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Development of Random Amplified Polymorphism DNA Markers Linked to CMV-B2 Resistance Gene in Melon

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Two random amplified polymorphic DNA (RAPD) markers linked to CMV-B2 resistance gene (*Creb-2*) in melon cultivar Yamatouri were cloned and sequenced to design sequence characterized amplified region (SCAR) markers for detection of CMV-B2 resistance gene (*Creb-2*) in melon. SCOPE14 derived from OPE-14 yielded a single DNA band at 541 bp, while SCAPB05 derived from APB-05, yielded a single DNA band at 1,046 bp, respectively. Segregation of SCOPE14 and SCAPB05 markers in bulk of F_2 plants demonstrated that they were co-segregated with RAPD markers from which the SCAR markers were originated. Furthermore, results of SCAR analysis in diverse melons showed SCAPB05 primers obtained a single 1,046 bp linked to *Creb-2* in resistant cultivars Sanuki-shirouri and Kohimeuri. However, SCOPE14 failed to detect *Creb-2* in diverse melons. Results of this study revealed that SCAR analysis not only confirmed melons that had been clearly scored for resistance to CMV-B2 by RAPD markers, but also clarified the ambiguous resistance results obtained by the RAPD markers.

Key words: *Cucumis melo* L., *Creb-2*, RAPD, SCAR

INTRODUCTION

Recently, PCR-based genetic markers have become available. These markers have been identified by either specific primers determined from known DNA sequences or arbitrary primers. Random amplified polymorphic DNAs (RAPDs) have been widely used and are one of the most powerful and fastest ways for tagging resistance genes (Michelmore *et al*. 1991; Paran *et al*. 1993; Reiter *et al*. 1992; Haley 1994; Wechter *et al*. 1995; Baudracco-Arnas & Pitrat 1996; Meyers *et al*. 1999; Zheng *et al*. 1999; Zheng & Wolff 2000). However, RAPD have some disadvantages. Paran and Michelmore (1993) reported that RAPD amplified products often contained repetitive DNA sequences and therefore can not be used as a hybridization probes. In addition, the RAPD technique is sensitive to changes in the reaction conditions and the results may be unstable. Therefore, there is a gap between the ability to obtain linked markers to a gene of interest in a short time and the use of these markers for map-based cloning approaches and for routine screening procedures.

Two RAPD markers (OPE-14_{550} and APB-05_{1050}) linked to CMV-B2 resistant melon have been previously reported in cultivar Yamatouri (Daryono & Natsuaki 2002). Furthermore, inheritance of resistance to CMV-B2 in cultivar Yamatouri was studied and it is controlled by a single dominant gene to which the symbol *Creb-2* assigned for CMV-B2 resistance gene (Daryono *et al*. 2003). OPE-14 primer yielded 550 bp RAPD marker, while APB-05 primer yielded 1,050 bp RAPD marker that were linked to CMV-B2 resistant melon in cultivar Yamatouri. By using these primers under similar PCR conditions, the 550 and 1050 bp were also detected not only in a few other resistant genotypes such as Mawatauri, Kohimeuri, Sanuki-shirouri, and PI 161375, but also sometimes detected in susceptible melons belonging to Makuwa and Conomon melon such as New-melon, Kintarou, and Katsura-shirouri. Although OPE-14_{550} and APB-05_{1050} were found to be conservative across diverse melon genotypes, they were sometimes either inconsistent or difficult to score and it is a characteristic of RAPD markers (Weeden *et al*. 1992; Staub *et al*. 1996).

Because of the disadvantages of RAPD markers, investigators have further characterized and converted the RAPD to more reliable and score-able markers such as Sequence-Characterized Amplified Regions (SCARs). A SCAR is a genomic DNA fragment at a single genetically defined locus that is identified by PCR amplification using a pair of specific oligonucleotides primers (Paran & Michelmore 1993). In this study, SCARs were derived by cloning and sequencing the two ends of the amplified products of RAPD markers. The sequence was used to design pairs of 23 to 24-mer oligonucleotide primers that resulted in the reproducible amplification of single loci when high annealing temperatures were used.

Therefore, we aimed to develop SCAR markers linked to *Creb-2* and apply the SCAR markers for detection of *Creb-2* in diverse melons.

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MATERIALS AND METHODS

Plant Materials and Bulk Segregant Analysis. Eighteen melon cultivars (Gulf stream, PI 371795, PI 125976, PI 125977, Mi Tang Ting, PI 230185, PI 169379, Sanuki-shirouri, OU641, Blewah-Bhisma, PI 161375, Kohimeuri, PI 414723, Action 434, Olive, and Shinjong) including P_1 Yamatouri, P_2 Vakharman (Table 1), bulk resistant and susceptible of F_2 individual plants were used in this study. The bulk resistant and susceptible DNAs of F_2 plants were preliminary used to evaluate SCAR markers tightly linked to *Creb-2* in melon. Seeds of each cultivar and F_2 individual plants were planted in plastic pots in growth chamber under continuous illumination (8,000 lux) at 26-28 °C. Healthy leaves were harvested from seedling at 3 to 5 leaves of each individual plant. One gram of fresh leaves of each plant was collected and immediately stored frozen at -20 °C. Genomic DNAs of 16 melon cultivars were used as templates for PCR amplification with the SCAR primers.

Cloning and Sequencing RAPD Products. RAPD reaction were performed and analyzed as described by Daryono and Natsuaki (2002). The amplified products of the linked RAPDs were excised from agarose gels and the DNA was purified by the QIA Quick Gel Extraction Kit (QIAGEN, USA). Three micro liters of purified DNA sample were mixed with 5 ìl of 2x Rapid ligation buffer, 1 ìl of 1/10 pGEM T-vector (pGEM T-vector, Promega, USA), and 1 ìl of T4 DNA ligase and incubated at room temperature for 1 h as described in the manufactures protocol. The ligated products were transformed into competent cell of *Escherichia coli* strain DH5á (Gibco BRL), and cells were placed on Luria-Bertani (LB) plates containing ampicillin and the chromogenic substrate, X-Gal. Transformed bacteria shown as white bacterial colonies were picked and grown in small-scale cultures. Plasmid DNA was isolated by standard alkaline lysis minipreparation method (Sambrook *et al*. 1989) and analyzed for presence of insert DNA in 1.5% electrophoresis gel. The plasmids with desired length of insert were selected and sequenced. DNA sequence was obtained by automatic sequencer ABI PRISM™ 377 (Applied Biosystems, USA) at least three for each independent clone. Nucleotide sequence was analyzed using MacVector 6.5 software (Oxford Molecular Ltd., USA) and search for sequence similarities was performed with BLASTX programs of DDBJ network service.

SCAR Design and Analysis. For each cloned RAPD amplification product, two oligonucleotides were designed to be used as SCAR primers. Each primer contained the original 10 bases of the RAPD primer plus the next 15 and 14 nucleotides of internal bases from the end for SCAPB05-1/ SCAPB05-2, and 13 to 14 nucleotides for SCOPE14-1/ SCOPE14-2 (Table 2). Primers were synthesized by Invitrogen-Japan. Amplification of genomic DNA (10 ng/ì l) with SCOPE14-1/ SCOPE14-2 primers was performed in a standard PCR reaction and consisted of 30 cycles of 1 minute at 95 °C, 1 minute at 67 °C and 2 minute at 72 °C, whereas with SCAPB05-1/SCAPB05-2, PCR conditions were as follows: 95 °C for 5 minutes, followed by 30 cycles of 1 minute at 95 °C, 1 minute at 67 °C and 2 minute at 72 °C. The amplified products were fractionated on 1.5% agarose gel in 1xTAE buffer and ethidium bromide stained bands of interest were excised and electro-eluted using standard procedures (Sambrook *et al*. 1989).

Table 1. Melon cultivars used in this study

Cultivars	Reaction against CMV-B2*
Gulf stream	S
PI 371795	S
PI 125976	S
PI 125977	S
Mi Tang Ting	R
PI 230185	S
PI 169379	S
Sanuki-shirouri	R
OU641	S
Blewah- Bhisma	S
PI 161375	R
Kohimeuri	R
PI 414723	S
Action 434	S
Olive	S
Shinjong	R
Yamatouri	R
Vakharman	S

*(Daryono *et al*. 2003), R: Resistant, S: Susceptible.

RESULTS

Cloning and Sequencing of RAPDs Linked to the CMV-B2 Resistance Gene. Two RAPD markers linked to CMV-B2 resistance gene in melon Yamatouri (OPE-14_{550} and APB-05_{1050}) were cloned and sequenced. Polymorphic DNA bands amplified by APB-05 marker obtained 1,046 base pairs of nucleotide sequences, while OPE-14 obtained 541 base pairs of nucleotide sequences (Figure 1). The terminal 10 bases exactly matched the primer sequences as the sequences were determined from the amplified products.

Amplification of Genomic DNA Using SCAR Primers. A pair of 23 to 25-mer SCAR primers was synthesized from each cloned RAPD product (Table 2). Genomic DNA from the resistant parent Yamatouri and susceptible parent Vakharman was used as the template for PCR amplifications with each pair of SCAR primer. In case of SCAPB05 and SCOPE14 primer, a single band of the same size as the progenitor RAPD fragment was amplified only in resistant Yamatouri. A single band of 1,046 bp was obtained by SCAPB05 primer, while

Table 2. Sequence of oligonucleotide primers for each SCAR locus derived from RAPD markers linked to *CMV*-B2 resistance gene in melon

Locus	Primer	Sequence (5' →3')	Polymorphism
$SCAPB05_{1046}$	SCAPB05-1	AACGCGCAACTTGATACAAATATAG	Dominant
	SCAPB05-2	AACGCGCAACAATAGAAGAACATC	
$SCOPE14_{541}$	SCOPE14-1	TGCGGCTGAGGACGGTTGGAGGTC	Dominant
	SCOPE-14-2	TGCGGCTGAGCATTCTCGAGCAG	