

Genetic Mapping of Resistant Genes in *Brassica pekinensis* Against *Plasmodiophora brassicae* Race 6

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Inbred lines of Chinese cabbage KU-101 (resistant line against *Plasmodiophora brassicae* race 6) and CS-113 (susceptible line) were crossed and their progeny lines F₁, BC₁F₁, F₂, and F₃ were produced for the construction of the genetic linkage map of *P. brassicae* race 6-resistant *Brassica campestris* ssp. *pekinensis* genome. Restriction fragment length polymorphism (RFLP) was applied to compare between parents and their F₂ progenies with a total of 192 probes and 5 restriction enzymes. The constructed RFLP map covered 1,104 cM with a mean distance between genetic marker of 8.0 cM, and produced 10 linkage groups having 121 genetic loci. The loci of *P. brassicae* race 6 (CR6)-resistant *Brassica* genome were determined by interval mapping of quantitative trait loci (QTL), which resulted from bioassay using the same race of the fungi in F₃ population. Resistant loci were estimated in numbers 1 (G1) and 3 (G3) linkage groups. In the regression test, G1 had a value of 4.8 logarithm of odd (LOD) score, while G3 had values of 4.2-7.2. Given these results, the location of the CR6-resistant loci within the *Brassica* genome map can now be addressed.

Keywords : *Brassica pekinensis*, clubroot, genetic mapping, *Plasmodiophora brassicae*, QTL.

Plasmodiophora brassicae causing clubroot disease of cruciferous plants has now become a serious disease in Korea (Chang et al., 2000; Sung et al., 2001). In many intensive Chinese cabbage growing areas, clubroot disease is an increasing problem. Given the rapid spread of the fungus and its generally lethal symptom, developing resistant cultivar of Chinese cabbage is considered the most effective and economical means of controlling the disease. However, few breeding programs for resistance to clubroot have been tried in Korea due to difficulties in *P. brassicae* single spore isolation and its race (Williams, 1966) or

isolate (Buczacki, et al., 1975) determination, as well as, to the poor knowledge of the genetic determinism of the resistance. Hence, this study attempted to generate genetic information on clubroot for the construction of resistance linkage map using genetic marker system.

Genetic markers make it possible to follow segregating genes or genomic segments and to evaluate genetic potential for crop improvement. Restriction fragment length polymorphisms (RFLPs) are genetic markers detected by hybridizing cloned DNA sequences to DNA fragments from restriction enzyme digests. As these length variations segregate in Mendelian fashion and can be mapped as loci to specific locations in the genome (Bostein et al., 1980), they have been used to construct genetic linkage maps in many species. Advantages of RFLP markers include abundance, phenotypic neutrality, and co-dominance, particularly when compared with other types of genetic markers (Burr et al., 1983; Tanksley, 1983).

To facilitate the construction of extensive genetic linkage maps in *Brassica* species, considerable research efforts were done on *Brassica oleracea* (Slocum et al., 1990), *B. napus* (Diers and Osborn, 1994; Landry et al., 1991), and *B. rapa* (Chyi et al., 1992; Song et al., 1991). Such consistent and comparative *Brassica* mapping program with RFLP has resulted in consolidated linkage maps for A (*B. rapa*, 2n=2x=20), B (*B. nigra*, 2n=2x=16), and C (*B. oleracea*, 2n=2x=18) genomes. Information from these types of studies can be used to develop better breeding strategies for a crop. Also, linkage map is a first step toward detailed genetic analysis that can culminate with the cloning of specific genes.

Regarding the clubroot resistance gene, Landry et al. (1992) reported the detailed genetic map of resistance genes to race 2 of *P. brassicae*. They showed 201 loci mapped in *B. oleracea* covering 1112cM including the identification of two dominant quantitative trait loci (QTLs) for resistance to race 2 of the clubroot disease. In addition, Fuchs and Sacristan (1996) identified the controlling resistance gene to clubroot and characterized the resistance response in *Arabidopsis thaliana*. Hypersensitive reaction associated

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pathogen-specific resistance to clubroot was demonstrated for the first time (Klement, 1982). This study was conducted with the objective of constructing a consolidated linkage map to include the clubroot resistance loci.

Materials and Methods

Populations for inheritance study for clubroot resistance. To determine the inheritance of clubroot resistance, two segregating populations were constructed. A single plant of line No. KU-101 (resistant line against *P. brassicae* race 6) was used as the female parent while a single plant of line No. CS-113 (susceptible line against *P. brassicae* race 6) was used as the male parent to produce the F₁ hybrids. Two parental plants were propagated and their leaves were used as DNA sources. Two F₁ hybrids were selfed to produce two F₂ populations. All F₂ plants were selfed and the F₃ seed was collected. Two hundred (200) F₂ individuals from each F₂ population were grown for their DNA extraction. A disease test with *P. brassicae* race 6 was performed for six F₃ lines of 8-week-old plants. *P. brassicae* infection severity was scored based on the classes. The F₃ observations were averaged and transformed to infection severity class units for comparing with the F₂ disease test results. The parents, F₂ and F₃, were grown by a randomized block design in the clubroot disease nursery and were inoculated with *P. brassicae* race 6.

Inoculum preparation and plant inoculation. Severely infected roots with previously confirmed *P. brassicae* race 6 were prepared as the inoculum source by the following methods. The roots were washed and stored at -20°C. To prepare the inoculum, the galled roots were allowed to thaw in distilled water and the spores were extracted by using a homogenizer. The extracted material was diluted with sterile water and then filtered through five layers of gauze. The resting spores were counted with a hemacytometer and the spore concentration was adjusted to 5×10^8 spores/ml with sterile water. Sterile 1:2 (v/v) peat/sand substrates, as recommended by Naiki et al. (1978), were mixed with previously prepared inoculum to 10^6 spores/g dry soil. Seeds of the parents, F₂, F₃, and susceptible host (*B. pekinensis* cv. Seoul) were sown on these inoculation soils.

Root hair infection with the fungus was confirmed in 8-week-old seedlings by the method described by Williams (1966). The roots were scored for infection severity according to the following index: class 0=no infection symptom or necrosis local region; class 1=1-25% of infected roots; class 2=26-50%; class 3=50-75%; and class 4=76-100%. Severity scoring of F₂ plants was calculated as the average of the observations of the four replicates. A low or high infection severity was interpreted as resistance or susceptibility, respectively. Per disease test, an analysis of variance was carried out to calculate environmental variance and the wide-sense heritability (H²). $H^2 = S_g^2 / (S_g^2 + S_e^2)$, where S_g² is the variance between genotypes and S_e² is the variance within genotypes based on four observations per genotype.

Isolation of DNA from leaves. Leaves were harvested and bulked for 30 individuals of each progeny of the self-pollinated parents, line No. KU-101 and No. CS-113, and for 200 individuals of F₂ families, respectively. The leaves were kept on ice

throughout harvesting, lyophilized immediately, and stored at -20°C. DNA was extracted from each family following the procedures described by Kidwell and Osborn (1992). DNAs were further purified by ultracentrifugation in cesium chloride-ethidium bromide (Murray and Thompson, 1993).

RFLP analysis. Total genomic DNA of each accession was hydrolysed independently with *EcoRI*, *HindIII*, *EcoRV*, *PstI*, and *BamHI* restriction endonucleases. The fragmented genomic DNAs (15 µg) were fractionated on a 1.0% agarose gel, transferred to a nylon membrane (positively charged; Boehringer Mannheim), and hybridized with a digoxigenin-labeled probe. The filter was washed at high stringency (Sambrook et al., 1989) and used for immunological detection according to the manufacturer's instructions by using CSDP (Boehringer Mannheim) as a substrate of alkaline phosphatase.

The F₂ population between line No. KU-101 and No. CS-113, tested with *P. brassicae* race 6, was used as the mapping population. The bulked extreme approach was followed to quickly locate the gene at its chromosome (Zhang et al., 1994). A resistant bulk was made by mixing equal amounts of DNA from 30 highly resistant plants in the F₂ population, and a susceptible bulk was made by mixing equal amounts of DNA from 40 highly susceptible plants in 200 plants. The DNA samples of the two parents and two bulks were digested with five restriction enzymes (*PstI*, *EcoRI*, *BamHI*, *HindIII*, and *EcoRV*) and assayed for RFLP with 192 cloned fragments, which were kindly provided by Dr. T. C. Osborn of Wisconsin University, USA. The probes were constructed using total genomic DNA and cDNA extracted from *B. rapa*, *B. napus*, and *B. nigra*, and digested with *PstI* and *EcoRI*. The location of the resistance gene on the RFLP linkage map was determined by Mapmaker/Exp 3.0 (Lander et al., 1987; Lincoln et al., 1992).

An additional sample of 100 plants in six lines taken at random from the same F₃ population was assayed with the markers that were likely to be linked to the clubroot resistance gene, as determined by the bulked extreme analysis. In addition, these were inoculated by *P. brassicae* race 6 for comparing the results of QTL analysis in F₂ population.

Results and Discussion

Inheritance of resistance genes. The resistance of inbred line No. KU101 to *P. brassicae* race 6 was confirmed by three times infection with *P. brassicae* race 6. The segregation ratio of F₁ and BC₁F₁ plants from the cross between inbred lines No. KU101 and No. CS113 did not show any Mendelian dominant fashion, and no clubroot disease was shown to be resistant and susceptible of clearance (Fig. 1). Thus, the clubroot resistance gene to *P. brassicae* race 6 seemed to be controlled by more than two genes. In this study, the interval mapping method of QTL was tried depending on RFLP with F₂ and F₃ progenies (Lander and Bostein, 1989).

Construction of map and determination of QTLs for the clubroot resistance genes. Of the 192 RFLP probes

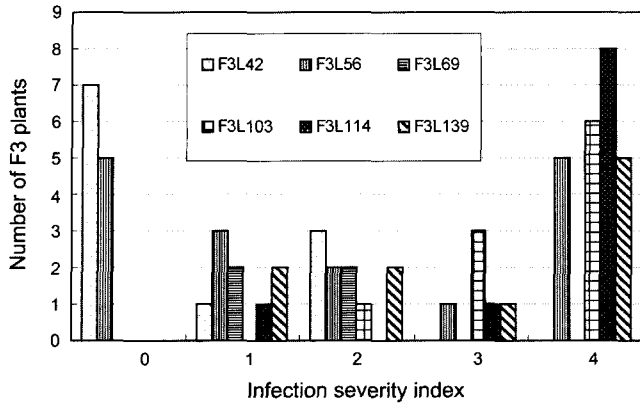


Fig. 1. Number of six F₃ plant lines (10 plants assayed in each line) according to infection severity index after inoculating *Plasmodiophora brassicae* race 6 to roots. Each index represents the following: index 0=no symptom or local lesion; 1=1-25% infection; 2=26-50% infection; 3=51-75% infection; 4=76-100% infection. Legends mean the line number of F₃ plants.

used in this study, 176 probes could show polymorphic bands from one to ten between the two parents. In the segregation test, 6 probes each were placed to 3 loci, and 12 probes each were placed to 2 loci (Fig. 2). Similarly, 121-

marker loci could be determined to 10 linkage groups and this grouping result was identical to actual 10 chromosomes in *Brassica* (Chyi et al., 1992). Finally, these probes covered approximately 1104 centimorgans (cM) (80%) of the RFLP linkage map (Teutonico and Osborn, 1994) and the mean distance between genetic markers was 8.0 cM (Fig. 2). With five restriction enzymes (*Pst*I, *Eco*RI, *Bam*HI, *Hind*III, and *Eco*RV), at least more than 70% of RFLP was obtained between parents and 5 loci were identified to non-linked ones. Determination of each locus was approved with > 3.0 value of LOD (log₁₀ of the odds ratio) score, which was a calculated ratio of the phenotypical effect QTL in each locus and a proof of the QTL presence (Fig. 3).

All loci were significantly examined by chi-square test in segregation, where each of the 98 and 23 loci was significant at 5% and 1% level, respectively. Duplicated genetic loci were also found, but they did not show wide-range homogeneity. Through the Mapmaker/QTL program used in this study, interval mapping which can make an estimate of each locus in the overall genome was carried out. Thus, the elimination of additive phenotypic effect by genetic recombination could be possible in progeny segregation (Teutonico and Osborn, 1994). In addition,

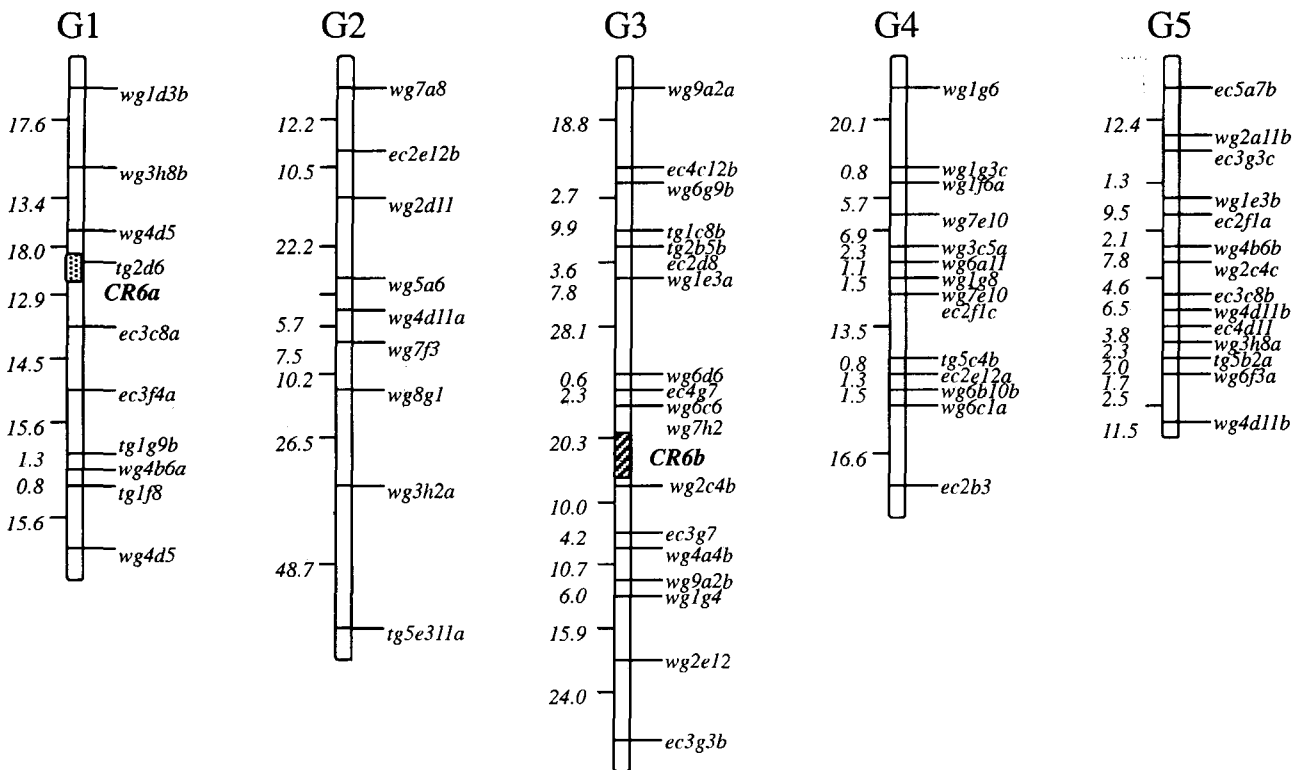


Fig. 2. *Plasmodiophora brassicae* race 6 resistance quantitative trait loci (QTLs) in *Brassica campestris* ssp. *pekinensis* genome. Linkage group number is indicated on top. Distances between markers are in centimorgans (left). Since this map was constructed from a multipoint mapping approach, distances between markers will vary and acquire an increased precision as more markers are added. The final order of the markers within each linkage group was assigned on the basis of the best logarithm of odd (LOD) score. Other orders were possible in certain areas and may also change as more markers are added to the map.

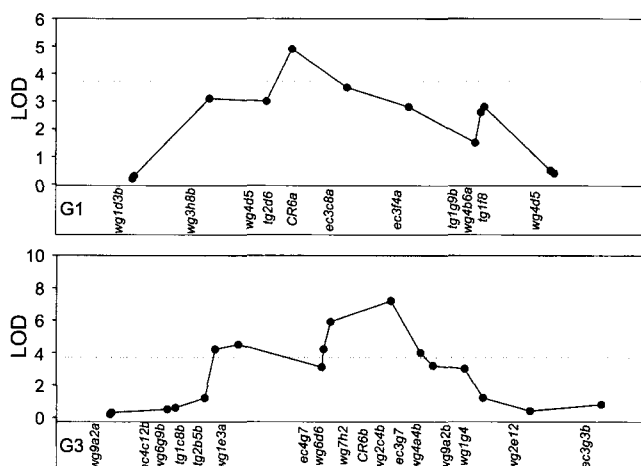


Fig. 3. Logarithms of odd (LOD) plots of quantitative trait loci (QTLs). LOD scores for QTLs affecting clubroot disease resistance are presented along the framework maps of linkage group 1 (G1) and 3 (G3). The LOD threshold of 3.7 is indicated. Percentage of variance explained by the QTL is indicated at left. The model used for this calculation fits QTL to the data simultaneously.

interval mapping was also applied for this QTL identification; with ANOVA and non-parametric tests, the QTLs can be rechecked.

Although a genetic map for *Brassica oleracea* based on RFLP markers was reported, which located the loci of resistance genes to race 2 of *P. brassicae* (Landry et al., 1992), authors of this study propose molecular map of two QTLs such as CR6a and CR6b conferring resistance to *P. brassicae* race 6 in *Brassica campestris* spp. *pekinensis*. A few digenic interactions were found in these QTLs, and for clubroot resistant loci additive QTLs were also found. Manzaneres-Dauleux et al. (2000) reported epistatic QTLs involved in clubroot resistance in *B. napus* populations, but no epistatic effects were found regarding clubroot resistance QTLs.

To verify the QTLs in F_2 population, F_3 lines were tested for infection of the fungi (Fig. 3). This was supported by the homozygous state of that locus, by which the resistant alleles of a QTL could be placed in QTL interval, if all markers are in this interval. Six F_3 lines consisting of eight plants were used for the clubroot test for QTLs verification. Based on resistant QTLs present inferred by flanking markers, two lines were selected and tested. These lines showed resistance level (Fig. 3) after *P. brassicae* infection to roots. Also, resistance segregated from F_2 to F_3 plants were found with heterozygous QTLs in the lines such as F3L56 and F3L114 (Fig. 3).

It was assumed that this quantitative resistance is race specific, where unique epistatic QTLs and QTLs location in different chromosomes have been reported (Landry et al.,

1992; Manzaneres-Dauleux et al., 2000), and shows additive trait in the population of *B. campestris* spp. *pekinensis*. In mapping the resistance loci to further characterize the resistance mechanism against *P. brassicae*, high-resolution linkage maps will be required. This map will surely be useful for the cloning of clubroot resistant genes such as map-based cloning strategy. In addition, the molecular dissection approach for this fungi resistance locus will provide good information on the genomic organization of similar resistance genes.

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