Development of SCAR Markers Linked to L^3 gene in *Capsicum*

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Random amplified polymorphic DNA (RAPD) markers linked to the L^3 locus were developed by applying the bulked segregant analysis method to two doubled haploid (DH) populations in *Capsicum*. The codominant RAPD markers, E18₂₇₂ and E18₂₈₆, were converted into sequence-characterized amplified region (SCAR) markers by molecular cloning and nucleotide sequencing. A PCR analysis using DH (n=176) and backcross (n=190) populations revealed that all the SCAR markers, PMFR11₂₆₉, PMFR11₂₈₃ and PMFR21₂₀₀, co-segregated with the original RAPD markers, and were mapped at a distance of 4.0 cM from the L^3 locus. Furthermore, after confirmation of their validity in 18 accessions of *Capsicum* spp. using the codominant SCAR marker pair, PMFR11₂₆₉ and PMFR11₂₈₃, it was suggested that the SCAR markers developed here could become effective in marker-assisted selection programs for the introduction of the L^3 gene derived from PI159236 (*C. chinense*) into sweet pepper for breeding purposes.

Key Words: *Capsicum*, L^3 gene, random amplified polymorphic DNA (RAPD) marker, sequence-characterized amplified region (SCAR) marker, doubled haploid (DH) population.

Introduction

Pepper mild mottle virus (PMMoV) is one of the most common pathogens in sweet pepper (*Capsicum annuum* L.) cultivation. Further spread of the pathogen has become a cause for concern due to the anticipated restriction of the use of methyl bromide in the future. PMMoV is classified into tobamoviruses and is spread by mechanical transmission or through infected seeds. Symptoms include puckered and mottled yellow leaves as well as streaked, mottled and withered fruits. Boukema (1982) reported that four pathotypes, P₀, P₁, P_{1.2} and P_{1.2.3}, could be identified in PMMoV, while four types of allelic resistance genes, L^1 , L^2 , L^3 and L^4 , were found in *Capsicum* species. It was also reported that a clear

gene-for-gene response was observed. As a result, the L^3 gene derived from PI159236 (*C. chinense*) is assumed to confer resistance to P_{1.2} and the L^4 gene derived from PI260429 (*C. chacoense*) is assumed to confer resistance to P_{1.2} and P_{1.2.3}, while the species carrying only L^1 and L^2 genes are not resistant to P_{1.2} and P_{1.2.3}.

P_{1.2} occurs widely in many cultivation areas in Japan whilst P_{1.2.3} is seldom found. To prevent the breakdown of resistance, the introduction of cultivars with the L^4 resistance gene should be avoided wherever possible. Alternatively, the use of cultivars with the L^3 resistance gene, together with prevention based on methods of cultivation, is being encouraged. Although some cultivars resistant to P_{1.2} have already been bred, new cultivars with the L^3 resistance gene, which display improved fruit quality, yield and resistance to diseases such as phytophthora blight and bacterial wilt disease should be developed.

Selection of lines resistant to diseases is usually conducted by inoculation of the pathogen and subsequent visual selection of the plants lacking specific symptoms. This procedure is, however, labor-intensive and time-consuming, and various environmental factors such as temperature and growth stage of the plants may interfere with the results. Moreover, this practice may be associated with the dissemination of the pathogen to neighboring cropping areas. Therefore, the application of marker-assisted selection (MAS) using appropriate molecular markers is considered to be a suitable strategy for the breeding of pepper (McCouch and Tanksley 1991).

Although a RFLP marker using a tomato probe linked to the L^3 gene was developed by Lefebvre *et al.* (1995), a simpler sequence-characterized amplified region (SCAR) marker is required for pepper MAS programs. Matsunaga *et al.* (2003) developed a SCAR marker linked to the L^4 gene, which is the dominant marker specific to the L^4 gene. However, effective DNA marker linked to the L^3 gene has not yet been developed.

In this paper, the development of a random amplified polymorphic DNA (RAPD) marker linked to the L^3 locus using bulked segregant analysis (BSA) (Michelmore *et al.* 1991) is described. Furthermore, the RAPD marker was transformed into more specific and reliable dominant and codominant SCAR markers.

Communicated by A. Kojima

Received June 16, 2003. Accepted January 24, 2004.

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Materials and Methods

Plant materials

One doubled haploid (DH) population, designated as K9-DH, was obtained by anther culture of a PMMoV-resistant F₁ hybrid 'K9' (*C. annuum* L.) and was used in BSA. 'K9' that harbors the *L*³ gene derived from PI159236 was bred at Nagano Chushin Agricultural Experiment Station (Yanokuchi *et al.* 1993). Anther culture was performed by using the method described by Dumas de Vaulx *et al.* (1981) with a minor modification (Todoroki *et al.* 1994, Yamaguchi *et al.* 1994). Another DH population, designated as K9/AC-DH, was obtained by anther culture of an F₁ hybrid between 'K9-11', a resistant DH line derived from a plant selected between K9-DH and a susceptible line 'AC2258' (*C. annuum* L.), and was used for developing RAPD and SCAR markers linked to the *L*³ gene. A BC₁ population was obtained from the crossing of 'K9-11' with 'AC2258' as the recurrent parent.

Furthermore, 18 accessions of *Capsicum* spp. were used to confirm the validity of the developed SCAR markers. Although, among nine accessions harboring the *L*³ gene except for PI159236, 'Berumasari', 'Himukamitori', 'T-143', 'Tosahime-R' and 'Spirit' harbored the *L*³ gene derived from PI159236, for the other accessions, the sources of the *L*³ gene could not be confirmed (Yanokuchi *et al.* 1993, Matsumoto *et al.* 1999).

PMMoV inoculation

P_{1,2} used in this study was collected in Miyazaki Prefecture. Young leaves were excised and mechanically inoculated with the sap extract, which was prepared by grinding P_{1,2}-infected leaves by rubbing with #500 carborundum powder. The inoculated leaves were kept in a growth chamber under moist conditions at 25°C for 24 hr under light. Local lesions appeared on the leaves of the plants harboring the *L*³ gene after 3 to 6 days.

RAPD analysis

Genomic DNA was extracted from young leaf tissues by the procedure described by Murray and Thompson (1980). BSA was carried out by pooling the DNA of 10 resistant and 10 susceptible plants from a K9-DH population separately.

The bulked DNA samples were screened with a total of 809 combinations of arbitrary two primers (A set to Z set, Operon Technologies, Calif.). The amplification conditions were adopted for RAPD analysis with minor modifications. The PCR reaction mixture consisted of 20 ng of template DNA, 1 × reaction buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl), 200 μM of dNTPs, 0.2 μM of 10-base random primers and 2 units of *rTaq* DNA polymerase (Boehringer Mannheim, Mannheim) in a total 20 μl reaction volume. DNA amplification was performed on a Perkin-Elmer GeneAmp PCR System 9600. The mixture was initially denatured at 95°C for 2 min, followed by 45 cycles of PCR

amplification with denaturation for 1 min at 94°C, primer annealing for 2 min at 35°C, primer extension for 3 min at 72°C, and finally incubated at 72°C for 7 min. The PCR products were separated by electrophoresis on a 2% agarose gel (SeaKem GTG agarose, FMC BioProducts, Rockland) with 0.5 × TBE buffer (44.5 mM Tris-borate, 1 mM EDTA), and stained with ethidium bromide. Linkage analysis using the RAPD markers and *L*³ gene was performed with the program MAPMAKER ver. 3.0 (Lander *et al.* 1987).

Cloning of RAPD markers and design of PCR primers

The RAPD fragments, putatively linked to the *L*³ gene, were excised from the gel, and purified by the glass powder method (GeneClean, BIO101, La Jolla). The purified fragments were introduced to a pT7BlueT-vector (Stratagene, La Jolla) using a DNA Ligation Kit ver. 1 (Takara Biomedicals, Otsu) and transformed into the JM109 strain of *E. coli* (Toyobo, Osaka). After molecular cloning, the DNA fragments of the resultant plasmid were purified using a QIAprep Spin Miniprep Kit (QIAGEN, Hilden) and the nucleotide sequence of the insert was determined by BEX, Tokyo.

The PCR primers were designed using the DNASIS software (Hitachi Software Engineering, Yokohama). The PCR reaction mixture consisted of 20 ng of template DNA, 1 × reaction buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂ and 50 mM KCl), 200 μM of dNTPs, 0.2 μM of designed primers and 1 unit of *rTaq* DNA polymerase (Boehringer Mannheim, Mannheim) in a total 20 μl reaction volume. The mixture was initially denatured at 95°C for 1 min, followed by 35 cycles of PCR amplification with denaturation for 30 sec at 94°C, primer annealing for 20 sec at 55°C, primer extension for 40 sec at 72°C, and finally incubated at 72°C for 7 min. The amplified products were separated by electrophoresis on a 13% polyacrylamide gel or 2% agarose gel with 0.5 × TBE buffer and stained with ethidium bromide.

Results and Discussion

*L*³-linked RAPD marker

It was found that two RAPD markers, E18₂₈₆ and E18₂₇₂, amplified with the E18 primer were polymorphic between the two bulks and reproducible. Segregation of these bands was further verified using a K9/AC-DH population (Fig. 1A). The E18 primer generated two polymorphic bands. Since they did not co-exist in each DH line, it was assumed that they were allelic (Table 1). Based on linkage analysis, the map distance between the marker and *L*³ was calculated to be 4.0 cM.

Conversion of RAPD marker to SCAR marker

The length of the RAPD fragment was 272 bp for the 'K9-11' allele or 286 bp for the 'AC2258' allele. These two fragments shared over 95% homology with 14 bp insertions at the 'AC2258' allele (Fig. 2).

Based on the sequences obtained, three types of oligonucleotides, PMF1, PMF2 and PMR1, were synthesized as

Fig. 1. DNA amplification patterns with primers, E18 (A), PMF1 and PMR1 (B), PMF2 and PMR1 (C). M: Molecular marker (ϕ X174/*Hae*III digest); K: 'K9-11', A: 'AC2258', F₁: 'K9-11' $\times$ 'AC2258', 1-13: Segregating K9/AC-DH individuals. Arrowheads indicate the RAPD (A) and the SCAR markers (B and C) linked to the *L³* gene. R: resistant individuals. S: susceptible individuals.

Table 1. Segregation of RAPD and SCAR markers in DH¹⁾ and BC₁²⁾ populations

DNA markers	DH				BC ₁			
	resistant		susceptible		resistant		susceptible	
	+ ³⁾	-	+	-	+	-	+	-
E18 ₂₇₂	94	0	4	0	nt ⁴⁾	nt	nt	nt
E18 ₂₈₆	3	0	72	0	nt	nt	nt	nt
PMFR11 ₂₆₉	88	0	4	0	96	0	3	0
PMFR11 ₂₈₃	3	0	68	0	4	0	87	0
PMFR21 ₂₀₀	88	10	3	72	nt	nt	nt	nt

¹⁾ DH population consisted of K9/AC-DH. 173, 163 and 173 individuals were examined for the E18₂₇₂ and E18₂₈₆, PMFR11₂₆₉ and PMFR11₂₈₃, and PMFR21₂₀₀ markers.

²⁾ One hundred-ninety individuals of the BC₁ population were examined for the PMFR11₂₆₉ and PMFR11₂₈₃ markers.

³⁾ + and -: presence and absence of the DNA markers.

⁴⁾ Not tested.

SCAR primers (Fig. 2). With the combination of PMF1 and PMR1 primers, the 269 bp fragment, designated as PMFR11₂₆₉, was amplified for the resistant allele, and the 283 bp fragment, designated as PMFR11₂₈₃, for the susceptible allele (Fig. 1B). With the combination of PMF2 and

PMR1 primers, the 200 bp fragment, designated as PMFR21₂₀₀, was amplified for the resistant allele (Fig. 1C). These results indicate that the PMFR11₂₆₉ and PMFR11₂₈₃ pair is a codominant marker, and that PMFR21₂₀₀ is a dominant marker.

Fig. 2. Nucleotide sequences of E18₂₇₂ and E18₂₈₆ fragments. Arrows indicate the positions of SCAR primers. Underlined parts indicate the sequence of the E18 primer for RAPD. The upper sequence indicates the 'K9-11' allele, and the lower indicates the 'AC2258' allele. Asterisk indicates an area with coincidental parts. Hyphen indicates an area with insertion parts in the 'AC2258' allele.

Table 2. Resistant genotypes to PMMoV and amplification with SCAR markers, WA31-1500S¹⁾, PMFR11₂₆₉ and PMFR11₂₈₃ in 18 accessions of *Capsicum* spp.

Accessions	Species	WA31-1500S	PMFR11		Resistant genotypes	Reaction ²⁾ to	
			269	283		P _{1,2}	P _{1,2,3}
AC2258	<i>C. annuum</i>	— ³⁾	—	+	L ⁺	S	S
Papri Gold	<i>C. annuum</i>	—	—	+	L ⁺	S	S
Kyouyutaka	<i>C. annuum</i>	—	—	+	L ¹	R	S
Tosahikari D	<i>C. annuum</i>	—	—	+	L ¹	R	S
Tabasco ⁴⁾	<i>C. frutescens</i>	—	—	+	L ²	R	R
PI159236 ⁴⁾	<i>C. chinense</i>	—	+	—	L ³	R	R
Berumasari	<i>C. annuum</i>	—	+	—	L ³	R	R
Himukamidori	<i>C. annuum</i>	—	+	+	L ³	R	R
T-143	<i>C. annuum</i>	—	+	+	L ³	R	R
Tosahime R	<i>C. annuum</i>	—	+	+	L ³	R	R
Spirit	<i>C. annuum</i>	—	+	+	L ³	R	R
Mihata 1	<i>C. annuum</i>	—	—	+	L ³	R	R
Sarara	<i>C. annuum</i>	—	—	+	L ³	R	R
Miogi	<i>C. annuum</i>	—	—	+	L ³	R	R
Fiesta	<i>C. annuum</i>	—	—	+	L ³	R	R
PI260429 ⁴⁾	<i>C. chacoense</i>	+	—	+	L ⁴	R	R
Leira	<i>C. annuum</i>	+	—	+	L ⁴	R	R
Special	<i>C. annuum</i>	+	—	+	L ⁴	R	R

¹⁾ This dominant SCAR marker was developed by Matsunaga *et al.* (2003).

²⁾ 'R' and 'S': 'resistant; local lesions' and 'susceptible; no symptoms' on inoculated leaves, respectively.

³⁾ '+' and '—': presence and absence of the DNA markers.

⁴⁾ These resistant genotypes were indicated by Boukema (1982).

PCR analysis in a K9/AC-DH population revealed that PMFR11₂₆₉, PMFR11₂₈₃ and PMFR21₂₀₀ cosegregate with the original RAPD marker, and that these SCAR markers are mapped at a distance of 4.0 cM from the L³ locus.

To verify the validity of the SCAR markers, the relation

between the codominant SCAR marker, PMFR11 and the resistance to PMMoV was examined by using a BC₁ population. The results showed a good agreement with the case where a K9/AC-DH population was used (Table 1).

The validity of PMFR11 was further examined with 18

accessions of *Capsicum* and WA31-1500S, the dominant SCAR marker for the *L*⁴ locus developed by Matsunaga *et al.* (2003) (Table 2).

As a result, although PMFR11₂₆₉ was detected in six among ten accessions harboring the *L*³ gene, it was not detected in the accessions harboring the *L*⁺, *L*¹ and *L*² genes. This suggests that PMFR11₂₆₉ could be effective in the detection of the *L*³ gene derived from PI159236. The examination with PI159236 and 'Berumasari', which harboring the *L*³ gene in a homozygous state detected only PMFR11₂₆₉. The examination with 'Himukamidori', 'T-143', 'Tosahime-R' and 'Spirit', which harboring the *L*³ gene in a heterozygous state resulted in the detection of both PMFR11₂₆₉ and PMFR11₂₈₃. On the other hand, PMFR11₂₆₉ was not detected in 'Mihata 1', 'Sarara', 'Miogi' and 'Fiesta' regardless of the presence of the *L*³ gene. This result was probably caused by either the presence of different sources of the *L*³ gene or recombination between PMFR11₂₆₉ and the *L*³ gene. WA31-1500S was detected only in the accessions harboring the *L*⁴ gene, and was not detected in the accessions harboring the *L*³ gene.

In the present study, a codominant SCAR marker pair, PMFR11₂₆₉ and PMFR11₂₈₃, and a dominant SCAR marker, PMFR21₂₀₀, were developed and the effectiveness of the codominant marker in the selection of homozygous individuals was confirmed. By utilizing useful characteristics of the K9/AC-DH populations, further studies will be directed to the construction of a high density map by applying new methods, such as AFLP (Vos *et al.* 1995), and to the development of the DNA markers linked to the useful characters for sweet pepper cultivation.

Acknowledgments

We are grateful to Dr. M. Hirai, Kyoto Prefectural University, and to Dr. M. Yano, National Institute of Agrobiological Sciences, for their suggestions and encouragement. We also thank Dr. T. Yabuya, Dr. M. Adachi, Dr. H. Kurata and Mr. S. Sugiyama for their critical review of the manuscript. It should be mentioned that this work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan.

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