Indonesian Journal of Biotechnology, December, 2011 Vol. 16, No. 2, pp.76-82

Development of Random Amplified Polymorphism DNA Markers Linked to Powdery Mildew Resistance Gene in Melon

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Abstract

A random amplified polymorphic DNA (RAPD) marker linked to powdery mildew resistance gene (*Pm-I*) in melon PI 371795 was reported. However, the RAPD marker has problem in scoring. To detect powdery mildew resistance gene (*Pm-I*) in melon accurately, the RAPD marker was cloned and sequenced to design sequence characterized amplified region (SCAR) markers. SCAPMAR5 marker derived from pUBC411 primer yielded a single DNA band at 1061 bp. Segregation of SCAPMAR5 marker in bulk of F_2 plants demonstrated that the marker was co-segregated with RAPD marker from which the SCAR marker was originated. Moreover, results of SCAR analysis in diverse melons showed SCAPMAR5 primers obtained a single 1061 bp linked to *Pm-I* in resistant melon PI 371795 and PMAR5. On the other hand, SCAPMAR5 failed to detect *Pm-I* in susceptible melons. Results of this study revealed that SCAR analysis not only confirmed melons that had been clearly scored for resistance to *Pm-I* evaluated by RAPD markers, but also clarified the ambiguous resistance results obtained by the RAPD markers.

Keywords : *Cucumis melo* L., *Pm-I*, RAPD, SCAPMAR5

Introduction

Melon (*Cucumis melo* L.) is a valuable cash crop grown throughout the world. It is a cross-pollinated diploid (2x = 2n = 24) species of African origin and a member of the genus *Cucumis,* in the family Cucurbitaceae (Robinson and Decker-Watters, 1999). Recently, melon is widely cultivated in Indonesia, mainly in Java including Ngawi, Magetan, Madiun, Sukoharjo and Kulonprogo. However, melon cultivation has been faced with plant diseases. One of the most damaging diseases affecting melon crops is powdery mildew (Alvarez *et al*., 2000). Powdery mildew attacking melon in Indonesia was caused by fungal species *Podosphaera xanthii* that reduces fruit quality and yield (Aristya and Daryono, 2007). Powdery mildew first appears as white, powdery spots that may form on both surfaces of leaves, on shoots, and sometimes on flowers and fruit. These spots gradually spread over a large area of the leaves and stems. Leaves infected with powdery mildew may gradually turn completely yellow, die, and fall off, which may expose fruit to sunburn. Severely infected plants may have reduced yields, shortened production times, and fruit that has little flavor (Davis *et al*., 2006). The use of genetically resistant cultivars will be good option for control fungi. Furthermore, new source of resistance to an Indonesian isolate of powdery mildew and a locus (*Pm-I*) that confers resistance to the powdery have been successfully determined as a single dominant gene in melon PI 371795 (Daryono and Qurrahman, 2009).

Recently, PCR-based genetic markers 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

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widely used and are one of the most powerful and fastest ways for tagging resistance genes (Michelmore *et al*., 1991; Paran and Michelmore, 1993; Zheng and Wolff, 2000). A RAPD marker ($pUBC411_{1050}$) linked to powdery mildew resistant melon has been previously reported in PMAR5 cultivar. Although $pUBC411_{1050}$ was found to be conservative across diverse melon genotypes, it was 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 and Michelmore, 1993). In this study, SCAR was derived by cloning and sequencing the two ends of the amplified products of a RAPD marker. The sequence was used to design pairs of 25 to 27-mer oligonucleotide primers that resulted in the reproducible amplification of single loci when high annealing temperatures were used.

The objective of this study was to develop SCAR markers linked to *Pm-I* and apply the SCAR markers for detection of *Pm-I* in diverse melons.

Materials and Methods

Plant materials and bulk segregant analysis

Fifteen melon cultivars (PMAR5, Harukei, WMR-29, PMR45, PMR5, Nigeashi-1, PI 414723, PI 124112, PI 124111, Sunrise, Kohimeuri, PI 161375, Nigeashi-2) including P_1 PI 371795, P_2 Action 434, and 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 marker tightly linked to *Pm-I* in melon. Seeds of each cultivar and F_2 individual plants were planted in plastic pots in growth chamber under continuous illumination (8000 lux) at 26°C-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 15 melon cultivars were used as templates for PCR amplification with the SCAR primers.

Cloning and sequencing RAPD products

A RAPD reaction was performed and analyzed as described by Daryono and Natsuaki (2002) using pUBC411 to amplify DNA of PI 371795. The amplified product of the linked RAPD was 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 5µl of 2x Rapid ligation buffer, 1µl of 1/10 pGEM T-vector (pGEM T-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 17 nucleotides of internal bases from the end for SCAPMAR5-F/SCAPMAR5-R (Table 1). Primers were synthesized by Invitrogen-Japan. Amplification of genomic DNA (10 ng/µL) with SCAPMAR5-F/SCAPMAR5-R primers was done in a standard PCR reaction and consisted of 30 cycles of 1 min at 95°C, 1 min at 60°C and 2 min at 72°C with 10 min extension 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 powdery mildew resistance gene

A RAPD marker ($pUBC411_{1061}$) linked to powdery mildew resistance gene in melon PI 371795 was cloned and sequenced. Polymorphic DNA bands amplified by pUBC411 marker obtained 1058 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 25 to 27-mer SCAR primers was synthesized from a cloned RAPD product (Table 1).

Genomic DNA from the resistant parent PI 371795 and susceptible parent

$pUBC114_{1061}$:

SCAPMAR5F →

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1    CAGACAAGCC CAGATAATTA ACATCTCACA CTCACCCCTT CACAAATACC AAAAAAAAAA GAAAAACAAA
71   AAAACCCACA AACTTGCACT TAGTAACATT TGAAGCCACT TAAATGAATC TTACAACCAA TTCTAAGACA
141  CTAAACAAGC CCAAATCATT AATAGTTCAC ACCTACCTTC CCATAAGCAC AAAAGCAACA AACTTACACT
211  TTTAGTAATT ACAATTCAAT GCGGTTAACT TACACATTGT TGGTGTCAAA AACTTGATTT AAATGCAAGT
281  GTATGTATCG AGTGTAATAT AACAATGCGA CGACCTAAAA GATTGAGTAT CGTTCTTTGG AGATCAAATG
351  TTTGGATTAT GTTTAACTAT ATATCCTCGA ATTAGATTGA ATTTTATTTA AAGATTTGAA TCTAGATGAG
421  GTGATTTTTG AAAATAAAGA AAACATAGGT AAAGTAAAAG GTTGAAATTT AAGTTTAAAA GTGTTGATTT
491  CTTTAATTAT TTCATGTCAT CCAAAGAAGA TTTTTGTATG GAATATTGTG TCAATTACTT GTTTAAGTCT
561  AAAAACTATT TTTCGGATTA TTATCTTTCC ATGACATTTT TTTTCATACA AACATAATTT AGTACTGGTT
631  TCTTGAAATC AAATTCCTTT AATTGATTTA AGATAGAAAA GGATATACAT TATTGAATTT ATTAGTCAGT
701  ACTTACCATT TCTCTTTTAA GTCTTTCTTA GTTGACAATA TGTACACATT CTCAAGATGA TCTCATGTAT
771  TCAAATCTGA TATTGTTTCA TATATACACA CCAACTTTCT CTGCATGGTT GTTCCTCTAA TAATATCATA
841  ATAAGTAGAA GTATGCTTTG ATACCATTTA TAACATCTCA GTTGTTAAAT CCTCTCATGA CAAGACAAGT
911  TAGTTTAAGA TTCAAGTTTT GACGAAGTTC GAATAGTGTC ATGAAGGTTT ACAAATTTGT TTTTCCCTTG
981  GGCTTATAAT TGTCAAAGAA TGGGAACAAC CCATGATAAA TTGGATATGC TACTTTAGCC CACAACTCCT
1051 AGGCTTGTCTG
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← SCAPMAR5R

Figure 1. The nucleotide sequence of $pBUC114_{1061}$. Arrows and bold characters indicate the sequences location for construction of SCAPMAR5 marker.

Table 1. Sequence of oligonucleotide primers for each SCAR locus derived from RAPD markers linked to powdery mildew resistance gene in melon

Locus	Primer	Sequence (5 ——— 3)	Polymorphism
$SCAPMAR5_{1061}$	SCAPMAR5F	CAGACAAGCCCAGATAATTAACATCTC_	Dominant
	SCAPMAR5R	CAGACAAGCCTAGGAGTTGTGGGCT	

*Underlined sequences indicate those nucleotides from arbitrary primers pUBC114

Action 434 was used as the template for PCR amplifications with each pair of SCAR primer. In case of SCAPMAR5 primers, a single band of the same size as the progenitor RAPD fragment was amplified only in resistant PI 371795. A single band of 1061 bp was obtained by SCAPMAR5 primers (Figure 2). Therefore this locus could also be readily scored as dominant marker.

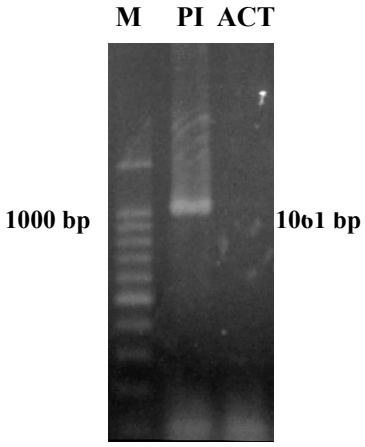

Figure 2. Amplification of genomic DNA using SCAPMAR5 primers. PI: resistant P_1 PI 371795; ACT: susceptible P_2 Action 434. M: 100 bp DNA marker (Promega). Single arrow shows 1061 bp of $SCAPMAR5_{1061}$.

To determine whether $SCAPMAR5_{1058}$ represented single loci in F_2 population, their segregation was initially analyzed in bulk F_2 individual plants from the cross PI 371795 x Action 434. Furthermore, $SCAPMAR5_{1061}$ was obtained a single locus only in resistant PI 371795 and resistant F_2 individual plants (Figure 3).

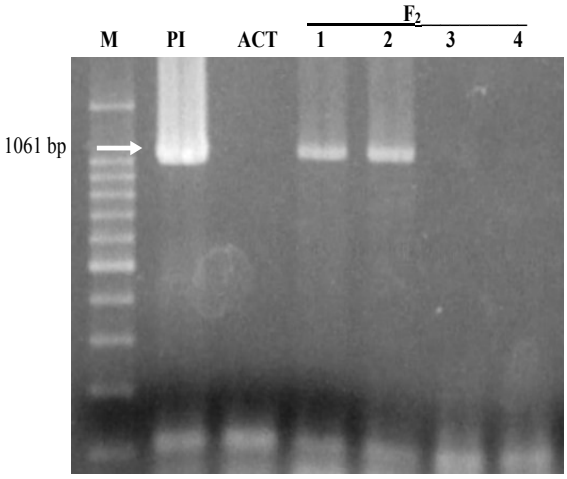

Figure 3. Segregation of $SCAPMAR_{1058}$ in F_2 progeny from crossed PI 371795 x Action 434. PI: resistant P_1 PI 371795; ACT: susceptible P_2 Action 434; 1 to 2: F_2 resistant individual plants; 3 to 4: F_2 susceptible individual plants. Single arrow shows 1061 bp of $SCAPMAR5_{1061}$.

Variability detected by SCARs

Genomic DNAs of 15 melon cultivars (PMAR5, Harukei, WMR-29, PMR45, PMR5, Nigeashi-1, PI 414723, PI 124112, PI 124111, Sunrise, Kohimeuri, PI 161375, Nigeashi-2, PI 371795 and Action 434) were used as

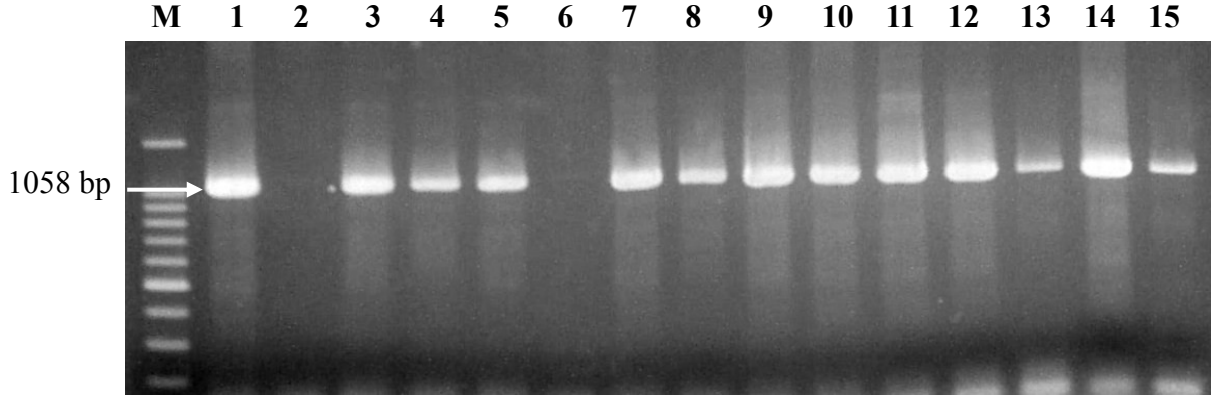

Figure 4. Gel electrophoresis of amplified genomic DNA melons using SCAPMAR5 primers. 1: PMAR5, 2 Harukei, 3. WMR-29, 4.PMR45, 5.PMR5, 6.Action 434, 7.PI 414723, 8.PI 124112, 9.PI 124111, 10.PI 371795, 11.Sunrise, 12.Nigeashi-2, 13.Kohimeuri, 14.PI 161375, 15.Nigeashi-2; M: 100 bp DNA step ladder (Promega). Single arrow shows 1061 bp of $SCAPMAR5_{1061}$.

templates for PCR amplification with the $SCAPMAR5_{1061}$ primers. The SCAR primers did not detect a high level of variation in diverse melon cultivars. For $SCAPB05_{1061}$ no additional alleles were detected as length variants in 1.5% agarose gels (Figure 4). $SCAPB05_{1061}$ primers detected two susceptible cultivars (WMR-29 and Action 434) and 13 resistant cultivars in diverse melons (Figure 4). These results indicated that $SCAPB05_{1061}$ primers could be used for screening and detection of *Pm-I* 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 powdery mildew resistance gene (*Pm-I*) in parental plants, bulk F_2 individual plants, and in diverse melons that included resistant and susceptible genotypes against powdery mildew. Using $SCAPMAR5_{1058}$ primers, *Pm-I* locus was amplified in resistant PI 371795 and not obtained in susceptible Action 434. The results suggest that such sequences containing the *Pm-I* regions of the genome are amplified in the resistant PI 371795, whereas these sequences are not readily amplified in susceptible Action 434.

Furthermore, $SCAPMAR5_{1058}$ markers displayed dominant segregation in bulk F_2 individual plants 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 PMAR5. Therefore these markers could be demonstrated to be derived from a single locus.

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 and Michelmore, 1993) and in apple (Evans and James, 2003), resistance gene (*Tm-2*) to *Tomato mosaic virus* (ToMV) in the genus *Lycopersicon* (Sobir *et al*., 2000), resistance gene (*snbTM*) to *Septnoria nodurum* blotch in durum wheat (Cao *et al*., 2001), resistance gene (*Fom-2*) to *Fusarium oxysporum* f. sp. *Melonis* race 1 in melon (Burger *et al*., 2003) and resistance gene (*Creb-2*) to *Cucumber mosaic virus* in melon (Daryono *et al*, 2009 and 2010). In this study, SCAR primers were applied for detection of resistance gene (*Pm-I*) to powdery mildew in bulk of F_2 individual plants and in diverse melons.

Results from SCAR analysis showed more accurate and easier to score for powdery mildew resistant cultivar contained *Pm-I* 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, *Pm-I*. Since there is no report on SCAR primers for detection of powdery mildew 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.

Acknowledgment

The authors would like to express special thanks to Prof. Keiko T. Natsuaki, Laboratory of Tropical Plant Protection, Tokyo University of Agriculture, Japan for kindly support laboratorial facilities to conduct this research. This research was supported by Research Grant from Ministry of Research and Technology for 2007 to 2010 and Hibah Kompetensi Research Grant from Ministry of National Education, Republic of Indonesia for 2009 to 2011.

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