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Mapping quantitative trait loci (QTLs) for resistance to *Cercospora* leaf spot disease (*Cercospora beticola* Sacc.) in sugar beet (*Beta vulgaris* L.)

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Abstract The breeding of sugar beet varieties that combine resistance to *Cercospora* and high yield under non-diseased conditions is a major challenge to the breeder. The understanding of the quantitative trait loci (QTLs) contributing to *Cercospora* resistance offers one route to solving this problem. A QTL analysis of *Cercospora* resistance in sugar beet was carried out using a linkage map based on AFLP and RFLP markers. Two different screening methods for *Cercospora* resistance (a field test at Copparo, Italy, under natural infection, and a newly-developed leaf disc test) were used to estimate the level of *Cercospora* resistance; the correlation between scores from the field (at 162 days after sowing) and the leaf disc test was significant. QTL analysis was based on F_2 and F_3 (half-sib family) generations derived from crosses between diploid single plants of 93164P (resistant to *Cercospora* leaf spot disease) and 95098P (susceptible). Four QTLs associated with *Cercospora* resistance (based on Lsmean data of the leaf disc test) on chromosomes III, IV, VII and IX were revealed using Composite interval mapping. To produce populations segregating for leaf spot resistance as a single Mendelian factor, we selected for plants heterozygous for only one of the QTLs (on chromosome IV or IX) but homozygous for the others.

Key words *Beta vulgaris* · *Cercospora beticola* · AFLP · QTL

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Introduction

One of the most serious and widely distributed foliar diseases of sugar beet in the world is *Cercospora* leaf spot disease, caused by the fungus *Cercospora beticola* Sacc. (Smith and Ruppel 1974). Resistance breeding can help to maintain crop yield even under severe disease pressure while reducing the levels of fungicide used. The development of tolerant or resistant varieties can increase sugar yield by up to 45% in the presence of *Cercospora* infection (Schäufele and Wevers 1996). Koch (1972) reported that under severe disease pressure higher sugar content and juice purity was achieved by *Cercospora* resistant varieties. However, the breeding of highly resistant hybrids that have a root yield potential equal to that of susceptible hybrids grown in the absence of *Cercospora* infection is still a major challenge for plant breeders (Smith and Campbell 1996).

The genetics of *Cercospora* resistance is not well understood; it is inherited quantitatively, with the main effects controlled by at least four or five major genes (Smith and Gaskill 1970). Realised heritabilities of approximately 0.25 and nearly identical narrow-sense heritabilities were reported by Smith and Ruppel (1974). Based on variance analyses, two different broad-sense heritabilities, i.e. 0.379 and 0.555, were found from crosses between a resistant and two different susceptible inbred lines (Smith and Ruppel 1974). Progress in breeding for resistance to *Cercospora* has been slower than for *Rhizoctonia* resistance in sugar beet, primarily because the resistance to *Rhizoctonia* has a higher heritability (Panella 1998).

Two factors often cause disappointingly slow progress during selection for resistance to broadly adapted pathogens: first, no sufficiently reliable screening method is available and second, resistance may be associated with undesirable agronomic traits (Parlevliet 1989). The KWS scoring scale (Anonymous 1970) is generally adopted as the standard method to screen for *Cercospora* resistance genotypes. Although this method may suffice for a crude ranking of genotypes (resistant, medium and susceptible),

a more precise quantitative scale is needed to facilitate a deeper genetic analysis of *Cercospora* resistance. In order to solve the second problem, plant breeders can use molecular markers as a tool to improve the efficiency of plant breeding. Based on a linkage map generated from molecular markers it is possible to locate the gene of interest [quantitative trait locus (QTL) localisation] and ultimately try to clone individual QTLs.

The aim of the investigation reported here was to localise putative QTL associated with *Cercospora* resistance in sugar beet. The mapping of QTLs for *Cercospora* resistance may provide an approach to the problem stated by Smith and Campbell (1996) of how to create a sugar beet that combines resistance with high yield in the presence or absence of *Cercospora* infection.

Materials and methods

Plant material

The F_2 population was derived by selfing a single F_1 plant of the cross between single diploid plants of line 93164P (as mother plant) and line 95098P (as pollinator). Parental line 93164P is male-sterile and resistant to *Cercospora* leaf spot disease, and pollinator line 95098P is susceptible to *Cercospora* leaf spot but has a good yielding ability.

Leaf spot resistance tests

Field trials were carried out in Copparo, north of Bologna, Italy, a location well known to have severe *Cercospora* epidemics on a regular basis. For the field trial, 89 F_3 half-sib families were used, obtained from the open pollination of F_2 plants. The experimental design was a lattice design (5×5) with two replications. The plot size was 8.1 m^2 with a final plant density of 84 plants per plot (=103,700 plants/ha). Seeds were planted using a commercial planter in March 1996, 24 cm apart within the row; harvest was in October 1996. The visual symptoms of the leaf spot severity ratings were recorded according to the KWS-scale index (1=no disease to 9=fully diseased) as described previously (Anonymous 1970). The data were averaged over the replications.

For the leaf disc resistance test, 196 individual F_2 plants (including the 89 F_2 plants used for the half-sib family production and testing described above) were cloned *in vitro*. From each F_2 individual, 2 cloned plants (designated A and B) were tested independently. The plants were maintained in the greenhouse at 20°C and 16 h of light. No pesticides or fungicides were applied during the growing period of the plants.

The leaf disc test was done principally according to Koch (1997). The leaf discs (approx. 14 mm in diameter) were cut from single fresh, healthy, fully developed sugar beet leaves and placed in single rows (8 leaf discs/row) in plastic petri dishes (20×20 cm) on the surface of 5% (w/v) water agar. The leaf discs were inoculated with aqueous suspensions of *C. beticola* spores from the isolate Pi maintained on potato dextrose agar (PDA agar, Difco Lab, Detroit). One-milliliter aliquots of the spore suspension, adjusted to an inoculum density of 100,000 spores/ml, were evenly sprayed onto the surface of the leaf discs surface using an atomiser connected to an air-compressor. The inoculated leaf discs were kept in a growth chamber (Weis Technik, Weis Umwelt Technik GmbH, Reiskirchen) for 9–11 days (18°C, 16 h of light, ca. 100% relative humidity). After 9–11 days, leaf spot infection was measured as the percentage of infected leaf area, by visual observation.

Two independent experiments representing two different environments were made with leaf discs from clones A and B. Each of the experiment used a randomised incomplete block designs with 14 blocks (=14 petri dishes, each containing 14 genotypes) nested in eight replications. The mean value of *Cercospora* leaf spot infection used for QTL analysis was calculated from both tests. Leaf discs from the resistant sugar beet line 93164P and the susceptible line KWS 1171 were used as controls.

Procedure CORRELATION (SAS institute 1991) was used to calculate correlation coefficients between the field and leaf disc test. A mixed model implemented by using *Procedure MIXED* (Littel et al. 1995) of the SAS programme package was applied to calculate Lsmeans (least squares means) values for the data of the leaf disc test with genotype×block×replications and clone×block×replications considered as random effects. Lsmeans are usually called the adjusted mean in standard textbooks (Littel et al. 1995).

DNA extraction, AFLP and RFLP

From each F_2 individual 5 g of fresh leaves was taken for DNA isolation, which was performed essentially as described by Saghai-Maroof et al. (1984). For AFLP analysis, the DNA concentration was adjusted to 10–12 ng/µl by comparison with phage λ DNA of known concentration. Radioactive AFLP marker analysis was performed essentially as described by Vos et al. (1995). The AFLPs were principally scored as dominant markers. RFLP analysis was carried out as described by Barnes *et al.* (1996), with probes selected to give an approximately even coverage of the genome to provide a framework of codominant markers.

Construction of linkage map and QTL analysis

The UNIX version of MAPMAKER/EXP v. 3.0 (Lander et al. 1987; Lincoln et al., 1993) on a Sun sparc 10 workstation was used to construct a genetic map of the F_2 population. Grouping of the markers were done in two steps: (1) the "group" command with a LOD score=11.0 and 25 cM (Haldane 1919) as proposed by Hallden et al. (1996) was used to determine all pairs of linked markers for the statistical acceptance of linkage and as critical distance between two linked markers; (2) after excluding unlinked markers produced from the first steps, grouping of the markers was done once more by using the "group" command with a LOD score of 3 and a critical distance of 25 cM. The numbering of linkage groups I–IX was according to Schondelmaier and Jung (1997). With error detection on, a framework marker was established based on the output of the LOD 1 of "order" command. The framework markers were used for QTL analysis by combining them with the data from the field trials and leaf disc test. A PC version of PLABQTL v. 4.0 (Utz and Melchinger, 1996) was used to analyse QTLs. In order to avoid false positives, we set a stringent LOD score of 2.4 as the threshold value for the detection of QTLs. *Cov SELECT* (used selected markers as cofactor) was used in calculating QTLs using PLABQTL.

Results

Field tests

Eighty-nine F_3 half-sib populations were grown in the field under natural infection pressure. Figure 1 shows the results of infection ratings. Leaf spot infection was determined at three different stages of development, 114 (c1), 149 (c2) and 162 (c3) days after sowing. In 1996 leaf spot infection began late, as shown by the low infection rates at the first two observations, which were 3.6 and 4.1, respectively. At the third developmental stage (c3), leaf spot infection increased dramatically to 8 on the KWS scale. A high variation in the disease rating was

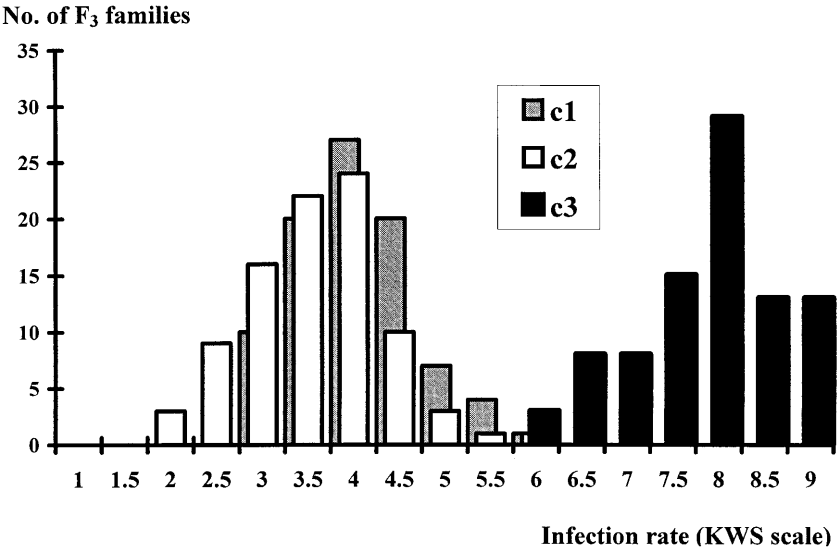

Fig. 1 *C. beticola* infection of 89 F_3 half-sib families from field tests under natural infection at three stages of development, c1, c2 and c3. The KWS scale system was used for determining the severity of infection with 1=uninfected and 9=fully diseased. Each column represents mean values from two replications

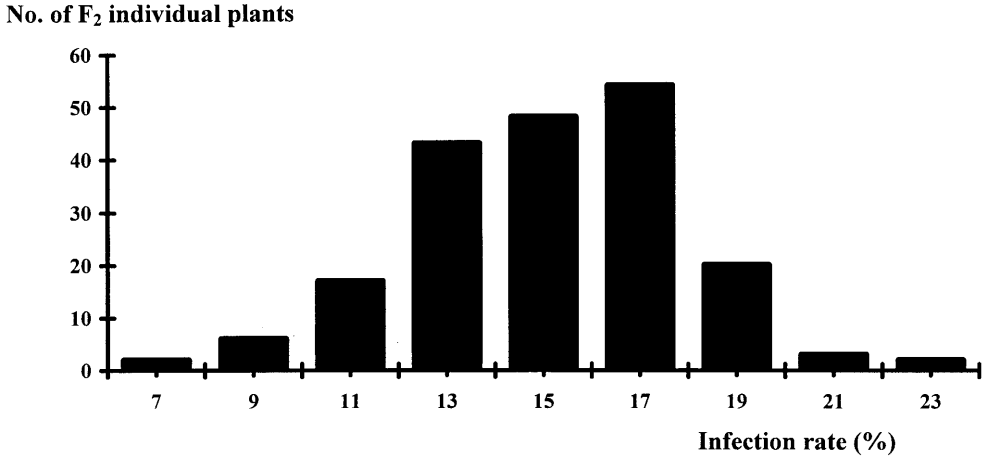

Fig. 2 Infection with *C. beticola*, measured as the percentage of diseased leaf area using the leaf disc test with 196 clonal plants from the F_2 population. From each F_2 individual two clones (A and B) were tested. Lsmean values were calculated from eight replicated experiments of each clonal plant

found among F_3 families at all stages of development (Fig. 1).

Leaf disc test

A newly-developed leaf disc test (Koch 1997) was applied to determine the infection rate of *C. beticola* in sugar beet. The test system was optimised with respect to spore concentration under the inoculation and environmental conditions (humidity, temperature) used for the test.

Results were obtained from tests with 196 F_2 clonal plants. From each individual genotype two different clones were tested. In general, the diseased area ranged between 7% and 23% of the leaf surface, with a mean value of 17% (Fig. 2). The observed variation is comparable to that found in the field test.

Using the leaf disc test, we require only 1 plant of each genotype for the *Cercospora* severity scoring, and evaluation can be finished in a relatively short time (about 11 days). In contrast, a large amount of seed is needed for standard field test methods (the production of sufficient seed can be a problem, especially when the genotypes under study are wholly or partially self-incompatible).

Correlation between rates of *C. beticola* infestation resulting from leaf disc and standard field tests

The artificial environment used for the leaf disc test, and the influence that this may have on host/pathogen-interactions, led us to question of the extent to which the results of the test are good indicators of *Cercospora* resistance under field conditions. Therefore, we compared the rates of infection resulting from the two methods using correlation analysis. Except between c2 and c3, the correlations between variables used to measure rates of infection from the field test were not significant (Table 1). The correlation between infection rates resulting from the leaf disc and standard field test (c3) proved to be significant (Table 1), with the highest correlation (0.51) between Lsmean and c3. No correlation was seen, however, between the leaf disc test results and either c1 or c2.

Table 1 Coefficient of correlation between all variables used in field and leaf disc tests to estimate the degree of plant susceptibility to *Cercospora* leaf spot disease. P values of the correlation coefficients are indicated in *italics*

c2[a]	c3[a]	Clone A[b]	Clone B[b]	Lsmeans[c]	Mean[d] (A-B)	
0.1603	0.04994	0.18314	0.0866	0.08194	0.15406	c1
0.1452	*0.6519*	*0.0954*	*0.4334*	*0.4587*	*0.1618*	
	0.39402	0.14436	0.15107	0.14995	0.16884	c2
	0.0002	*0.1901*	*0.1702*	*0.1734*	*0.1247*	
		0.27915	0.50527	0.50743	0.44772	c3
		0.0101	*0.0001*	*0.0001*	*0.0001*	
			0.53338	0.45757	0.87628	Clone A
			0.0001	*0.0001*	*0.0001*	
				0.9414	0.87493	Clone B
				0.0001	*0.0001*	
					0.79821	Lsmeans
					0.0001	

[a] c1, c2, c3, Mean values of *Cercospora* leaf spot rating in the field experiment at the first, second and third time points
[b] Clone A, Clone B, Mean values, respectively, of *Cercospora* leaf spot rating of clonal plant A or plant B determined by leaf disc test
[c] Lsmeans, Least squares means for genotype effect [=adjusted mean (A–B)] generated by *Proc Mixed* of SAS programme based on data of clone A and clone B. Clonal plant A and B are clonal plants, generated *in vitro*, of single F_2 plants

Table 2 Location of QTLs associated with *C. beticola* leaf spot resistance. Infection rate was determined by the leaf disc test method (Lsmeans) or standard field test (c3). QTLs were identified by composite interval mapping using computer program PLABQTL ver. 4.0 (Utz and Melchinger 1996). Chromosome designation was according to Schondelmaier and Jung (1997). Lsmeans=least squares means for genotype effect [=estimated mean (A–B)] calculated by *Proc Mixed* of the SAS computer programme package, based on data of clonal plant A and B. Clonal plant A and B are clonal plants of single F_2 individuals, produced by *in vitro* micropropagation. c3 is the third observation of leaf spot infection using the KWS scale

Test method	Chromosome	Left marker	LOD Score	R^2	Additive	Dominant	d/a
Field	IV	E335905d	5.32	25.1	–0.522	–0.107	0.205
Field	VII	M31	4.04	19.7	–0.411	–0.020	0.048
Field	VIII	P324708 C	3.36	17.6	0.083	–0.447	–0.037
Field	VIII	P324701d	4.10	19.9	–0.524	–0.415	0.217
Field	IX	E355111 C	3.68	18.1	–0.307	0.249	–0.811
Leaf disc	III	M30	2.66	6.9	–0.824	0.171	–0.208
Leaf disc	IV	M17	8.56	20.2	–1.497	–0.003	0.002
Leaf disc	VII	M34	2.40	6.2	0.710	–0.256	–0.361
Leaf disc	IX	E355111 C	8.06	18.8	–1.441	–0.584	0.405

QTL analysis

A total of 261 polymorphic loci were evaluated in the F_2 population. Of these, 226 (182 AFLPs and 44 RFLPs) could be mapped, giving rise to a linkage map of 744 cM covering the nine chromosomes of sugar beet. On average, 25 marker loci were located on each chromosome, with an average spacing of 3.1 cM. We used AFLP anchor markers to allocate the linkage groups to the nine chromosomes of beet (Schondelmaier and Jung 1997) thus facilitating later comparisons of QTL results.

For QTL analysis, a final sparse map was created by selecting only framework markers using the ”order” command of MAPMAKER/EXP with LOD>1. This strategy was used always keeping several points in mind: (1) tightly linked markers with a distance ≤1 cM which had been placed on the map using the ”place” command could generally only be ordered on the chromosomes with LOD <1; (2) there is a significant risk of mapping loci in the wrong order if they are within a short distance (<2 cM) of each other (Hallden et al., 1996); (3) PLABQTL gives more reliable results when the distances between markers are greater than 1 cM.

QTLs were first identified on the basis of field test data using a LOD score of 2.4 as the threshold. Altogether, five QTLs were found, one on each of chromosomes IV, VII and IX and two on chromosome VIII (Table 2). The highest explained phenotypic variance was shown by the QTL located on chromosome IV (R2=25.1%). Based on a dominant/recessive model, all QTLs had additive effects except the locus on chromosome IX, which showed partially recessive gene action.

QTLs were also sought based on data from the leaf disc tests, again using a LOD score of 2.4 as the threshold. Four QTLs were located, on chromosomes III, IV, VII, and IX (Table 2). The highest explained phenotype variation was again shown by the QTL located on chromosome IV (R^2=20.2%). Two QTLs showed partially recessive allele effects (chromosomes III and VII), one QTL was partially dominant (chromosome IX) and the locus on chromosome IV showed additive effects.

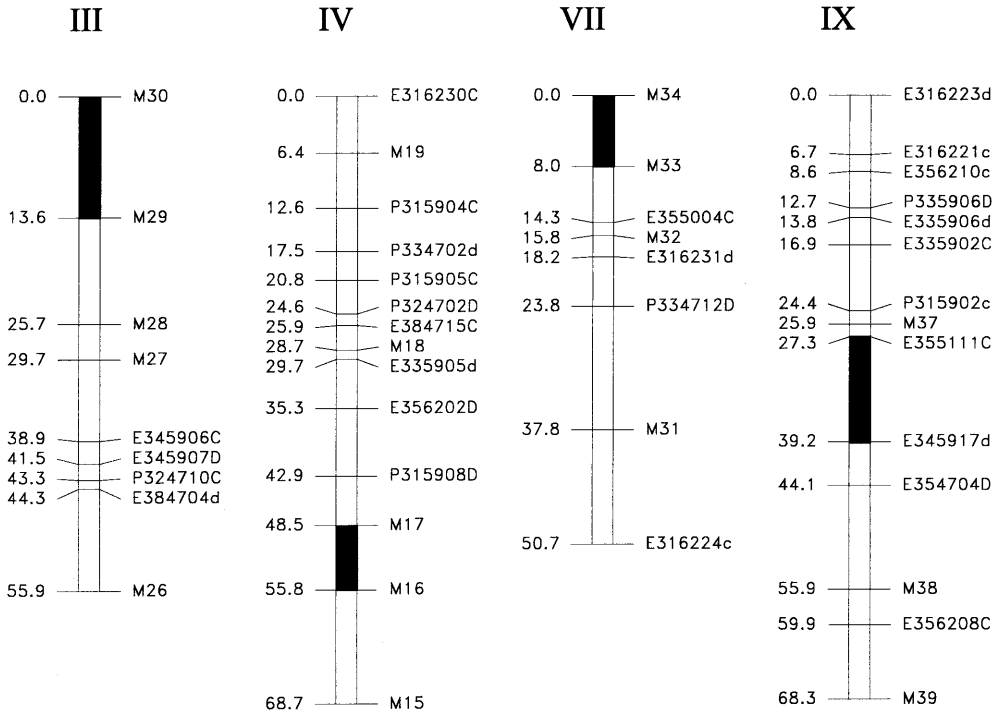

Fig. 3 Graphical presentation of QTLs associated with leaf spot resistance as determined from leaf disc test data. Chromosome numbers in *Roman numerals* are according to Schondelmaier and Jung (1997). The codes of marker loci are listed on the *right*, and the map distances are listed on the left (Haldane centiMorgans). The map was constructed using MAPMAKER/EXP v. 3.0 (Lander et al. 1987; Lincoln et al. 1993). PLABQTL v. 4.0 (Utz and Melchinger 1996) was used for QTL detection. *Shaded columns* represent QTLs for *Cercospora* resistance. *M* codes for RFLP markers. *E* and *P* code for AFLP markers generated using primer combinations of *Eco*RI/*Mse*I and *Pst*I/*Mse*I, respectively

The resistance alleles derived from the resistant parent was contributed in reducing leaf spot infection except for the QTL on chromosome VII (marker locus M34) detected from leaf disc test data (Table 2). The resistance alleles with the highest effects were from QTLs located on chromosomes IX (marker E355111 C) and IV (marker M17); these reduced leaf spot infection by –2.03% and –1.5% respectively (Table 2). Graphical presentation of QTL associated with resistance to *C. beticola* disease based on the leaf disc test is presented in Fig. 3.

Discussion

We have measured leaf spot resistance in a F_2 population derived from a cross between a susceptible and a resistant sugar beet. In contrast to previous reports, we have determined infection rate at the single plant level using a modified leaf disc test procedure. These data were compared to data from a field test under natural inoculation.

For field scoring of the leaf spot disease we used the KWS method, which has been frequently applied by beet breeders in the past. Disease severity was scored at 114 (c1), 149 (c2) and 162 (c3) days after sowing (which correspond to stage C on the beet scale of development – Winner 1974). Equal means between the first two observations between July and August indicate the slow development of *C. beticola* populations in the field. The correlation between c1 and c2 was not significant. This demonstrates that any differentiation between resistant and susceptible genotypes was not possible at early stages of development.

However, *C. beticola* severities increased dramatically within the 13 days following c2, resulting in an average infection score of 8 on the KWS scale. The maximum score of 9 was achieved by more than 12% of the F_3 populations tested, suggesting that the highest degree of infection had been reached 162 days after planting. No further observations were considered necessary. Natural inoculation at this site was severe enough to give a clear differentiation between suseptible and resistant F_3 lines, thus providing the possibility to map corresponding resistance genes. Artificial inoculation with *C. beticola* spore suspensions, however, had to be carried out at locations with a low infection pressure (Ruppel and Gaskill 1971; Adams et al. 1995). Another method for determining leaf spot resistance in the field has been described by Rossi and Battilani (1989a); by this method, the severity is evaluated for each leaf and the rate computed as leaf area affected/total leaf area×100.

Although the KWS scale gives reliable results for the screening of whole populations, the repeatability is low at the single plant level. This was demonstrated by Rossi and Battilani (1989b) in a field experiment with beet populations. After rating the population with 7 on the KWS scale they found a large variation between individual plants when measuring the diseased leaf area.

There is obviously a need for an accurate measurement of leaf spot infection. This is one of the reasons why the new leaf disc test method was developed. From a practical point of view, its greatest advantage over testing in the field is that we can determine resistance at the single plant level. Significant correlation coefficients between different experiments with different clonal plants strongly suggest that this test gives reliable results. These results, determined as percentage diseased leaf ar-

ea, were positively correlated with the third field observation (c3). The highest coefficient of correlation was between Lsmean and c3. It can be concluded that selection for resistance to *Cercospora* based on c3 and on the leaf disc test (lsmean) will both be highly efficient. Because of this result, we decided to use c3 and lsmean for exploring QTLs for *Cercospora* resistance.

By combining genetic marker information with data from disease scoring, we were able to estimate the locations of QTLs for leaf spot resistance by composite interval mapping (CIM) (Jansen 1993; Zeng 1994) using the PLABQTL computer sofware program (Utz and Melchinger 1996). This algorithm was proposed to improve precision of QTL mapping (Jansen 1993; Zeng 1994). Based on Lsmean data, four QTLs were found, located on chromosomes III, IV, VII and IX, respectively. The putative locations of QTLs on chromosomes IX based on lsmean were confirmed by the result of QTL analysis based on c3 data, and *vice versa*.

To confirm these results, we also used MAPMAKER/QTL v.1.1 (Lander et al. 1987; Lincoln et al. 1993) and QTL CARTOGRAPHER (Basten et al. 1997) to detect QTLs (data not shown). Based on Lsmean trait data, MAPMAKER/QTL (LOD score >3) identified significant QTLs on chromosome IV (M17) and IX (E355111 C), similar to PLABQTL. In addition a second significant QTL was found on chromosome IV (E345904d). QTL CARTOGRAPHER (using CIM or model 6 (LOD score >3) revealed QTLs on chromosome IV (M17), VII (M33), and IX (E355111 C). Two additional QTLs were found on chromosome IX (E335906d and E354704D). The MAPMAKER/QTL and QTL CARTOGRAPHER programmes are based on likelihood procedures in detecting QTL; the difference between them is that QTL CARTOGRAPHER uses CIM or IM alternatively, whereas MAPMAKER relies only on IM. PLABQTL is different from QTL CARTOGRAPHER in the algorithm used to detect QTLs. PLABQTL uses regression procedures (Haley and Knott 1992) instead of likelihood.

QTLs for leaf spot disease in sugar beet have not been identified before. Based on field experiment data of F_2 and F_1 populations, the number of genes controlling resistance was estimated by Smith and Gaskill (1970) using the Castle-Wright formula. They found that at least four to five loci were probably involved in leaf spot resistance, which is in rough accordance with our findings (based on leaf disc test). Given the low heritability of this trait (Smith and Ruppel 1974), it is important to confirm the robustness of QTLs associated with leaf spot resistance in different genetic backgrounds and environments.

The molecular markers presented here can be valuable tools both for marker assisted selection and for attempts at cloning the QTLs. For breeding, the QTLs located on chromosome IV and IX are of the greatest interest because these loci have the largest LOD scores (8.56 and 8.08) and R^2 values. The resistance alleles at both of these loci contribute the strongest resistance effects (–2.3% and –1.5%) with gene actions partially dominant and additive for the loci on chromosome IX and chromosome IV, respectively. The existence of significant additive gene action for leaf spot disease in sugar beet was also reported by Smith and Ruppel (1974). As a first step towards cloning the QTLs, plants have been selected which are heterozygous for only one of the loci (on either chromosome IV or IX) but homozygous for all of the others. After selfing the selected plants, populations will be created segregating for leaf spot resistance as a single Mendelian factor. Fine mapping can be carried out to select markers for use as probes to screen our beet YAC library (Kleine et al. 1995). A similar strategy has recently been presented by Yano et al. (1997) for genes controlling heading date in rice.

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