

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 F₂ 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-14₅₅₀ and APB-05₁₀₅₀) 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-14₅₅₀ and APB-05₁₀₅₀ 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 P₁ Yamatouri, P₂ Vakharman (Table 1), bulk resistant and susceptible of F₂ individual plants were used in this study. The bulk resistant and susceptible DNAs of F₂ plants were preliminary used to evaluate SCAR markers tightly linked to *Creb-2* in melon. Seeds of each cultivar and F₂ 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 µl of 2x Rapid ligation buffer, 1 µl of 1/10 pGEM T-vector (pGEMT-vector, Promega, USA), and 1 µl 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 DH5α (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/µl) 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).

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-14₅₅₀ and APB-05₁₀₅₀) 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 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.

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	<u>AACGCGCAAC</u> TTGATACAAATATAG	Dominant
	SCAPB05-2	<u>AACGCGCAACA</u> ATAGAAGAACATC	
SCOPE14 ₅₄₁	SCOPE14-1	<u>TGCGGCTGAGG</u> ACGGTTGGAGGTC	Dominant
	SCOPE-14-2	<u>TGCGGCTGAGC</u> ATTCTCGAGCAG	

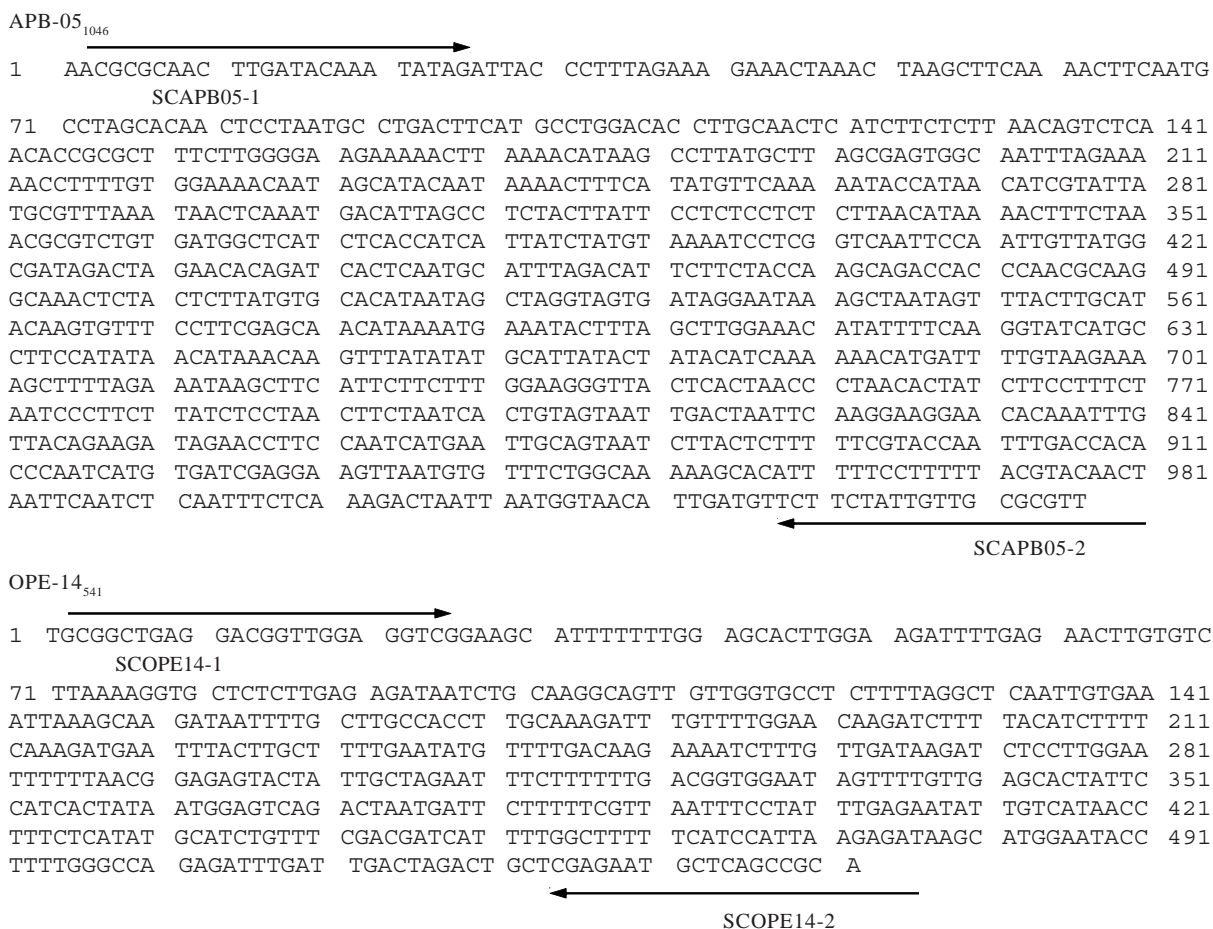


Figure 1. The nucleotide sequence of APB-05₁₀₄₆ and OPE-14₅₄₁, (AB167771 and AB167772). Arrows characters indicate the sequences location for construction of SCAR markers (AB167767 to AB167770).

another single band of 541 bp was amplified by SCOPE14 primer (Figure 2). Therefore this locus could also be readily scored as dominant marker.

To determine whether SCAPB05₁₀₄₆ and SCOPE14₅₄₁ represented single loci in F₂ population, their segregation was initially analyzed in bulk F₂ individual plants from the cross Yamatouri x Vakharman. Furthermore, SCAPB05₁₀₄₆ and SCOPE14₅₄₁ were obtained a single locus only in resistant Yamatouri and resistant F₂ individual plants (Figure 3).

Variability Detected by SCARs. Genomic DNAs of 16 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) were used as templates for PCR amplification with the SCAPB05₁₀₄₆ and SCOPE14₅₄₁ primers. These SCAR primers did not detect a high level of variation in diverse melon cultivars. For by SCOPE14₅₄₁, two additional alleles were detected in susceptible cultivars Gulf Stream, PI 125976, PI 125977, PI 230185, Blewah-Bhisma, and Olive (Figure 4a). However, primers SCOPE14₅₄₁ could not detected polymorphism between the amplification products from resistant and susceptible in diverse melons i.e. in case of susceptible cultivar PI 169379 and OU641, where a 541 bp DNA fragment linked to *Creb-2* was also obtained in these cultivars. On the other hand, SCAPB05₁₀₄₆, no additional alleles

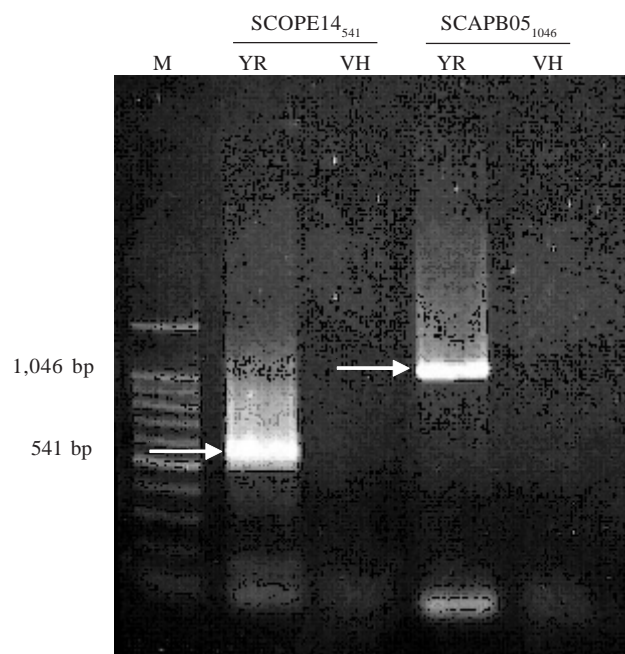


Figure 2. Amplification of genomic DNA using two SCAR primers. YR: resistant P₁ Yamatouri; VH: susceptible P₂ Vakharman. M: 100 bp DNA marker (Promega). Single arrow shows 541 bp of SCOPE14₅₄₁ and double arrow indicate 1,046 bp of SCAPB05₁₀₄₆.

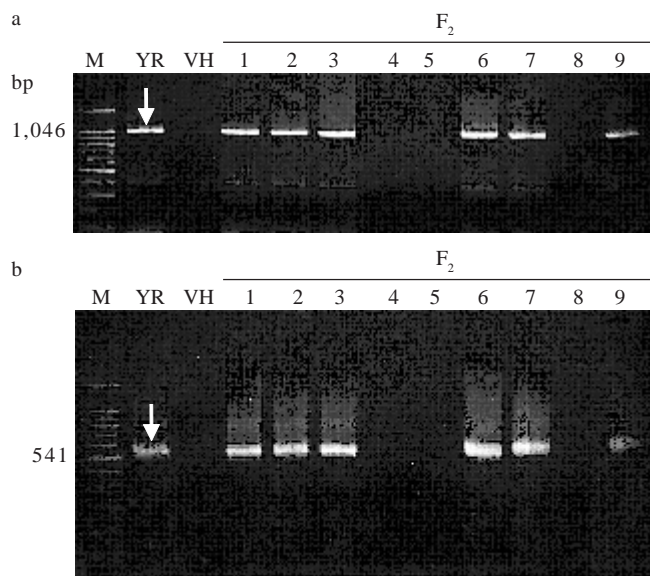


Figure 3. Segregation of SCAPB05₁₀₄₆ and SCOPE14₅₄₁ in F₂ progeny from crossed Yamatouri x Vakharman. a. SCAPB-05 primers; b. SCOPE-14 primers; YR: resistant P₁ Yamatouri; VH: susceptible P₂ Vakharman; 1 to 9: F₂ individual plants.

were detected as length variants in 1.5% agarose gels (Figure 4b). SCAPB05₁₀₄₆ primer could detect two resistant cultivars in diverse melons and obtained a 1046 bp in resistant cultivars Sanuki-shirouri and Kohimeuri respectively. These results indicated that SCAPB05₁₀₄₆ primer could be used for screening and detection of *Creb-2* in diverse melons.

DISCUSSION

In this study two SCAR primers were developed from the RAPD markers and they initially applied to detect polymorphism linked to CMV-B2 resistance gene (*Creb-2*) in parental plants, bulk F₂ individual plants, and in diverse melons that included resistant and susceptible genotypes against CMV-B2. Using SCOPE14₅₄₁ and SCAPB05₁₀₄₆ primers, *Creb-2* locus was amplified in resistant Yamatouri and not obtained in susceptible Vakharman. The results suggest that such sequences containing the *Creb-2* regions of the genome are amplified in the resistant Yamatouri, whereas these sequences are not readily amplified in susceptible Vakharman.

Furthermore, SCOPE14₅₄₁ and SCAPB05₁₀₄₆ markers displayed dominant segregation in bulk F₂ individual plants

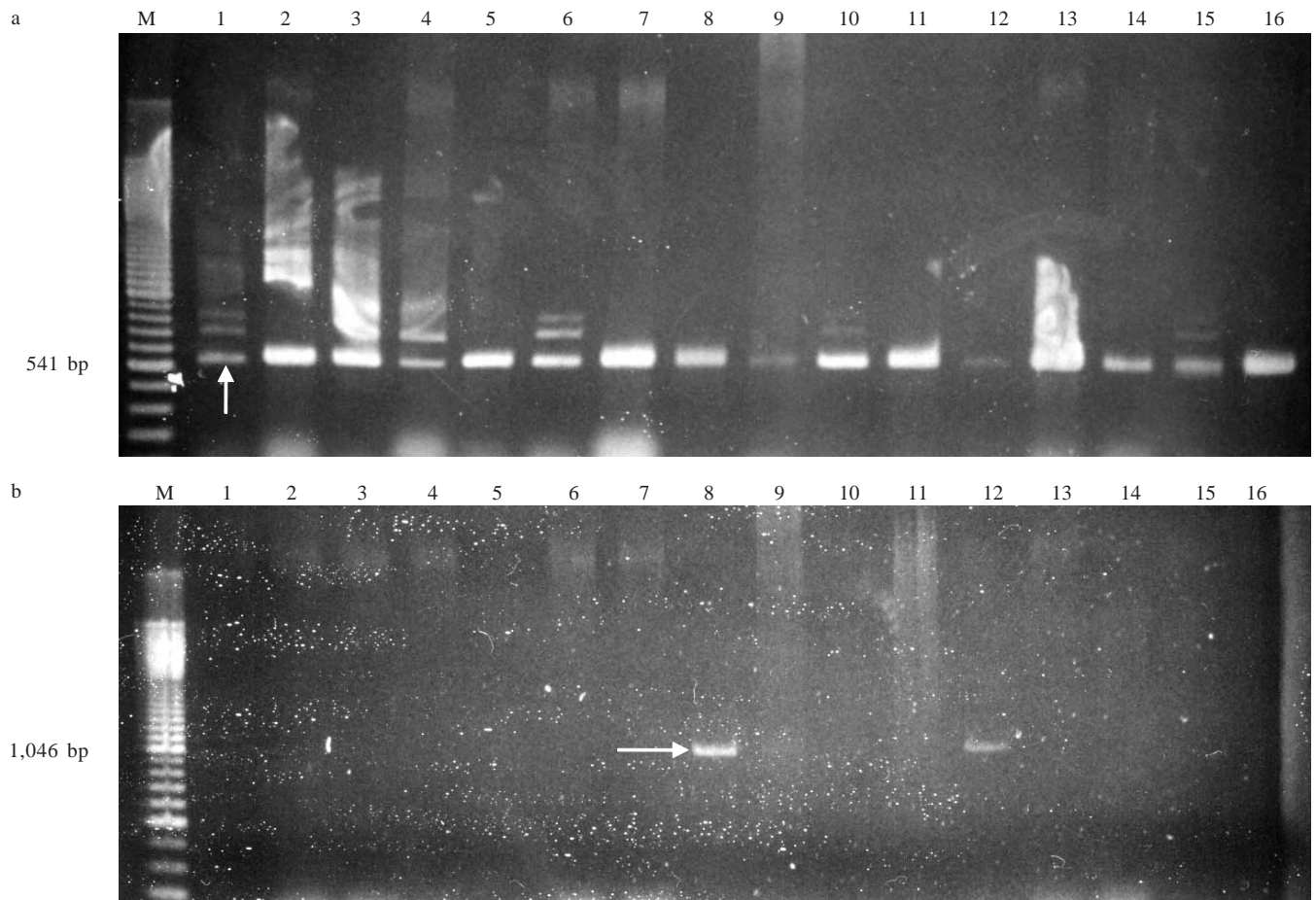


Figure 4. Gel electrophoresis of amplified genomic DNA melons using two SCAR primers. a. amplified by SCOPE14₅₄₁; b. amplified by SCAPB05₁₀₄₆. 1: Gulf stream; 2: PI 371795; 3: PI 125976; 4: PI 125977; 5: Mi Tang Ting; 6: PI 230185; 7: PI 169379; 8: Sanuki-shirouri; 9: OU641; 10: Blewah- Bhisma; 11: PI 161375; 12: Kohimeuri; 13: PI 414723; 14: Action 434; 15: Olive; 16: Shinjong; M: 100 bp DNA step ladder (Promega).

and the segregation of these SCAR markers in bulk F_2 plants demonstrated that they co-segregated with the RAPD markers from which they were originated. Polymorphism could be detected in bulk F_2 individual plants between the products of the extended primers derived from APB-05 and OPE-14. Therefore these markers could be demonstrated to be derived from a single locus.

The level of DNA band variation detected by the SCARs, SCOPE14₅₄₁ and SCAPB05₁₀₄₆ in diverse melon cultivars was low. This is contrast to the level variation detected by RFLPs and AFLPs in melon (Baudracco-Arnas & Pitrat 1996; Wang *et al.* 1997; Zheng *et al.* 1999). The difference probably reflects the ability of the two methods to detect divergent alleles. Several types of genetic changes that are detectable by RFLP and AFLP analysis may prevent amplification by PCR-SCAR.

SCARs have several advantages over RAPD markers. The use of RAPD allowed identifying molecular markers linked to the resistance gene (s) within a few months. As the annealing conditions are more stringent for SCARs than for RAPDs, only one locus was detected by the SCAR primers. Also the use of longer oligonucleotide primers for SCARs allowed a robust and more reproducible assay than could be obtained with the short primers used for RAPD analysis. Although the co-dominant SCARs are the more useful for genetic studies, the dominant SCARs may be ultimately more useful in breeding applications if a quick presence (plus) or absence (minus) assay can be developed to detect the product. This would be eliminating the need for electrophoresis to resolve the products as well as decreasing the cost and increasing the speed of analysis such as decrease the use of ethidium bromide.

Furthermore, SCAR primers have been used for identification of the resistance gene to pathogens in many plants, such as identification of resistance genes to downy mildew in lettuce (Paran & Michelmore 1993) and in apple (Evans & James 2003), resistance gene (*Tm-2*) to *Tomato mosaic virus* (ToMV) in the genus *Lycopersicon* (Sobir *et al.* 2000), resistance gene (*snbTM*) to *Septoria nodurum* blotch in durum wheat (Cao *et al.* 2001), and resistance gene (*Fom-2*) to *Fusarium oxysporum* f. sp. *Melonis* race 1 in melon (Burger *et al.* 2003). In this study, SCAR primers were applied for detection of resistance gene (*Creb-2*) to CMV-B2 in bulk of F_2 individual plants and in diverse melons.

Results from SCAR analysis showed more accurate and easier to score for CMV-B2 resistant cultivar contained *Creb-2* locus in diverse melons than the RAPD analysis. SCAR analysis not only confirmed all samples that had been clearly scored by RAPD analysis, but also clarified the ambiguous results obtained by the RAPD analyses. In summary, SCARs used in this study are advantageous over RAPD markers as they detect only a single locus, *Creb-2*. Since there is no report on SCAR primers for detection of CMV resistant cultivar in melon, these SCAR markers could be useful for plant breeding application such as marker assisted selection (MAS), finger-printing, and cultivar identification in melon.

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