Identification of a RAPD Marker Linked to the Susceptible Gene of Black Spot Disease in Japanese Pear

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Summary

Two hundred fifty random sequence primers were used to screen a pair of bulked DNA samples derived from open-pollinated progenies of Japanese pear ‘Osa Nijisseiki’ to identify RAPD (randomly amplified polymorphic DNA) markers linked to the susceptible A gene of black spot disease. Only one primer, CMNB41, among three primers which generated DNA fragments, was present in the susceptible bulk, but not in the resistant one. CMNB41 generated the DNA fragment, CMNB41/2350, which co-segregated with the susceptible A gene among the self-pollinated F₁ progenies of ‘Osa Nijisseiki’. This RAPD marker CMNB41/2350 is at a distance of about 3.1 cM from the susceptible A gene. Moreover, the frequency of occurrence of the CMNB41/2350 marker in susceptible cultivars and selected progenies of ‘Osa Nijisseiki’ x ‘Oharabeni’ was 96%.

Key Words: black spot disease, Japanese pear, RAPD marker, linkage analysis.

Introduction

Black spot disease of Japanese pear (Pyrus pyrifolia Nakai), caused by Alternaria alternata (Fr.) Keissler Japanese pear pathotype, is a serious fungal disease for the sensitive cultivars such as ‘Nijisseiki’, ‘Shinsui’ and ‘Nansui’ (Kozaki, 1973; Nishimura et al., 1978). The susceptibility of this fungal disease is controlled by a single dominant gene, A proposed by Kozaki (1973). Moreover, the susceptible cultivars are generally heterozygous (Aa) at this locus, whereas the resistant ones are homozygous recessive (aa) (Kozaki, 1973). Recently, several resistant mutants to black spot disease were selected from ‘Nijisseiki’ by chronic irradiation with gamma rays (Sanada et al., 1988); ‘Gold Nijisseiki’ is one of these mutants (Sanada et al., 1993). However, the resistance in ‘Gold Nijisseiki’ is incomplete and intermediate because the recessive mutation is confined to the L₁ II cell layer (Sanada et al., 1994).

To isolate and map the dominant A gene on the pear chromosome, closely linked molecular markers are needed for any genetic investigation in the future. In apple, linkage maps have been developed using methods, such as the bulked segregant analysis (BSA) (Michelmore et al., 1991), to identify molecular markers linked to major genes controlling scab resistance (Koller et al., 1994; Yang and Krüger, 1994; Yang et al., 1997), powdery-mildew resistance (Markussen et al., 1995), fruit color (Cheng et al., 1996) and morphological traits (Lawson et al., 1995). Randomly amplified polymorphic DNA (RAPD) markers combined with BSA have been useful in such genetic analysis.

In this paper, we describe a RAPD marker closely linked to the susceptible A gene of black spot disease in Japanese pear.

Materials and Methods

Plant material

To obtain susceptible and resistant bulk to black spot disease, 20 open-pollinated progenies of ‘Osa Nijisseiki’ were assayed in advance for tolerance of black spot disease. Sixty-five self-pollinated progenies of ‘Osa Nijisseiki’, growing in the field of Faculty of Agriculture, Shinshu University, were used for segregation analysis. Moreover, 31 commercial pear cultivars, susceptible or resistant to black spot disease and 19 susceptible hybrid progenies of ‘Osa Nijisseiki’ x ‘Oharabeni’ were checked for potential co-segregation of DNA fragments with the susceptible phenotypes. Fresh young leaves of these progenies and cultivars were prepared and stored frozen at -85 °C until DNA extraction.

Assay for black spot disease

All progenies used in this study were assayed for the tolerance to black spot disease using the leaf-necrosis assay of AK-toxin 1 (Nakashima et al., 1985) and done the spore-inoculation test by a virulent isolate (Kozaki,
1973). For the leaf-necrosis assay of AK-toxin 1, disks of young leaves were administered 5 μl of AK-toxin 1 at 10^{-3} to 10^{-5} M concentration on a portion of the leaf scarted faintly with a broken pasteur pipette. These leaf disks were incubated in a petri dish at 25°C for 2 days and the tolerance to black spot disease was judged from the degree of necrotic symptoms at each concentration of AK-toxin 1. For the spore inoculation test, the spore suspension of virulent isolate strain No.15A at the density of 5 × 10^6 spores/ml was sprayed to the unfolded first leaf. These leaves were also incubated in a petri dish at 25°C for 2 days, and the tolerance was evaluated as above.

**DNA extraction**

DNA extraction protocol was a modified cetyltrimethyl ammonium bromide (CTAB) procedure from those of Wagner et al. (1987) and Bousquet et al. (1990) with the additional steps of RNase treatment and phenol-chloroform extraction. Five hundred mg of leaf material was pulverized in liquid nitrogen to which 7 ml of washing buffer (100 mM Tris- HCl (pH 8.0), 0.35M sorbitol, 10% polyethylene glycol (MW 6,000) and 5% 2-mercaptoethanol) were added and homogenized. The homogenate was centrifuged at 14,000 rpm for 10 min. The supernatant was discarded and the pelvis was resuspended in 3 ml of extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris- HCl (pH 8.0), 20 mM EDTA, 0.05% 2-mercaptoethanol, 1% PVP) and brought to 1% N-lauroylsarcosine. The mixture was incubated at 65°C for 1 hr. After chloroform extraction, 6 ml of the precipitation buffer (1% CTAB, 50 mM Tris- HCl (pH 8.0), 10 mM EDTA) was added to the supernatant and the mixture was centrifuged at 10,000 rpm for 10 min. The pellet was resolved in 3 ml of high salt TE buffer (1 M NaCl, 10 mM Tris- HCl (pH 8.0), 1 mM EDTA) and 10 μl 2-mercaptoethanol was added to the mixture. After the mixture was incubated at 65°C for 30 min., 3 ml of isopropanol was added to the mixture and centrifuged; the pellet was resuspended in 700 μl of TE buffer (50 mM Tris- HCl (pH 8.0), 5 mM EDTA) and incubated with 10 μl of 0.01% RNase solution at 55°C for 30 min. The mixture was extracted with phenol-chloroform and the DNA in the aqueous phase precipitated with ethanol. The DNA in the residue was resuspended in the TE buffer and quantified using gel electrophoresis and compared to known DNA concentration.

**RAPD analysis**

For BSA, an equal amount (2 ng) of DNA from each 10 individual was pooled before being subjected to polymerase chain reaction (PCR). Two hundred fifty primers of single 10-mer kits (OPA01-J20, Operon Technologies Inc.) and 12-mer kits (CMNB40-89, Bex Co. Ltd.) were used to compare DNA profiles.

The PCR was conducted in 20 μl containing 20 ng of genomic DNA template, 0.4 μM of a primer, 0.5 U of Taq polymerase (TaKaRa Taq, Takara Biochemical, Tokyo, Japan), 10 mM Tris- HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, and 200 μM dNTPs. The reaction mixture was overlaid with two drops of mineral oil. Template DNA was initially denatured at 94°C for 1 min, followed by 40 cycles of PCR amplification using the following parameters: 45 s denaturation at 94°C, 75 s primer annealing at 37°C, and 2 min primer extension at 72°C. A final 8 min incubation at 72°C was allowed to complete primer extension. Amplification was performed on a programmable thermal cycler (model: OmnipGene HB-TR3-CM, Hybaid Ltd.). Amplified products were electrophoretically separated on 2.0 % agarose gels and stained with ethidium bromide; the gels were photographed on a transilluminator using Polaroid film. DNA digested with Hind III and pUC18 digested with Dra I, HintI and Eco O109I were used as molecular markers. Each fragment size of RAPD marker was determined by a sizer software program developed by Fujita N. (1990).

**Results and Discussion**

**Susceptibility and resistance of progenies of 'Osa Nijisseiki' and 'Osa Nijisseiki' × 'Oharaben'**

All progenies of the open-pollinated and selfed progenies of 'Osa Nijisseiki' and hybrids of 'Osa Nijisseiki' × 'Oharaben' were assayed for the tolerance to black spot disease with AK-toxin 1 and spore inoculation of virulent isolate strain No.15A. AK-toxin 1 at 10^{-3} to 10^{-5} M concentration caused veinal necrosis with varying degrees on the leaf disks of susceptible progenies. Higher concentration of AK-toxin 1 induced higher degree of necrotic symptom. However, the toxin did not cause such symptom on the disks of resistant ones because it is a host-specific nature (Nakashima et al., 1985). The spore inoculation also caused the same symptoms on the whole leaf of susceptible progenies.

The numbers of susceptible and resistant progenies were 43 and 22 among 65 self-pollinated progenies of 'Osa Nijisseiki' tested, respectively (Table 2). The number of susceptible progenies was slightly fewer than the expected ratio of 3 (susceptible) : 1 (resistant). This result suggests that the homozygous progenies susceptible to black spot disease may easily succumb to the disease (Kozaki, 1973).

On the contrary, the numbers of susceptible and resistant hybrids of 'Osa Nijisseiki' were 19 and 20 among 39 progenies tested, respectively (Table 3, Table 4). The segregation ratio corresponded the expected ratio of 1 (susceptible) : 1 (resistant) because 'Oharaben' is resistant to the disease.

**Primer screening and identification of CMNB41/2350**

Initial screening of two 250 primers against the susceptible and resistant bulk revealed three primers that generated DNA fragments which were present in the
susceptible bulk, but absent in the resistant bulk (Fig. 1). However, two of these primers, CMNB71 and OPB08, were subsequently shown to generate DNA fragments that did not correspond or co-segregate with the susceptibility of black spot disease when applied to the susceptible, self-pollinated progenies of ‘Osa Nijisseiki’. These DNA fragments generated from CMNB71 and OPB08 may have originated from pollen-derived DNA because open-pollinated progenies of ‘Osa Nijisseiki’ were used for the BSA study.

Only one primer, CMNB41, co-segregated with the polymorphic fragment (2350 bp) that was found to be closely linked to the susceptible A gene of black spot disease (Fig. 2). Thus, this fragment was designated as CMNB41/2350. The primer sequence of CMNB41 is 5’GACAGCGTCTCA3’ and the GC content is 58%. With the CMNB41 primer, six DNA fragments with and without the marker segregated with varying ratios. CMNB41/2350 obtained from the self-pollinated progenies of ‘Osa Nijisseiki’ segregated with a 43 : 22 ratio (Table 1). Therefore, these RAPD markers may be heterozygous in ‘Osa Nijisseiki’ because they, except for CMNB41/2350, did not co-segregate with the susceptible A gene.

Table 2 shows the result of linkage analysis between the susceptibility of black spot disease and the RAPD marker CMNB41/2350 in 65 self-pollinated progenies of ‘Osa Nijisseiki’. Out of the 43 susceptible progenies, 42 contained the CMNB41/2350 fragment, whereas 22 resistant progenies except one did not. The recombination ratio between the RAPD marker CMNB41/2350

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**Fig. 1.** Electrophoresis gel of RAPD markers which detect polymorphisms between the susceptible and resistant bulk by three different primers, CMNB41, CMNB71 and OPB08. The polymorphisms distinguishing the bulks are indicated by a solid arrowhead. S: Susceptible bulk, R: Resistant bulk, M: Molecular size marker (bp).

**Fig. 2.** Electrophoresis gels of RAPD patterns with the presence or absence of the CMNB41/2350 marker in the self-pollinated progenies of ‘Osa Nijisseiki’. A solid arrowhead indicates the CMNB41/2350 marker, and a hollow arrowhead indicates the absence or presence of the marker in the susceptible or resistant progenies. S: Susceptible bulk, R: Resistant bulk, M: Molecular size marker (bp).
Table 1. Segregation analysis of CMNB41 markers in the self-pollinated progenies of 'Osa Nijisseiki'.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Segregation of seedlings for CMNB41 makers</th>
<th>Expected ratio</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>B41/2790</td>
<td>Present 46 : Absent 19</td>
<td>3 : 1</td>
<td>0.62</td>
<td>0.43</td>
</tr>
<tr>
<td>B41/2690</td>
<td>Present 49 : Absent 16</td>
<td>3 : 1</td>
<td>0.01</td>
<td>0.94</td>
</tr>
<tr>
<td>B41/2350</td>
<td>Present 43 : Absent 22</td>
<td>3 : 1</td>
<td>2.71</td>
<td>0.10</td>
</tr>
<tr>
<td>B41/1720</td>
<td>Present 43 : Absent 22</td>
<td>3 : 1</td>
<td>2.71</td>
<td>0.10</td>
</tr>
<tr>
<td>B41/1240</td>
<td>Present 54 : Absent 11</td>
<td>3 : 1</td>
<td>2.26</td>
<td>0.13</td>
</tr>
<tr>
<td>B41/1160</td>
<td>Present 44 : Absent 21</td>
<td>3 : 1</td>
<td>1.85</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* Molecular weight

Table 2. Linkage analysis between the susceptibility of black spot disease and the RAPD marker CMNB41/2350 in the self-pollinated progenies of 'Osa Nijisseiki'.

<table>
<thead>
<tr>
<th>Phenotype to black spot disease</th>
<th>Plants (no.)</th>
<th>Segregation of seedlings for CMNB41/2350</th>
<th>$\chi^2$ (9 : 3 : 3 : 1)</th>
<th>$P$</th>
<th>Recombination ratio (%) ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>43</td>
<td>Present 42 : Absent 1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistant</td>
<td>22</td>
<td>Present 1 : Absent 21</td>
<td>92.0</td>
<td>&lt;0.001</td>
<td>3.1 ± 0.6</td>
</tr>
</tbody>
</table>

and the susceptible A gene of black spot disease has a linkage of 3.1 cM. Therefore, the cloning and sequencing of the RAPD marker will be needed for map-based cloning and isolation of the susceptible A gene.

 Screening different genotypes with CMNB41/2350

Analysis of the RAPD marker CMNB41/2350 in the susceptible selected lines and cultivars to black spot disease showed a close linkage between the RAPD marker and the susceptibility (Fig. 3). The presence or absence of CMNB41/2350 fragment in the genotypes tested is summarized in Table 3. Of the 19 susceptible progenies of 'Osa Nijisseiki' x 'Oharabeni', and 6 cultivars, including 'Gold Nijisseiki' which is a chimeric mutant of 'Nijisseiki' (Sanada et al., 1994), 24 had the RAPD marker CMNB41/2350. This corresponds to a 96% frequency of occurrence which strongly indicates that this RAPD marker is closely linked to the susceptible A gene at a genetic mapping distance of 3.1 cM. There-

 Fig. 3. Electrophoresis gel of RAPD patterns amplified by CMNB41 in the susceptible cultivars and selected lines. S : Susceptible bulk, R : Resistant bulk, 1–6 are 'Nijisseiki', 'Osa Nijisseiki', 'Gold Nijisseiki', 'Shinsui', 'Kimizukawase', and 'Nansui'; 7–14 are progenies of 'Osa Nijisseiki' x 'Oharabeni'; R-1, 2, 4, 5, 8, 11, 12, and 18. M : Molecular size marker (bp).

Table 3. Relationship between CMNB41/2350 marker and susceptible genotypes to black spot disease.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cultivars and lines with (+) or without (−) CMNB41/2350 marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible lines</td>
<td>Progenies of 'Osa Nijisseiki' G' - 4, 9, 26, 40, 41, 45, 53,</td>
</tr>
<tr>
<td>x 'Oharabeni'</td>
<td>R' - 1, 2, 4, 5, 8, 11, 12, 18, 19, 31, 39</td>
</tr>
<tr>
<td>Susceptible cultivars</td>
<td>Nijisseki, Osa Nijisseki, Gold Nijisseki, Shinsui, Kimizukawase, Nansui</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
</tr>
</tbody>
</table>

* G is the line with green leaves, R is one with red leaves.
Table 4. Relationship between CMNB41/2350 marker and resistant genotypes to black spot disease.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cultivars and lines with (+) or without (−) CMNB41/2350 marker</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resistant lines</strong></td>
<td></td>
</tr>
</tbody>
</table>
| Progenies of ‘Osa Nijisseiki’ × ‘Oharabeni’                    | G 2−1, 5, 6, 8, 10, 11, 16, 20, 24, 28, 30, 39, 42, 51, 54,  
| R − 21                 | R − 14, 15, 16, 40                                            |
| **Resistant cultivars** |                                                               |
| Hosui, Shinko, Echigoshiki, Okusankichi, Niitaka, Waseaka, Chojujo, Imamuraaki | Kosui, Kikusui, Kikusui, Yasato, Suisei, Ishiwase, Shugyoku,  
|                        | Kamoi, Hakko, Choju, Wasekozo, Tama, Taihaku, Yahri, Inomiyahakuri, Oharabeni, Max Red Bartlett |
| **Total**               |                                                               |
| 9                       | 36                                                            |

G is the line with green leaves, R is one with red leaves.

Fig. 4. Electrophoresis gels of RAPD patterns amplified by CMNB41 in the resistant cultivars.

fore, CMNB41/2350 provides a useful genetic marker for the susceptible A gene of black spot disease.

However, the RAPD marker CMNB41/2350 was also found in the 8 resistant cultivars ‘Hosui’, ‘Shinko’, ‘Niitaka’, ‘Chojujo’, ‘Echigoshiki’, ‘Okusankichi’, ‘Waseaka’, and ‘Imamuraaki’ among the 25 resistant cultivars tested (Fig. 4, Table 4). Most of these cultivars with the RAPD marker are old cultivars except for ‘Hosui’ and ‘Shinko’ which suggests that recombination may have occurred between the RAPD marker CMNB41/2350 and the susceptible A gene as a result of artificial selection and transmitted to their descendants. For example, the pollen parent of ‘Shinko’ is presumed to be ‘Waseaka’ with the RAPD marker (Kajiura and Sato, 1990). ‘Amanokawa’, which is a parent of ‘Echigoshiki’ and ‘Niitaka’, is presumed to be a seedling of ‘Imamuraaki’ which has the RAPD marker (Kajiura and Sato, 1990). When a recombination occurs, 50 % of the progenies should inherit the marker regardless of the tolerance to black spot disease. The possibility of such recombination will be elucidated by the segregation analysis of the RAPD marker CMNB41/2350 in these cultivars.

Acknowledgment

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ナシ黒斑病感受性遺伝子に連鎖するRAPDマーカーの同定
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要約

ナシ黒斑病感受性A遺伝子に連鎖するRAPDマーカーを同定するために、ニホンナシ品種‘おさ二十世紀’の自然交配実生の中から感受性および抵抗性のDNAパルクを設計し、250種類のランダムプライマーを用いてスクリーニングした。黒斑病感受性パルクにのみ存在し、抵抗性パルクには存在しないDNA断片を有する3種類のプライマーのうち、‘おさ二十世紀’の自家交配後代の解析により、CMNB41のプライマーだけが、黒斑病感受性A遺伝子と連鎖するDNA断片CMNB41/2350を有することが明らかになった。このRAPDマーカーCMNB41/2350と黒斑病感受性A遺伝子の遺伝距離は約3.1 cMであった。さらに、黒斑病感受性品種および‘おさ二十世紀’×‘大原紅’の交配系統におけるこのCMNB41/2350マーカーの出現頻度は96%であった。