LINKAGE ANALYSIS AND MAPPING OF SCAR MARKERS LINKED TO CMV-B2 RESISTANCE GENE IN MELON

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SUMMARY

Two random amplified polymorphic DNA (RAPD) markers linked to Cucumber mosaic virus resistance gene (Creb-2) in melon “Yamatouri” have been cloned and sequenced to design sequence characterized amplified region (SCAR) markers. SCOPE14 derived from OPE-14333 yielded a single DNA band (541 bp), while SCAPB05 derived from APB-051059 yielded a single DNA band (1046 bp). SCOPE14 and SCAPB05 were applied for targeted linkage mapping of Creb-2 in 154 F2 progeny of a cross between “Yamatouri” and susceptible “Vakharman”. Linkage analysis of these SCAR markers showed that genetic distances of SCOPE14 and SCAPB05 to the Creb-2 were 20 and 13.5 cM, respectively. Furthermore, SCAPB05 showed a distinct polymorphism when used as a probe for restriction fragment length polymorphism (RFLP) analysis against “Yamatouri” and “Vakharman” for the Creb-2.

Key words: Cucumis melo, CMV-B2, Creb-2, linkage map, RFLP, SCAR

Melon (Cucumis melo L.) is an important fruit grown throughout the world and a member of the family Cucurbitaceae. It is a cross-pollinated diploid (2n = 2x = 24) species which consists of 90 genera and 750 species (Robinson and Walters, 1999). To the molecular geneticist, melon offers the additional advantage of a very small genome size 4.5-5.0 x 10^8 bp, only three times that of Arabidopsis (Arumuganathan and Earle, 1991).

Cucumber mosaic virus (CMV) is a member of the genus Cucumovirus, and it is one of the common viruses attacking cucurbits including melons, having a larger host range than most viruses (Smith et al., 1988). Aphids transmit CMV in a non-persistent manner (Palukaitis et al., 1992). This virus frequently causes substantial loss in yield and inferior fruit quality, especially in the early melon seeding. To limit the incidence of CMV in melon, screening and breeding of resistant cultivars against the virus has been previously

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conducted. Resistance to CMV Indonesian isolate (CMV-B2) has been found in “Yamatouri”, and it is controlled by a single dominant gene to which the symbol 
Creb-2 was proposed (Daryono et al., 2003).

Random amplified polymorphic DNA (RAPD) analysis has been used for identification and mapping of diseases resistance genes in melon (Wechter et al., 1995, Baudracco-Arnas and Pitrat, 1996; Zheng et al., 1999; Zheng and Wolff, 2000; Burger et al., 2003; Daryono and Natsuaki, 2003). Furthermore, two SCAR markers (SCOPE14541 and SCAPB051046) linked to Creb-2 in resistant melon “Yamatouri” has been reported (Daryono et al., 2009).

Many plant genetic maps have been generated using F2 populations based on RAPD markers. The first molecular map of melon consisting of 102 loci was constructed using a F2 population with RFLP, RAPD, isozyme, disease resistance and morphological markers (Baudracco-Arnas and Pitrat, 1996). In this study, SCOPE14541 and SCAPB051046 markers linked to Creb-2 locus were applied for linkage mapping of Creb-2 in an F2 population consisting 154 F2 individual plants derived from the cross between resistant “Yamatouri” and susceptible “Vakharman”. The SCAR markers were also applied for detection of Creb-2 in diverse melons.

MATERIALS AND METHODS

Plant materials and F2 mapping population

The F2 mapping population was derived from a cross between resistant “Yamatouri” as female parent, and susceptible “Vakharman” as pollen parent. F1 individuals were self pollinated, and obtained 154 F2 individuals. They were grown in a growth chamber and one side of the cotyledon was sampled for DNA extraction at the cotyledon stage. The other side of cotyledon and true leaves were inoculated by CMV-B2 and scored their resistance and susceptibility by symptom development and DAS-ELISA (Daryono and Natsuaki, 2002). The DNAs of each F2 individual plant were used for linkage analysis and mapping population. In addition, six melons resistant to CMV infection including “Mawatari”, “Miyamauri”, “Shinjung”, “Sanuki-shirouri”, “Kohimeuri”, “Mi Tang Ting”, and PI 161375 were used to confirm the application of SCAR markers for detection of melon resistant to CMV.

SCAR analysis and scoring data

Two SCAR markers (SCOPE1441 and SCAPB051046) were applied in this study (Daryono et al., 2009). Amplification of 6 melon cultivars and F2 individual genomic DNAs (10 ng/μl) with SCOPE14 and SCAPB05 primers was done in a standard PCR reaction. The PCR conditions were as follows: 95°C for 5 minutes, followed by 30 cycles of 1 minute at 95°C, 1 minute at 60°C, and 2 minute at 72°C. PCR products were electrophoresed on 1.5% agarose gels and detected by staining with ethidium bromide. For linkage analysis, markers present in a progeny were scored as “1” and absent as “0”.

Restriction fragment length polymorphism (RFLP) analysis

DNA samples from “Yamatouri” and “Vakharman” were digested with five restriction enzymes (EcoRI, EcoRV, HindIII, BamHI, and XbaI) according to the manufacturer’s instruction (Amersham Pharmacia Biotech). Ten μg of the digested DNA was separated on a 1% agarose gel. DNA fragments were blotted on a Hybond N+
membrane (Amersham Pharmacia Biotech) according to the manufacture's instruction. Digoxigenin-labeled probes (SCAPB05_1046 and SCOPE14_241) were prepared using ECL direct nucleic acid labeling and detection systems (Amersham Biosciences, UK) and used for hybridization to melon DNA according to the manufacture's instruction. After overnight hybridization at 42°C, the membrane was washed once in primary wash solution of 20x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 20 minutes two times at 42°C, then in secondary wash solution for 5 minutes at room temperature. Detection reagent I and II (Amersham Biosciences, UK) were added into membrane for 1 min. The hybridization signal was detected by exposure to X-ray films according to the manufacturer's instruction.

**Linkage map construction**

The map was constructed using MAPMAKER version 3.0 (Lander *et al.*, 1987). Both SCAR markers were initially associated with two-point comparisons of the group command with a LOD of 3.0 and a recombination value of 0.3. Both SCAR markers were then placed in sequence via three-point analyses of the order command. Scoring errors were detected by checking for double cross-over. Finally, candidate orders were confirmed with the "ripple" and "compare" commands. Markers that could not be confidently located were placed via the "try" command. Recombinant frequencies or map units (cM) were calculated using the Kosambi distance.

**RESULTS**

**Inheritance of Creb-2 in F2 population**

The polymorphism between the resistant and susceptible parents to CMV-B2 in F2 population was assayed using SCOPE14 and SCAPB05 primers and compared with the previous results of symptom observation and viral accumulation evaluated by DAS-ELISA (Daryono *et al.*, 2003). Each SCAR primer produced a single band linked to Creb-2 in F2 resistant plants respectively (Figure 1). SCOPE14 primers produced a single DNA band of 541 bp and showed in 122 F2 individual plants, while 32 individual plants did not allow the amplification of PCR products with the expected size (Table 1). On the other hand, SCAPB05 primers obtained a single DNA band of 1046 bp and appeared in 125 F2 individual plants, whereas 29 individual plants did not obtain the band (Table 1). Segregation data of two SCAR markers conducted in this study revealed similar results with symptom development and ELISA conducted in the previous study (Daryono *et al.*, 2003). The Creb-2 locus fits the expected 3:1 ratio for single dominant gene in F2 population (Table 1).
Figure 1. Segregation of SCOPE1441 and SCAPB051046 markers in F2 progeny from crossed Yamatouri x Vakharman. Lines 1 to 16 are F2 progeny. (a): SCOPE1441; (b): SCAPB051046. M: 100 bp DNA ladders (Promega). A single arrow shows 541 bp and a double arrows show 1046 bp.

Table 1. Segregation for resistance to CMV-B2 in F2 population from crossed Yamatouri with Vakharman scored by symptom development and ELISA, SCAPB051046, and SCOPE14541 markers.

<table>
<thead>
<tr>
<th>Determined by</th>
<th>Number of plants</th>
<th>$\chi^2$ 3:1</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
<td>Susceptible</td>
<td>Total</td>
</tr>
<tr>
<td>Symptom and ELISA</td>
<td>119</td>
<td>35</td>
<td>154</td>
</tr>
<tr>
<td>SCAPB051046</td>
<td>125*</td>
<td>29*</td>
<td>154</td>
</tr>
<tr>
<td>SCOPE14541</td>
<td>122*</td>
<td>32*</td>
<td>154</td>
</tr>
</tbody>
</table>

$\chi^2$: Expected ratio
*: Including recombinant individuals judged by SCAR markers

Southern-blot analysis

SCAPB051046 and SCOPE14541 showed bands both in Yamatouri and Vakharman (Figure 2). By using SCOPE14541 probe, three to four bands shown in EcoRI, EcoRV, and HindIII digested melon DNAs, implying that three to four copies of SCOPE14541 marker were present in melon (Figure 2a). In contrast, the clone from SCAPB051046 marker was shown to hybridize to a small number of different DNA fragments in Yamatouri and Vakharman (Figure 2b). The numbers of bands produced by SCAPB051046 marker were less than those with SCOPE14541. Furthermore, a polymorphism band between “Yamatouri” and “Vakharman” was detected when SCAPB051046 marker was used as a probe and a single
major band was present in lanes of “Yamatouri” DNA digested with BamHI, whereas weak signal band was detected in “Vakharman” DNA (Figure 2b).

**Linkage analysis and mapping of the SCAR markers**

A total of two SCAR markers, SCAPB05<sub>1046</sub> and SCOPE14<sub>541</sub> were placed on the map. Recombinants were detected between Creb-2 and SCOPE14<sub>541</sub> in 19 F<sub>2</sub> individual plants, including 8 recombinant plants in 119 F<sub>2</sub> resistant individual plants and 11 recombinant plants in 35 F<sub>2</sub> susceptible individual plants. On the other hand, other recombinants were detected between Creb-2 and SCAPB05<sub>1046</sub> in 18 F<sub>2</sub> individual plants, including 6 recombinant plants in 119 F<sub>2</sub> resistant individual plants and 12 recombinant plants in 35 F<sub>2</sub> susceptible individual plants (Table 2).

By using the MAPMAKER, SCAPB05<sub>1046</sub> and SCOPE14<sub>541</sub> were mapped at 13.5 cM and 20 cM, respectively, from the Creb-2 locus on the same side of the chromosome. The linkage of SCAPB05<sub>1046</sub> and SCOPE14<sub>541</sub> with the Creb-2 locus is presented in Figure 3.

![Figure 2](image-url)

**Figure 2.** Southern hybridization with DNA fragments cloned from the SCOPE14<sub>541</sub> (a) and SCAPB05<sub>1046</sub> (b) markers. The restriction endonucleases used are indicated above the lanes.
**Table 2. Phenotypic frequencies of 154 F₂ individual plants**

<table>
<thead>
<tr>
<th>Loci</th>
<th>Phenotypes</th>
<th></th>
<th>(Susceptible F₂ individuals)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Resistant F₂ individuals)</td>
<td>s/++</td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>r/+</td>
<td>r/-</td>
</tr>
<tr>
<td>Creb-2</td>
<td>SCAPB051046</td>
<td>113</td>
<td>6</td>
</tr>
<tr>
<td>Creb-2</td>
<td>SCOPE14541</td>
<td>111</td>
<td>8</td>
</tr>
</tbody>
</table>

*Resistant and susceptible were judged by SCAR markers

**Figure 3. Linkage map of SCAR markers with the Creb-2 locus**
Conservation of the Creb-2 locus in CMV melon cultivars

SCOPE14_{541} and SCAPB05_{1046} were applied for detection of Creb-2 in CMV resistant melons. SCOPE14_{541} was present in resistant “Mi Tang Ting”, PI 161371, “Kohimeuri”, “Yamatouri”, “Mawatauri”, and “Miyamauri”, while SCAPB05_{1046} was conserved only in “Mi Tang Ting”, PI 161371, “Kohimeuri”, “Yamatouri”, and “Mawatauri” (Figure 4).

(a)

(b)

Figure 4. Detection of SCOPE14_{541} and SCAPB05_{1046} in CMV resistant melon cultivars. A: SCOPE14_{541}; B: SCAPB05_{1046}. YR: Yamatouri; VH: Vakharman; MT: Mawatauri; MR: Miyamauri; SJ: Shinjorg; SU: Sanuki-shirouri; MIT: Mi tang Ting; KH: Kohimeuri; 161: PI 161375. M: 100 bp DNA step ladder (Promega). A single arrow shows 541 bp and a double arrows show 1046 bp.
DISCUSSION

The resistant interaction between melon and CMV is restricted to fairly limited area of research. Kobori et al. (2000) reported that resistance to CMV in Cucumis figarei is caused by restriction of CMV movement at the interface between mesophyll and phloem pathway. While Hirai and Amemiya (1990) demonstrated the restriction of virus spread in the leaves of resistant melon might not be caused by a direct inhibition of cell-to-cell movement but might be caused by an inhibition of virus replication in the cells around the early-infected cells. However, the resistance mechanisms acting in cultivar Yamatouri was demonstrated to be related to the existence of mechanisms that inhibit movement of virus from inoculated leaves to upper leaves and this type of resistance would affect long distance movement of viral particles (Daryono et al., 2003).

Furthermore, the genetic analysis of the interaction resulted in the identification of the Creb-2 locus in the resistant melon cultivar Yamatouri as a single dominant gene (Daryono et al., 2003). Therefore, to tag the Creb-2 gene with molecular markers, a map-based cloning approach was applied. Due to the inconsistency of the RAPD markers linked to Creb-2 locus in melon, SCAR markers was developed and apply to map markers linked to the target locus. Using SCOPE14 and SCAPB05 primers, Creb-2 locus was amplified in resistant Yamatouri and not obtained in susceptible Vakharman (Daryono et al., 2009). This result suggests 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.

The comparison between phenotypic scoring of a segregating population and molecular evaluation of the genotype highlights the difficulty in identifying resistant plants on the basis of inoculation alone. Plants that might have been considered resistant on the basis of symptom evaluation were identified as susceptible by the molecular marker test. In the present study, the SCOPE14s41 and SCAPB05s1046 markers successfully identified the F2 population tested as well as the recombinant individuals that could not detected by symptom development and ELISA. As described in the results, among 154 F2 individual plants, 20 recombinant plants were detected by SCOPE14s41 marker, while 18 recombinant plants were detected by SCAPB05s1046 marker. In addition, the dominant SCAR markers SCOPE14s41 and SCAPB05s1046 are simpler to analyze and resulted in good identification of the F2 population tested.

By using 154 F2 individual plants, the genetic map of two SCAR markers linked to Creb-2 was constructed with 13.5 and 20 cM of genetic distances between Creb-2 and SCOPE14s41 and SCAPB05s1046. The genetic distances of these markers to Creb-2 is relative far comparing with the molecular markers linked to the CMV-Y resistance gene (Cry) in cowpea (Vigna unguiculata) which has been reported by Chida et al. (2000) and the genetic distance of their markers to the Cry were 0.7, 5.2, 11.5 and 24.5 cM, respectively.

Furthermore, the genome size of melon has been estimated to be 454-502 megabase pairs (Mb) as reported by Arumuganatham and Earle (1991) and a number of genetic maps have been constructed for melon in recent years using some molecular markers (Baudracco-Arnas and Pitrat, 1996; Wang et al., 1997; Oliver et al., 2001; Perin et al., 2002; Danin-Poleg et al., 2000). Although, this mapping population is different from theirs,
13.5 cM and 20 cM between Creb-2 and SCOPE14541 and SCABP051046 markers correspond to a physical distance of about 6.2 and 7.5 Mbp. These values are shorter in the total length of the linkage groups compared to other published melon maps as reported by Boudraeco-Arnas and Pitrat (1996), 110 marker map with total length of the linkage groups is 1,390 cM; Wang et al. (1997) with 204 markers spanning 1942 cM; Oliver et al. (2001) with 412 markers and 1197 cM; Perin et al. (2002), 668 markers and 1654 cM; Danin-Poleg et al. (2000) with 74 markers and 610 cM; and Silberstein et al. (2003) with 179 markers spanning 1421 cM. Moreover, linkage maps of other cucurbit crops have been constructed in cucumber (Kennard et al., 1994; Meglic and Staub, 1996; Serquen et al., 1997), and watermelon (Hashizume et al., 1996) using different molecular marker technology.

Some melon cultivars have been reported to be resistant to CMV such as Mi Tang Ting and Shirouri-nigo (Takada, 1979), PI 161375 (Pitrat & Lecoq, 1980), Kohimeuri (Hirai and Amemiyia, 1989), Yamatouri, Mawatauri, Miyamauri, Shinjong, and Sanukishirouri (Daryono et al., 2003). The resistance in these cultivars showed almost similar to that on Yamatouri with no visible symptoms in upper leaves after inoculation with CMV. Hence, the resistant cultivars were tested whether they maintained or not molecular markers linked to Creb-2. Frequency of marker detection was higher for SCOPE14541 than for SCABP051046. However, SCOPE14541 sometimes detected not only in CMV-B2 resistant cultivars but also in susceptible melons, while SCABP051046 constantly detected in CMV-B2 resistant cultivars (unpublished data). These results suggested that conservation of these markers in melon genomes was not dependent on the distance of markers to the Creb-2 locus.

The utility of the map will be located markers linked to disease resistance gene on the map for example in this study, Yamatouri possesses gene conferring resistance to CMV-B2 (Creb-2) which is controlled by a single dominant gene. Using F2 progenies of the same cross, the locus of Creb-2, can be readily identified in a similar manner and placed on the map. Therefore, our map is particularly useful to study the disease resistance gene organization in the genome of melons.

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REFERENCES


