Korean J. Plant Res. 20(6) : 511~517(2007)

Strain Distinction and Their Distribution of *Barley Yellow Mosaic Virus* Base on RAPD Analysis in Korea

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Abstract - A stable method for strain distinction using viral RNA 1 structures analyses was developed and compared with the combined RT-PCR and RAPD methods. Seven out of 61 random primers were found to be polymorphic based on RAPD analysis resulting on the differentiation of the 33 BaYMV isolates into four distinct groups according to geographical districts. The first and largest group includes 13 isolate and consists mainly of two-rowed malting barley in Haenam area. The second group had ten collections from inland in west southern. The third group had seven isolates from west southern coastal region, where mainly six-rowed naked barley is cultivated. The last fourth group included three isolates from Gyungnam region in east southern area. Conclusively, RNA 1 analysis proved to be stable and efficient method for strain distinction for Korean BaYMV isolates. Further, results of pathogenicity and RNA 1 structure analyses revealed four groups BaYMV strains and were distributed all over Korea, represented by Naju, Haenam-okcheon, Iksan and Milyang.

Key words - BaYMV strain, RNA 1 structure, Differential variety, Pathogenicity

Introduction

Barley yellow mosaic virus (BaYMV) causes serious problems to barley growth at heading date and yield not only in Korea but in other temperate countries worldwide (Adams *et al.*, 1987; Lee, 1981; So *et al.*, 1997). The virus belongs to *Bymovirus* genus (Barnett, 1991) and is transmitted in barley plants by fungal vector, *Polymyxa graminis*. BaYMV is generally destructive when seeded during autumn than in spring season. The infection develops systemic disease symptoms, yellowing and mosaic spots in leaves, manifested by short culm length, delayed heading date and finally reduced yield to 40~100% (Frahm, 1989; So *et al.*, 1990, 1991).

After first report of BaYMV occurrence in Japan (Miyamoto, 1958), their strain distribution was reported in Japan (Kashiwazaki, 1989), England (Adams *et al.*, 1987) and Germany (Huth *et al.*, 1984). Until now there is only a suspicion of the strain existence in Korea through cultivar pathogenicity investigation. Unfortunately, BaYMV strain distinction in Korea had been done by using Japan's differential varieties only (Lee *et al.*, 1998). However, BaYMV isolates in Korea had more different pathogenic characteristics than those of Japan's probably due to different climatic factors that generally affect the viral infection, symptom expression and viral replication in field test (Matthews, 1991).

In addition, nucleic acid analysis was suggested as an effective method for strain distinction of *Potyvirus* (Gibbs and McIntyre, 1970). The pathogenic gene of BaYMV was found to be located on viral RNA 1 (Kashiwazaki and Hibino, 1996) and that RNA 2 was not related to BaYMV strain diversity (Shi *et al.*, 1995). Therefore, in this study, strain distribution in Korea by different pathogenic responses was further investigated. Also, an efficient method for strain distinction using viral RNA 1 structure analyses was developed in order to establish the BaYMV strains distribution in domestic barley fields.

Materials and Methods

Field pathogenic test

The pathogenicity test was carried out in field from 1995 to 1997 in four regions such as Iksan, Naju, Milyang and Haenam-okcheon. These locations have been known showing significantly different pathogenic responses in testing of barley breeding lines for several years. Nine differential varieties that used for distinction for BaYMV strains in Japan were seeded from 20 to 30 in October from 1995 to 1997 above regions to distinct the strains. These varieties and their resistant genes were listed in Table 1.

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Table 1. List of the differential barley varieties used in the pathogenicity test

Type	Variety[a]	Type	Variety
Two-rowed	New Golden	Two-rowed	Ishikushirazu *(rym3)*
	Agaki Nijo	Six-rowed	Kashimamugi
	Amagi Nijo *(rym6)*		Joshuhiro Hadaka
	Haruna Nijo *(rym6)*		Mokusekko-3 *(rym1+rym5)*
	Misato Golden *(rym5)*[b]		

[a]Variety: a barley varieties used for BaYMV strain differentiation in Japan.
[b]Resistant gene(s) possessed by variety.

Sample collection, RNA extraction and detection

A total of 75 samples were collected from 36 barley fields nationwide from March 10 to 25, 2004 (Table 2). Plant samples showing typical symptoms as yellow and or mosaic spots on leaves were collected and tested by ELISA and RT-PCR to detect the presence of BaYMV. ELISA was done following prior reported method (So *et al.*, 1997). For RT-PCR, total RNA was extracted from leaves using Rneasy plant mini kit (QUIAGEN. Inc). cDNAs were synthesized by mixing 3㎕ total RNA and 17㎕ mixtures [25mM $MgCl_2$, AMV/TFL 5x reaction buffer, 10mM dNTPmix, AMV reverse transcriptase, RNase inhibitor, Nuclease free water, downstream primer (50pmol)] with the following temperature cycles such as 45℃/30min, 97℃/5min and 4℃/5min. The amplification process was conditioned to 94℃/1min, 60℃/1min and 72℃/2min for 38 cycles after denaturation at 94℃/5min. BaYMV specific primers reported by Lee *et al.* (1998) were used in this study.

Viral RNA 1 analysis

Samples detected for BaYMV by both ELISA and RT-PCR were subjected to RAPD (Randomly amplified polymorphic DNA) analysis and viral RNA 1 structures were examined. sixty one primers were used for RAPD analysis with modified cDNA amplification conditions; 94℃/1min, 55℃/1min and 72℃/2min for 35 cycles after denaturation at 94℃/5min. Banding patterns were investigated on 1.2% agarose gel and were analyzed by NTsys program for their genetic relationships.

Results and Discussion

Field pathogenicity test

Table 3 showed the different pathogenic responses of differential cultivars tested in 4 regions. Results indicated that different strains were distributed in domestic fields, however, some cultivars such as Misato Golden, Ishikushirazu, Kashimamugi, Joshuhiro Hadaka and Haganemugi showed varying pathogenic responses yearly. These confused results possibly related to different environmental conditions yearly. Some reports revealed that unstable pathogenic responses is most likely related to different viral infection and the symptom caused by climatic conditions (Frahm *et al.*, 1989; Park *et al.*, 2003) and mixed infection with another soil borne viruses (Park *et al.*, 2004). On the other hand, mechanical inoculation is one way to avoid mixed infections by natural infection. However, because of the lower mechanical inoculation efficiency of BaYMV than BaMMV (Huth *et al.*, 1984; So *et al.*, 1997; Lee *et al.*, 2002; Werner *et al.*, 2003), this method could not use the BaYMV strain differentiation. Even though Japan already differentiated the 6 type

Table 2. Places for the collection of barley leaf samples

Province	Region	Collection
Jeonbuk	Jeongeup, Gochang, Buan, Gimje Namwon, Imsil, Iksan, Daeya, Samrye	17
Jeonnam	Haenam, Gangjin, Hwasoon, Jindo, Wando, Mooan, Naju, Jangheung, Younggwang, Hwasun, Youngam, Hampyung	28
Gyungnam	Sacheon, Muan, Milyang, Goseong	13
Chungnam	Yuseong, Nonsan	7
Gangwon	Samcheok	5
Gyunggi	Suwon	5
	Total (29 regions)	75

Table 3. Pathogenic responses of the ten differential varieties in different regions in 1995-1997 field test

Varieties	Ik			Nj			Hn			My
	95	96	97	95	96	97	95	96	97	97
New Golden	+	+	-	+	+	+	-	+	+	+
Agaki Nijo	+	+	+	-	+	+	+	+	+	+
Amagi Nijo	+	+	+	+	+	+	+	+	+	-
Haruna Nijo	+	+	+	+	+	+	+	+	+	-
Misato Golden	+	+	+	-	+	-	+	-	+	-
Ishukushirazu	+	-	-	+	-	-	-	+	+	-
Kashimamugi	-	-	+	-	-	+	-	-	+	-
Joshuhiro Hadaka	+	-	+	+	+	-	+	+	+	+
Haganemugi	nt	-	+	nt	-	-	nt	-	-	-
Mokusekko-3	-	-	-	-	-	-	-	-	-	-

Differential varieties used to differentiate strains in Japan. Ik; Iksan, Nj; Naju, Hn; Haenam-okcheon and My; Milyang. + and indicates with infection and no infection of BaYMV, respectively. nt = not tested.

of BaYMV strain (Kashiwazaki *et al.,* 1989), Ordon *et al.* (2004) suggested that developing and using of DNA marker will be better to distinct them than relying on pathogenic differences in fields. Which also considered the mistake of pathogenic variation that could be occurred in field test. Moreover, Lee *et al.* (1998) mentioned the difficulty of using the Japan's differential varieties in Korea due to the differences of serological and pathological properties of BaYMV isolates between Korea and Japan. For this reason, So *et al.* (1997) emphasize on the necessity of more accurate differential system for Korean BaYMV strain distinction. Considering to above the results and previous reports, relying on pathogenic only has difficulty to BaYMV strain distinction in Korea.

Analysis and comparison of viral RNA 1 structures

A total of 33 isolates were detected to have BaYMV infection solely out of 75 materials tested by ELISA and RT-PCR (Fig. 1A and Table 4). These 33 isolates were used in the RAPD analysis and compared their RNA 1 structure. Seven random primers (Table 5) out of 61 primers tested showed polymorphism in banding patterns (Fig. 1B). The genetic relationship of the 33 isolates revealed four groups indicating the presence of genetic variation (Fig. 2). Each group possibly corresponds to distinct BaYMV strain and their distribution was revealed (Table 6). Interestingly, each group was related to regional districts. The first group is the largest group consisting of 13 isolates mainly from Haenam, Gangjin and Naju distributed in west southern areas and a major two-rowed malting barley were cultivated. The second one had ten collections from inland in west southern where in major fields were six rowed naked barley. The third includes seven isolates occupied in west southern coastal region where cultivated with mainly six-rowed naked barley. The last fourth group had three isolates from only east southern area, Gyungnam. Seo (1995) reported that the two Japan's

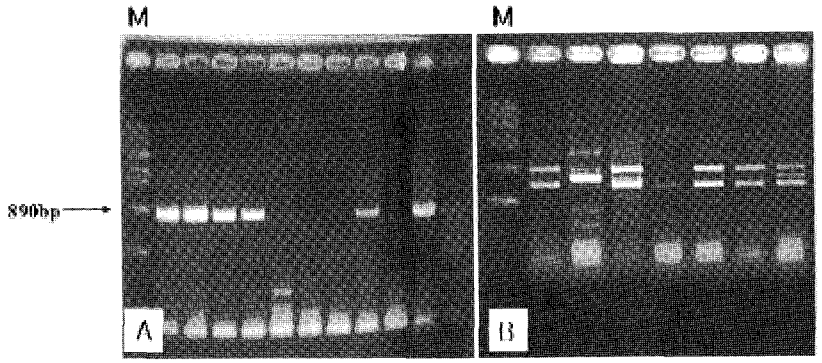

Fig. 1. RT-PCR products of BaYMV CP region (A) using specific primer from collections and partial RAPD patterns (B) of viral RNA 1 of BaYMV isolates using URP 6 primer from domestic regions. M is 100bp DNA size marker.

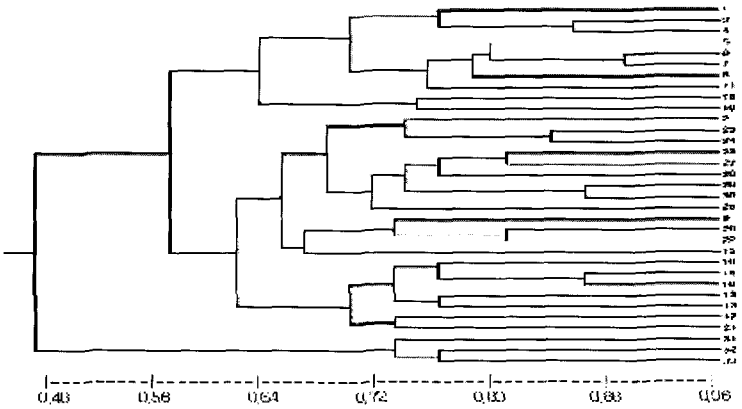

Fig. 2. Phenogram based on the relationship of RAPD polymorphism of the viral RNA 1 among 33 BaYMV isolates. The isolate numbers are inside the Table 6 as number in phenogram columns. The numerical value below the phenogram means coefficient among the isolates.

Table 4. BaYMV isolates collected from different provinces tested by ELISA and RT-PCR

Province	Isolate	ELISA	RT-PCR	Province	Isolate	ELISA	RT-PCR
Jeonbuk	Gochng-gosu-2	+	+	Jeonnam	Gangjin-eup	+	+
	Buan-sangseo	+	+		Jindo	+	+
	Gimje-juksan	+	+		Wando-gunnae	+	+
	Namwon-2	+	+		Muan-cheongcheon	+	+
	Imsil-osu-1	-	+		Muan-mongtan	+	+
	Iksan-1	+	+		Naju-2	+	+
	Daeya	+	+		Naju-saeji	+	+
	Samrye-eup	+	+		Jangheung-eup	+	+
	Chunpo	-	+		Hampyung-hakgyo	+	+
Jeonnam	Haenam-miam	+	+	Gyungnam	Sacheon-gonayang-1	+	+
	-bukpyung	+	+		-2	-	+
	-masan-1	+	+		Milyang-1	+	+
	-masan-2	+	+	Chungnam	Yuseong-1	+	+
	-okcheon	+	+		Nonsan-1	+	+
	Gangjin-gundong	+	+	Gangwon	Samcheok-3	+	+
	-sinjeon-1	+	+		-5	+	+
	-sinjeon-2	-	+				
Total					75	46	33

+ and - indicates positive and negative infection with BaYMV. nt = not tested.

Table 5. Random primers and their nucleotide sequences used in RAPD analysis

Primer*	Nucleotide sequence
URP 6	5' ATGTGTGCGATCAGTTGCTG 3'
URP 10	5' GATGTGTTCTTGGAGCCTGT 3'
RP 19	5' GTGACGTAGG 3'
RP 27	5' GGACTGGAGT 3'
RP 51	5' ATACGGCGTC 3'
RP 59	5' CAGCACCCAC 3'
Takara pd (N) 9	-

Selection of polymorphic random primers out of 61 pimers tested. Takara pd(N)9 is a product name from Takara Korea Biomedical Inc.

Table 6. Differentiation of BaYMV isolates by difference of RNA 1 structure

Strain type	Isolate	Number in Phenogram	Strain type	Isolate	Number in Phenogram
I	Gangjin-gundong	1	II	Gangjin-eup	2
	Gangjin-sinjeon-1	3		Haenam-okchun	9
	Haenam-masan-1	4		Muan-cheongcheon	15
	Gangjin-sinjeon-2	5		Imsil-osu-1	20
	Haenam-masan-2	6		Samcheok-3	22
	Haenam-bukpyung	7		Samcheok-5	23
	Haenam-miam	8		Yuseong-1	24
	Gochang-gosu-2	11		Daeya	25
	Naju-2	18		Nonsan-1	26
	Naju-saeji	19		Samrye	27
III	Buan-sangseo	10		Choonpo	28
	Wando-gunnae	12		Jindo	29
	Hampyung-hakgyo	13		Muan-mongtan	30
	Gimjae-juksan	14	IV	Sacheon-gonyang-1	31
	Namwon-2	16		Sacheon-gonyang-2	32
	Iksan-1	17		Milyang	33
	Jangheung-eup	21			

BaYMV strains, BaYMV-I and -II, were also present in Korea. However, in his study field test was conducted only in 1 year which could not consider to the pathogenic variation in different years as shown in this study. Therefore, several problems were encountered in BaYMV strain distinction in Korea. Several papers reported the existence of BaYMV strains based on different pathogenicity in Korea, however there is no any efficient differentiation method and information for their distribution. BaYMV RNA 1 analysis has been considered as efficient method for strain distinction and more searching pathogenic related genes based on the reports that pathogenic gene of BaYMV located on viral RNA 1 (Kashiwazaki and Hibino, 1996) and that RNA 2 was not related to BaYMV strain diversity (Shi *et al.*, 1995).

The viral RNA structure had been used for genetic classification of virus and the strain distinction (Skotnicki *et al.*, 1996; Ryu *et al.*, 2000; Goetz and Huth, 2005). In addition, nucleic acid analysis was suggested as an effective method for strain distinction of Potyvirus (Gibbs and McIntyre, 1970). Viral coat protein (CP) encoding region has been known as related to symptom manifestation (Saito *et al.*, 1987; Dawson *et al.*, 1988) and used strain distinction or its possibility (Xiao *et al.*, 1994; Tracy *et al.*, 1992). BaYMV and BaMMV was identified as independent group by its homology analysis (Kashiwazaki *et al.*, 1992). However, others reported that BaYMV CP analysis only can be use for inter specific distinction (Kashiwazaki *et al.*, 1989) and unappropriate using it to strain differentiaion in Potyvirus (Meckern *et al.*, 1992). In Korea, analyzed coat protein encoding region of 11 BaYMV isolates and showed more than 98.2%～99.9% homology in CP region indicating that this gene is highly conserved thus difficult to differentiate the strain (Lee *et al.* 2002). On the other hand, NIa-Pro is another gene encoded in RNA1 and showed lower homology compared to coat protein and NIb regions suggesting that this region is the possible source of variation.

This study reports an efficient method and survey distribution of BaYMV strains using modified RAPD method as demonstrated by distinct grouping of BaYMV isolates tested. BaYMV isolates in Korea revealed four groups related to geographical districts namely; Iksan, Naju, Hanam-okcheon and Milyang. So we suggest that these isolates as four BaYMV strain named BaYMV-I, N, H and M respectively. This study also serves some information to search for pathogenic related gene and developing DNA marker available for strain distinction.

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(Received 1 August 2007 ; Accepted 20 September 2007)