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Mapping of two genes for resistance to clubroot (*Plasmodiophora brassicae*) in a population of doubled haploid lines of *Brassica oleracea* by means of RFLP and AFLP markers

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Abstract A genetic map covering 615 cM in 12 linkage groups was assembled based on 92 RFLP and AFLP markers segregating in a population of 107 doubled haploid lines (DH lines) of *Brassica oleracea*. The DH-line population was obtained through microspore culture from the F_1 of two homozygous parents: DH-line Bi derived from the cabbage landrace Bindsachsener, and DH-line Gr from broccoli cv 'Greenia'. Sixty-five percent of the loci, and in some cases complete linkage groups, displayed distorted segregation ratios, a frequency much higher than that observed in F_2 populations of the same species. DH-line Bi was resistant to clubroot, which is caused by a Dutch field isolate of *Plasmodiophora brassicae*. Resistance in the DH-line population was determined in two ways: by assigning symptom grades to each plant, and by measuring the fresh weights of the healthy and affected parts of the root system of each plant. Using a multiple QTL mapping approach to analyze the fresh weight data, we found two loci for clubroot resistance; these were designated *pb-3* and *pb-4*. The additive effects of these loci were responsible for 68% of the difference between the parents and for 60% of the genetic variance among DH-line means. Also, indications for the presence of two additional, minor QTLs were found. Analysis of symptom grades revealed the two QTLs *pb-3* and *pb-4*, as well as one of the two minor QTLs indicated by analysis of the fresh weight data.

Key words Microspore culture · Quantitative trait locus (QTL) · Multiple QTL models (MQM)

Abbreviations *AFLP* Amplified fragment length polymorphism · *DH line* doubled haploid line · *MQM* multiple QTL model · *RFLP* restriction fragment length polymorphism · *QTL* quantitative trait locus

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Introduction

In the past decade, molecular genetic markers have found their way into the study of *Brassica* genomes. RFLP maps have been published for *B. napus* (Landry et al. 1991; Ferreira et al. 1995b), *B. oleracea* (Slocum et al. 1990; Kianian and Quiros 1992; Landry et al. 1992) and *B. rapa* (Song et al. 1991; Chyi et al. 1992; Teutonico and Osborn 1994). These markers have also been used to clarify evolutionary and taxonomic relations within the genus *Brassica* (Song et al. 1988a, b, 1990; Dias et al. 1991; Nienhuis et al. 1993). For plant breeders, the linkage of markers with morphological, physiological and biochemical characters (e.g. Figdore et al. 1993; Kennard et al. 1994; Teutonico and Osborn 1994; Song et al. 1995) is useful, as are markers closely linked to disease resistance genes. Linkage of genes for disease resistance with molecular markers has been described in *Brassica* species by Landry et al. (1992), Figdore et al. (1993), Ferreira et al. (1995a) and Dion et al. (1995).

Breeding for clubroot resistance in *B. oleracea* is difficult as the expression of resistance is often quantitative and inocula generally consist of mixtures of pathotypes (Voorrips 1996). For these reasons, single plant evaluations of resistance are often unreliable, and indirect selection based on markers closely linked to resistance genes would be a great improvement. Indirect selection would also facilitate the incorporation of multiple resistance genes into cultivars. The study of the genetics of resistance can also greatly benefit from the linkage information provided by molecular markers.

In earlier reports we described classical genetic analyses of clubroot resistance in several resistant doubled haploid lines (DH lines) of *B. oleracea* (Voorrips and Kanne 1996a, b). For one of these lines: DH-line Bi, derived from the cabbage landrace Bindsachsener, the

segregation data did not allow us to draw conclusions concerning the number and effects of segregating resistance genes. The present report deals with the molecular marker analysis of a population of DH lines derived from the F_1 between resistant DH-line Bi and susceptible DH-line Gr, the construction of a molecular marker map of the *B. oleracea* genome and the mapping of QTLs for clubroot resistance.

Materials and methods

Plant material

Clubroot resistant DH-line Bi was obtained via microspore cuture from a cabbage line selected by I.R. Crute (HRI, Wellesbourne, UK) from the German landrace Bindsachsener. Clubroot susceptible DH-line Gr was similarly derived from broccoli cv 'Greenia' (Hammenhögs Frö AB, Hammenhög, Sweden). From three plants of the F_1 (Bi × Gr) a population of doubled haploids was obtained through microspore culture according to the procedure described by Duijs et al. (1992). About $2.2 \cdot 10^7$ microspores from 218 flower buds yielded 390 embryos from which 120 flowering plants were regenerated; of these, 107 produced seeds upon selfing.

Pathogen

The *P. brassicae* isolate used in this study was obtained from an infested field at the Brabant Experimental Station at Breda, The Netherlands, and characterized as ECD 16/3/30 (Buczacki et al. 1975; Voorrips and Visser 1993). Inoculum was prepared according to Voorrips and Visser (1993) from clubs stored at − 20°C.

Resistance tests

Resistance tests were carried out in a phytotron chamber, at 22°C, with a photoperiod of 16 h at 110 $\mu E \cdot m^{-2} \cdot s^{-1}$ HPI-T illumination, according to Voorrips and Visser (1993). One seed was sown per pot and inoculated with $2 \cdot 10^7$ spores. After 6 weeks the root symptoms were evaluated. Three experiments were performed in which both parents, the F_1 and the population of DH lines were tested in eight blocks among a number of other populations not reported in this study (Voorrips and Kanne 1996a).

Symptom evaluation

In all experiments disease symptoms were graded visually: grade 0, no symptoms; grade 1, small, separated globular or spindle-shaped clubs on tap root or side roots, not more than 2 mm in diameter; grade 2, intermediate symptoms; grade 3, clubs larger than 1 cm in diameter or affecting more than half of the root system. Symptoms of grade 3 always affected the tap root and the proximal part of lateral roots, and sometimes extended into the hypocotyl.

In two of the three experiments the fresh weights of the affected parts (the so-called clubs) and healthy parts of the root system of each plant were also measured. From these two measurements, club weight (CW) and healthy root weight (HRW), each measured in grams, the measure of resistance $R = \ln (HRW + 0.5)/(CW + 0.5))$ was calculated. This value was shown in a previous study to correlate well with the symptom grade and to have a normally distributed error term with an approximately constant error standard deviation of 0.44 (Voorrips and Kanne 1996b). The R value was corrected for test and block effects before further analysis.

RFLP markers

All RFLP procedures were as described by Van der Beek et al. (1992, 1996). Genomic DNA was digested with restriction endonuclease *Eco*RI. Probes sized 0.35–2.5 kb were obtained from a *B. napus* embryo cDNA library and cloned into the *Pst*I site of vector pBR322 (Harada et al. 1988); these were made available by B.S. Landry (Agriculture Canada Research Station, St-Jean-Sur-Richelieu, Canada). Cloned inserts were polymerase chain reaction (PCR)-amplified and radiolabelled (Bernatzky and Tanksley 1986).

RFLP markers were named after the probe (1NB8–4NF12) detecting them. If several polymorphic loci were detected with the same probe the loci were designated by appending different lowercase letters to the probe code. This nomenclature is similar to that followed by Landry et al. (1992), but identically named markers in their and our work do not necessarily refer to the same loci.

AFLP markers

AFLP (amplified fragment length polymorphism) is a recently developed molecular marker technique based on selective PCR amplification of restriction fragments (Vos et al. 1995). Genomic DNA was digested with restriction endonucleases *Eco*RI and *Mse*I. After ligation of double-stranded adapters to the ends of the restriction fragements, preamplification was performed with primers specific for the *Eco*RI and *Mse*I adapters, including one selective nucleotide (underlined), followed by amplification with similar primers with three selective nucleotides (italics) 5′ GAC TGC GTA CCA ATT C*A A C*3′, and 5′ GAT GAG TCC TGA GTA *A C C T* 3′. Dependent on the amount of amplified DNA after preamplification the reaction mix was diluted 1/20 or 1/40 in TE, and 10 µl was used in the final amplification. The E35 primer was end-labelled with [γ^{33}P] ATP (Feinberg and Vogelstein 1983). Amplification fragments were separated on 5% denaturing polyacrylamide gels. The gels were dried, and autoradiography was carried out with Kodak XOMAT AR X-ray film for 1–7 days at − 70°C using intensifier screens. Segregating AFLPs were labelled A01 to A26 in the order of decreasing fragment size.

Linkage analysis

The JOINMAP 2.0 package (Stam 1993; Stam and Van Ooijen 1995) was used to assign markers to linkage groups and to calculate the most probable order and distances of the markers within each linkage group. Map distances were based on Kosambi's (1944) mapping function. The basis for assigning markers to linkage groups was a LOD (log of odds) score ≥ 4.0 with one or more other markers in the group.

Quantitative trait locus (QTL) analysis

Since the error terms of the R value were approximately normally distributed (Voorrips and Kanne 1996b), the mean R values of the DH lines could be analyzed with the Multiple-OTL Model (MQM) method (Jansen 1994; Jansen and Stam 1994). This is a two-step method, where the first step consists of the selection of markers to be used as cofactors, and the second step consists of the estimation of the effects of putative QTLs throughout the genome after correction for the effects of the selected markers. In the first step, cofactors were selected from 38 markers distributed over the linkage map at approximately 25-cM intervals, the criterion being a significance of $P \geq 0.02$ for the marker effect on R. In the second step, a LOD threshold of 2.7 was used to obtain a confidence level of 0.95 throughout the genome. This threshold was determined based on 1000 simulations with the actual molecular marker data, according to the method of Jansen (1994). The MQM analysis was carried out with a GENSTAT5 program developed by R.C. Jansen (CPRO-DLO, Wageningen, The Netherlands).

The error terms of the mean symptom grades of the DH lines were not normally distributed, and the mean symptom grades were there-

fore not suitable for MQM analysis. A non-parametric Kruskal-Wallis test was performed at each marker locus for significance of a QTL at that locus. In order to obtain a confidence level of 0.95 throughout genome, the tests per marker locus were considered significant if $P \leq 0.001$. The Kruskal-Wallis analysis was carried out using the MAPQTL 3.0 package (Van Ooijen and Maliepaard 1995).

Since the segregating population consisted of DH lines no dominance effects could be assessed using either analysis method.

Results

RFLP and AFLP markers

Sixty-six RFLPs between the parental lines were detected with 37 probes, using restriction endonuclease *Eco*RI. Three further probes (1NH12, 2NA11, 3NG10) did not detect any RFLPs. Five RFLP clusters without recombination were found (1NE1c/3ND12, 1NG9/2NA8a/3NB4b, 1NF2b/2NA1a, 1NF2a/1NH3a/2NF10 and 3NB4c/3NC3b). Because the *Eco*RI restriction fragment sizes detected were unequal within all these groups these markers detected different but closely linked loci.

With one set of selective primers 26 AFLPs were detected. Five AFLP pairs without recombination were found. In four pairs (A11/A12, A13/A14, A17/A18 and A22/A23) both amplified fragments were inherited from the same parent. Since the sizes of both fragments in these pairs were very similar, it is possible that they represent double bands, as reported by Vos et al. (1995), and not different markers. In one pair (A04/A05) each of the two parents contributed one amplified fragment.

Construction of the linkage map

Ninety-two markers were scored for 107 DH lines. The grouping of the markers in linkage groups was determined at a range of LOD scores. Preliminary mapping within the linkage groups at each LOD score was performed. If gaps larger than 25 cM occurred within a group, or fewer than 3 markers were responsible for linking subgroups, the coherence of the group was checked by comparing the recombination between the markers on each side of the gap with their calculated map order. In this way, it was found that linkage groups assembled at LOD values ≥ 4.0 were coherent with one exception: groups 3 and 6 appeared to be linked based on the LOD score (with 1NG1b on group 3 joined to 4NE11c on group 6), but the recombination values between markers in the two groups were not in agreement with the map order. Therefore, these two groups were separated (Fig. 1).

Of the 92 markers, 87 could be assembled into 12 linkage groups. The 5 remaining markers were not linked to any other markers. The linkage groups were numbered in decreasing order of map length (Fig. 1). The total map length covered by the 12 linkage groups was 615 cM.

Since the RFLP probes used were a subset of those used by Landry et al. (1992) for the construction of a *B. oleracea* map, it was possible in some cases to identify corresponding linkage groups. When we used the five single-copy probes 2NB10, 2NE4, 3NB3, 3NF4 and 4ND3 as common markers between the two maps, our linkage groups 1, 2 and 3 could be identified with their groups 5, 4 and 1, respectively. From the relative map positions of multicopy probe RFLPs, our linkage groups 5 and 12 could further be tentatively associated with two regions of their linkage group 3.

A high frequency of segregation distortion was observed. The segregation of 59 markers (64%) deviated significantly ($P \leq 0.05$) from the expected 1:1 ratio. Almost all loci on linkage groups 3, 8, 9 and 12 segregated an excess of Bi alleles, and all loci in group 11 showed an excess of Gr alleles. On all linkage groups except group 10 single loci or groups of loci showed segregation distortion. The most extreme segregation ratios were observed for markers 1NF2b and 2NA1a on linkage group 8 (93 Bi:9 Gr: 5 missing) and unlinked marker 2NA8b (8 Bi: 96 Gr: 3 missing).

Mapping of genes for clubroot resistance

The severity of clubroot symptoms was measured by the R value as well as by assigning a symptom grade. Means of the R values and the symptom grades were calculated for all lines. The number of tested plants per line varied considerably due to the often low fertility of the DH lines resulting in a very limited seed supply, as well as to a sometimes very slow or weak plant development. Data from plants with a total root system weight of less than 0.3 g were rejected, since these plants generally lacked the resources to develop clubroot symptoms (Voorrips and Kanne 1996b). For 91 DH lines symptom measurements were available. One very fertile DH line was used as a partially resistant standard genotype in the experiments; therefore, many data (179) were available for this line. For the other 90 DH lines the numbers of evaluated plants ranged from 1 to 16, with an average of 7.4. The variance of 91 DH-line means of R was 0.55. The error variance per plant, calculated from all DH lines, the original F_1, the two parents and several other genetically uniform populations, was 0.19 (Voorrips and Kanne 1996b). The mean error variance of DH-line means was calculated as 0.04, leaving an (additive) genetic variance of DH-line means of 0.51. The narrow-sense heritability in the DH population was therefore $0.51/0.55 = 0.93$.

The mean R values of the DH lines were analyzed using the MQM method. As the first step, 3 markers were selected as cofactors: 4NE11a, 2NA8c and 3NH5a on linkage groups 3, 1 and 10, respectively. From the total variance of DH-line means of 0.55, a residual variance of 0.18 was not absorbed by the cofactors. In the second step of MQM analysis, the LOD scores of possible QTLs throughout the genome were deter-

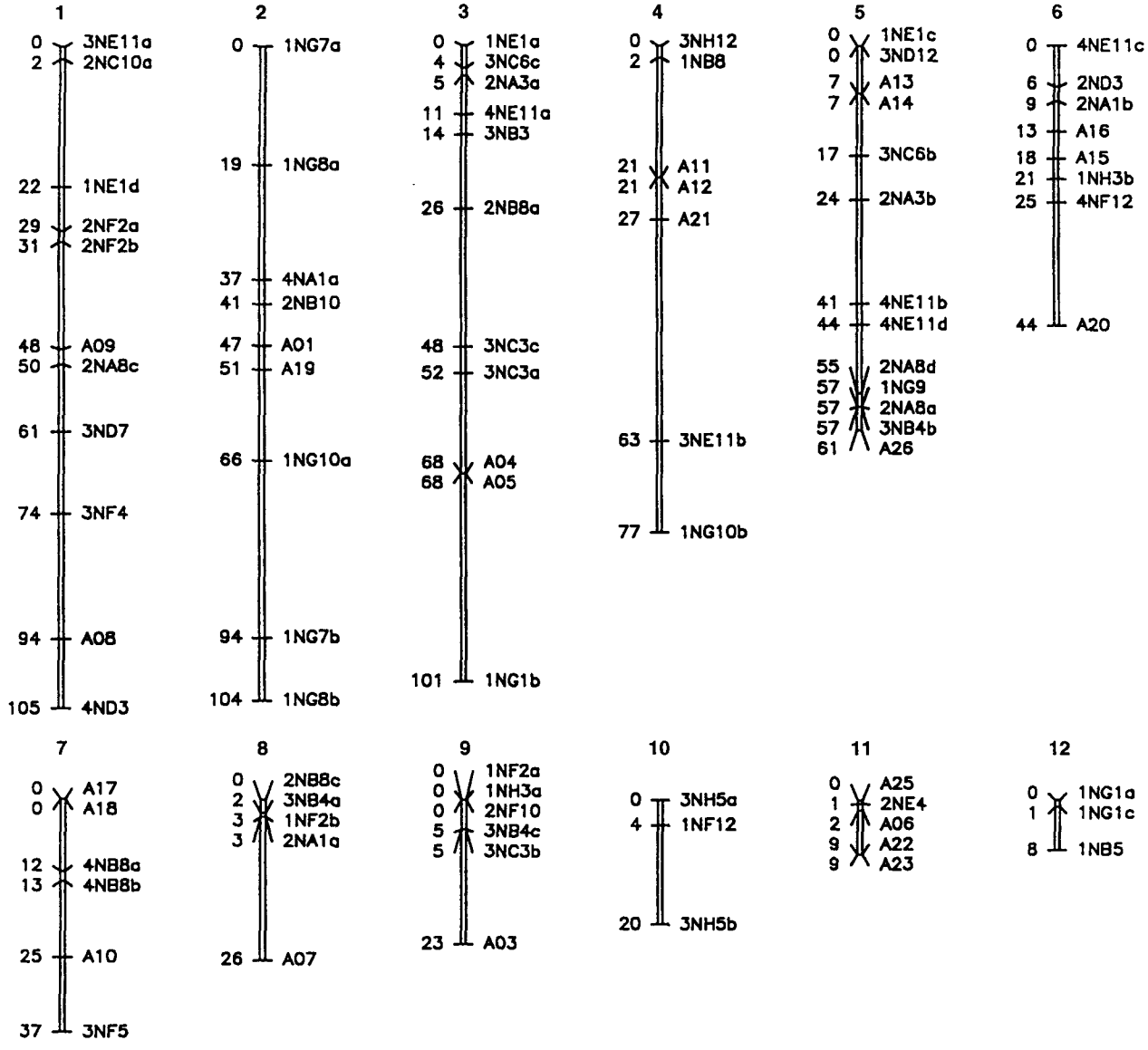


Fig. 1 Linkage map of *Brassica oleracea* based on 92 RFLP and AFLP marker loci segregating in a population of doubled haploid lines. Five markers (2NA8b, 2NB8b, 4NA1b, A02 and A24) were not linked to other loci

mined. Two QTLs, designated *pb-3* and *pb-4*, with LOD scores above the threshold of 2.7 were found near 2 markers used as cofactors, 4NE11a and 2NA8c (Fig. 2). Near the third cofactor (3NH5a) and over a large part of linkage group 6 between marker loci 2NA1b and A20 elevated LOD scores of about 2.0 were found, but these remained below the LOD threshold value. The effects of substituting the homozygous resistant genotype for the homozygous susceptible one were estimated to be 1.17 and 0.41 (50% and 18% of the difference between the parental line means) for *pb-3* and *pb-4*, respectively; these two QTLs explained 54% and 6%, respectively, of the total genetic variance of the DH-line means. As one of the four possible homozygous QTL genotypes (with the Gr allele of *pb-3* and the Bi allele of *pb-4*) was scarcely represented among the DH lines, a possible epistasis effect could not be estimated.

For *pb-3* and *pb-4* and for the possible QTLs near marker 3NH5 and on linkage group 6, the alleles conferring resistance were inherited from the resistant parent. The frequency distributions of mean R values of DH lines with the different genotypes at the peak marker loci near *pb-3* and *pb-4* (4NE11a and 2NA8c, respectively) are shown in Fig. 3A. Both 4NE11a and 2NA8c showed significantly distorted segregation ratios of 70:34:3 and 30:70:7 (Bi : Gr:missing), respectively.

QTL analysis of mean symptom grades of the DH lines involved Kruskal-Wallis tests for the probability of finding a QTL linked with each marker locus. As expected, significant effects were found for markers closely linked to *pb-3* and *pb-4* (Fig. 2). The frequency distribu-

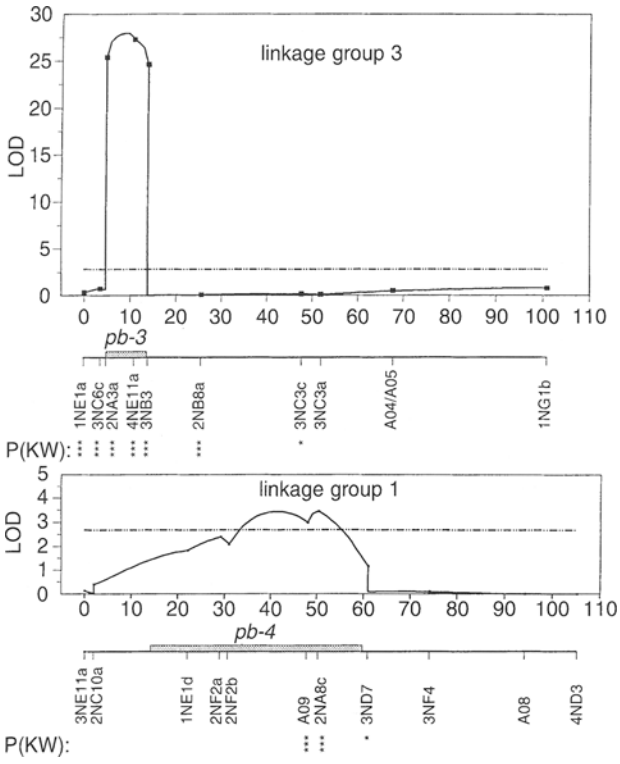
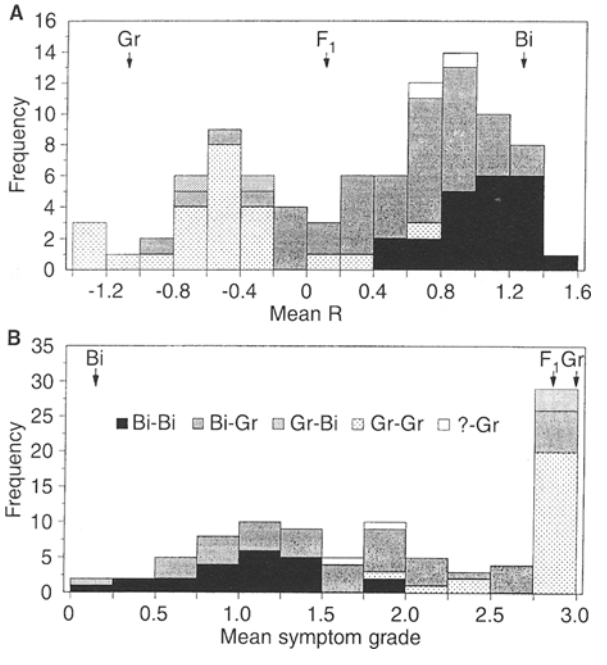


Fig. 2 QTL likelihood maps for clubroot resistance on linkage groups 3 (*pb-3*) and 1 (*pb-4*). LOD scores were obtained by MQM mapping of the measure of resistance, *R*; the *horizontal line* represents the threshold LOD value of 2.7. Two - LOD support intervals are shown as *grey boxes*. The significance of the Kruskal-Wallis tests P (KW) for mean symptom grades is indicated for each marker locus as * ($P < 0.01$), ** ($P < 0.001$) and *** ($P < 0.0001$)

Fig. 3A, B Frequency distributions for the means of the measure of clubroot resistance, *R* (**A**) and of the symptom grade (**B**) in a population of doubled haploid lines, in relation to genotypic composition (Bi or Gr allele) for the two marker loci, 4NE11a and 2NA8c, that are the most closely linked to clubroot resistance loci *pb-3* ande *pb-4*, respectively



tion of mean symptom grades of DH lines with the different genotypes at loci 4NE11a and 2NA8c is shown in Fig. 3B. As in the analysis of the R values, notable but not quite significant effects were detected at some loci on linkage group 6. The possible QTL near marker 3NH5a indicated by MQM mapping was not revealed by the analysis of the symptom grades.

Discussion

The genetic map presented here is the first one based on both RFLP and AFLP markers. For a DH-line population as used in this study, the dominant marker type of AFLPs is as informative as the codominant type of most RFLPs. The RFLPs were detected with cDNA clones and thus show homology with expressed sequences, while the AFLPs presumably represent a random sample from the genome. There was no obvious difference in the distribution of both types of markers over the genome, although AFLPs and RFLPs never mapped at the same locus.

The length of the linkage map, 615 cM, was less than the length of other genomic maps published for *B. oleracea*. This is most likely due to the smaller number of markers (92) in comparison to the other maps: 820 cM with 258 loci (Slocum et al. 1990), 747 cM with 108 loci (Kianian and Quiros 1992), 1112 cM with 201 loci (Landry et al. 1992).

On the basis of multicopy RFLP probes, our linkage groups 3 and 5 show homology over part of their length, presumably indicating an interchromosomal duplication. Recombination in the segment on linkage group 3 is apparently reduced in comparison with the homologous segement on group 5, as indicated by their different map lengths. Although these linkage groups are also homologous to linkage groups 1 and 3 of Landry et al. (1992), respectively, those authors found no evidence for this duplication due to an accidental lack of relevant polymorphisms in their mapping population (Fig. 4). Two other duplicated regions were found: 1NG7–1NG8 twice in linkage group 2, and 1NF2–3NB4 in groups 8 and 9. Also, some closely linked duplications of the same probe were noted: 2NF2 in linkage group 1, 3NC3 in group 3, 2NA8 in group 5, 4NB8 in group 7, 3NH5 in group 10 and 1NG1 in group 11.

The frequency of distorted segregation, affecting 65% of the loci, is much larger than that observed in intraspecific F_2 populations in *B. oleracea:* 5% (Slocum et al. 1990), 5–12% (Kianian and Quiros 1992) and 12% (Landry et al. 1992) of the RFLP marker loci, respectively. In populations of *B. napus* DH lines 20–34% of the marker loci showed skewed segregation (Tanhuanpää et al. 1994; Cloutier et al. 1995; Ferreira et al. 1995b). In contrast, Orton and Browers (1985) found significant deviations from the expected 1:1 ratios at all segregating isozyme loci in four DH populations of *B. oleracea* (respectively 3, 2, 1 and 1 loci). The most

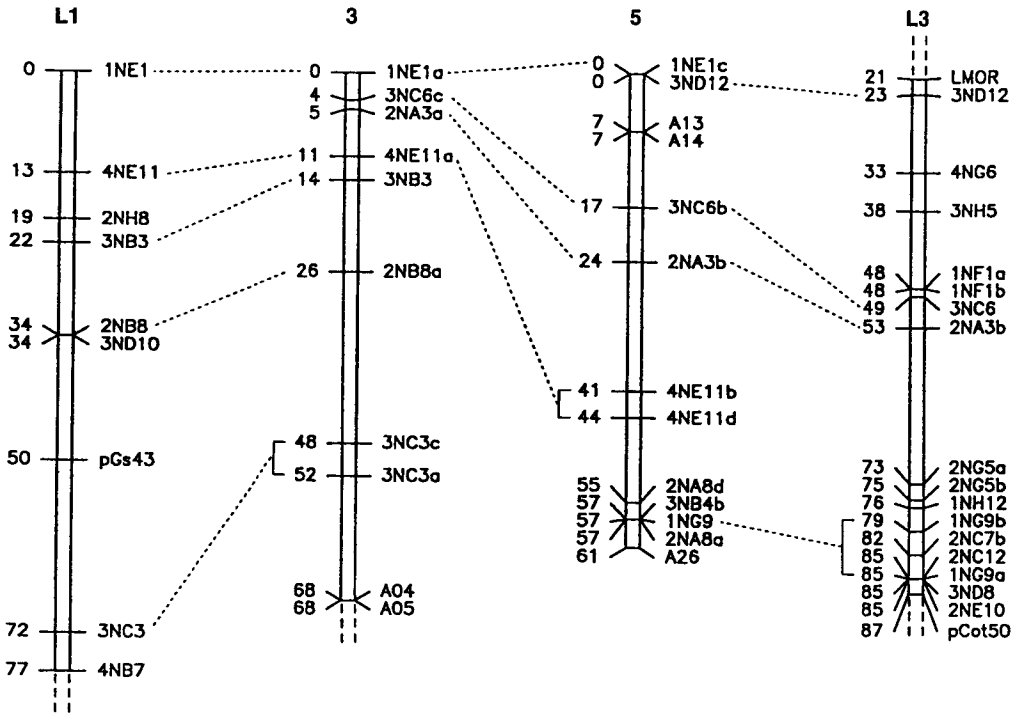


Fig. 4 Duplication between linkage groups 3 and 5, and parts of the corresponding linkage groups published by Landry et al. (1992) (labelled L1 and L3) not showing this duplication

extreme segregation ratios mentioned in Tanhuanpää et al. (1994), Ferreira et al. (1995b) and Orton and Browers (1985) were 9:26, 17:81 and 24:76, respectively, while we found even more extreme ratios (9:93 and 96:8). Presumably, the increased frequency of distorted segregation ratios in *B. oleracea* DH populations reflects selection pressure due to the microspore culture and plant regeneration process and, possibly, also an increased selection pressure on deleterious recessive alleles in a DH population compared to an F_2. In *B. oleracea* the regeneration of plants from microspores generally occurs at a lower frequency than in *B. napus*; this may account for the higher frequency of segregation distortion found in DH populations in this species.

Voorrips and Kanne (1996a,b) studied the segregation of clubroot resistance in populations descended from the same cross (Bi × Gr) that yielded the population of DH lines discussed here. Neither the analysis of symptom grades nor that of R values determined for individual plants in F_2 and backcross populations showed evidence for a simple inheritance of resistance. In retrospect, this is not surprising since the QTL analyses presented here indicate that at least three QTLs are involved in the resistance of DH-line Bi. One QTL, *pb-3*, can be considered a major gene and is responsible for half of the total additive resistance effect in DH-line Bi. However, apart from *pb-3* and *pb-4* at least one further QTL must be present to account for the unexplained 32% of the difference between the parental lines. Further, in the population of DH lines both *pb-3* and *pb-4* showed significant segregation distortion. If this should also be the case in the F_2 or backcross populations the genetic analyses carried out by Voorrips and Kanne (1996a, b) would be invalid. This illustrates two important advantages of QTL analysis: distorted segregation is easily observed and compensated for, and even a partial explanation of observed segregation of traits yields meaningful results.

Landry et al. (1992) were the first to report the linkage of clubroot resistance genes with genetic markers. They studied an F_2 population from a cross between a cabbage breeding line resistant to race 2 (Williams 1966) and a susceptible rapid-cycling *B. oleracea* line. The parentage of the resistant line included *B. napus* cv 'Wilhelmsburger' which is resistant to races 2, 3, 6 and 7, as well as cv 'Badger Shipper', which is resistant to races 1, 3 and 6. The resistance tests were performed with a field isolate designated as race 2. Two QTLs for clubroot resistance were mapped (*CR2a* and *CR2b*) in linkage group 6 and 1, respectively. Intriguingly, *CR2a* was linked to marker 2NA8, while our gene *pb-4* was linked to marker 2NA8c detected with the same probe. However, *CR2a* probably originates from *B. napus* based on the resistance of the progenitors of the segregating population, while *pb-4* originates from *B. oleracea*. The magnitude of the gene effects could not be compared as a different measuring scale was used in their study. Landry et al. (1992) estimated that *CR2a* and *CR2b* together explained 61% of the variation for clubroot resistance in the F_2 population.

Figdore et al. (1993) studied the segregation of RFLP markers and clubroot resistance to race 7 (Williams 1966) in an F_2 from a cross between a susceptible cauliflower cultivar and broccoli line OSU CR-7 (Baggett and Kean 1985), which is resistant to race 7. They found strong evidence for one resistance gene, and pos-

sibly spurious indications for two other resistance genes. Treating symptom grades as quantitative data, they estimated that this gene accounted for 12.6% of the variance in the F_2. Since different markers were used in their study, the map position of this resistance gene cannot be compared with the positions of *pb-3* and *pb-4*.

The nomenclature of clubroot resistance genes in *B. oleracea* is not standardized at this time. Chiang and Crête (1970) hypothesized the existence of two recessive genes for resistance to race 6 (Williams 1966), which they labelled *pb-1* and *pb-2*. However, in a later study (1976) they found only evidence for one such gene. The work of Landry et al. (1992) was based on the same resistant parent used by those authors, but since they used another test method and another race of *P. brassicae* it is not surprising that they discovered different, in their case dominant, genes for resistance. Other authors have also presented evidence for mono- and oligogenic inheritance of clubroot resistance in *B. oleracea*, but they have not labelled those genes (reviewed in Voorrips 1995). The approach of Landry et al. (1992) to name the genes after the race to which they confer resistance is confusing, because there is not yet a universally recognized nomenclature for races and because one gene is likely to confer resistance to multiple races. Therefore, we prefer to follow the nomenclature system used by Chiang and Crête (1970), who added sequential numbers to the prefix *pb-*. Since the R value of our F_1 was intermediate between that of the parents and the mean symptom grade of the F_1 close to the susceptible parent, we use the recessive symbol to indicate the resistant alleles.

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