Identification and Precise Mapping of Resistant QTLs of Cercospora Leaf Spot Resistance in Sugar Beet (Beta vulgaris L.)

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ABSTRACT The complex inheritance of resistance to Cercospora leaf spot (CLS), the most severe fungal foliar disease in sugar beet, was investigated by means of quantitative trait loci (QTL) analysis. Over a three year period, recombinant inbred lines (RILs) of sugar beet (Beta vulgaris L.), generated through a cross between lines resistant ('NK-310mm-O') and susceptible ('NK-184mm-O') to CLS, were field-tested for their resistance to the pathogen. Composite interval mapping (CIM) showed four QTL involved in CLS resistance to be consistently detected. Two resistant QTL (qcr1 on chromosome III, qcr4 on chromosome IX) bearing 'NK-310mm-O' derived alleles promoted resistance. Across 11 investigations, the qcr1 and qcr4 QTL explained approximately 10% and over 20%, respectively, of the variance in the resistance index. Two further QTL (qcr2 on chromosome IV, qcr3 on chromosome VI) bearing 'NK-184mm-O' derived alleles each explained about 10% of the variance. To identify the monogenic effect of the resistance, two QTL derived from 'NK-310mm-O' against the genetic background of 'NK-184mm-O', using molecular markers. The qcr1 and qcr4 were precisely mapped as single QTL, using progenies BC5F1 and BC2F1, respectively. The qcr1 that was located near e11m36-8 had CLS disease severity indices (DSI) about 15% lower than plants homozygous for the 'NK-184mm-O' genotype. As with qcr1, heterozygosis of the qcr4 that was located near e17m47-81 reduced DSI by about 45% compared to homozygosis. These two resistant QTL might be of particular value in marker-assisted selection (MAS) programs in CLS resistance progression.

KEYWORDS Cercospora leaf spot disease resistance mapping QTL sugar beet

Caused by the fungus Cercospora beticola Sacc., Cercospora leaf spot (CLS), one of the most serious and widespread foliar diseases of sugar beet, typically provokes necrotic lesions, leading to a rapid and progressive destruction of the plant’s foliar apparatus (Holtschulte 2000). The continued replacement of new leaves occurs at the expense of reserve substances stored in sink tissues, and leads to a reduction in yield and sugar content. Yield losses of as much as 42 to 50% have been reported for CLS-infected beet crops (Smith and Martin 1978; Verreet et al. 1996). CLS control programs have sought to prevent disease infection by using resistant cultivars, applying fungicides, and rotating beets with non-host crops. As part of these efforts, sugar beet (Beta vulgaris L.) geneticists and breeders have sought to breed CLS resistance.

Lewellen and Whitney (1976) identified monogenic resistance to the C. beticola race C2-induced CLS in a sugar beet cultivar; however, this resistance proved to be unstable and the cultivar was abandoned (Koch and Jung 2000). Some wild relatives of sugar beet have shown CLS resistance; B. procumbens C. Sm. shows CLS resistance, but is sexually incompatible with B. vulgaris (Panella and Frese 2000). However, some B. vulgaris spp. maritima accessions showing strong resistance to CLS have served as a source of CLS resistance in sugar beet (Leuterbach et al. 2004). An accession of B. vulgaris spp. maritima
collected in the Po River delta by Dr. Munerati was backcrossed with sugar beets, and their resultant offspring became breeding material (Coons et al. 1955). These offspring reached the United States, were propagated there, and were then redistributed to the world (Skaracis and Biancardi 2000). The resistance achieved was effective in lowering the rate of infection in sugar beets, or in delaying the infection process (Rossi et al. 1996). However, the introduction of these resistance traits to other breeding lines was difficult given that their inheritance did not follow a simple Mendelian pattern, but rather was quantitative (Saito 1966). The resultant resistance was assumed to be controlled by at least four or five genes whose effects varied depending on the severity of infection (Smith and Gaskill 1970). Broad-sense heritability and realized heritability were estimated to be 60–70% and 25%, respectively, while variation caused by environmental factors ranged from 44 to 62% (Smith and Ruppel 1974). Due largely to environmental factors that affect the expression of resistance at the field level, mass selection for resistant phenotypes, either by natural infection or artificial inoculation, has made little progress. An apparent negative correlation between the CLS resistance and sugar yield (Saito 1966; Koch 1970) further complicated the task of these breeding programs. Genetic approach of CLS resistance, which can be aided by molecular markers, can help break the potential linkage between CLS resistance and unfavorable traits. Schäfer-Pregl et al. (1999) and Nilsson et al. (1999) conducted quantitative trait loci (QTL) analyses of CLS intensity in sugar beet lines consisting of an F2 population and F3 families. They reached a similar conclusion to Smith and Gaskill (1970), namely that at least four or five QTL were involved. Similarly, two QTL analyses conducted by Setiawan et al. (2000)—one based on a field test under natural infection condition and another using a leaf disk test—detected at least four QTL. While these studies were pivotal in elucidating the genetics of CLS resistance in sugar beet, questions remained as to the precise map positions of the QTL, as well as their respective gene products and effects.

We launched a multitask research program on the genetic analysis of sugar beet resistance to multiple diseases, including Aphanomyces root rot, CLS and Rhizoctonia root rot. An initial field-based screening of Japanese sugar beet lines identified breeding line ‘NK-310mm-O’ as a source of a high level of resistance to multiple diseases (Taguchi et al. 2007). This prompted us to characterize the genetic nature of these resistance traits, with a goal of establishing a marker-assisted selection (MAS) system for resistance to these diseases. This would enable the rapid development of a sugar beet line with all known resistances under field, or in delaying the infection process.

Field trials were carried out in HARC fields, in Memuro, Japan. For initial QTL analysis, the experiment was set up as a randomized block design with four replications (2005 and 2006) or two replications (2007). Individual plot size was 1.35 m², and the final plant density was ten plants per plot (= 70000 plants ha⁻¹). For selection of backcross progenies, a similar design, with four replications, was used in 2008. For precise QTL mapping, (conducted in 2009), sugar beets were planted in a zigzag pattern; each subject plant was enclosed by a barrier of resistant plants, to prevent them from contacting each other (Taguchi et al. 2002). The initial plant density was 70000 plants ha⁻¹, and declined to roughly 3500 plants ha⁻¹ after the removal of barrier plants, before the evaluation of CLS resistance. Individual plot size was 32.4 m², and the final plant density was 120 plants.

Seeds were sown in paper pots (19 mm diameter and 13 cm height, Nippon Beet Sugar Mfg. Co., Ltd.) in early April. One month later, seedlings were transplanted to the field. Cercospora beticola inoculum was prepared as follows: petioles of sugar beet leaves expressing severe CLS symptom were collected from HARC fields, dried, and ground to a powder. In early July of the subsequent year, inoculum (5 g) was applied at the foot of each plant. Initial symptoms were observed roughly one month after inoculation. Visual symptoms of CLS were rated on an index ranging from zero for no symptoms, to one for fully destroyed main leaves in each 10 plants per replication. Data for RILs, NILs and BLs were averaged across replications in each investigation time.

DNA isolation and genotyping with molecular markers

Total cellular DNA was extracted from fresh leaves according to the procedure of Roger and Bendich (1988). Amplified Fragment Length Polymorphism (AFLP) was detected using an AFLP Analysis System I (Invitrogen, Carlsbad, CA, USA). The restriction endonucleases EcoRI and MseI were used in this analysis. The adapter-ligated DNA was pre-amplified with primers having a single selective nucleotide. For selective amplification, EcoRI-NNN and MseI-NNN primers were used to amplify the desired endonucleases. Amplified products were electrophoresed using a High Efficiency Genome Scanning (HEGS) system (Hori et al. 2003; Kikuchi et al. 2003), in which discontinuous non-denatured polyacrylamide gel and TBE buffer were used. The gels were scanned after staining with Vistra Green I (GE Healthcare UK, Amersham Place, England) or Sybr green I (Molecular Probes, Eugene, OR, USA) and photographed under a UV transilluminator (ATTO, Tokyo, Japan). Cleaved Amplified Polymorphic Sequence (CAPS) markers were developed as follows. PCR products were generated using primer sets, as described by Möhring et al. (2005), Hunger et al. (2003), and Schneider et al. (1999), then digested with one of thirteen restriction endonucleases: HaellI, Hhat, TaqI, HapII, Mbol, AfaI, Xspi, AluI, and AcclII (Takara Bio, Ohtsu, Japan);
Table 1 Disease severity indices (DSI) means for Cercospora leaf spot (CLS) under inoculated field evaluation

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Monohomare (medium-weak)</td>
<td>9.05</td>
<td>9.12</td>
<td>9.20</td>
<td>8.08</td>
<td>8.17</td>
<td>8.23</td>
</tr>
<tr>
<td>Monohikari (medium)</td>
<td>—</td>
<td>—</td>
<td>1.3</td>
<td>3.7</td>
<td>4.4</td>
<td>4.7</td>
</tr>
<tr>
<td>Yukihinode (strong)</td>
<td>2.5</td>
<td>3.1</td>
<td>4.1</td>
<td>0.4</td>
<td>2.5</td>
<td>3.4</td>
</tr>
<tr>
<td>NK-310mm-O (very strong)</td>
<td>1.3</td>
<td>1.5</td>
<td>2.1</td>
<td>0.2</td>
<td>1.7</td>
<td>2.7</td>
</tr>
<tr>
<td>NK-184mm-O (weak)</td>
<td>4.3</td>
<td>4.6</td>
<td>4.9</td>
<td>1.2</td>
<td>4.0</td>
<td>4.9</td>
</tr>
<tr>
<td>F1</td>
<td>2.0</td>
<td>2.6</td>
<td>3.0</td>
<td>1.0</td>
<td>2.8</td>
<td>3.5</td>
</tr>
<tr>
<td>F-test</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
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<tr>
<td>LSD(5%)</td>
<td>0.7</td>
<td>0.7</td>
<td>0.6</td>
<td>0.3</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>LSD(1%)</td>
<td>1.0</td>
<td>0.9</td>
<td>0.8</td>
<td>0.4</td>
<td>0.6</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*p < 0.01. LSD, least significant differences.
The actions of the four QTL were contrasting: qcr1 and qcr4 decreased DSI when plants had ‘NK-310mm-O’ alleles, and their effects were additive: 20.12 to 20.45 for qcr1 and 20.17 to 20.64 for qcr4. The CLS resistance conferred by qcr4 was always greater than that derived from qcr1 (Table 3). QTL qcr2 and the qcr3 were also additive, but increased DSI in plants bearing ‘NK-310mm-O’ alleles. The additive effects of the qcr2 and the qcr3 were estimated to be 0.11 to 0.57, and 0.14 to 0.44, respectively. The ‘NK-310mm-O’ genotypes with respect to the marker mp0117/HaeIII around qcr2 had DSI that was an average of 14% higher than the ‘NK-184mm-O’ genotype in RILs. Around the qcr3 BvATT6, it had DSI that was an average of 18% higher than the ‘NK-184mm-O’ genotypes.

**Verifying the allelic differences on the detected resistance QTL**

In examining the phenotype of plants having either of the two resistant QTL, qcr1 and qcr4, qcr1, it was discovered to be located near Acr1, a resistance gene for Aphanomyces root rot whose source was ‘NK-310mm-O’ (Taguchi et al. 2010). The hypothesis that map positions of qcr1 and Acr1 being so close meant that plants selected for

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**Table 2 Frequency distribution for CLS indices in RILs of ‘NK-310mm-O’ × ‘NK-184mm-O’**

<table>
<thead>
<tr>
<th>Year</th>
<th>Date</th>
<th>Intensity</th>
<th>Mean DSI</th>
<th>Frequency of DSI for Cercospora Leaf Spot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>0  1  2  3  4  5</td>
</tr>
<tr>
<td>2005 (n = 80)</td>
<td>9.05</td>
<td>medium-early</td>
<td>2.3 1.1</td>
<td>15 17 29 15 4</td>
</tr>
<tr>
<td></td>
<td>9.12</td>
<td>medium-late</td>
<td>3.0 1.1</td>
<td>3  17 21 22 17</td>
</tr>
<tr>
<td></td>
<td>9.02</td>
<td>late</td>
<td>4.0 1.0</td>
<td>0  4 13 18 45</td>
</tr>
<tr>
<td>2006 (n = 80)</td>
<td>8.08</td>
<td>early</td>
<td>0.7 0.3</td>
<td>68 12 0 0</td>
</tr>
<tr>
<td></td>
<td>8.17</td>
<td>medium-early</td>
<td>2.8 0.7</td>
<td>0  14 31 33 2</td>
</tr>
<tr>
<td></td>
<td>8.23</td>
<td>medium-late</td>
<td>3.8 0.6</td>
<td>0  0 8 42 30</td>
</tr>
<tr>
<td></td>
<td>8.28</td>
<td>late</td>
<td>4.1 0.6</td>
<td>0  0 4 28 48</td>
</tr>
<tr>
<td>2007 (n = 80)</td>
<td>8.13</td>
<td>early</td>
<td>1.3 0.9</td>
<td>43 21 15 1  0</td>
</tr>
<tr>
<td></td>
<td>8.20</td>
<td>medium-early</td>
<td>2.4 1.1</td>
<td>16 19 31 11 3</td>
</tr>
<tr>
<td></td>
<td>8.28</td>
<td>medium-late</td>
<td>3.3 1.1</td>
<td>5  9 19 32 15</td>
</tr>
<tr>
<td></td>
<td>9.02</td>
<td>late</td>
<td>3.9 1.0</td>
<td>0  0 8 9 22 41</td>
</tr>
</tbody>
</table>

DSI, disease severity index (0–5 scale [0 = no symptoms, 5 = almost complete necrosis]); SEM, standard error of the mean.

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**Figure 1** Linkage map based on RILs (‘NK-310mm-O’ × ‘NK-184mm-O’). Markers labeled “e-” were AFLP markers, while markers labeled “/C3/C3” were CAPS markers. Marker intervals are indicated in cM. The total map length is 867 cM.
Acr1 were also CLS resistant despite the linked qcr1 was tested. Five near isogenic lines (NILs; BC3F3) and five back-crossed lines (BLs; BC3F3) selected for a probable chromosomal region containing the Acr1 QTL but having an otherwise 'NK-184mm-O' nuclear background (Taguchi et al. 2010) were planted to examine CLS resistance. With a mean DSI (Sept. 10) of 2.4, 'NK-310mm-O' plants exhibited a lower DSI than the strong resistant variety 'Stout' on (Table 4). Under the same conditions, 'NK-184mm-O' plants were severely damaged (mean DSI of 4.6). The DSIs of the five NILs and the five BLs-CMS lines ranged from 3.5 to 4.8, of which NIL-2, NIL-3, BL-CMS-2, and BL-CMS-3 were the most resistant (Table 4). One of the two resistant backcross lines, BL-CMS-2, which was absent from tk/Xsp1 to e10m37-9 (Taguchi et al. 2010), was selected and crossed with 'NK-184mm-O' to generate a population segregating the CLS resistance likely governed by the qcr1. In 2009, QTL analysis of CLS resistance in this population (BC3F3) was conducted using a SIM method. The LOD score and the estimated additive effect assessed through the SIM method are presented in Table 5. The qcr1 was located near the PCR marker e11m36-8, on chromosome III (Figure 2A). The explained variance was roughly 45% (Table 5). Plants heterozygous with respect to genotype marker around qcr1 had Cercospora leaf spot disease severity indices (DSI) about 15% lower than plants homozygous for the 'NK-184mm-O' genotype. To assess the effect of qcr4, graphical genotypes of the 80 RILs derived from 115 molecular markers were used to select lines bearing the qcr4 but not the qcr1 QTL. Line 'RIL 56' was selected as meeting this criterion, and was crossed with 'NK-184mm-CMS'. The resultant B1F1 was back-crossed with 'NK-184mm-O', to generate two BC2F1-CMS populations each segregating the qcr4. In 2009, plants of the population were planted for QTL analysis of CLS resistance by the SIM method. The qcr4 was located near the PCR marker e17m47-81, on chromosome IX (Figure 2B). The explained variance was roughly 46%. Plants heterozygous with respect to genotype marker around qcr1 had Cercospora leaf spot disease severity indices (DSI) about 45% lower than plants homozygous for the 'NK-184mm-O' (Table 5).

**DISCUSSION**

Genetic analysis of CLS resistance in sugar beet, carried out in a number of previous studies, revealed the quantitatively inherited nature of the resistance (Smith and Ruppel 1974; Saito 1966), but the
chromosomal location of the genes responsible remained obscure. This occurred because the CLS resistance introduced from wild relatives may have been constituted by multiple genes with weak effects, making it difficult to identify individual genes as Mendelian factors. To overcome this difficulty, genetic analysis of a resistant source using well-characterized genetic stocks, such as RILs and NILs, may be a solution. Consequently, in the present study we sought to identify QTL for CLS resistance from ‘NK-184mm-O’ under the field conditions.

The four QTL identified (qcr1, qcr2, qcr3 and qcr4) affected CLS resistance differently. The qcr4 QTL was the most stable of the four, showing large LOD scores in all eleven trials, and explained over 20% of phenotypic variance in all investigations. The other three QTL were rather unstable compared to the qcr4, their LOD peaks sometimes being beneath the threshold of significance. This might be attributable to environmental factors and/or plant conditions. For example, Saito (1966) pointed out that variation in CLS resistance was influenced by leaf age. Moreover, alleles of the qcr2 and the qcr3 of ‘NK-310mm-O’ appeared to confer CLS susceptibility, which led us to infer that, although ‘NK-310mm-O’ was the highly resistant line, there might be room to improve its resistance to CLS.

The qcr1 and the qcr2 were mapped to chromosomes III and IX, respectively. Various sugar beet chromosomes have been associated with QTL for CLS resistance; Schäfer-Pregl et al. (1999) detected LOD peaks on chromosomes II, III, VI, and IX in F1 families and on chromosomes IV and V in F2 data. Nilsson et al. (1999) mapped five QTL on chromosome I, II, III, and IX, of which two were on chromosome III (T.

Kraft, personal communication). The QTL mapped by Setiawan et al. (2000) were on chromosomes IV, VII, VIII (two QTL), and IX in their field test, and III, IV, VII, and IX in their leaf disc test. Because their experimental conditions and genetic model differed from ours, a direct comparison of results may be inappropriate; however, it seems significant that chromosomes III and IX have always been associated with the QTL of CLS resistance. Thus, it appears possible that CLS resistance in sugar beet involves genes located on chromosomes III and IX.

For MAS selection or molecular investigation of the qcr1 and qcr4 QTL, knowledge regarding their individual effects and precise map position will be useful. We genetically approached the CLS resistance to clarify the contribution of the individual qcr1 and qcr4 QTL. A similar approach was used in dissecting rice heading QTL (Yano et al. 1997; Yamamoto et al. 2000). Assuming that molecular markers are a sufficiently stringent criterion for the introduction (as is now feasible) of a candidate chromosomal region of interest into progeny for verifying the detected QTL, particular care must be taken to accurately evaluate phenotypes, as the effect of a single QTL may be small. Therefore, individual subject plants were isolated from one another by planting CLS resistant beets as barriers. This procedure was expected to prevent subjects from touching infected beets. Subsequently, it caused easier observation of the whole plant phenotype. As a result, map positions of the qcr1 and qcr4 were confined to chromosomal segments, and could be estimated as single QTL. Expained variance figures for the qcr1 and qcr4 QTL suggested that plants having one or both QTL in the heterozygous form exhibited higher resistance than ‘NK-184mm-O’.

The map position of the qcr1 is consistent with the location of the resistance gene cluster in the sugar beet genome. A number of important sugar beet disease resistance genes have been mapped to chromosome III: CLS resistance QTL (Schäfer-Pregl et al. 1999; Setiawan et al. 2000; Nilsson et al. 1999), Rhizomania resistance genes Rz1 to Rz5 (Barzen et al. 1999; Scholten et al. 1999; Gidner et al. 2005; Grimmer et al. 2007), and Aphomomyces root rot resistance gene Acr1 (Taguchi et al. 2010). In addition, a number of resistance gene analogs (RGAs) have been cloned from sugar beet, some of which have been mapped to a gene cluster on chromosome III (Lein et al. 2007). Further study of sugar beet genomics is forthcoming to clarify the evolution of sugar beet resistance gene clusters.

The chromosome III also contains another important gene, X, a restorer of fertility for Owen CMS (Owen 1945; Higahara et al. 2005). The map position of the X is represented by a molecular marker MP-A16, a PCR marker which co-segregates with X. The close linkage of the X, qcr1, and Acr1 suggests a possible linkage drag in the breeding of ‘NK-310mm-O’. This line, which originated from breeding line ‘Tmm-1’, a Japanese donor source of the monogerm trait, was conferred by the m gene on chromosome IV (Barzen et al. 1995; Schondelmaier and Jung 1997). Although it appeared to be a highly heterogeneous population, no one had recognized any disease resistance traits in ‘Tmm-1’. In the 1960s, ‘Tmm-1’ was subjected to the selection for maintainer genotypes to obtain a breeding line having both the monogerm trait and maintainer genotype. After the second-
round maintainer selection, a pedigree whose Aphanomyces root rot resistance was as high as that of 'NK-310mm-O' emerged (data not shown). The maintainer selection increased the frequency of a non-restoring allele of $X$, known to be very rare in the sugar beet population (Bosemark 2006). Since $Acr1$ and $qcr1$ are linked to the non-restoring allele (i.e., 'NK-310mm-O' is a maintainer line), the maintainer selection may have resulted in an increased frequency of $Acr1$ and $qcr1$ QTL, which likely lurked as infrequent alleles in 'Tmm-1'. Although our speculation needs to be supported by additional data and other scenarios are possible, there may be concern that maintainer

**Figure 2** Precise linkage map around the detected two resistant QTL, $qcr1$ (A) and $qcr4$ (B). The frequency distribution for CLS indices in BC$_2$F$_1$ (A) and BC$_3$F$_1$ (B). Different genotypic classes, as defined at the nearest marker locus for the QTL peak in each population. White represents individuals heterozygous for the allele from the resistant parent, and gray represents individuals homozygous for allele from the susceptible parent. **p < 0.01. m, mean ± SE.**
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