K. Perry
	Cornell, New York, USA	Professor G. Bergstrom
	Warsaw, Virginia, USA	Professor E. Stromberg
	Kent County, Ontario, Canada	ATCC PV-116
	Montpellier, Hérault, France	Dr. P. Signoret
	Seine-Maritime, France	Ms. F. Gitton/SPRV-Orleans
	Loiret, France	Ms. F. Gitton/SPRV-Orleans
	Landes Le Gaulois, Loir-et-Cher, France	Dr. M. Bonnefoy
	Chambon sur Cisse, Loir-et-Cher, France	Dr. M. Bonnefoy
	Koenigslutter, Braunschweig, Germany	Dr. W. Huth
	Parma, Parma, Italy	Dr. C. Rubies-Autonell
	Cadriano, Bologna, Italy	Dr. C. Rubies-Autonell
	Ozzano, Bologna, Italy	Dr. C. Rubies-Autonell
	Rome, Roma, Italy	Dr. V. Vallega
WYMV	Dengzhou, Henan, China	Professor J. Yu
	Huangchan, Henan, China	Professor J. Yu
	Luotian, Hubei, China	Professor J. Yu
	Ishioka, Ibaraki, Japan	Dr. S. Kashiwazaki
	Morioka, Iwate, Japan	Dr. Y. Ohto
BaYMV	Royston, Hertfordshire, UK	Mr. T. Whitehead
	St. Neots, Cambridgeshire, UK	Ms. E. Metcalfe
	Newark, Nottinghamshire, UK	Ms. E. Metcalfe
BaMMV	Royston, Hertfordshire, UK	Mr. T. Whitehead
	Streatley, Bedfordshire, UK	Ms. E. Metcalfe
OMV	Llanishen, Monmouthshire, UK	Mr. E. Broom
	Salisbury, North Carolina, USA	ATCC PV-167
SBWMV	Stillwater, Oklahoma, USA	Professor R. Hunger
	Chambon sur Cisse, Loir-et-Cher, France	Dr. M. Bonnefoy
	Minerbio, Bologna, Italy	Dr. C. Rubies-Autonell
	Trowbridge, Wiltshire, UK	Ms. D. Wright

Table 2. Nucleotide sequences of the primers used for WSSMV and WYMV detection

Primer	Nucleotide sequence (5′–3′)	Expected amplicon size (nucleotides)
WMVCPR	$^{4412/\mathit{7374}}$GGTTAGCTCTGGRTGTCCATCAG$^{4390/\mathit{7352}}$	
WMVCPF	$^{3531/\mathit{6496}}$GCTGCGGACACACAAACWGACG$^{3552/\mathit{6517}}$	882, *879*
Oligo-d(T)Not1	CAATTCGCGGCCGC$(T)_{15}$	
WSSMVF	^{3694}CAGCAACCAAAGTYRCAGCAAC^{3715}	982
WYMVF	$^{\mathit{7122}}$CCTCCTTCAGGAACACAAGATTGTCA$^{\mathit{7147}}$	*544*
WYMVF1	$^{\mathit{6641}}$ATGACAAGAAAGCCAGGGACC$^{\mathit{6661}}$	*1025*

Numbers at termini indicate nucleotide positions in the 3′-terminal half of the WSSMV RNA1 genome (EMBL accession number X73883) (normal text), and in the WYMV RNA1 genome (EMBL accession number D86634) (italic text).

is four WSSMV isolates (EMBL accession numbers AJ237925, AJ237926, X73883 and AB010578), three WYMV isolates (Yu et al., 1995; EMBL accession numbers AJ237924 and D86634) and two BaYMV isolates (EMBL accession numbers D01091 and X69757), was carried out using the Clustal V method from the MegAlign package (DNA Star, Madison, USA). This sequence alignment revealed that the WSSMV and WYMV isolates fell into two separate, homogenous groups representing the two species and confirmed the taxonomic identification of the four cloned isolates. The WSSMV isolates from Montpellier, New York, and Arkansas had 99.4%, 98.8%, and 93.9% nucleotide sequence homology with the corrresponding region of WSSMV-F respectively. None of these substitutions were coding apart from those in the Arkansas isolate which coded for 6 amino-acid substitutions. This isolate has recently been shown to have different biological properties to the New York isolate (E.A. Milus, pers. comm.). The Henan isolate of WYMV shared 98.5% nucleotide sequence homology with the corrresponding region of WYMV-J, 4 of these 13 nucleotide substitutions coded for amino-acid substitutions. In this region of RNA 1, WSSMV-F and WYMV-J share only 68.2% nucleotide homology. This data provides further evidence for the hypothesis proposed by Namba et al. (1998) that WSSMV and WYMV are distinct species rather than strains of the same virus.

From the sequence alignment, areas of divergent sequence between WSSMV and WYMV were identified and three primers, WSSMVF, WYMVF, and WYMVF1, were designed which were complementary to part of the CP gene of either WSSMV or WYMV. For two-step multiplex RT-PCR, production of cDNA was performed using 0.5 µl of template RNA, 2 µl of Oligo-d(T)Not1 (5µM) and 100 units of MMLV reverse transcriptase (Promega, Southampton, UK) in a 10 µl volume at 37 °C for 1 h according to the manufacturer's instructions. PCR amplification was performed in 50 µl reaction volumes containing 10 µl of cDNA, 5 µl of 10× *Taq* reaction buffer (100 mM Tris–HCl, 500 mM KCl, pH 9.0), 2.5 µl of $MgCl_2$ (25 mM), 1 µl each of dATP, dCTP, dGTP, and dTTP (10 mM), 2.5 units of *Taq* DNA polymerase (Promega, Southampton, UK), 2 µl of WSSMVF and 1 µl of WYMVF (5 µM) upstream primers. Thermocycling was performed as follows: 94 °C for 2 min, then 30 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 3 min. Amplified products were observed after electrophoresis in ethidium bromide-stained 1.2% agarose gels.

By using two-step multiplex RT-PCR and total RNA purified from either leaf or root tissue infected with WSSMV or WYMV, a PCR product of the expected size (982 and 544 nucleotides respectively) was obtained from all of the eighteen WSSMV and five WYMV isolates which were tested (Table 1, Figure 1, lanes 2–14). Following gel electrophoresis, there was no discernible difference in the size of the products obtained from different isolates of WSSMV or WYMV, and no non-specific bands were observed. No samples were naturally infected with WSSMV and WYMV but two bands of the expected size were produced if RNA from both was used as a template in a single reaction. No PCR products were produced using total RNA extracted from the leaf tissue of healthy wheat or wheat infected with BaYMV, BaMMV, OMV or SBWMV (Figure 1, lanes 15–19). This protocol successfully detected infection by either WSSMV or WYMV in all plants which gave positive ELISA results. It also detected infection in plants reared in the controlled environment rooms up to 3 weeks before they either showed symptoms or infection could be diagnosed by ELISA.

For one-step RT-PCR, the Titan One Tube RT-PCR system (Roche Molecular Biochemicals, Lewes, UK) was used according to the manufacturers's protocols. In each reaction, 1 µl of template RNA, 3 µl of $MgCl_2$ (25 mM), 2 µl of Oligo-d(T)Not1 (5 µM) and 2 µl of WSSMVF, WYMVF or WYMVF1 (5 µM) primers were used. Reverse transcription was performed at 50 °C for 30 min and the thermocycling conditions were as follows: 94 °C for 2 min, then 10 cycles of 94 °C for 30 s, 56 °C for 30 s and 68 °C for 1 min, then 25 cycles of 94 °C for 30 s, 56 °C for 30 s and 68 °C for 2 min, followed by 68 °C for 7 min. Reaction products were visualized in ethidium bromide-stained agarose gels. This method satisfactorily amplified a single PCR product of the expected size from all isolates of WSSMV using the WSSMVF primer (982 nucleotides) and all WYMV isolates using either the WYMVF (544 nucleotides) or WYMVF1 (1025 nucleotides) primers. After gel electrophoresis, the WYMVF1 primer gave stronger, more intense bands with all WYMV isolates than the WYMVF primer. However, in all cases the PCR products obtained with this one step protocol were not as intense or discrete as those obtained using the two step method. Furthermore, although the WSSMVF

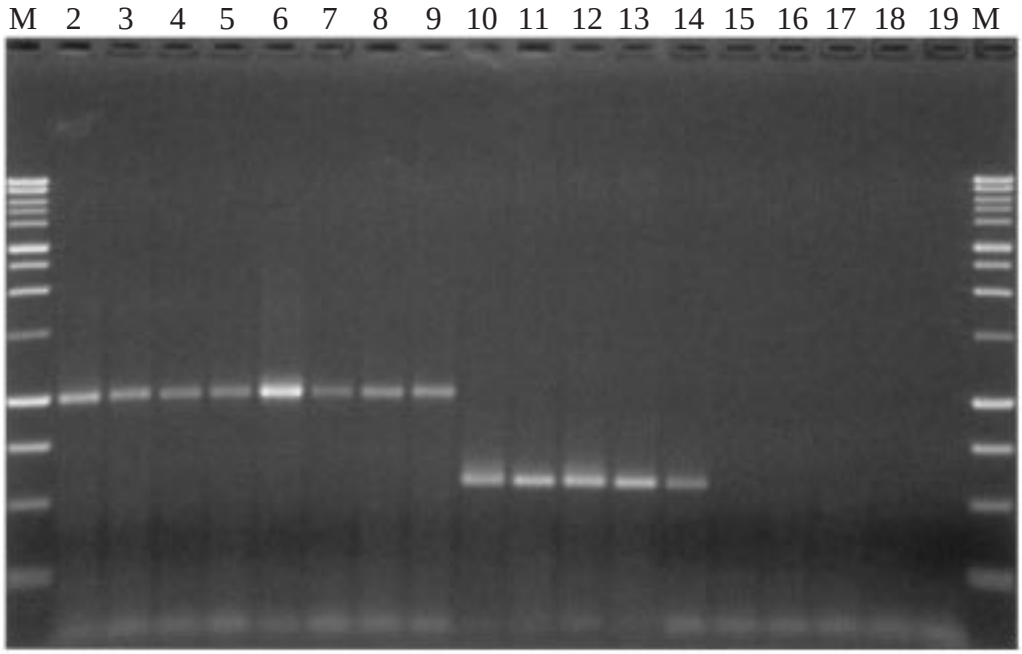

Figure 1. Agarose gel electrophoresis of two step RT-PCR amplification products obtained with specific primers to WSSMV and WYMV. Lanes M,1kb DNA ladder (Promega, Southampton, UK) consisting of 13 fragrents (0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9 and 10kb); lanes 1-9, WSSMV isolated from Montpellier, France; Bay, USA; Parma, Italy; Cornell, USA; Kent County, Canada; Hesston, USA; Warsaw, USA and Chambon sur Cisse, France respectively and extracted from either leaf (2–6) or root (7–9) tissue; lanes 10–13, WYMV isolated from Dengzhou, China; Ishioka, Japan; Morioka, Japan; Huangchan, China and Luotian, China respectively and extracted from either leaf (10–12) or root (13–14) tissue; lane 15, BaYMV isolated from Newark, UK; lane 16, BaMMV isolated from Royston, UK; lane 17, SBWMV isolated from Minerbio, Italy; lane 18, healthy wheat; lane 19, no template.

and WYMVF1 primers could be used in a multiplex reaction, the similarity in the size of the PCR products obtained with positive samples (982 and 1025 nucleotides respectively) meant that these products could not be distinguished by electrophoresis. In common with the two-step method, no non-specific bands were observed nor were products produced using total RNA extracted from healthy wheat or wheat infected with other viruses.

Therefore to conclude, from the sequence data collected during this study and that previously published, specific primers for both WSSMV and WYMV and an appropriate multiplex RT-PCR protocol were designed. Using eighteen WSSMV and five WYMV isolates, these primers were shown to be specific for either WSSMV or WYMV. This molecular protocol was not only as reliable as DAS-ELISA, it was also more sensitive and could detect infection up to three weeks earlier than serological methods. Furthermore, unlike DAS-ELISA, this method was also able to distinguish between isolates of WSSMV, WYMV, and BaYMV. The molecular protocol could be adopted for use in a one-step reaction, with its innate advantages of convenience and decreased contamination risks. However in the one-step method, each reaction was considerably more costly, the positive reaction products were less distinct and the protocol was unsuitable for use as a multiplex reaction since the PCR products from both primers were similarly sized.

Using this new molecular protocol it was possible to begin to define the geographical distribution of the two viruses. WYMV appears to be confined to China and Japan whereas WSSMV has a wider distribution and occurs throughout France, Germany and Italy in Europe, and in Canada and throughout the USA in North America. Although six French wheat bymovirus isolates were tested, these were all shown to be WSSMV and no evidence was found for the occurrence of WYMV in France despite its reported presence by Hariri et al. (1987).

It is anticipated that this RT-PCR assay will greatly facilitate WSSMV and WYMV testing for both

quarantine and breeding purposes. In particular the increased sensitivity of this molecular assay compared to serological methods will help to identify truly resistant wheat breeding lines rather than those which are merely tolerant or 'field resistant'.

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