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Short communication

Simultaneous detection of wheat dwarf virus, northern cereal mosaic virus, barley yellow striate mosaic virus and rice black-streaked dwarf virus in wheat by multiplex RT-PCR

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ABSTRACT

Wheat dwarf virus (WDV), barley yellow striate mosaic virus (BYSMV), rice black-streaked dwarf virus (RBSDV) and northern cereal mosaic virus (NCMV) are four viruses infecting wheat and causing similar symptoms. In this paper, a multiplex reverse transcription polymerase chain reaction (m-RT-PCR) method has been developed for the simultaneous detection and discrimination of these viruses. The protocol uses specific primer set for each virus and produces four distinct fragments (273, 565, 783 and 1296 bp), detecting the presence of RBSDV, BYSMV, WDV and NCMV, respectively. Annealing temperature, concentrations of dNTP, Taq polymerase and Mg^{2+} were optimized for the m-RT-PCR. The detection limit of the assay was up to 10^{-2} dilution. The amplification specificity of these primers was tested against a range of field samples from different regions of China, where RBSDV, BYSMV, WDV have been detected. This study fulfills the need for a rapid and specific wheat virus detection that also has the potential for investigating the epidemiology of these new viral diseases.

Wheat (*Triticum aestivum*) is the most widely grown food crop in the world and the national staple food of 43 countries, feeding at least one third of the world's population. China is the main producer of wheat, with approximately 15% total cultivating area and 17.1% of total world production (http://faostat3.fao.org/download/Q/QC/E). Wheat production is challenged by many pathogens (Liu et al., 2015), including more than 50 viruses that cause typical symptoms including mosaic, yellowing, dwarfing, stunting or excessive tillering, and subsequently decreasing yield (Wang et al., 2015).

The incidence of wheat viral diseases varies from year to year and region to region, depending on agricultural practices, environment and vector populations (Liu et al., 2014b; Wu et al., 2015). Yellow dwarf viruses (YDVs) are the most widespread and damaging group of cereal viruses worldwide and, together with wheat yellow mosaic virus (WYMV), they constitute the major economically important group of viruses in China (Liu et al., 2013; Zhao et al., 2010). Epidemics of wheat rosette dwarf disease caused by northern cereal mosaic virus (NCMV) were reported in northern China during the 1970s but rarely develop during the last 30 years. On the other hand, several other viruses, including wheat dwarf virus (WDV), barley yellow striate mosaic virus (BYSMV) and rice black-streaked dwarf virus (RBSDV), although reported elsewhere around the world (Vacke, 1961; Conti, 1969; Luisoni et al., 1973), were only found recently to infect wheat in northern China (Xie et al., 2007; Di et al., 2014; Ren et al., 2016).

Although WDV transmitted by leafhopper (*Psammotettix alienus* Dahlbom) and BYSMV, RBSDV and NCMV by the small brown planthopper (*Laodelphax striatellus* Fallén), they cause very similar symptoms on wheat plants such as severe dwarfing, increased tillering and reduced heading, so it is very difficult to distinguish the four viruses from each other in the field. For this reason, a rapid method for accurate diagnosis of the four viruses is needed. Although the polymerase chain reaction (PCR), combined with reverse transcription (RT-PCR) is a key technology for plant virus detection (James et al., 2006), individual RT-PCR to detect only one virus is laborious and expensive when several viruses have to be detected in one or numerous samples. To overcome this problem, multiplex RT-PCR (m-RT-PCR) assays have been developed for the simultaneous detection of multiple viruses in a single reaction by combining several pairs of primers (e.g., Ito et al., 2002; Liu et al., 2012; Panno et al., 2012). For wheat viruses, an m-RT-PCR system for barley/cereal yellow dwarf viruses (B/CYDVs), wheat spindle streak mosaic virus (WSSMV), soil-borne wheat mosaic virus (SBWMV) and wheat streak mosaic virus (WSMV) (Deb and Anderson 2008) and for BYDVs (GAV, GPV and PAV), barley stripe mosaic virus (BSMV), wheat yellow mosaic virus (WYMV), wheat dwarf virus (WDV)

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Table 1
Primers for the m-RT-PCR to detect RBSDV, WDV, BYSMV and NCMV.

Primer	Sequence (5′-3′)	Tm (°C)	Product size (bp)	Target gene	Accession number
RBSDV-F	TTTCAGACTCTAACGAACAACGAC	56.9	273	Major outer capsid protein	AJ297433
RBSDV-R	TTGAGCAGGAACTTCACGAC	58.0			
BYSMV-F[a]	GACAGTCCGGGAAGCAAGAA	59.8	565	L protein	KM213865
BYSMV-R[a]	GGTCGGAGTACCCCCTTAGT	61.9			
WDV-F	ATGGTGACCAACAAGGACTCC	60.0	783	Coat protein	EF536864
WDV-R	TTACTGAATGCCGATGGCTTTG	58.2			
NCMV-F[a]	ATGGCAAATGAGCACAAGAG	55.8	1296	N protein	NC_002251
NCMV-R[a]	TTACATTCCGAACACTTCATCC	56.3			

F: forward primer; R: reverse primer.
[a] Primers described previously (Duan et al., 2010; Di et al., 2016).

and wheat blue dwarf phytoplasma (WBD) (Tao et al., 2012) have been developed. The aim of our study was to develop and optimize an m-RT-PCR assay for the simultaneous detection of NCMV, RBSDV and BYSMV, which are RNA viruses, and WDV, a DNA virus that produces mRNA during its transcription.

Wheat plants infected by one of the virus (RBSDV, WDV, NCMV or BYSMV) were maintained in our greenhouse. Field samples were collected from the major wheat-producing areas in China, including Shandong, Shanxi, Henan, Shaanxi and Hebei Provinces in May 2016. Total RNA of wheat leaf (about 0.1 g) was extracted using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instruction.

The cDNA for simplex RT-PCR (s-RT-PCR) was synthesized in a total volume of 20 μL of using 1 μL of RNA (about 1 μg), 2 μL of reverse primer (10 μM − see Table 1), 2 μL of dNTP Mix (each at 2.5 mM; TaKaRa, China), 4 μL of 5 × MMLV-buffer, 1 μL of M-MLV reverse transcriptase (200 U/μL; Promega, USA), 0.5 μL of Recombinant RNase Inhibitor (40 U/μL; TaKaRa) and 8.5 μL of DEPC water. The s-PCR for individual detection of the viruses contained 2 μL of cDNA, 2.5 μL of 10 × Buffer (Mg^{2+} plus, 15 mM), 2 μL of dNTP Mix (each at 2.5 mM; TaKaRa), 0.5 μL of forward and reverse primer (see Table 1), 0.2 μL of rTaq polymerase (5 U/μL; TaKaRa) and 17.3 μL of ddH_2O. The s-PCR was performed using S1000 thermal cycler (Bio-Rad, USA), as follows: 94 °C for 3 min; 35 cycles at 94 °C for 30 s, 58 °C for 45 s and 72 °C for 80 s; final extension at 72 °C for 10 min. PCR products were electrophoresed in 1% agarose gel in Tris-acetate-EDTA (TAE) buffer, stained with ethidium bromide and then viewed under UV. The protocol for the m-RT-PCR was the same as the s-RT-PCR except the four primer pairs and RNA were mixed equally together.

Specific primers targeting each one of the four viruses were designed according to the genome sequences of each virus retrieved from GenBank (AJ297433; KM213865; EF536864; NC_002251) using Primer Premier 5 (Premier, Canada). The development of a PCR multiplex assay, allowing simultaneous detection of several targets in a single amplification, is often complex and challenging. Interference and (or) competition between the individual amplifications have to be avoided. The primers are therefore a crucial factor for successful multiplexing (Bertolini et al., 2001). They need to have similar *Tm* values and cross-hybridization between primers (primer-dimer) must be avoided. Thus, each primer pair was designed with the following additional criteria: similar annealing temperature, different size of each PCR product, absence of any secondary structures and primer–primer interaction.

Several pairs of primers were tested for the specificity of each virus; one set of primers (Table 1) amplified the target for each virus without any nonspecific bands (Fig. 1). The expected bands were obtained (273 bp for RBSDV, 565 bp for BYSMV, 783 bp for WDV and 1296 bp for NCMV). The assay was repeated three times independently and reproducibly detected the four viruses using the infected wheat samples grown in greenhouse.

Adapting the primer concentration of each target is particularly important as the efficiency of amplification is generally better for short sequences. Their primer concentration often needs to be lower than

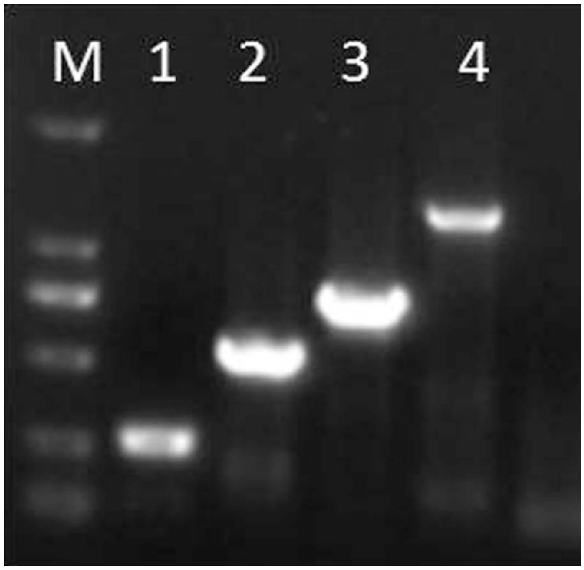

Fig. 1. Detection of four wheat viruses by simplex RT-PCR. Lanes 1: RBSDV (273 bp); 2: BYSMV (565 bp); 3: WDV (783 bp); 4: NCMV (1296 bp). M: DL 2000 marker (2000/1000/750/500/250/100 bp). Wheat plants infected respectively by RBSDV, WDV, NCMV or BYSMV were maintained in our greenhouse.

longer PCR products. To test the compatibility of these primers in m-RT-PCR, the RNA (each 1 μg) from the four viruses were mixed together equally. The RT-PCR protocol was applied using either a mix of the four pairs of primers (each 2 μL, 10 μM) or the single pair of primer for each virus. However, the NCMV could not be detected and the band intensity of WDV was lower compared to s-PCR (single pair of primer from the targeted virus).

The concentration of the primers was very important and needed to be adjusted. Liu et al. (2014a) found that when changing the primers' concentration, amplification efficiencies of tobacco bushy top virus (TBTV), and satellite RNA of TBTV (Sat-TBTV) varied, while those of tobacco vein distorting virus (TVDV) and tobacco vein distorting virus associate RNA (TVDVaRNA) remained stable. Here, firstly, several concentrations of each primer pair (put the concentration in μM) were tested by s-PCR (Fig. 2). All the viruses were still detected at the lowest primer concentration. The concentration of each primer was further optimized for m-PCR. The four specific bands can be obtained reproducibly when 0.1 μL of WDV primer, 0.2 μL of RBSDV and BYSMV primers and 0.5 μL of NCMV primer were used for the PCR (Fig. 3).

The amplified PCR products were further sequenced to validate the results. The PCR products were extracted using Wizard SV Gel and PCR Clean-Up System (Promega, USA) and cloned in pEASY-T5 vector (Transgen, China). The positive clones were sequenced by Sanger sequencing at Sangon (China). The data showed that the sequences of each band matched the designed region and the calculated sizes (unpresented data).

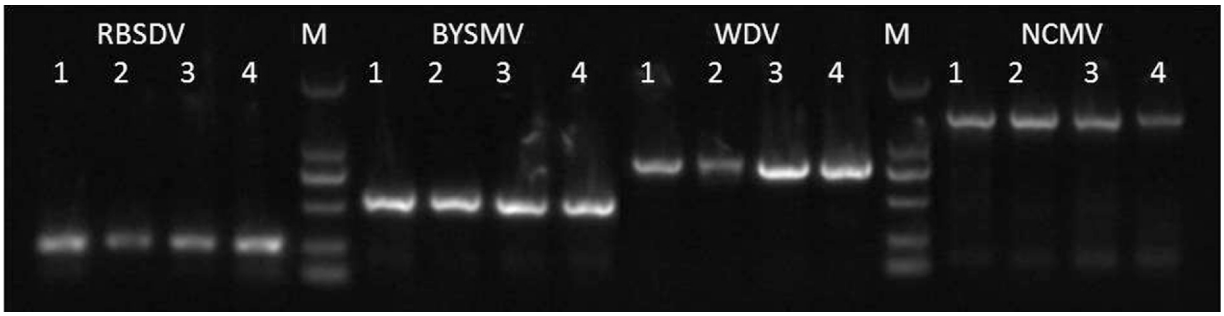

Fig. 2. Amplification of the four wheat viruses (RBSDV–273 bp, BYSMV–565 bp, WDV–783 bp and NCMV–1296 bp) with different concentrations of primers. Lanes 1: 0.04 μM; 2: 0.08 μM; 3: 0.12 μM; 4: 0.16 μM; M: DL 2000 marker (2000/1000/750/500/250/100 bp).

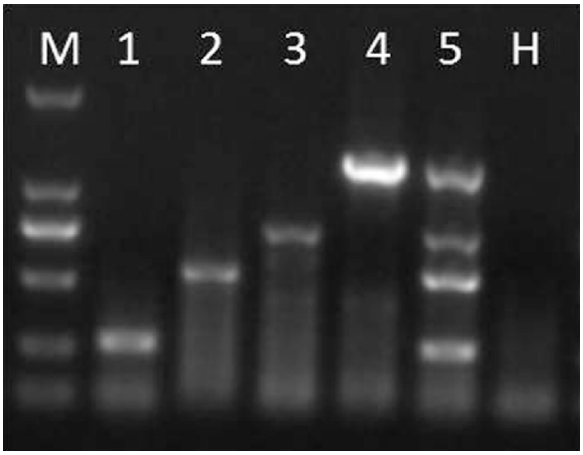

Fig. 3. Detection of four wheat viruses by multiplex RT-PCR. Lanes 1: RBSDV (273 bp); 2: BYSMV (565 bp); 3: WDV (783 bp); 4: NCMV (1296 bp); 5: mixture of four viruses; H: uninfected, healthy control. M: DL 2000 marker (2000/1000/750/500/250/100 bp). Wheat plants infected respectively by RBSDV, WDV, NCMV or BYSMV and uninfected were maintained in our greenhouse.

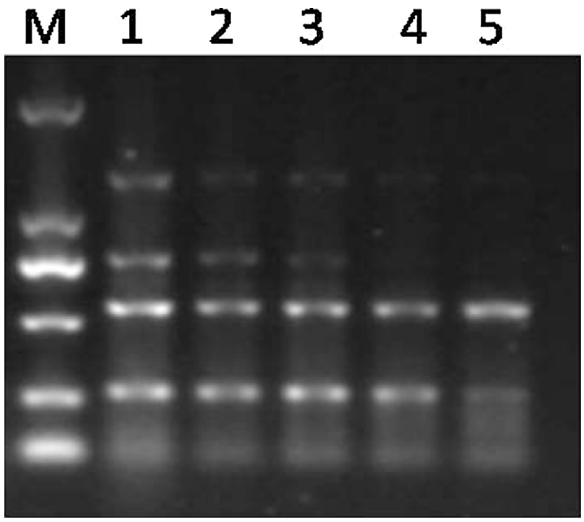

Fig. 5. Sensitivity of m-RT-PCR using 10-fold serial dilutions of cDNA, the cDNA was obtained using equally mixed reverse primers and RNAs of the wheat samples infected with the four viruses (RBSDV–273 bp, BYSMV–565 bp, WDV–783 bp and NCMV–1296 bp). Lane 1: 10^0 cDNA; 2: 10^{-1} cDNA; 3: 10^{-2} cDNA; 4: 10^{-3} cDNA; 5: 10^{-4} cDNA. M: DL 2000 marker (2000/1000/750/500/250/100 bp).

In the m-PCR detection system developed by Tao et al. (2012), developed for five RNA viruses, one DNA virus and one phytoplasma in wheat, they extracted separately RNA and DNA in the experimental procedure. In this study, we detected three RNA viruses and one DNA virus in wheat starting from RNA extract only. The DNA virus, WDV, was also detected through its transcribed RNA. Therefore, extracting only RNA is sufficient for one sample to detect the three RNA viruses and one DNA virus; there is no need to extract DNA, saving cost and time.

The optimization of PCR conditions is a key step to achieve multiplex detection. Therefore, different annealing temperatures (52/54/56/58/60 °C), different concentration of dNTP Mix (each at 2.5 mM; 1/2/4/6/8 μL), rTaq (5 U/μL; 0.1/0.2/0.3/0.4/0.5 μL) and Mg^{2+} (25 mM, 0.5/1.0/1.5/2.0/2.5 μL) were tested. The results showed that the tested annealing temperatures and rTaq concentrations did not affect the amplification efficiency for the four viruses (Fig. 4A and C). When using a high concentration of dNTP Mix, all the four bands could not be found, and using low concentration of Mg^{2+}, bands of NCMV, BYSMV and WDV were not obtained (Fig. 4 B and D). The optimal PCR conditions were therefore determined as follow: 2 μL of dNTP Mix (each at 2.5 mM), 0.2 μL of rTaq (5 U/μL) and 1.5 μL of Mg^{2+} (25 mM) and an annealing temperature of 58 °C.

Sensitivity is another criterion to evaluate the multiplex system: different viruses could have different detection limits (Meena and Baranwal, 2016). The sensitivity of the m-RT-PCR protocol was therefore evaluated using a 10-fold serial dilution of cDNA. The cDNA was synthesized with a total of 4 μL of RNA mixture (1 μL of RNA of each virus). The detection limit of m-RT-PCR to detect the 4 viruses was 10^{-2} (Fig. 5). The NCMV and WDV bands were indeed very faint at higher dilution. RBSDV band intensity dropped at 10^4-fold dilution while the BYSMV detection signal remained intense.

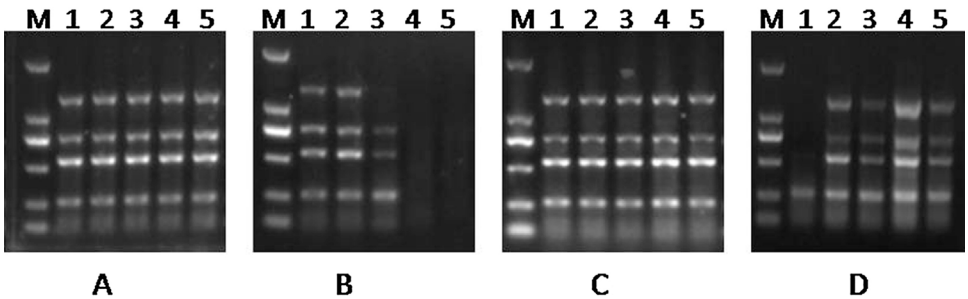

Fig. 4. Optimization of m-RT-PCR to detect the four wheat viruses (RBSDV–273 bp, BYSMV–565 bp, WDV–783 bp and NCMV–1296 bp). (A): Annealing temperature, lanes 1: 52 °C; 2: 54 °C; 3: 56 °C; 4: 58 °C; 5: 60 °C. (B): dNTP Mix (each at 2.5 mM), lanes 1: 1 μL; 2: 2 μL; 3: 4 μL; 4: 6 μL; 5: 8 μL. (C): rTaq (5U/μL), lanes 1: 0.1 μL; 2: 0.2 μL; 3: 0.3 μL; 4: 0.4 μL; 5: 0.5 μL. (D): Mg^{2+} (25 mM), lanes 1: 0.5 μL; 2: 1.0 μL; 3: 1.5 μL; 4: 2.0 μL; 5: 2.5 μL. M: DL 2000 marker (2000/1000/750/500/250/100 bp).

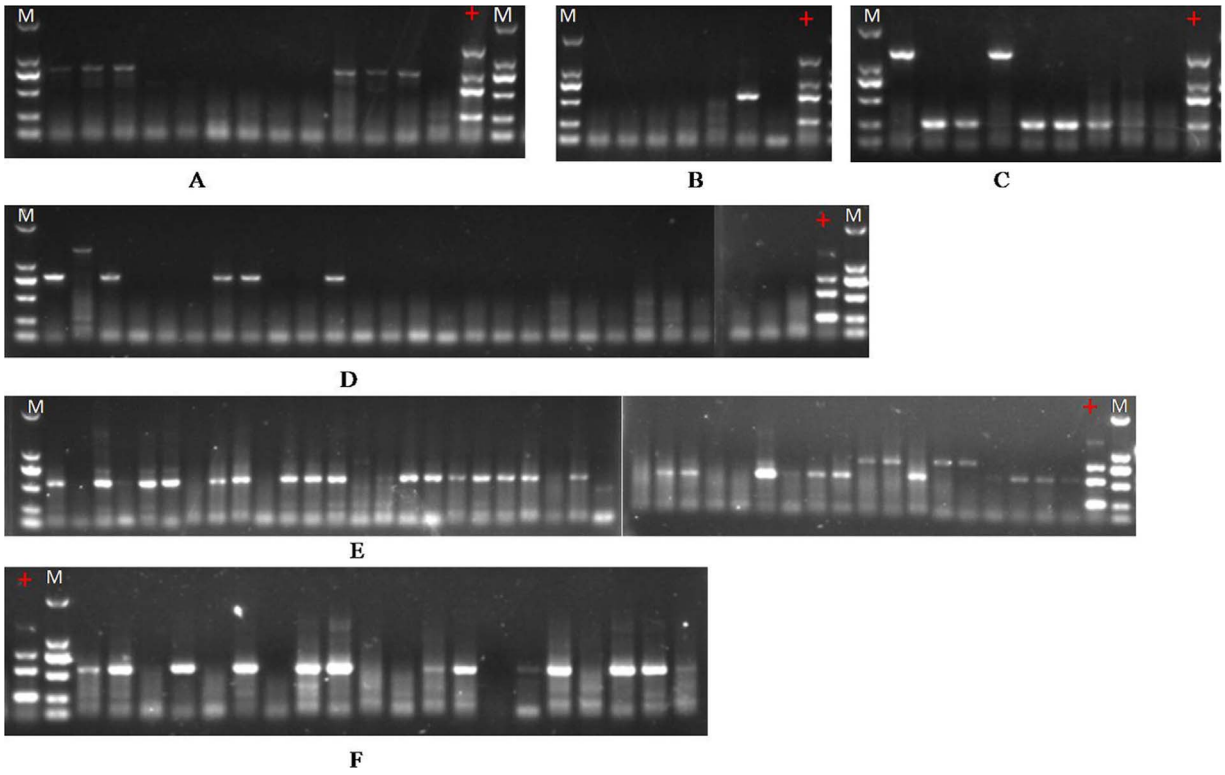

Fig. 6. m-RT-PCR detection of wheat viruses from wheat samples collected from fields in five provinces in China. A: Taiyuan, Shanxi; B: Zhengzhou, Henan; C: Jinan, Shandong; D: Hancheng, Shaanxi; E and F: Shijiazhuang, Hebei. Lanes M: DL 2000 marker (2000/1000/750/500/250/100 bp); +: positive control, wheat samples infected RBSDV (273 bp), BYSMV (565 bp), WDV (783 bp) and NCMV (1296 bp) were mixed together.

Table 2
Detection of four viruses in field samples collected from five provinces of China in 2016.

Location	No. of samples	No. of positive samples			
		RBSDV	BYSMV	WDV	NCMV
Hancheng, Shaanxi	27	0	0	5	1
Zhengzhou, Henan	6	0	1	0	0
Jinan, Shandong	9	5	0	0	2
Shijiazhuang, Hebei	62	0	38	4	0
Taiyuan, Shanxi	13	0	0	6	0
Total	117	5	39	15	3

From April to May 2016, 117 diseased wheat samples with dwarfing, lack of heading, increased tillering, and some with slight yellowing were collected from five provinces of China. The optimized m-RT-PCR system was applied on these 117 samples (See example in Fig. 6). RBSDV was detected in five samples from a single province, BYSMV was detected in 39 samples from two provinces, WDV was detected in 15 samples from three provinces, and NCMV was detected in three samples from two provinces (Table 2). In total, 62 samples were positive for one of the viruses, but none had a mixed infection even though RBSDV, BYSMV and NCMV are all transmitted by *L. striatellus*. More samples need to be tested to know whether there are naturally mixed infections or not. Importantly, the symptoms observed in the negative samples might be caused by other viruses or environmental factors; they deserve further analysis.

In summary, we developed a reliable, convenient, rapid and economical m-RT-PCR method to simultaneously detect three RNA viruses and one DNA virus from wheat plants that are very difficult to distinguish them from each other in the field. This method will improve the detection of these viruses, allowing to better understand their etiology, incidence and disease development.

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