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Multiplex real-time RT-PCR for detection of *Wheat streak mosaic virus* and *Tritcum mosaic virus*

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Wheat streak mosaic virus (WSMV) and *Triticum mosaic virus* (TriMV) are widespread throughout the southwestern Great Plains states. When using conventional diagnostics such as enzyme-linked immunosorbent assays (ELISA), these two viruses are commonly found together in infected wheat samples. Methods for molecular detection have been developed for wheat viral pathogens, but until recently no multiplex method for detection of both WSMV and TriMV within a single sample was available. Therefore, the objective of this study was to develop a multiplex real-time PCR technique for detection of both pathogens within a single plant sample. Specific primers and probe combinations were developed for detection of WSMV and TriMV, single and multiple reactions were run simultaneously to detect any loss in sensitivity during the multiplex reaction, as well as any cross-reaction with other common wheat viruses. The multiplex reaction was successful in detection of both pathogens, with little difference between single and multiplex reactions, and no cross-reaction was found with other common wheat viruses. This multiplex technique not only will be useful for diagnostic evaluations, but also as a valuable tool for ecological and epidemiology studies, and investigations of host/pathogen interactions, especially when the host is infected with both pathogens.

1. Introduction

Each year, viral pathogens of wheat (*Triticum aestivum* L.), such as *Wheat streak mosaic virus* (WSMV), and *Barley yellow dwarf virus* (BYDV) cause severe losses throughout the Great Plains. Significant losses due to wheat streak have been reported in Colorado, Kansas, and Oklahoma (Shahwan and Hill, 1984; Hunger, 2004). For this reason, improvement in disease resistance is an important aspect of wheat production. The development of disease resistant cultivars has helped to increase production in high disease areas, but also has led to the discovery of previously unidentified pathogens. In a 2006 regional variety trial, Ron-L, a newly released wheat streak mosaic (WSM) resistant cultivar, displayed disease symptoms resembling WSM. After further testing, it was determined that the symptoms were caused by a previously unknown viral pathogen, which was later named *Triticum mosaic virus* (TriMV) (Seifers et al., 2008). *Triticum mosaic virus* is a member of the family Potyviridae (Tatineni et al., 2009; Fellers et al., 2009), and like WSMV and *Wheat mosaic virus* (WMoV), another common viral pathogen of wheat in the Great Plains, it is transmitted by the wheat curl mite (*Aceria tosichella* Keifer) (Seifers et al., 2009). Symptoms of TriMV are identical to WSMV, therefore it is impossible to tell them apart only by visual examination.

During a 2008 wheat virus survey conducted by members of the Great Plains Diagnostic Network, WSMV and TriMV were found to be widespread across much of the Great Plains region (Burrows et al., 2009). These two viruses were most commonly found as multiple infections of the same plant. At the time, a multiplex method was not available for detecting both viruses in the same sample. Such a method would be extremely valuable in ecological and epidemiological studies of mite-vectored viruses. Therefore, the objective of this work was to develop a multiplex real-time PCR technique to detect WSMV and TriMV, from a single sample. The following describes specific PCR primers that can be used in detection assays.

2. Materials and methods

2.1. Plant materials and RNA extraction

Wheat (*T. aestivum* L.), cultivar 'Karl 92', was grown in 3.7 L pots in the greenhouse and inoculated with a mixture of WSMV, isolate

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Sidney 81, and TriMV, isolate U06-123. Two seedlings, Feeke's scale 3 (Large, 1954), were inoculated using a 0.1 M potassium phosphate inoculation buffer/WSMV or buffer/TriMV mixture (Martin, 1978). After approximately 12 days post-inoculation, infected plant material was collected and stored at −80 °C until RNA extraction. Single and multiple virus infected samples were identified using double antibody sandwich enzyme linked immunosorbent assay DAS-ELISA (Agdia Inc.). Total RNA was extracted using an RNeasy plant mini kit from Qiagn (Qiagen Inc., Valencia, CA, USA) and then quantified using a GeneQuant Pro (Cambridge, UK). Samples were diluted to 20 ng/μl total RNA for each sample before analysis by real-time PCR.

2.2. Design of virus specific primer/probe combinations

Specific primer and probe combinations were created using Primer express software (Applied Biosystems Inc., Foster City, CA, USA) for individual detection of WSMV and TriMV. Sequence alignments were performed using the Clustal V method from the MegAlign program (DNAStar Inc., Madison, WI). Specific primers for WSMV were developed from the NIb region of the viral genome (GenBank accession no. AF057533) and labeled with 6FAM as a fluorescent detector (Stenger et al., 1998). Sequences for WSMV forward primer were WsmF7288 (5′-CAAAGCTGTGGTTGATGAGTTCA-3′), reverse primer WsmR7343 (5′-TTGATTCCGACAGTCCATGGT-3′) (IDT, Coralville, IA); and probe WsmP7312 (5′-FAM6-CAAATTCTTCTACACAAAGCATTTGCGCG-MGBFQ-3′) (Applied Biosystems). Primer/Probe combinations for TriMV, TriF272 (5′-CATGCACATTGGAGCAATTTG-3′), TriR337 (5′-GCATGCTCAATCCAAGTCCAT-3′) (IDT); and probe Trip295 (5′-NED-CGTTGGGCTAATGCGGCAGCA-MGBFQ-3′) (Applied Biosystems), were developed using sequence from the coat protein (GenBank accession no. FJ669487) region of the viral genome and labeled with NED as the fluorescent dye. A 3′ TaqMan MGB non-fluorescent quencher (NFQ) was used to lower background signal.

2.3. Multiplex real-time polymerase chain reaction

Three different reactions were run on a single 96 well plate to detect any differences between single and multiplex reactions in virus detection. One reaction contained both the TriMV and WSMV primer/probe combinations, a second contained the WSMV primer/probe only and, the third had the TriMV primer/probe only. Forward and reverse primers for both viruses were added to reaction mixtures, at 200 nM concentration, and probe concentrations were 250 nM. Reactions were run using One-step TaqMan master mix (Applied Biosystems), consisting of 1× Master Mix, 1× MultiScribe and RNase inhibitor mix. For each reaction, 2 μl of 20 ng/μl RNA extract were added for a total volume of 25 μl per reaction. No template controls consisted of 2 μl of nuclease-free water instead of RNA. Real-time PCR was conducted using an ABI Prism7000 Sequence Detection System (Applied Biosystems). Reaction parameters were set at 48 °C for 30 min, 95 °C 10 min, and 40 cycles of 95 C for 15 s and 62 °C for 1 min. The C_T value for each sample reaction was calculated using the Sequence Detection Software's default threshold setting of 0.2 ΔRn (fluorescence) units. Samples containing WMoV, BYVD-PAV, *Cereal yellow dwarf*-RPV, and *Sugarcane streak mosaic virus* were also included to test for cross-reaction with other common viruses. The tests were repeated twice and C_T values were averaged between the two tests. An additional six samples containing both WSMV and TriMV were collected from a field site near Amarillo, TX (USA) and verified by ELISA to contain both WSMV and TriMV. These samples were tested to determine variability in virus titers. *Triticum mosaic virus* samples from Oklahoma and Kansas also were included to determine applicability of the methodology for samples from a wide geographic area.

Table 1
Results for samples tested by EUSA and multiplex PCR.

Sample	ELISA		PCR	
	WSMV	TriMV	WSMV	TriMV
TriMV only	–	+	–	+
WSMV only	+	–	+	–
TriMV/WSMV	+	+	+	+
WMoV	–	–	–	–
BYDV-PAV	–	–	–	–
CYDV-RPV	–	–	–	–
SCSMV	–	–	–	–

+ sign indicates a positive result for ELISA or PCR test; – sign indicates a negative result for ELISA or PCR test.

2.4. Determination of amplification efficiencies

A dilution series was included to determine amplification efficiencies for the WSMV and TriMV primer/probe combinations within the multiplex reaction. Total plant RNA, containing both WSMV and TriMV, was diluted to 10 ng/μl and this was used to create a dilution series, using 10-fold serial dilutions to 1×10^{-5} ng/μl. Each dilution was tested, using real-time PCR amplification, as previously described. The C_T values for each dilution were plotted against total RNA concentration. Dilutions with C_T values greater than 35 were not included in the results because C_T values greater than 35 are considered to approach the sensitivity limits of the PCR detection system. Amplification efficiency (Ex) was calculated from the slope of the graph using the equation $Ex = 10^{(-1/slope)} - 1$ (Anonymous, 2006).

3. Results

3.1. Detection of TriMV

Increased fluorescence was found for the TriMV probe in both the TriMV only sample and the TriMV/WSMV samples. The multiplex and single primer reactions for the TriMV only sample gave a mean C_T value of 17.67 for the multiplex and 17.23 for the single TriMV probe reaction. The WSMV/TriMV sample gave a mean C_T value of 21.80 and 18.79 for the multiplex and single reactions, respectively (Fig. 1). No amplification was detected when the TriMV probe was used to test WSMV, WMoV, BYDV-PAV, CYDV-RPV, and SCSMV samples. The TriMV reactions were also negative for both the negative control and no template control (Table 1).

3.2. Detection of WSMV

Amplification with the WSMV probe was detected in both the WSMV/TriMV and WSMV only samples. Virus detection was found in the multiplex and single probe reactions, for the WSMV only sample, with a mean C_T of 18.41 and 16.56, respectively. The WSMV/TriMV sample had mean C_T values of 15.65 and 14.61 for the multiplex and single probe reactions, respectively (Fig. 1). The WSMV primer/probe combination was highly specific to WSMV and did not react with any of the other viruses that were tested.

3.3. Relation of WSMV and TriMV in multiple infected samples

Wheat streak mosaic virus and TriMV also were detected in the multiple infected field samples included in the study. In all but one of the samples tested, WSMV had a lower C_T value than TriMV. The five other samples tested had a mean C_T value of 20.84 for WSMV with a standard deviation of 0.78. Amplification of TriMV within