Horticulture, Environment, and Biotechnology
ll Volumes & Issues
SSN: 2211-3452 (Print) 2211-3460 (Online)

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Hort. Environ. Biotechnol. 52(1):65-73. 2011.
DOI 10.1007/s13580-011-0142-4

Research Report

Development of Gene-based Markers for the *Bs2* Bacterial Spot Resistance Gene for Marker-assisted Selection in Pepper (*Capsicum* spp.)

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Received July 13, 2010 / Accepted September 13, 2010

Abstract. The *Bs2* gene of pepper (*Capsicum* spp.) confers resistance to the most commonly detected races of *Xanthomonas campestris* pv *vesicatora* (*Xcv*). Using information from a published database, we developed gene-based markers for selection of *Bs2* in pepper. High polymorphism was detected in exon 3, intron 2 and 3'-UTR of the *Bs2* gene. The three regions were sequenced and numerous SNPs and In/Dels were identified between resistant and susceptible inbred lines. Seven SNPs were selected to develop SNP markers using the tetra-primer amplification refractory mutation system-PCR methodology (Tetra-primer ARMS-PCR). One SNP (G/C) was used to develop a molecular marker that can perfectly identify the presence of the bacterial spot resistance. The gene-based markers were further validated to test association with resistance in 80 lines and cultivars. The markers successfully identified a bell pepper commercial cultivar 62-2 that harbors the *Bs2* gene. The successful development of the markers in this study provides a useful tool for MAS to screen the *Bs2* gene in pepper.

***Additional key words*:** MAS, single nucleotide polymorphism, SNP, tetra-primer ARMS-PCR, *Xanthomonas campestris*

Introduction

Bacterial spot, caused by the bacterium *Xanthomonas campestris* pv. *vesicatora* (*Xcv*), is a severe disease of pepper (*Capsicum* spp.) that results in reduced yield and quality in many pepper production areas in warm-temperate and tropical countries, especially where overhead irrigation is applied. The traditional method for controlling bacterial spot consists of frequent applications of copper-based pesticides; however, genetic resistance has been described as a more desirable method (Cook and Guevara, 1984; Kim and Hartmann, 1985). Three dominant, non-allelic resistance genes, *Bs1*, *Bs2* and *Bs3*, have been identified conferring resistance to *Xcv* (Hibberd et al., 1987), and the corresponding avirulence genes *avrBs1*, *avrBs2*, and *avrBs3* have been cloned from *Xcv* and shown to induce race specific resistances. While *Bs1* and *Bs3* confer resistance to races 2 and 1 of *Xcv*, respectively (Hibberd et al., 1987), the *Bs2* gene confers resistance to most *Xcv* races inculuding race 0, 1, 2, and 3 (Kousik and Ritchie, 1996) suggesting that *Bs2* may provide more durable resistance in the field. This gene has been deployed in the commercial pepper varieties through simple backcrossing. Availability of molecular genetic markers for this gene may help in selection of the resistance genotypes in a breeding program without disease screening. Availability of co-dominant markers that distinguish between heterozygous and homozygous plants facilitates easy and fast selection. Although the *Bs2* gene was cloned (Tai et al., 1999b), there is no marker suitable for marker-assisted selection (MAS). Two RAPD markers, F1 and B3, located 0.6 and 0.3 cM away from the locus, respectively, and one AFLP marker, A2, co-segregated with *Bs2* were reported previously (Tai et al., 1999a). However, those markers are dominant markers and not suitable for MAS, because heterozygous plants can not be distinguished from homozygotes. After cloning the *Bs2* gene sequenced (Tai et al., 1999a), only two co-dominant PCR-based markers, L1 and R1, were developed from the sequence of the *Bs2* gene. Among them, marker L1 was successfully used in identification of pepper genotypes possessing the *Bs2* gene (Park and Crosby, 2007), but information about the marker

is not publicly available. In this paper, we redesigned and developed new markers based on the *Bs2* gene, which can be used for MAS.

Materials and Methods

Plant Materials

Four inbred lines for each of bacterial spot resistance and susceptibility were used for genotyping analysis provided by Pepper Breeding Unit, National Institute of Horticultural & Herbal Science (NIHHS), Rural Development Administration (RDA), Korea. Resistant lines included 8NH1, 8NH2 (progenies derived from a cross between *C. annuum* 3-25-27 (resistant parent) and *C. annuum* LV2319 (susceptible parent)), 8NH3, and 8NH4 (progenies derived from a cross between *C. annuum* 3-25-27 and *C. annuum* LF (susceptible parent)). Susceptible lines were 8N1, 8N2, 8N3, and 8N4. The resistant parental line 3-25-27 was selected from Florida 3-27 for bacterial spot resistance at AVRDC-The World Vegetable Center, Taiwan. In addition, 74 lines and cultivars from NIHHS germplasm and six commercial pepper cultivars, 62.2, 72.1, Tisana, 99G54, Early Calwonder (ECW) and 00G49, provided by the Pepper & Breeding Institute, Korea, were also tested. Most of the *Capsicum* genotypes belong to *C. annuum*; two were *C. baccatum*, four were *C. chinense*, and one was *C. frutescens* (Table 1).

DNA Extraction and Primer Design

Genomic DNA was extracted from young leaves of field-grown plants following the protocol described by Murray et al. (1980) and slightly modified by Fulton et al. (1995). The genomic sequences for *C. annuum* YAC clone YCA22D8 (accession number: AY702979) and *C. chacoense Bs2* (accession number: AF202179) were obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sites/entrez). Intron/exon boundaries were determined using the SPIDEY tool available at http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/spideyweb.cgi. A total of 30 primers were designed from exons, introns and 3'-untranslated region (3'-UTR) of the *Bs2* gene with the program Primer3 version 4.0 (Rozen and Skaletsky, 2000).

Oligonucleotide primers were synthesized by Bioneer Corp. (Korea). The primers were first screened for polymorphism on the resistant inbred line 8NH1 known to carry the *Bs2* and the susceptible inbred line 8N1. PCR conditions were optimized before genotyping using a set of pepper lines and cultivars.

PCR Amplification

Each PCR reaction was carried out in a total reaction volume of 25 μL containing 15-20 ng of genomic DNA, 200 μM deoxyribonucleotide triphosphate mix (Roche, Korea), 1X PCR buffer and 1U of DNA *Taq* polymerase (Roche, Korea), 0.25 μM of each primer for polymorphism screening and 0.1 μM of each primer for tetra-primer ARMS-PCR. Reactions were performed on an Eppendorf Mastercycler Gradient. The amplification profile consisted of an initial denaturation for 5 min at 94℃ followed by 35 cycles of PCR amplification under the following parameters: 1 min at 94℃, 1 min at the annealing temperature 40-65℃ (depending on melting temperature of the primers), and 1 min of primer elongation at 72℃. A final incubation at 72℃ for 5 min was programmed to allow completion of primer extension. For touchdown PCR reactions, samples were incubated for 5 min at 94℃, followed by 35 cycles of 1 min at 94℃, 1 min at the annealing temperature, 1 min at 72℃, followed by a final extension period for 5 min at 72℃. The annealing temperature was 55 or 65℃ (depending on melting temperature of the primers) for the first cycle, and decreased by 1℃ for each subsequent cycle. Amplified products were separated on a 2% ethidium bromide-agarose gels using 1X TBE buffer for 1.5 hours at 120V and photographed under UV light. A 100 bp ladder was used as a molecular weight marker. Amplified fragments were excised from the gel and purified with the Agarose Gel DNA extraction kit (Bioneer, Korea) for sequencing.

Sequencing and SNP Detection

Sequencing was performed directly from the PCR products by MacroGene (Seoul, Korea). Sequences of the resistant and susceptible inbred lines, together with 24 tested lines and cultivars were aligned using programs Xmanager Enterprise 3.0 (NetSarang Computer Inc., Korea), BioEdit version 7 (Hall, 1999), and SNPSS (Dr. Hee-Ju Yu, personal communication). SNP variants were identified by visual inspection of the alignments, and polymorphism was determined if the sequence trace was high and the nucleotide variation was clear between the two sets of the resistant and susceptible inbred lines. Alignment of the amino acid sequences for the gene was performed by using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Development of SNP Markers

The tetra-primer amplification refractory mutation system-PCR (tetra-primer ARMS-PCR) methodology (Ye et al., 2001) was used to develop SNP markers. The SNP primers were designed from the sequence containing the identified SNPs using program Tetra-primer ARMS-PCR (http://cedar.genetics.soton.ac.uk/public_html/primer1.html). The program composed of 10 primer combinations for each SNP. Of those, one to two was selected on the basis of the expected size of the amplicons and on the smallest difference between the

Table 1. Genotyping and phenotyping results of the *Capsicum* accessions tested.

Spicies	Accession	Origine	Phenotype	Genotype		
				M1	M2	M3
C. annuum	3-25-27	AVRDC	R	R	R	R
C. annuum	8avs1	AVRDC	S	S	S	S
C. annuum	8avs2	AVRDC	S	S	S	S
C. annuum	8avs3	AVRDC	S	S	S	S
C. annuum	8avs4	AVRDC	S	S	S	S
C. annuum	8avs5	AVRDC	S	S	S	S
C. annuum	De Cheiro	Brazil	-	S	S	S
C. annuum	Valentina	Commercial var., Isreal	-	S	S	S
C. annuum	Baltasar	Commercial var., Isreal	-	S	S	S
C. annuum	Amadeo	Commercial var., Isreal	-	S	S	S
C. annuum	PBY-16000.F1	Commercial var., Isreal	-	H	H	S
C. annuum	PL-4007	Commercial var., Isreal	-	S	S	S
C. annuum	PD7000	Commercial var., Isreal	-	S	S	S
C. annuum	PD 7001	Commercial var., Isreal	-	S	S	S
C. annuum	PBL-10004	Commercial var., Isreal	-	H	H	S
C. annuum	PBL-14005.F1	Commercial var., Isreal	-	H	H	S
C. annuum	PL-4010.F1	Commercial var., Isreal	-	S	S	S
C. annuum	Goal	Commercial var., Korea	S	S	S	S
C. annuum	Score	Commercial var., Korea	S	S	S	S
C. annuum	ECW	PBI, Korea	-	S	S	S
C. annuum	62.2	PBI, Korea	-	R	R	R
C. annuum	72.1	PBI, Korea	-	S	S	S
C. annuum	Tisana	PBI, Korea	-	S	S	S
C. annuum	99G54	PBI, Korea	-	S	S	S
C. annuum	00G49	PBI, Korea	-	S	S	S
C. annuum	8N2	NIHHS, Korea	-	S	S	S
C. annuum	LV2319	AVRDC	S	S	S	S
C. annuum	AC2258	AVRDC	S	S	S	S
C. annuum	PI201234	AVRDC	S	S	S	S
C. annuum	LF	AVRDC	S	S	S	S
C. annuum	Blue Dragon	Local var., Korea	S	S	S	S
C. annuum	Emsung	Local var., Korea	S	S	S	S
C. annuum	Bungeo	Local var., Korea	S	S	S	S
C. annuum	Kangwong Kalcho	Local var., Korea	S	S	S	S
C. annuum	Kalmi	Local var., Korea	S	S	S	S
C. annuum	Mac min	Local var., Vietnam	-	S	S	S
C. annuum	Ot Thoc	Local var., Vietnam	-	S	S	S
C. annuum	YCM334	NIHHS, Korea	S	S	S	S
C. annuum	Tean	NIHHS, Korea	S	S	S	S
C. annuum	7HR3	NIHHS, Korea	S	S	S	S
C. annuum	7HR5	NIHHS, Korea	S	S	S	S
C. annuum	7HR6	NIHHS, Korea	S	S	S	S
C. annuum	7HR27	NIHHS, Korea	S	S	S	S
C. annuum	LE5	NIHHS, Korea	S	S	S	S
C. annuum	8NH1	NIHHS, Korea	R	R	R	R
C. annuum	8NH2	NIHHS, Korea	R	R	R	R
C. annuum	8NH3	NIHHS, Korea	R	R	R	R
C. annuum	8NH4	NIHHS, Korea	R	R	R	R
C. annuum	8N1	NIHHS, Korea	S	S	S	S

Table 1. (continued).

Spicies	Accession	Origine	Phenotype	Genotype		
				M1	M2	M3
C. annuum	8N3	NIHHS, Korea	S	S	S	S
C. annuum	8N4	NIHHS, Korea	S	S	S	S
C. annuum	8N5	NIHHS, Korea	S	S	S	S
C. annuum	K134203	Turkey	-	S	S	S
C. annuum	K134214	Turkey	-	S	S	S
C. annuum	K134237	Turkey	-	S	S	S
C. annuum	Konchowang	Commercial var., Korea	S	S	S	S
C. annuum	Gold Medal	Commercial var., Korea	S	S	S	S
C. annuum	Golden Light	Commercial var., Korea	S	S	S	S
C. annuum	Dangcho	Commercial var., Korea	S	S	S	S
C. annuum	Dangcho Welbeeing	Commercial var., Korea	S	S	S	S
C. annuum	Dogbul King	Commercial var., Korea	S	S	S	S
C. annuum	Dogyachungchung	Commercial var., Korea	S	S	S	S
C. annuum	Manita	Commercial var., Korea	S	S	S	S
C. annuum	Mansahyungtong	Commercial var., Korea	S	S	S	S
C. annuum	Bukang	Commercial var., Korea	S	S	S	S
C. annuum	Bukang Q	Commercial var., Korea	S	S	S	S
C. annuum	Buja	Commercial var., Korea	S	S	S	S
C. annuum	Buchon	Commercial var., Korea	S	S	S	S
C. annuum	Palita	Commercial var., Korea	S	S	S	S
C. annuum	Gumtap	Commercial var., Korea	S	S	S	S
C. annuum	Super Manita	Commercial var., Korea	S	S	S	S
C. annuum	Super Bigarim	Commercial var., Korea	S	S	S	S
C. annuum	Shinhong	Commercial var., Korea	S	S	S	S
C. annuum	Omchungna	Commercial var., Korea	S	S	S	S
C. annuum	Yuggkang Red King	Commercial var., Korea	S	S	S	S
C. annuum	Wanggun	Commercial var., Korea	S	S	S	S
C. annuum	Wangdaebag	Commercial var., Korea	S	S	S	S
C. annuum	Urigun	Commercial var., Korea	S	S	S	S
C. annuum	Chammani	Commercial var., Korea	S	S	S	S
C. annuum	Chunhajeil	Commercial var., Korea	S	S	S	S
C. annuum	Tesan	Commercial var., Korea	S	S	S	S
C. annuum	PR Mujug	Commercial var., Korea	S	S	S	S
C. annuum	PR Chohong	Commercial var., Korea	S	S	S	S
C.baccatum	GA	NIHHS, Korea	S	S	S	S
C.baccatum pendulum	C-130	NIHHS, Korea	S	S	S	S
C.chinense	Bode Rosinha	Brazil	-	S	S	S
C. chinense	Biquinho	Brazil	-	S	S	S
C. chinense	Murupi	Brazil	-	S	S	S
C. chinense	Habanero	NIHHS, Korea	S	S	S	S
C. frutesens	Malagueta	Brazil	-	S	S	S

R: resistance, S: susceptibility, '-': not tested, M1: marker 14F/14R, M2: marker 25-1, M3: marker 25-2.

predicted annealing temperatures. Inner primers and forward outer primers were designed within the sequence of the exon 3. Outer reverse primers were designed based on the sequence of the 3'-UTR, as the sequence of exon 3 comprised only 313 bp, and most of SNPs located at the end of the sequence. Primers were designed so that fragment sizes were to be within a range of 100-400 bp. Primer sets were first tested on 8NH4 and 8N4 to optimize PCR conditions before performing genotype analysis.

Results

Polymorphism Detection

In order to amplify the *Bs2* gene, a set of sixteen primers were designed based on sequence of the *Bs2* gene. Genomic DNA of the resistant inbred line 8NH1 and the susceptible inbred line 8N1 were screened for polymorphisms. No polymorphisms were detected for exon 2 and intron 1, but high polymorphisms were observed for exon 3, intron 2 and 3'-UTR sequences (data not shown). As such, another set of fourteen primers were designed based on these regions, and 60 primer combinations using these primers were screened for polymorphism. Of these, four primer combinations detected codominant polymorphisms between susceptible and resistant inbred lines, while 26 showed dominant bands, 22 were monomorphic, and eight primer combinations produced no amplicons. To develop co-dominant markers for MAS, all dominant markers were discarded. Among the four polymorphic primers that amplified codominant bands, primer combinations of 6F/6R and 15F/15R produced multiple bands in the susceptible genotypes, indicating presence of paralogs (data not shown). The primer combinations that amplified a single band (14F/14R and 15F/10R) were finally selected. While the susceptible genotype produced fragments of about 1500 and 600 bp, the resistant genotype generated fragments of about 700 and 500 bp by 15F/10R and 14F/14R, respectively (Fig. 1). These markers were then confirmed with another three resistant lines (3-25-27, 8NH3 and 8NH4), three susceptible lines (LV2319, 8N3 and 8N4). However, susceptible lines 8N3 and 8N4 amplified a fragment the same size as amplified from the resistant genotypes by primer combination 15F/10R (Fig. 1). Primer 14F/14R amplified a 500 bp fragment from all resistant genotypes and a 600 bp fragment from all susceptible genotypes. Thus, the developed marker 14F/14R can be used in MAS.

Identification of the SNPs

Since polymorphisms were only detected in exon 3, intron 2 and 3'-UTR, we next employed an alternative approach to search for additional SNPs in these regions. The two polymorphic primer pairs, 15F/10R and 14F/14R, were used to identify SNPs between resistant and susceptible inbred lines. However, because, PCR products were directly sequenced without subcloning, the end of exon 3 was incomplete. Therefor, primer pair 11F/18R was used to get the full sequence of exon 3. Primer sequences are listed in Table 2. Fragments

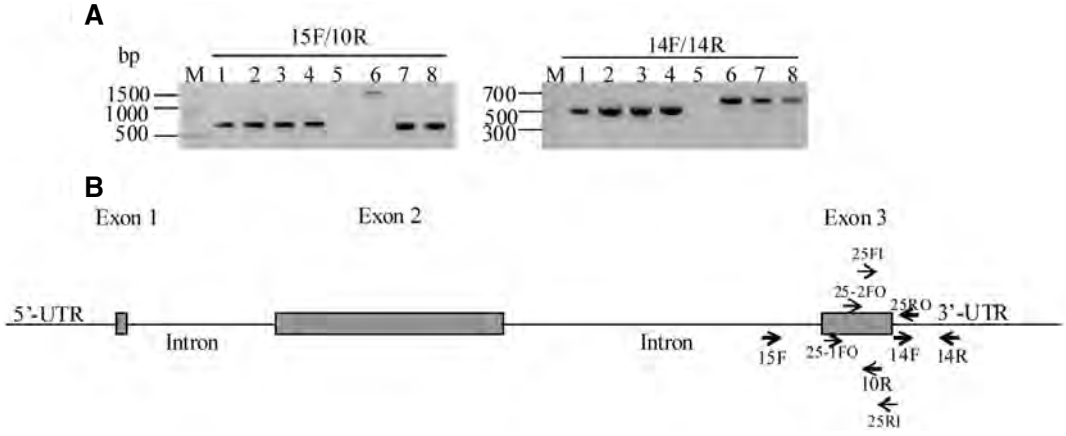

Fig. 1. Confirmation of polymorphic primers. (A) Agarose gel electrophoresis of the amplification products of selected polymorphic primers. Primers are indicated on the top; (B) Structure of the Bs2 gene and positions of polymorphic primers are indicated by arrows (25-1FO, 25-1 forward outer, 25-2FO, 25-2 forward outer, 25FI, 25 forward inner, 25RI, 25 reverse inner, 25RO, 25 reverse outer. Markers 25-1 and 25-2 shared primers 25RI, 25RO, and 25FI). Lanes M, 100 bp molecular ladder, 1, resistant parent *C. annuum* '3-25-27'; 2-4, resistant inbred lines '8NH1', '8NH3', '8NH4'; 5, susceptible parent *C. annuum* 'LV2319'; 6-8 susceptible inbred lines, '8N1', '8N3', '8N4'.

Table 2. List of selected PCR primers used for screening polymorphism.

Primer	Sequence (5' - 3')	T_m[z] (°C)	Position[y]
10R	TTGTTTCTTCTCAGTGAAGGAGAGT	54.4	34620
11F	GTACATCACCAGAGTCATAAGAGCA	54	34192
14F	ACAAGTCACATTATTCAGATGCAGA	54.4	34641
14R	GGTCACATATCCAATGTGTTCATAA	54.3	35240
15F	ATGTCACGACCAATCCCATT	53.6	33976
18R	TGTTAGGATCAAATATCGTGTCTCA	54.4	35007

[z]Melting temperature.
[y]Forward and reverse primer positions (5'-3') on the sequence of the clone YCA22D8.

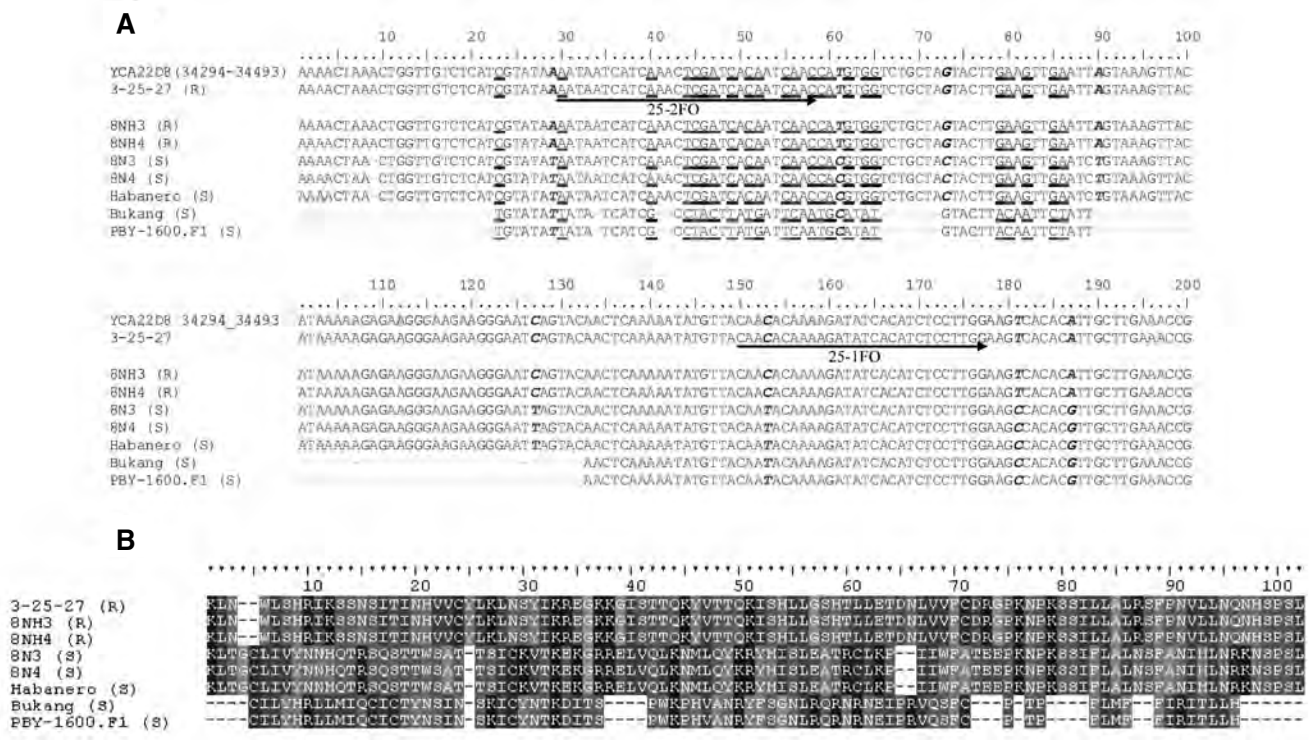

Fig. 2. Part of sequence alignment of the clone YCA22D8 which selected from 34294 bp to 34493 bp containing 200 bp of the exon 3 (A) and amino acid sequence alignment of the exon 3 (B) of the resistant parent (*C. annuum* 3-25-27), two resistant inbred lines (8NH3 and 8NH4), two susceptible inbred lines (8N3 and 8N4), two commercial cultivars (*C. annuum*: Bukang and PBY-1600.F1) and *C. chinense* Habanero. *Dashes lines and underlined* capital letters are SNPs and In/Dels, respectively, between inbred lines and commercial cultivars. Bold and italic capital letters are SNPs between the resistant inbred lines and the susceptible inbred lines. Arrows are locations of forward outer primers (FO) 25-1 FO and 25-2 FO from a G/C polymorphism. R, resistance, S, susceptible.

obtained from additional 28 lines and cultivars were used for sequencing. A total of 21 SNPs were identified in exon 3, and insertions/deletions between inbred lines and commercial cultivars were observed (Fig. 2A).

Development of SNP Markers

Among the twenty-one SNPs identified in the exon 3 between resistant and susceptible inbred lines, seven SNPs were selected to design primers using the tetra-primer ARMS-PCR methodology that was described in the Materials and Methods. The nine primer combinations were screened on 8NH4 and 8N4 with different annealing temperatures and touchdown PCR to improve amplification efficiency as suggested by others (Chiapparino et al., 2004; Okayama et al., 2004; Rincón and Medrano, 2003; Rubio et al., 2008). Only two primer combinations produced co-dominant bands as expected, while two generated dominant bands, three were monomorphic, and two were no amplicons (data not shown). The two primer combinations that generated co-dominant bands were 25-1 and 25-2 which were designed from a G/C polymorphism (Table 3). These primer combinations differed only in the sequence of the forward outer primers (Fig. 2A). Their amplification patterns were then confirmed with another three resistant lines (3-25-27, 8NH1 and 8NH3), three susceptible inbred lines (LV2319, 8N1 and 8N3). These SNP markers distinguished the C allele (resistance), which generates a fragment of 196 bp by the both primers, from G allele (susceptible), which amplifies fragments of 159 bp by 25-1 and 279 bp by 25-2. The fragments of 299 and 419 bp were produced from outer primers 25-1 and 25-2, respectively (Fig. 3). However, LV2319 and 8N1 produce no G-allele specific fragment by 25-1, while none of G-allele specific fragment and outer amplicon were produced in LV2319 and 8N1 using the primer combination 25-2.

Marker-assisted Selection

The three polymorphic primer sets, 14F/14R, 25-1 and 25-2, were used to assay 80 pepper genotypes. The results showed that these markers identified a bell pepper line 62-2 possessing the *Bs2* gene and three commercial varieties such as PBY-16000.F1, PBL-10004, and PBL-14005.F1 showed heterozygous patterns with primer combinations 25-1 and 14F/14R, but not by 25-2 (Fig. 4). Surveys of NIHHS genotypes using markers 14F/14R and 25-1 are shown in the Fig. 4. Most genotypes were phenotyped previously (Dr. Myoung-Cheol Cho, personal communication); and the genotyping results were consistent with the phenotypes observed (Table 1). These new allele-specific and co-dominant markers make it possible

Table 3. List of selected polymorphic tetra-primer ARMS-PCR primers.

Primer		Sequence (5 – 3)	T_m[z]	Expected amplicon size (bp)
25-1	Forward inner	AATCTTTCTGGCCTTGAACTCCTATC	58.3	C allele: 196
	Reverse inner	AGAGTTTTTACGATTCAGATGAATATTTGC	58.7	G allele: 159
	Forward outer	CAATACAAAAGATATCACATCTCCTTGGA	58.8	
	Reverse outer	CTTGTATCTGTCATTGTGTGTGTTCTCA	58.2	Two outer primers: 299
25-2	Forward inner	AATCTTTCTGGCCTTGAACTCCTATC	58.3	C allele: 196
	Reverse inner	AGAGTTTTTACGATTCAGATGAATATTTGC	58.7	G allele: 279
	Forward outer	AATAATCATCAAACTCGATCACAATCAAC	58.7	
	Reverse outer	CTTGTATCTGTCATTGTGTGTGTTCTCA	58.2	Two outer primers: 419

[z]Melting temperature.

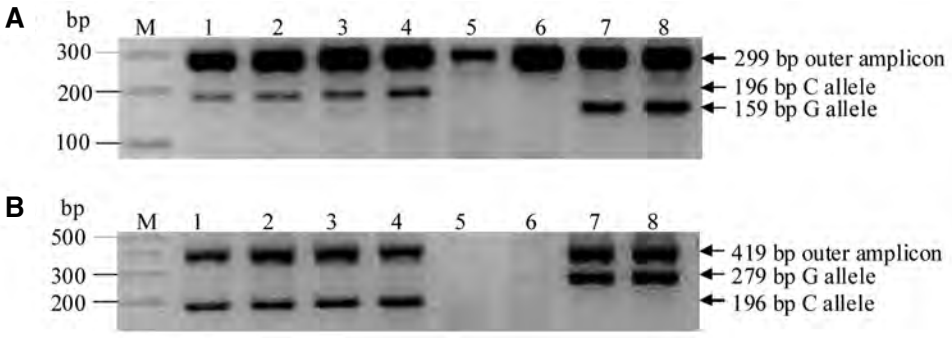

Fig. 3. Amplification of tetra-primer ARMS-PCR primers 25-1 (A) and 25-2 (B). M, 100 bp molecular ladder; 1, resistant parent *C. annuum* '3-25-27'; 2-4, resistant inbred lines '8NH1', '8NH3', '8NH4'; 5, susceptible parent *C. annuum* 'LV2319'; 6-8, susceptible inbred lines '8N1', '8N3', ' 8N4'.

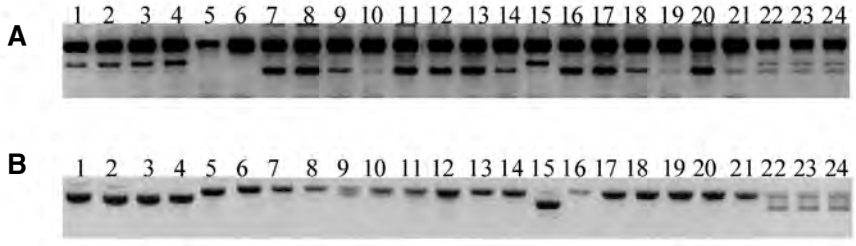

Fig. 4. Agarose gel electrophoresis of genotyping of 24 pepper lines and cultivars using the markers 25-1 (A) and 14F/14R (B). 1, Resistant parent '*C. annuum* 3-25-27'; 2-4, resistant inbred lines '8NH1', '8NH3', '8NH4'; 5, susceptible parent *C. annuum* 'LV2319'; 6-8 susceptible inbred lines, '8N1', '8N3', '8N4'; 9, susceptible parent *C. annuum* 'LF'; 10-24, selected genotypes representing NIHHS germplasm; 15, bell pepper commercial cultivar 62-1 harboring Bs2 gene; 22-24, commercial cultivars PBY-16000. F1, PBL-10004, PBL-14005F1, which showed heterozygous pattern.

to monitor all the *Bs2* alleles commonly used in breeding programs. When the parental genotypes are unknown, plants should be tested for homozygosity or heterozygosity of *Bs2* using primers 25-1, 25-2 and 14F/14R together.

Discussion

The concept of DNA marker-assisted selection (Tanksley et al., 1989) and recent studies of its applications in pepper (Garces-Claver et al., 2007; Rubio et al., 2008) show that the target genes can be identified efficiently in a segregating population at any plant growth stage with the use of DNA molecular markers. In this study, we developed codominant gene-based markers of the *Bs2* bacterial spot resistance gene for MAS. The primers were designed based on the sequence of the *Bs2* gene. Thirty out of 60 primer combinations showed polymorphism. These polymorphic primers were designed from the sequences of exon 3, intron 2 and 3'-UTR, while primers designed from the exon 2 sequence were not polymorphic. Thus, the invariant parts of the sequence will have a higher selection pressure and/or are indispensable for *Bs2* function. Sequencing results showed that numerous SNPs and In/Dels were present in these regions. They were also in very close proximity to other SNPs, which were often signif-

icantly associated with a particular phenotype (Daetwyler et al., 2008). Noncoding regions such as introns and 3'-UTR regions usually provide up to threefold higher frequency of SNPs than coding regions (Zhu et al., 2003). Nevertheless, we found a total of twenty-one SNPs in the 316 bp sequence of the exon 3 between resistant and susceptible lines. This resulted in changing amino acid in coding region (Fig. 2B). The relationship of amino acid transition with disease resistance should provide more valuable insight into the function of the *Bs2* gene.

Many methods have been developed for SNP genotyping and a variety of platforms have been used, including microarrays, mass spectrometry, fluorescence plate readers and flow cytometry (Giancola et al., 2006; Lee et al., 2004; Paris et al., 2003). However, these methods were developed for high throughput with high costs; therefore, the practical application of this DNA marker type is limited. In this study, we used tetra-primer ARMS-PCR methodology (Ye et al., 2001) for development of SNP markers, as it is a simple and economical method for SNP genotyping within a single PCR run. Without the requirement for enzymatic digestion, the tetra-primer ARMS-PCR markers decrease genotyping cost and permit the direct screening of heterozygous plants (Garcés-Claver et al., 2007; Rubio et al., 2008). Of the nine SNP markers that we developed, two showed the expected bands, two showed dominant bands, three were monomorphic, and two lost the amplicons. Fail in amplification has been reported previously (Rubio et al., 2008; Ye et al., 2001). The polymorphism rate (22.2%) in the present study was lower than in the Alves et al. (2008) study (40%) which obtained two markers showing expected band sizes among the five SNP markers developed for candidate resistance gene in wild peanuts (*Arachis* spp.) using tetra-primer ARMS-PCR procedure. Thus, non-specificity was likely due to a 3'-terminal mismatch and is not refractory to extension in some cases (Newton et al., 1989; Ye et al., 2001).

The primer combinations 25-1 and 25-2 designed from a G/C polymorphism and located at the end of exon 3, generated different band patterns when different forward outer primers used. For example: G allele specific fragment was absent in LV2319 and 8N1and the specific fragments were not insensitive as the outer fragments using primer combination 25-1, while 25-2 did not generate the G allele specific fragment and amplicon of outer primers in LV2319 and 8N1. The loss and weakness of specific fragment or outer amplicons in the susceptible genotypes could be due to competition between fragments during their amplification (Rubio et al., 2008).

Among the four co-dominant markers developed, three markers, 14F/14R, 25-1 and 25-2, were used to identify the *Bs2* gene in additional 80 pepper genotypes. Markers 14F/14R and 25-1 identified heterozygous plants, but not 25-2. A large number of genotypes did not generate specific fragments using primer combination 25-2. It may be that the outer primer was located in the SNP-rich region among inbred lines and commercial cultivars and leading primer binding failed. However, marker 25-2 resulted in a more intense band than the others in all of the resistant inbred lines and the commercial cultivar 62-2. This makes these markers capable of screening resistant plants in a wide range of crosses in pepper breeding programs. In addition, markers 14F/14R and 25-1 have the advantage of identifying homozygous individuals from heterozygous plants in F2 populations, which is impossible to achieve by evaluation of disease phenotype. Importantly, the methods described here are simple and amenable to large numbers of samples.

Previously Tai et al. (1999a) had mapped two RAPD markers, F1 and B3, as being 0.6 and 0.3 cM from the gene and one AFLP marker, A2, was found to cosegregate with the locus. The genetic distance between the marker and the gene determines the accuracy of marker assisted selection when the marker is used in the breeding program. Therefore, since markers F1, B3 and A2 are close to the gene, it would be appropriate to use in MAS. However, those markers are dominant; they cannot differentiate homozygous plants from the heterozygous plants. Thus, they are not applicable in MAS. For MAS, it would be ideal to develop so-called 'perfect Markers' where the DNA fragments of the markers are actually on the gene of interest, in which case the linkage between the markers and the gene is almost unbreakable (Ellis et al., 2002). Park and Crosby (2007) have developed a codominant PCR-based marker (L1) based on the *Bs2* gene sequence, which was successfully used in identification of pepper genotypes possessing the *Bs2* gene, but information about the marker is not publicly available. The three codominant markers reported in this study are like-wise perfect and provide new codominant and allele-specific SNP markers for the identification of bacterial spot resistance in *Capsicum*. The results have clearly demonstrated the feasibility of these markers, and its application for germplasm screening and MAS.

Acknowledgments: The authors acknowledge Drs. Hee-Ju Yu, Soo-Min Park and Sung-Chan Lee for their helpful assistance in sequence alignment, and Pepper & Breeding Institute for providing part of materials for this study. The authors are grateful to Prof. Dr. Thomas Debener and Dr. Peter Hanson for reading various drafts of the paper.

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