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Detection and Classification of Barley Yellow Dwarf Virus Strains Using RT-PCR

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ABSTRACT : Barley Yellow Dwarf Virus (BYDV), an aphid-borne luteovirus, is a major plant pathogenic disease causing a huge economic loss in the grain production of a wide range of *Gramineae* species throughout the world. It has been recently reported that BYDV also occurred frequently in wheat field of Korea. Here, we performed to develop the detection and classification methods of BYDV strains that were accomplished by reverse transcription-polymerase chain reaction (RT-PCR). Since there are high variations among BYDV strains, three pairs of primers were designed to detect BYDV strains such as PAV (Vic-PAV and CN-PAV) and MAV (primer A) simultaneously, specifically Vic-PAV (primer B), and MAV (primer C) based on the genomic RNA sequences of BYDV strains previously published. The validity of the primers was confirmed using several BYDV strains obtained from CIMMYT. Though three BYDV strains were able to be detected using primer A, PCR products were not distinguished between two PAV strains. It was possible to separate them with a restriction enzyme, *Eco*RI, whose restriction site was present in the amplified DNA fragment from Vic-PAV, but not from CN-PAV.

Keywords : BYDV, detection, classification, PAV, MAV, RT-PCR, *Eco*RI restriction enzyme

Barley yellow dwarf virus (BYDV), a member of the luteovirus group (Matthews, 1982), is a single-stranded positive-sense RNA virus occurring worldwide in wheat, barley, oats, and other *Gramineae* (Rochow, 1969). Early infection by BYDV can cause severe damages such as stunting, reduced tillering, leaf yellowing or reddening, delayed heading or ripening, increased sterility, and fewer and lighter kernels (Baltenberger *et al.*, 1987; Hoffman & Kolb, 1997, 1998). Since BYDV was first recognized as the causative agent by Oswald & Houston (1951), its infection has been assumed to be one of the most economically important plant pathogenic diseases worldwide with yield losses being recorded as high as 70% in wheat (Paul & Warren, 1967).

BYDV was divided into five strains (PAV, MAV, SGV, RPV, and RMV) which were classified by their transmission through different species of aphids (Rochow, 1969; Rochow & Muller, 1971). Among them, it has been known that the most prevalent strain is PAV, a non-vector-specific strain that can be also transmitted by the principal vectors of MAV (*Macrosiphum avenae* Fabr.) and RPV (*Rhopalosiphum padi* L.). Five strains of BYDV can be classified into two strains by serological detection methods, subgroup I containing PAV, MAV, and SGV, and subgroup II containing RPV and RMV (Rochow & Carmichael, 1979).

Infection of BYDV can be transmitted by aphids only. The verification of the presence or absence of BYDV in plants used to require manipulations of aphid vectors, which makes the identification of BYDV strains more difficult and also requires several tests of virus acquisition and transmissibility by specific aphid vectors for each sample tested (Rochow, 1969). Moreover, since BYDV is phloem-limited and present at low concentrations in the extracts of infected tissues (D'Arcy *et al.*, 1983; Stanley, 1969), the sensitivity of a detection technique is very important to verify this virus in field samples.

Enzyme-linked immunosorbent assays (ELISA) have been widely used to detect BYDV so far, and other techniques such as reverse transcription-polymerase chain reaction (RT-PCR) and nucleic acid hybridization have been recently reported to be more sensitive and capable of distinguishing some virus strains (Fouly *et al.*, 1992; Habili *et al.*, 1987). Although nucleic acid hybridization was considered as a powerful technique for the detection of plant virus (Hull & Al-Hakin, 1988), the risks of handling radioactive materials and the probe problems of nonspecific hybridization to healthy samples have discouraged its wide application. RT-PCR, a highly sensitive method for amplification of viral RNA genome, has become widely used in the detection and differentiation of RNA viruses in plant (Kim *et al.*, 1999; Langeveld *et al.*, 1991). The application of RT-PCR method for BYDV detection was initially performed by Robertson *et al.* (1991), but the primers have not been available for Korean BYDV isolates.

In this study, we present the development of RT-PCR assay and restriction enzyme (RE) digestion analysis as detection and classification methods of BYDV strains. By these assays,

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the distribution of BYDV strains which have not been reported in Korea is to be investigated, and the RT-PCR and RE analysis can be used as the earlier detection method for selection BYDV-resistant lines in small grain cereals.

MATERIALS AND METHODS

Virus isolates and plant materials

From 1999 to 2000, wheat, barley, and oats plants showing yellowing or reddening in leaves were collected from various regions in Korea. These plants were stored at -70°C for the isolation of viral RNA genome. BYDV strains, Vic-PAV, CN-PAV, and MAV-PS1, as a positive control in this study were obtained from Dr. M. Henry (CIMMYT, Mexico).

Total RNA extraction, primers, and RT-PCR amplification

Viral RNA was isolated from the leaf tissues of collected plants using total RNA extraction kit (Promega, USA) according to the manufacturer's instructions. The primers were designed based on the nucleotide sequences of Vic-PAV (accession number : X07653), CN-PAV (AF192967), and MAV-PS1 (D012130). The primer sequences, the positions, and the expected sizes of their respective PCR products are shown in Table 1.

Detection of viral RNA genome was performed using RT-PCR System (Promega, USA) consisting of one step with cDNA synthesis and PCR amplification. RT-PCR reactions were conducted in a reaction containing 10 μl AMV/Tf*i* 5X Reaction Buffer, 1 μl dNTP mix (10 mM each), 2 μl $MgSO_4$ (25 mM), 1 μl each primer (50 pmol), 1 μl AMV reverse transcriptase (5 units/μl), 1 μl Tf*i* DNA polymerase (5 units/μl), 5 template RNA in a final volume of 50 μl made up to the volume with nuclease-free water. Thermocycling was performed in a PTC 200 (MJ Research, Inc., USA) and programmed as follows; cDNA synthesis of 1 cycle at 48°C for 45°C min, AMV RT inactivation and RNA/cDNA/primer denaturation of 1 cycle at 94°C for 2 min; followed by 30 cycles for template denaturation at 94°C for 30 sec, primer annealing at 60°C for 1 min, extension at 68°C for 2 min in case of primer A, C or 30 cycles for template denaturation at 94°C for 30 sec, primer annealing at 63°C for 1 min, extension at 72°C for 2 min in case of primer B; followed by 1 cycle for final extension at 68°C for 7 min. The PCR products were stored at -20°C until analysis and visualized by electrophoresis on a 1.2% TBE-agarose gel after staining with 250 ng/ml ethidium bromide (EtBr) solution for 10 min.

Restriction enzyme digestion

After precipitation of the PCR products with ethanol and 3 M sodium acetate and collection by centrifugation, they were resuspended in nuclease-free water. A 12 μl volume of PCR products were digested with 2 μl of restriction enzyme, *Eco*RI (12 units/μl). The PCR fragments were analyzed by electrophoresis on a 4% Nusieve 3 : 1 agarose gel (FMC, USA) stained with EtBr after cleavage with *Eco*RI at 37°C for 3 hours.

Cloning and sequencing of RT-PCR products

The PCR products were purified by using PCR purification kit (QIAGEN, Germany) before cloning. After ligation of the PCR fragments into the digested pGEM-T easy vector (Promega, USA), they were transformed to *Escherichia coli* JM109 and cloned. The sequencing reactions and analyses were carried out using BigDye Terminator Cycle sequenc-

Table 1. Primers used to detect and classify BYDV strains.

Primer	Orientation	Position (strain)	Sequences	Amplified DNA (bp)
Primer A	Sense	3182-3202 (Vic-PAV)	5'-GTTGAGTTTAAGTCACACGCG-3'	388, 302
	Antisense	3551-3569 (Vic-PAV)	5'-TGGGGACAGGTGCAGGAGT-3'	
Primer B	Sense	4433-4452 (Vic-PAV)	5'-GTTTTGCCCTCCAAGGAACA-3'	694
	Antisense	5102-5126 (Vic-PAV)	5'-GGGTCGTTAATCCCCTCACTGAGCC-3'	
Primer C	Sense	3143-3163 (MAV-PS1)	5'-GTTGAGTTTAAGTCACACGCG-3'	783
	Antisense	3905-3925 (MAV-PS1)	5'-TTTACAGCTGTTTAATTCCAA-3'	

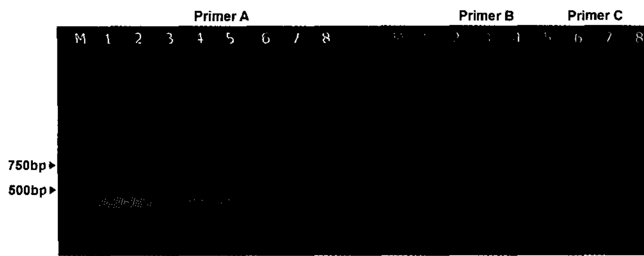

Fig. 1. Agarose gel electrophoresis of RT-PCR products amplified with primer A, B, and C from total RNA purified from wheat plants infected with BYDV. M: size marker (1kb ladder), 1~3: Vic-PAV, 4~5: CN-PAV, 6~7: MAV, 8: healthy plant.

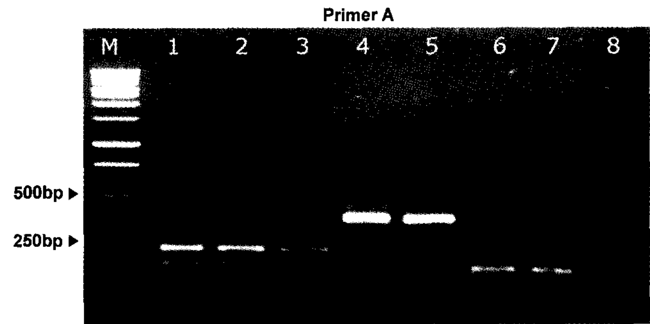

Fig. 2. Restriction enzyme *Eco*RI digestion of PCR products amplified with primer A. M: size marker (1kb ladder), 1~3: Vic-PAV, 4~5: CN-PAV, 6~7: MAV, 8: healthy plant.

ing kit (Perkin-Elmer/Applied Biosystems Inc., USA) in an automated Applied Biosystems 377 DNA sequencer.

RESULTS

Detection of BYDV strains using RT-PCR

The RT-PCR amplification was performed using primer A designed to detect Vic-PAV, CN-PAV, and MAV-PS1 simultaneously, primer B to only Vic-PAV, and primer C to only MAV-PS1, all of which were based on the nucleotide sequences of coat protein gene of Vic-PAV, CN-PAV, and MAV-PS1 in GenBank (http://www.ncbi.nlm.nih.gov/Entrez/index.html). In Fig. 1, an expected fragment of 388 bp was amplified from Vic-PAV and CN-PAV and that of 302 bp from MAV-PS1. The amplification with primer B produced a single fragment of 694 bp from Vic-PAV and that with primer C 783 bp from MAV-PS1. No corresponding fragment was amplified from total RNA extracted from healthy plants as a control.

Classification of BYDV strains using restriction enzyme *Eco*RI

When 388 bp of a single DNA fragment was amplified by primer A, the differentiation of Vic-PAV and CN-PAV could be accomplished by restriction enzyme digestion. The restriction enzyme digestion analysis is much simpler and more economical method, compared to RT-PCR reaction by primer B. The resulting amplification products by primer A were further characterized by digestion with restriction enzyme, *Eco*RI (Fig. 2). The 388 bp of DNA fragment amplified from Vic-PAV was divided into two fragments by *Eco*RI while that from CN-PAV was not. This indicates that there is an *Eco*RI site in the 388 bp of DNA fragment amplified from Vic-PAV and not in that from CN-PAV. In addition, the 302 bp of DNA fragment from MAV-PS1 was digested to two fragments, an indication that there is also an *Eco*RI site in the DNA fragment.

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Vic-P     1   GTTGAGTTTAAGTCACACGCGTCCGCCAATACGGCAGGCGCTATCTTTAT
Kor-P1    1   ----------------------------C---------------------
Vic-P    51   TGAGCTCGACACCGCGTGCAAGCAATCAGCCCTGGGTAGCTACATTAATT
Kor-P1   51   --------------------------------------------------
Vic-P   101   CCTTCACCATCAGCAAGACCGCCTCCAAGACCTTCCGGTCAGAGGCAATT
Kor-P1  101   --------------------------------------------------
Vic-P   151   AATGGGAAGGAATTCCAGGAATCAACGATAGACCAATTTTGGATGCTCTA
Kor-P1  151   ---------GAATTC-----------------------------------
Vic-P   201   CAAGGCCAATGGAACTACCACTGACACGGCAGGACAATTTATCATTACGA
Kor-P1  201   ---------------C----------------------------------
Vic-P   251   GTTGAGTTTAAGTCACACGCGTCCGCCAATACGGCAGGCGCTATCTTTAT
Kor-P1  251   --------------------------------------------------
Vic-P   301   CCAAAACCTGCACCGGAACCAACACCAACCCCCCAGCCAACGCCGGCTCC
Kor-P1  301   --------------------------------------------------
Vic-P   351   ACAGCCCACACCTGAACCAACTCCTGCACCTGTCCCCA
Kor-P1  351   -------G------------------------------ (388bp)

CN-P      1   GTTGAGTTTAGAACACACGCGTCCGCCACTACGTCGGGCGCTATGTTTAT
Kor-P2    1   ----------AGT-------------------------------------
CN-P     51   TGAGCTCGACACCTCGTGCAAGCAATCAGCCTTATCTAGCTACATTAACT
Kor-P2   51   ---A-------------------------------------T------T-
CN-P    101   CATTCACCATCAGCAAATCAGCCTCAAAGTCCTTCCGCGCAGCGGAGATT
Kor-P2  101   -------------------C------------------------------
CN-P    151   GGAGGGACCCAGTTCCAGGCGACATCGGTGAATCAGTTCTTTCTCTTGTA
Kor-P2  151   -----------A-----------------------A--------T-----
CN-P    201   CAAGGCAAATGGCACGACGACTGATATTGCGGCGCAGTTCATCATAAAAA
Kor-P2  201   ---------------T--A-----------A-G------------C-G--
CN-P    251   TTGAGCTTCA (no further sequence data available)
Kor-P2  251   ----------TCTAATGACTGCCAAATAGGTAGACTCCTCAACACCGGAA
Kor-P2  301   CCAAAACCCACACCAGCTCCAACACCTGCCCCAACACCTGAGCCAAAACC
Kor-P2  351   AGAGCCCACTCCTGCACCTGTCCCCA (376 bp)

M-PS1     1   GTTGAGTTTAAGTCACACGCGTCCGCCTCTACAGTCGGCGCAATGTTTAT
Kor-M     1   --------------------------------------------------
M-PS1    51   TGAACTCGACACTTGGTGCTCACAATCAACCTTGGGTAGCTACATTAACT
Kor-M    51   --------------C-----------------------------------
M-PS1   101   CATTCACCATCTCAAAATCAGCAACCAAAACCTTCACCGCCCAACAGATT
Kor-M   101   --------C---------------------A-------------------
M-PS1   151   GACGGGAAGGAATTCAGGGAGAGTACGGTGAACCAATTTTACATGCTATA
Kor-M   151   ---------GAATTC--------C--------T--------T--------
M-PS1   201   CAAGGCGAATGGTAGTACATCTGATACCGCCGGGCAATTCATCATTACAA
Kor-M   201   ------A-------------------------------------------
M-PS1   251   TACGCGTTGCCAACATGACTCCCAAATAGGTAGACTCCTCAACACCAGAA
Kor-M   251   ---------------------------------------GC--CTGTCCC
M-PS1   301   CC
Kor-M   301   -A (302 bp)
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Fig. 3. Nucleotide sequences alignments of PCR products of BYDV strains amplified by primer A. GAATTC: *Eco*RI restriction site.

To verify this result of restriction enzyme digestion, each fragment amplified from three isolates by primer A was sequenced and analyzed. Korean BYDV isolates were also sequenced to find the validity of the application results to them. Vic-PAV-like Korean isolates were designated as Kor-P1, CN-PAV-like isolates as Kor-P2, and MAV-PS1-like

isolates as Kor-M. The results of the sequences analysis show that there is an *Eco*RI site in the fragment from Kor-P1 and Kor-M and not in that from Kor-P2 (Fig. 3). Thus, it is considered that this restriction enzyme analysis after RT-RCR can be applied to Korean isolates though some variations exist in their nucleotide sequences.

DISCUSSON

It has been reported that RT-PCR method is appropriate for detection of viruses present at low concentrations such as BYDV due to its high sensitivity (Figueria *et al.*, 1997; Kim *et al.*, 1999; Langeveld *et al.*, 1991). However, the unexpected detection errors occur frequently due to denaturation and contamination of viral RNA during the procedure of total RNA extraction. The unfitness of PCR conditions, enzymes, and thermocyclers also add to detection errors. Thus, it is important to conduct RT-PCR reaction in optimum conditions for the accuracy of detection.

There are high variations even among PAV strains such as Vic-PAV, P-PAV, NY-PAV, JAN-PAV, and CN-PAV as well as other BYDV strains. This makes it difficult to design the primers to detect all BYDV strains. We developed the primers to detect Vic-PAV, CN-PAV, and MAV-PS1. It is thought that the development of the primers specifically to detect prevalent strains is invaluable in practice. The RT-PCR amplification was performed using three pairs of primers designed to detect BYDV strains. Expectedly, the amplification with primer A produced a single fragment of 388 bp from Vic-PAV and CN-PAV and that of 302bp from MAV-PS1. Also, a 694 bp of amplified DNA fragment was amplified by primer B from Vic-PAV and a 783 bp by primer C from MAV-PS1 (Fig. 1). When 388 bp size of DNA fragment was amplified by primer A, it was possible to distinguish between Vic-PAV and CN-PAV by restricting the PCR products with *Eco*RI (Fig. 2). Kim *et al.* (1999) also reported that soybean mosaic virus strains could be classified by restriction enzyme digestion after RT-PCR. The restriction enzyme digestion analysis is simpler and more economical method, compared to RT-PCR reaction by primer B, suggesting that the additional analysis of restriction enzyme can be generally used for classification of virus strains including BYDV.

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