Development of a multiplexed PCR detection method for Barley and Cereal yellow dwarf viruses, Wheat spindle streak virus, Wheat streak mosaic virus and Soil-borne wheat mosaic virus

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Abstract

Barley and Cereal yellow dwarf viruses (B/CYDVs), Wheat spindle streak mosaic (WSSMV), Soil-borne wheat mosaic virus (SBWMV) and Wheat streak mosaic virus (WSMV) constitute the most economically important group of wheat viruses. In this paper, a multiplex reverse transcription polymerase chain reaction (M-RT-PCR) method was developed for the simultaneous detection and discrimination of eight viruses: five strains of B/CYDVs, WSSMV, SBWMV and WSMV. The protocol uses specific primer sets for each virus producing five distinct fragments 295, 175, 400, 237, and 365 bp, indicating the presence of two strains of BYDVs, -PAV, -MAV, CYDV-RPV and two unassigned Luteoviridae BYDV-SGV and -RMV, respectively. This system also readily detected WSSMV, SBWMV and WSMV specific amplicons at 154, 219 and 193 bp, respectively. The amplification specificity of these primers was tested against a range of field samples from different parts of United States. The protocol also utilizes fluorescently tagged primers that can streamline the detection of each virus through capillary electrophoresis. This study fulfills the need for a rapid and specific wheat virus diagnostic tool that also has the potential for investigating the epidemiology of these viral diseases.

Keywords: Wheat viruses; Barley yellow dwarf virus; Cereal yellow dwarf virus; Wheat streak mosaic virus; Wheat spindle streak mosaic virus; Soil-borne wheat mosaic virus; Multiplex PCR

1. Introduction

Wheat serves as a natural host for many viruses that generally cause symptoms that are distinctive from other infectious diseases. Although the incidence of viruses in wheat (or other cereals like barley, oats, rye, triticale) in a given field may be relatively inconspicuous in some years, the viral infection may become very obvious leading to serious economic losses. Triticum aestivum is susceptible to approximately 55 viruses (Brunt et al., 1996) of which Barley and Cereal yellow dwarf virus (B/CYDV), Wheat spindle streak mosaic virus (WSSMV), Wheat streak mosaic virus (WSMV) and Soil-borne wheat mosaic virus (SBWMV) constitute the major economically important group of viruses because of their ability to cause a significant level of disease and yield losses worldwide.

Yellow dwarf viruses (YDVs), a group of Luteoviruses and Poleroviruses, constitute one of the most economically important viral pathogens of cereals because of their significant negative impact on worldwide cereal grain production (Plumb, 1983). Since first reported (Oswald and Houston, 1953), YDVs have been extensively described (Miller and Rasochová, 1997) and can infect over 150 species in the Poaceae family, including wheat, barley, oat, maize, rice, yam, sorghum, and triticale (D’Arcy, 1995). These single stranded positive-sense RNA viruses were initially classified as five strains based upon their aphid transmission phenotypes (Rochow and Muller, 1971). Recently, these viruses were re-classified into two different species: Luteovirus, which includes Barley yellow dwarf viruses (PAV, MAV, SGV and RMV) and Polerovirus, which includes Cereal yellow dwarf virus (CYDV-RPV) (Fauquet et al., 2005).

Abbreviations: BYDV, Barley yellow dwarf virus; CYDV, Cereal yellow dwarf virus; WSSMV, Wheat spindle streak mosaic virus; WSMV, Wheat streak mosaic virus; SBWMV, Soil-borne wheat mosaic virus; M-RT-PCR, multiplex reverse transcriptase PCR.

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B/CYDV infection, through aphid transmission, leads to stunting of the plant and a concomitant reduction in seed production (Burnett, 1990). Furthermore, several strains of BYDV/CYDV can co-exist in the same plant resulting in more severe symptoms (Haber, 1995). Studies have shown the importance of these viruses because of their ability to cause significant yield losses in cereals worldwide (Burnett, 1990; Lister and Ranieri, 1995).

*Polymyxa graminis* Ledingham, a worldwide biotrophic fungal parasite of many cereals, is a vector for plant viruses belonging to the genera *Furovirus*, *Bynmovirus*, and *Pecluvirus* (Adams, 1991). In the United States, *P. graminis* is the vector for SBWMV and WSSMV (Wiese, 1987), which can also cause significant yield losses in winter wheat. The symptoms include mild stunting of plants and yellow streaks in leaves. *Wheat streak mosaic virus*, another important pathogen of wheat (Brakke, 1987) is transmitted through the wheat curl mite (*Aceeria tosichella* Keifer) (Hollings and Brunt, 1981). This virus causes a severe mosaic and stunting of winter wheat, barley, oats, maize, rye and certain millets and is a serious threat to winter and spring wheat in the USA (Rochow and Muller, 1971).

To identify and characterize the different virus variants involved in the B/CYDV pathosystem, accurate, timely and cost-effective methods are essential for studying this disease on both local and regional scales. The use of enzyme-linked immunosorbent assays (ELISA) has been a cost-efficient means of detecting B/CYDV in a large number of samples (French, 1995). This technique has the disadvantage of being time-consuming with a detection limit of approximately one million virus particles per sample (Canning et al., 1996), which may produce false negative results. Additionally, identification of specific B/CYDVs by ELISA requires different antisera for each virus. Other techniques besides ELISA, such as polymerase chain reaction (PCR), nucleic acid hybridization and non-radioactive chemiluminescent methods are capable of distinguishing the different viruses (Fouly et al., 1992; Habili et al., 1987). However, the use of radioactive probes and the non-specific hybridization of probes with healthy samples have discouraged the use of nucleic acid hybridization. While the simplicity of the ELISA technique makes it quite practical, PCR-based technologies including reverse transcription polymerase chain reaction (RT-PCR) are becoming a key procedure in plant virus detection (James et al., 2006). The RT-PCR assays offer the possibility of improved sensitivity and a more rapid diagnosis of these viral infections. These PCR-based techniques have proven to be conceptually simple, highly specific, sensitive and are now common in research laboratories (Harrison, 1998). However, the use of PCR in diagnostic laboratories can be limited due to its cost. This has led to the recent development of RT-PCR assays that simultaneously detect multiple pathogens using primer pairs to different viruses in separate reactions that share single standard amplification conditions as well as combining multiple primer sets into single “multiplexed” amplification reaction. Thus, the multiplex RT-PCR overcomes the limitation of cost and is a rapid, efficient method with a high degree of specificity and sensitivity.

In multiplex PCR, two or more target sequences are simultaneously amplified within the same reaction. This technique has progressed to become a valuable tool for identification of viruses (Casas et al., 1999), bacteria (Nayak et al., 2005), fungi and other parasites. RT-PCR, a variant of PCR, has been successfully used to detect low levels of B/CYDVs (Figueira et al., 1997; Henson and French, 1993; Martin et al., 2000). Robertson et al. (1991) developed a novel pair of short primers, which amplified a B/CYDV specific 533 bp fragment for detecting B/CYDV in oat and wheat. This method served as a good tool to detect B/CYDV’s (+/− infection status) over a wide range of samples but could not eliminate the possibility of false negatives due to its tendency to produce non-specific bands (Malmstrom and Shu, 2004). It also failed to reveal the identity of specific B/CYDVs present in the samples. This problem was overcome by the development of a PCR-based method that could detect and distinguish between different B/CYDVs and potentially determine which virus was present (Malmstrom and Shu, 2004). The method uses a “basic multiplex” to distinguish between BYDVs and CYDVs and then an “enhanced multiplex” further discrimiates between BYDVs (BYDV-PAV, -MAV and -SGV), although MAV could not be distinguished in mixed infections. The method also could not discriminate between CYDV-RPV and BYDV-RMV.

PCR methods have been developed to reliably detect and distinguish between WSSMV and *Wheat yellow mosaic virus* (Clover and Henry, 1999) and quantify SBWMV (Claudio et al., 2004). In 1999, a PCR diagnostic protocol was developed that distinguished between WSSMV and SBWMV (Gitton et al., 1999), although the primers designed from the sequence of a French isolate of WSSMV could faintly detect *Barley yellow mosaic virus* in barley plants. A PCR diagnostic method for WSMV was developed using a primer derived from 3′-terminal sequences of five WSMV isolates and an oligo d(T)-based primer (French and Robertson, 1994). Adding proteinase K and boiling to the nucleic acid extraction procedure permitted PCR-based detection of both WSMV and BYDV in field samples, which suggested that a multiplex PCR approach was possible for wheat virus detection.

In this study we have reported the first multiplex RT-PCR (M-RT-PCR) based assay that can quickly, precisely and simultaneously detect these eight important wheat viruses BYDV-PAV, -MAV, -SGV, -RMV and CYDV-RPV, WSSMV, WSMV and SBWMV in a single lane of a high-resolution agarose gel or by fluorescent capillary electrophoresis. This demonstrated that the multiplex RT-PCR assay could be used for the sensitive and specific detection of wheat viruses in infected leaf tissue across a wide range of field samples. The diagnosis of mixed infections with wheat viruses in one single reaction will reduce the cost, significantly increase the number of samples that can be analyzed, which will allow an examination of the viral disease epidemiology in wheat.

2. Materials and methods

2.1. Plant materials

Oat leaf tissue infected with known B/CYDV isolates was kindly provided by Dr. Stewart. M. Gray, Ithaca, NY. Leaf tissue samples from different fields around the country were also
received to determine the presence of different viruses using our detection system. These tissue samples were kindly provided by Dr. Carl Griffey, Virginia; Dr. Jerry Johnson, Virginia; Dr. Bob Hunger, Oklahoma and Gail Ruhl from Purdue University.

2.2. Plant RNA extraction and reverse transcription

Total plant RNA, used as a template for the first strand cDNA synthesis (reverse transcription), was extracted from infected leaf samples using TRIzol (Invitrogen Life Technologies, Carlsbad, CA). Leaf tissue, including leaf tips, was frozen in liquid nitrogen, ground in an RNase- and DNase-free mortar and pestle, and RNA was extracted from approximately 250 mg tissue using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). RNA was re-suspended in 50 μl of diethyl pyrocarbonate treated water and quantified using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA was synthesized from this RNA using random primers rather than oligo-dT because the B/CYDV genomes are not polyadenylated. cDNA synthesis was carried out in a 20 μl reaction volume using 2 μg of total RNA using the iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA), following the manufacturer’s protocol. The cDNAs were diluted 10-fold in sterile distilled water before using them in the multiplex PCR virus detection reaction.

2.3. Design of virus-specific primers

For a multiplex reaction to work properly the primer pairs for all amplified regions need to be compatible. The primer annealing temperatures must be similar for all primer pairs and complementarity between primers avoided to prevent the formation of primer-dimers, which can significantly reduce the primers annealing to the template DNA. Table 1 shows the primers used in this study. The annealing/melting temperatures ($T_m$) for the primers listed in this table are very similar, which helped achieve uniform amplification across the different viruses detected. Sequences for different B/CYDV from the NCBI database (http://www.ncbi.nlm.nih.gov/blast) were aligned using ClustalW (http://www.ebi.ac.uk/Tools/sequence.html) for designing the virus primers. All three strains of BYDVs (PAV, MAV, and SGV) show very high sequence identity in the ORF3 region that encodes the coat protein. They also share homology with RPV and RMV in the coat protein region. From the multiple sequence alignment of several different isolates of the same virus, divergent sequences between the strains of YDVs were identified and primers were designed, which were specific for each virus. Table 1 gives the location of the primers in the different viruses. The sequences for WSSMV, SBWMV and WSMV were also studied to identify regions of sequence divergence from each other as well as the B/CYDV to design primers specific to these viruses. The candidate primer pairs for all of these viruses were further analyzed and then tested extensively in all different combinations. For capillary electrophoresis detection, the forward primer from each primer pair was 5'-end-labeled with the commercially available fluorescent dye “FAM” (MWG Biotech, High Point, North Carolina).

2.4. Multiplex polymerase chain reaction

Multiplex PCR amplification was carried out with different sets of virus-specific primers. The PCR conditions were optimized against a range of reagent concentrations and annealing and extension temperatures. Following optimization, each PCR reaction included 2 μl of diluted cDNA (approximately 40 ng), 6 μl of 25 mM MgCl2 (Promega, Madison WI), 2.5 μl of a dNTP mixture with each dNTP at 5 mM (Invitrogen Life Technologies, Carlsbad, CA), 4 μl of 10 × Polymerase Buffer (Promega, Madison WI) and 1.5 μl of 5 U/μl Hot-start Taq polymerase (Qiagen Inc., Valencia, CA) and the primers. To amplify all eight viruses in one tube, 2 μl of each primer set (a mixture of

<table>
<thead>
<tr>
<th>Target virus</th>
<th>Primer</th>
<th>Sequence</th>
<th>5’ Position</th>
<th>$T_m$ (°C)</th>
<th>NCBI accession</th>
<th>Amplicon size (bp)</th>
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<tr>
<td>BYDV-PAV</td>
<td>PAVL1</td>
<td>AGAGGAGGGGCAATCTCGT</td>
<td>2999</td>
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<td></td>
<td>SGVR2</td>
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<td>X73883</td>
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10 μM forward and 10 μM reverse primer) were added for a final reaction volume of 32 μl. The samples were amplified using the PTC-100 Peltier Thermal Cycler (Bio-Rad, Hercules, CA). To allow maximum amplification, while avoiding competing reactions and primer-dimer amplification, a touch-down PCR was performed. The Hot-start Taq polymerase was first activated at 95 °C (10 min) followed by 6 cycles of denaturation at 95 °C (30 s), annealing at 60 °C (30 s) with the annealing temperature decreasing by 1 °C in each successive step and extension at 72 °C (30 s). These 6 cycles were followed by 30 cycles of 95 °C (30 s), 55 °C (1 min), 72 °C (30 s) and a final extension at 72 °C for 10 min.

2.5. Detection of amplified products

Amplified PCR products were analyzed by gel electrophoresis in an ethidium bromide stained 2% metaphor-agarose gel (1:1 metaphor to agarose ratio) using sodium boric acid electrophoresis buffer (Brody and Kern, 2004). The PCR products were visualized under UV light using a gel documentation system (GelDoc Bioimaging System, UVP Inc., Upland, CA). A 100 bp EZ load molecular DNA marker (Invitrogen Life Technologies, Carlsbad, CA) was run in each gel to determine the fragment sizes of the amplified products.

2.6. Cloning and sequencing

To ensure that the PCR produced the expected viral sequences, the multiplexed PCR products were cloned into the pCR TOPO-4 vector system (Invitrogen Life Technologies, Carlsbad, CA). Clones containing an insert were selected by colony PCR using M13 and T7 primers and sequenced by the Genomics Facility (Purdue University, West Lafayette, IN) with M13 forward and reverse primers. The sequences were then verified by a BLAST search of the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/blast).

2.7. Capillary electrophoresis

Capillary electrophoresis (CE) analyses were performed with a 3130xl Genetic Analyser instrument (Applied Biosystems, Foster City, CA). Forward primers from the virus-specific primer sets were labeled at the 5′ end with 6-FAM (MWG Biotech, High Point, North Carolina). The multiplex PCR contained labeled forward and unlabeled reverse primers for the eight sets of primers and the samples subjected to capillary electrophoresis. The best peak resolution was achieved prior to CE by diluting the PCR reactions 50-fold in deionized sterile water. A 1 μl aliquot was then added to 9 μl of hi-diformamide (Applied Biosystems, Foster City, CA) containing a 100-fold dilution of the Genescan 500 LIZ size standard (Applied Biosystems, Foster City, CA). Following capillary electrophoresis, this fluorescent size standard was used to determine the size of the amplicons when the results were analyzed using the GeneMarker v1.5 software (SoftGenetics LLC, State College, PA).

Fig. 1. Multiplex RT-PCR with different wheat viruses. Individual PCRs in Lanes 2–9 using different YDV strains PAV, RPV, MAV, RMV, SGV (Lanes 2–6) and other wheat viruses WSSMV, SBWMV and WSMV (Lanes 7–9) produced specific virus amplicons. The M-RT-PCR, containing the eight virus-specific primer sets and virus templates, produced a ladder of DNA fragments (MLX, Lane 10) corresponding to the amplicons produced in Lanes 2–9. Lane 1 contains a 100 bp molecular marker ladder.

3. Results

The M-RT-PCR indicates the presence of five YDV strains, WSSMV, SBWMV and WSMV through the production of virus-specific amplicons each having a different product length. Individual virus-specific reactions (Fig. 1, Lanes 2–9) produced a single fragment at an expected size (Table 1). The multiplex reaction detected all eight viruses (Fig. 1, Lane 10) with amplicon sizes identical to that in the individual virus-specific PCR reactions when the cDNAs containing each virus were pooled together. Each of these amplifications was optimized by a series of assays that varied the DNA polymerases, concentration of primer pairs, MgCl₂ and dNTPs, annealing temperature, extension temperature and time and cycle numbers (data not shown). This optimization resulted in the control plant samples, containing specific virus, producing distinct amplicons for each virus in both multiplexed and non-multiplexed reactions, without the production of non-specific bands.

This multiplex detection assay was also tested on field samples collected from around the country and detected an array of virus combinations (Fig. 2). Most of these field samples showed different combinations of B/CYDVs. The plant samples from Georgia showed multiple infections with the presence of BYDV-PAV, -MAV, WSSMV (Fig. 2A, Lanes 2, 4 and 5) and CYDV-RPV (Fig. 2A, Lanes 3 and 6). The intensity of the bands suggests that the M-RT-PCR can detect virus from samples having a range in the titer of the virus. The faint band at about 242 bp (Fig. 2A, Lane 7) was cloned, sequenced and identified as a PAV isolate with high sequence identity to a PAV isolate from Iran (data not shown). Both of the Oklahoma field samples showed a heavy infestation with multiple viruses like CYDV-RPV, BYDV-PAV, -MAV, SBWMV, and WSSMV in sample G (Fig. 2B, Lane 2) along with an additional BYDV-SGV in sample H (Fig. 2B, Lane 3). Arkansas samples looked more dominantly
Fig. 2. Panel A: Multiplex RT-PCR analysis of field samples from Georgia. Field samples from different areas of Georgia (A–F) were tested for all eight wheat viruses. A positive control M-RT-PCR provides a reference ladder showing all eight virus amplicons. Lanes 1 and 8 contain a 100 bp molecular marker ladder. Panel B: Multiplex RT-PCR analysis of field samples from other parts of United States: Oklahoma (samples G and H, Lanes 2–3), Arkansas (samples I and J, Lanes 4–5) and Indiana (samples K and L, Lanes 6–7) along with a no template control (NTC, Lane 9). An M-RT-PCR positive control provides a reference ladder showing all eight virus amplicons (Lane 8). Lanes 1 and 10 contain a 100 bp molecular marker ladder.

infected by BYDV-PAV and -MAV than -SGV and WSSMV (Lanes 4 and 5) as indicated by the fluorescence intensity of the bands. Field samples from Indiana showed a different trend with the presence of BYDV-SGV, -MAV and WSSMV (Lane 6) and SBWMV and WSSMV (Lane 7). Following gel electrophoresis, there were no discernible differences in the size of the virus amplicons obtained from the different field samples indicating that the M-RT-PCR could detect the viruses efficiently from a wide range of field areas. The specific amplified fragments from control infected samples and field samples were validated by cloning and sequencing the fragments (data not shown).

Primer specificity was tested by performing a “drop-out” experiment where one of the primer pairs were removed at a time to see which, if any, of the primer pairs cross-react. When pooled cDNAs carrying all eight viruses were used and each virus-specific primer set was sequentially eliminated (Fig. 3), there was no BYDV-PAV band in absence of the PAV primers (Lane 2), or RPV band in absence of the CYDV-RPV primers (Lane 3). Similar results were observed for all other viruses (Lanes 4–9) when compared with the control multiplex reaction (Lane 10). This demonstrated that the primers used in the multiplex are very specific for each virus.

The results above were obtained using agarose gel electrophoresis. Capillary electrophoresis, however, has the potential to increase the throughput of this virus detection assay. Following single-plex (data not shown) and multiplex reactions using fluorescently labeled primer sets the amplicons were detected using CE (Fig. 4). In the electropherogram of the multiplex reaction (Fig. 4), the blue peaks corresponded to each of the eight viruses. The LIZ-labeled molecular weight standards (orange peaks) were used by the data analysis software (GeneMarker v1.4) to generate a standard curve from which the size of the virus amplicons was determined. The size of the virus amplicons was very close to the PCR amplified product size observed in an agarose gel and the expected sizes listed in Table 1. Field samples tested in agarose gel (Fig. 2A and B) were also run in CE and the identical results were obtained (data not shown) demonstrating that CE also accurately detected these eight viruses.

4. Discussion

For diagnosis of wheat viruses, it is essential to detect and differentiate between them and that has been the aim of this study. The technique developed in this study allowed, for the first time, unambiguous diagnosis of five yellow dwarf viruses (BYDV-PAV, BYDV-MAV, BYDV-SGV, BYDV-RMV, CYDV-RPV), Wheat spindle streak mosaic virus, Soil-borne wheat mosaic virus and Wheat streak mosaic virus using specific primers for
each of them in a multiplex RT-PCR in infected plant tissue coming from different geographical regions.

Traditional detection methods such as ELISA prove inefficient when used to distinguish between different types of viruses rapidly within a single sample. However, the ever-increasing availability of the viral sequences has permitted the development of nucleic acid diagnostic techniques to perform this kind of assay and multiplex PCR emerged as a significant one with the advantage of simultaneous detection. Multiplex PCR has been especially useful in plant pathology because different bacteria and/or RNA viruses frequently infect a single host and consequently sensitive detection is needed for the propagation of pathogen-free plants. There are several examples that have reported to use M-RT-PCR for detecting two or three plant viruses (Grieco and Gallitelli, 1999; Saade et al., 2000; Sharman et al., 2000). However, owing to the technical difficulties in designing reactions involving compatible primers, there are only a few examples which were successful in amplifying more than three plant viruses in a single PCR-based assay (Bariana et al., 1994; Nie and Singh, 2000; Okuda and Hanada, 2001) with one of them being successful in detecting six viruses in olive trees (Bertolini et al., 2001). Therefore, our method of detecting eight wheat viruses in a single test is unique.

Another extremely important criterion is to have the assay as universal as possible in detecting a wide range of isolates for the viruses. Due to the sequence variability reported for different isolates of the same virus like BYDV-PAV or CYDV-RPV it was important to have primers, designed for each virus, act as universal diagnostic tool. Although sequence alignments and comparisons were done, empirical testing and a trial-and-error approach was required to identify primers that were virus-specific and amplified efficiently. Parameters such as the extent of homology with the targets and between the primers, primer length, primer GC content, the melting temperatures and their concentrations in the PCR were considered. Additionally, the information on the genetic diversity and distribution of various variants of B/CYDVs (Bisnieks et al., 2004; Mastari et al., 1998; Ueng et al., 1992; Vincent et al., 1990) and the other wheat viruses (WSSMV, SBWMV and WSMV) (Clover and Henry, 1999; Hall et al., 2001) between host plant species, helped to design specific primers that have a wide geographical range. Our recent efforts could not identify PAV-129 or BYDV-PAS, a North American isolate of BYDV-PAV (Bisnieks et al., 2004; Chay et al., 1996) that has 86.5% nucleic acid similarity with the BYDV-NY-PAV isolate. We are currently doing further research to detect this PAS isolate within our system. However, the analysis of field samples from various regions in the US confirmed that this test has the ability to detect geographically diverse isolates.

This multiplex-PCR based assay is quite versatile as the set of primers included in the reaction can be varied depending upon the viruses one needs to detect. The test can be performed with a specific primer set if one wishes to detect a single virus or defined sets of primers to detect a group of virus. Consequently, this M-RT-PCR is versatile, convenient, cost effective and efficient. Similar to the recent development of several strain-specific multiplex PCRs for monitoring outbreaks of different viruses (Bertolini et al., 2001; James et al., 2006), this method also has the potential to observe virus infections in wheat, and other small grain cereals, in a single test.

Two previous studies developed B/CYDV PCR detection methods (Robertson et al., 1991; Malmstrom and Shu, 2004). Unlike the Lu-1/Lu-4 system developed by Robertson et al. (1991) which had the disadvantage of identifying a significant number of false positives, the method developed by Malmstrom and Shu (2004) used either a “basic” version to detect B/CYDVs or an “enhanced” version to determine which strains were present in a mixed infection. Our protocol readily detects the presence of any of those B/CYDVs and three more wheat viruses (WSSMV, SBWMV and WSMV) in a single PCR.
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