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Capsid Protein Gene Sequence Analysis and Development of Diagnostic Method by RT-PCR of Barley Yellow Mosaic Virus

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ABSTRACT

A rapid and sensitive assay for specific detection and identification of barley yellow mosaic virus(BaYMV) was set up using the reverse transcriptase polymerase chain reaction(RT-PCR). A couple of primers was select to discriminate the viruses. PCR fragments of BaYMV(ca. 0.9 kb) were obtained by using the method designed for BaYMV capsid protein. RT-PCR fragments were cloned with vector pT7 Blue and the resulting clones were sequenced. Capsid protein of BaYMV consisted of 297 amino acids and 891 nucleotides. The capsid protein sequence of BaYMV showed that 98% of nucleotides and 99% of amino acids homology.

Key words : BaYMV, RT-PCR, capsid protein, and sequence.

INTRODUCTION

Barley yellow mosaic virus(BaYMV), a kind of *bymovirus* in *potyviridae* is soil borne filamentous virus transmitted by *Polymyxa graminis*. Some viruses, such as barley mild mosaic virus(BaMMV), rice necrosis mosaic virus(RNMV), wheat spindle strick mosaic virus(WSSMV), oat mosaic virus(OMV), and soil-borne wheat mosaic virus(SbWMV), are also reported as the soil borne virus transmitted by *P. graminis* like BaYMV. The *Potyviridae* is classified as *Potyvirus* (aphid), *Bymovirus*(fungus), *Rymovirus*(mite), *Ipomo-virus*(whitefly) according to vectors(Salm et al., 1996, Richter et al., 1995, Ward and Shukla, 1991). The known hosts of the soil borne barley viruses are so narrow; those are cereal such as barley, wheat, rice, ray, and oat. The BaYMV has caused considerable damage to barley crops in Korea, Japan and China in east Asia and some European countries like Germany, England, France and Belgium(Adams et al., 1988, Huth et al., 1984, Huth and Adams, 1990, Ikata and Kawai, 1940, Lee et al., 1998, Usugi and Saito, 1976). The virus symptoms develop in early spring in case of seeding in fall. Main symptoms of this virus are developing mosaic, yellowshing, necrotic sporting in leaves, stunting, and withering to death in severe case. Once barley fields get infestation, the soil itself become a source of infection, therefore, it is impossible to control because of the virus soil transmission. For this reason, it had been encouraged to develop resistant varieties for the virus disease in many countries, but severe strain of virus harming the new resistant varieties arise other problems. For instance, six strains of BaYMV were

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isolated on the basis of pathogenecity to different barley cultivars in Japan(Adams et al., 1988, Shi et al., 1995, Usugi and Saito, 1976). The properties of BaYMV are filamentous particles of two lengths, 500-600㎚ and 250-300㎚ each, and 13㎚ in diameter both. The nucleic acid is ssRNA virus composed of RNA1 of 7.6kb and RNA2 of 3.5kb(Huth et al., 1984, Huth and Adams,1990, Lee et al., 1998). ELISA, a serological method, or immunoserological electron microscopic system with labeling indicator had been used for the diagnostic methods of Bymovirus for past years(Ehler and Paul, 1986, Hariri et al., 1996, Lee et al., 1996, Nomura et al., 1996, Schenk et al, 1993, Usugi and Saito, 1976). However, identification or classification of molecular level have been developed recently for analyzing of gene sequence(Berger et al, 1997, Salm et al., 1996, Schlichter et al., 1993). Even though we have analysis of gene sequence by PCR or RACE PCR(Poggi et al., 1995, Shi et al., 1995, 1996), comparative research of coat protein amplification and the relationship through reverse transcriptase polymerase chain reaction(RT-PCR) is not developed enough yet(Foulds et al., 1993, Kashiwazaki et al., 1989, 1990, 1996, Poggi et al., 1995, Shi et al., 1996). Therefore, we diagnosed the BaYMV with ELISA and RT-PCR, and cloned the cDNA of capsid protein to analyze gene sequence of the BaYMV in order to accomplish the sensitive diagnostic method system.

MATERIALS AND METHODS

Virus isolates

ELISA samples were yellowish barley plants as like virus symptoms collected from five cities in four province of South Korea during 1998-1999. Those cities are Iksan in Cheonbuck-pro., Naju in Cheonnam-pro., Millyang and Jinju in Kyungnam-pro., and Suwon in Kyungki-pro. Antisera were BaYMV-Ⅱ-1, BaMMV-Kor and SbWMV. Comparative viruses are BaYMV-HN reported by Lee et al.(1998) and BaYMV-Ⅱ-1 supplied from the Japan Agriculture Research Center. ELISA, complied with Clark and Adams(1977), detected whether they were infected by BaYMV, BaMMV, and SbWMV. The samples of RT-PCR were infected by only the BaYMV in ELISA(Table 1).

Table 1. Field samples used for sequence analysis of the BaYMV capsid protein gene

Location	Barley cultivar
Iksan	Baegdong
Naju	Bunong
Milyang	Saecchalssalbori
Jinju	Cchalbori
Suwon	Dongbori 1 ho

Total RNA extract

Total RNA was extracted from barley leaves infected by only BaYMV and used with Sepa Gene Kit. After the 10㎎ infected leaves with tris-buffer were homogenized by power homogenizer, they were mixed with acetate buffer and protein denaturant containing some guanidine thiocyanate. Then, they were centrifuged for ten minutes at 15,000 rpm with extractors, chloroform and sodium acetate. Supernant, that was mixed with glycogen and 2-propanol and stored in -80℃ for 20 minutes, was again centrifuged for 10 minutes at 15,000 rpm to deposit nucleic acid. The nucleic acid washed with ethanol of 70% and vacuum dried was diluted with sterilized-distilled-water to be RT-PCR using.

RT-PCR

This reaction process was complied with an instruction of RT-PCR high kit(Toyobo Co.). The extracted total RNA(1.5㎎/㎖) was mixed with 8㎖ compounding solution of 5x RNase buffer, 0.1M DDT, 5mM dNTPs, dH_2O, superscript RT(10U/㎕), and RNase inhibitor(10U/㎕). After the mixture was reverse

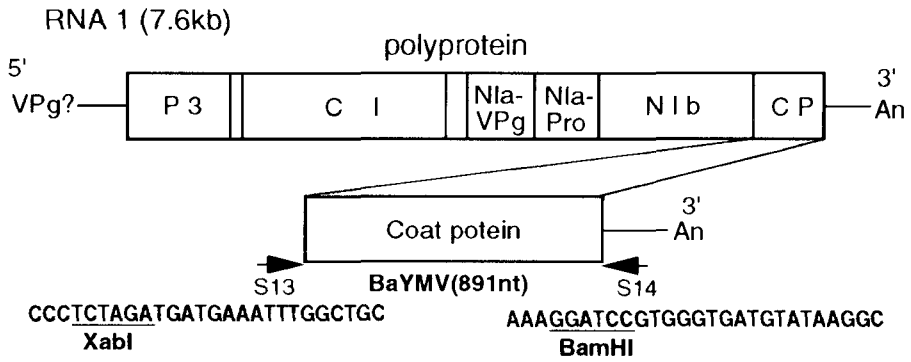

Fig. 1. The genetic map of the RNA 1(upper) and the oligonucleotide primers used to amplify of the BaYMV capsid protein gene(down).

transcription reacted for 30min. at 42℃ to synthesis the first strand cDNA, it was thermal treated for 5 min. at 99℃ to be used for PCR.

According to PCR process, the first strand cDNA was transcribed with Ampli. Tag DNA polymerase (2.5U/㎕) to amplified. Specific primer was designed to amplify and clone the capsid protein according to nucleotide sequence described by Kashiwazaki et al.(1989, 1990, 1996) and Lee et al.(1996). The primers of BaYMV were S14 and S13 which were designed to have cutting positions of *Bam*HI and *Xba*Iin this process(Fig.1).

PCR was done in condition of 94℃/1min, 55℃ /2min, 72℃/3min and amplifying of 30 cycles respectively, addition to amplifying reaction of 72℃ /7min. The separated electrophoresis bands were confirmed by electrophoresis of PCR amplifying products with 1.2% agarose gel and staining with ethium bromide.

Sequencing and gene analysis

Cloning was provided from purifying of products of RT-PCR with QIAquick PCR purification kit(QIAGEN Co.). The products(90ng/㎕) and pT7 Blue vector (Novagen Co., 60ng/㎕) were used for ligation reaction, then it was transformed to *Escherichia coli* JM109 competent cell. Three clones were selected from each five locations to perform sequencing. The sequence was used by ABI PRISM dye primer cycle sequencing core kit(Perkin elmer Co.) -21M13 and -M13 reverse primer in ABI PRISM 377 DNA sequencing system. Sequence data were analyzed with Hitachi Software Engineering DNASIS system.

RESULTS

RT-PCR

The RT-PCR results of BaYMV isolates from Iksan, Naju, Milyang, Jinju and Suwon in Korea and BaYMV-Ⅱ-Ⅰ sent from Japan were showed in Fig. 2. Five BaYMV isolates from each regions, BaYMV-HN and BaYMV-Ⅱ-1 from Japan showed single band of 0.9 Kb zone of electrophoresis with PCR amplifying products(Fig. 2), but any RT-PCR did not occur in BaMMV, analogous virus of BaYMV. The RT-PCR did not happen in lane 8 where health plants set up either.

Capsid protein sequencing and homology

Nucleotide sequence was determined from 3 clones that were selected in RT-PCR amplifying fragments cloned in pT7 Blue vector. Capsid protein genes of BaYMV supplied from every 5 isolates consisted of 297 amino acids and 891 nucleotides. When we compared

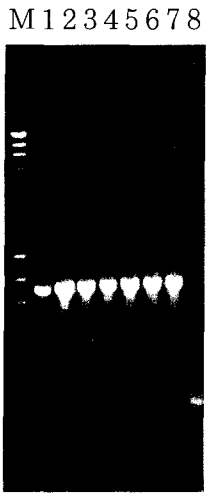

Fig. 2. Agarose gel electrophoresis of RT-PCR amplified products of BaYMV in total RNA extracted from infected barley leaves. M: Size marker(DRIgest Ⅲ, Toyobo Co.); Lane 1: BaYMV-Iksan, Lane 2: BaYMV-Naju, Lane 3: BaYMV-Milyang, Lane 4: BaYMV-Jinju, Lane 5: BaYMV-Suwon, Lane 6: BaYMV-HN, Lane 7: BaYMV-Ⅱ-1, Lane 8: Healthy barley(cv., Baegdong).

Table 3. Homologies between capsid protein sequences of isolates: amino acid level(upper right part of table) and nucleotide level(lower left part of table)

Isolate	% identity with isolate				
	Iksan	Naju	Milyang	Jinju	Suwon
Iksan	-	99.6	99.6	99.6	100
Naju	98.9	-	100	100	99.6
Milyang	99.6	99.4	-	100	99.6
Jinju	99.9	99.6	99.9	-	99.6
Suwon	99.2	98.9	99.2	99.3	-

the isolates collected from Iksan city with those from the other cities, we could find out 0-2 difference in amino acid and 7-11 difference in nucleotide level(Table 2). But any difference was not found among 3 clones. When sequence data of capsid protein of BaYMV were analyzed through DNASIS program, homology of nucleotide and amino acid were more than 98% and 99% respectively(Table 3).

DISCUSSION

We could achieve virus gene amplifying in processing of RT-PCR of BaYMV collected from barley fields in 5 differential regions by using the specific primer. Consequently, BaYMV separated single band of 0.9 kb zone of electrophoresis with PCR products. Because any reaction did not occur in the health plants, it was possible to conclude that RT-PCR by using S13 and S14 can tell the infection of BaYMV as rapid and sensitive diagnostic method. After cloning RT-PCR products in pT7 Blue vector and analyzing nucleotide sequence with 3 selected clones, we could know that capsid protein consisted of 891 nucleotides and 297 amino acids. This result has correspondence with facts reported by Kashiwazaki et al.(1989, 1990) and Foulds et al.(1993). It has been shown that homologies of nucleotides and amino acids are 98% and 99% respectively by analyzing sequence data of BaYMV capsid protein through DNASIS program.

Table 2. Sequence variations in the capsid protein sequences of five Korean isolates of BaYMV

BaYMV isolate	Amino acid position								Number of differences	
	1	12	14	15	20	28	62	74	a.a.	nt.
Iksan	A	D	R	I	R	A	M	S	-	-
Naju	-	-	-	-	K	-	-	-	1	10
Milyang	-	-	-	-	R	-	T	-	1	9
Jinju	-	-	-	-	K	V	-	T	2	7
Suwon	-	-	-	-	R	-	-	-	-	11

*Amino acid difference from the BaYMV-Iksan sequence are indicated. aa: amino acid, nt: nucleotide, -: Identical amino acid.

These facts suggest to think there is no remarkable difference between capsid protein and isolates of BaYMV occurred in all around Korea. Under prediction of possible enzyme cutting positions in capsid protein of BaYMV registered in DDBJ and Gene Bank, we enzyme treated RT-PCR amplifying products of the BaYMV capsid protein. So it was very difficult to distinguish differential isolates of each region for same cutting position. According to Kashiwazaki et al.(1990, 1995) and Huth et al.(1984, 1990), BaYMV capsid protein has more than 98% homology among strains. Therefore, we would like to propose the new method of using a pair of primers(BaYMV: S13+S14) designed to detect BaYMV in barley plants. Moreover, it was also possible to distinct stains of BaYMV occurred in every regions by RT-PCR and analyzing of pathogenicity with mechanical inoculation to host plants.

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