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Genetic Analysis of Clubroot Resistance in Chinese Cabbage using Single-spore Isolates of *Plasmodiophora brassicae* and Development of RAPD Markers Linked to its Resistance Gene

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Abstract

To identify inheritance of clubroot disease resistance genes in Chinese cabbage, seedling tests of BC_1P_1, BC_1P_2, and F_2 populations derived from F_1 hybrid (var. CR Saerona) using single spore isolate (race 4 identified with William's differential host) from *Plasmodiophora brassciae* were conducted. Resistance (R) and susceptible (S) plants segregated to 1:0 in backcross to the resistant parent. The F_2 population segregated in a 3(R):1(S) ratio. This result implied that the resistance of clubroot disease is controlled by a single dominant gene to the race 4 of *P. brassicae* in CR Saerona. To develop DNA markers linked to clubroot resistance genes, 185 plants of CR Saerona among F_2 populations were used. A total of 300 arbitrary decamer was applied to F_2 population using BSA-RAPD (Bulked segregant analysis-Randomly amplified polymorphic DNA). One RAPD marker linked to clubroot resistance gene in CR Saerona (OPJ_{1100}) was identified. This marker was 3.1 cM in distance from resistance gene in F_2 population. This marker may be useful for a marker-assisted selection (MAS) and gene pyramiding of the clubroot disease resistant gene in Chinese cabbage breeding programs.

Key words: *Brassica rapa.* spp. *Campestris*, Randomly amplified polymorphic DNA, Linkage analysis

Introduction

Chinese cabbage (*Brassica rapa* spp. *Campestris*) is not only one of the most important vegetables in Korea as a major ingredient of Korean traditional food 'Kimchi', it also is used as a model plant for studies of polyploidization.

Clubroot is a soil-borne disease caused by *Plasmodiophora brassicae* and on infested roots, it progresses into a distorted massive gall by plasmodia which prevents the roots from uptaking water and nutrients (Braselton 1995). It has been a major problem in *Brassica* crop cultivation areas and is a more serious problem in highland areas, mono-cropping. and extensively-cultivated areas in Korea (Cho et al. 2003).

Some agricultural practices such as crop rotation, utilization of catch crop and fungicide have been tried to prevent clubroot disease occurrence (Cho et al. 2002; Murakami et al. 2000; Tanaka et al. 1999). However, these methods are ineffective, expensive and there is a possibility to environmental contamination (Cho et al. 2003; Voorips 1995). Therefore, many efforts to breed *Brassica* crops resistant against clubroot have been accomplished (Hirai 2006). Genetic analysis of resistance CR has been thought to be controlled by a monogenic dominant gene (Hirai et al. 2004; Kuginuki et al. 1994; Piao et al. 2002; Suwabe et al. 2003; Yoshigawa 1981). But, these resistant

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hybrid cultivars have been susceptible to clubroot in some field trials (Hirai et al. 2004; Piao et al. 2004). This is thought to have occurred by the evolution of pathotypes or loss of resistance genes during breeding of CR cultivars (Kuginuki et al. 1999). Park (2002) suggested that the origin of the CR gene in CR Saerona was not identical to that of CR-Shinki. When the interaction between pathogen isolates collected in several regions and clubroot resistance cultivars sold in Korea was analyzed, the occurrence of disease in CR Saerona was different to that of the other CR-cultivars, including CR-Shinki. The resistance parent line (930WG) of CR Saerona was bred by the use of different genetic materials with CR-Shinki (personal communication with breeder).

More virulent pathotypes can be more prevalent in F_1 hybrids which were produced by the same resistance source in cultivation areas (Cho et al. 2003). In the infected field, the pathogens are possibly mixtures of different genotypes, so it is essential to prepare homogenous isolates using single-spore isolation for the reliable genetic analysis of resistance (Manzanares-Dauleux et al. 2000). In this study, a new genetic resource of clubroot resistance in Chinese cabbage, genetic analysis of resistance genes, and RAPD markers linked to resistance genes are reported.

Materials and Methods

Plant materials

A clubroot resistant line, 930WG (P_2), and a susceptible line, 332MS (P_1) were used for the parental lines to make the F_1 hybrid Chinese cabbage cultivar, CR Saerona. For the genetic analysis of resistance against clubroot, a backcross population (BC_1P_1, BC_1P_2) and an F_2 population consisting of 203 plants were made by bud pollination and self-bud pollination, respectively. Plant DNA was isolated from young leaves of the F_2 population using Plant DNA extraction kit (G-SpinIIp™, iNtRON Biotechnology, Korea) and DNA concentration was calculated by DNA fluorometer (NanoDrop ND-100, Nanodrop Tech, USA).

Clubroot resistance test

The *Plasmodiophora brassicae* of single spore isolates (SG110-2, Williams differential host (Williams 1966), race 4; European Clubroot Differential host (Buczacki et al. 1975), $ECD_{0/7/-}$) were kindly provided by the NHRI (National Horticultural Research Institute) and maintained in a susceptible Chinese cabbage cultivar, 'Chilseong' (Nongwoo Bio, Korea). The inoculums preparation and resistance test to *P. brassicae* were carried out with soil inoculation methods as reported by Cho et al. (2002). The infected root was ground five times with distilled water (w/v) and the impurities were removed by filtering through eight cheese cloths. The solution containing the resting spores was centrifuged at 3,000 rpm for 5 min and the sediments were re-suspended with distilled water. This centrifugation and re-suspension procedure was repeated three times. The final concentration of resting spores was adjusted to 5×10^6 spores/ml using a hemocytometer. The soil block containing the resting spores was made with a mixture of resting spore solution, Root Media (Nongwoo Bio, Korea), filtered soil with sieve, and Perlite in a ratio of 1.5:3:4:2(volume). The non-infected Root Media was filled in 50-pore-plug tray and the soil block (3 cm width x 3 cm length x 5 cm depth) was inserted into them. The seeds of the backcross and F_2 population were sown in the soil block with three seeds at the center and covered with Root Media at about 2 cm layer thick. The symptom grades of clubroot disease were scored as 0: no visible symptom, 1: very slight swelling, usually confined to lateral root (arrow indicated), 2: moderate swelling on lateral or tap roots, 3: severe swelling on lateral or tap roots 35 days after sowing (Fig. 1).

Fig. 1. Symptom grade of clubroot disease caused by *Plasmodiophora brassicae* in Chinese cabbage. Grade 0: No visible symptom, 1: very slight swelling, usually confined to lateral root (arrow indicated), 2: moderate swelling on lateral or tap roots, 3: severe swelling on lateral or tap roots.

RAPD analysis

For the RAPD analysis, we applied a bulked segregant analysis (Michelmore et al. 1991). Two bulks were made by mixture of DNA from each 10 F_2 lines showing no symptom grade 0, and highly susceptible lines showing symptom grade 3 as BR(bulked resistance) and BS(bulked susceptible), respectively. The parental DNA, two bulks DNA were used for RAPD analysis as follows: genomic DNA 20 ng, primer 5 pM, dNTP 200 μM, Taq polymerase (Bioneer, Korea) 1.0U, 10X buffer 2$\mu\ell$ (500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 0.01%

gelatin) in a total volume 20㎕. The reaction was performed in Perkin Elmer 9700 (Applied Biosystems, Foster City, USA) with following condition: pre-denaturation 5 min at 95℃, 40 cycles of 1 min at 94℃, 1 min 30 sec at 35℃, 1 min at 72℃, and post-extension 15 min at 72℃ A total of 300 OPERON arbitrary 10mer kits (Operon Tech., Alameda, USA) were used for the bulked segregant analysis to detect polymorphism between BR and BS.

Statistical analysis

To analyze the genetic resistance of clubroot disease, the plants that had symptom grade 1 were regarded as susceptible plants. The goodness-of-fit of the resistance in CR-Saerona was calculated in Chi-square test according to the segregation ratio of resistance and susceptible at a 1:1, 1:0, and 3:1, with respect to the corresponding populations, BC_1P_1, BC_1P_2, and F_2, respectively. Linkage between RAPD marker and resistance gene was calculated with 'MAPMAKER' vers. 3.0 (Lander et al. 1987) and converted to cM with Kosambi mapping function.

Results

Genetic analysis of clubroot resistance in a CR-Saerona cultivar

The segregation analysis of the clubroot resistance gene in Chinese cabbage cultivars against single spore isolates (SSI) of *P. brassicae* was performed using a backcross population and an F_2 population. The resistance (R) and susceptible (S) plants in the backcross population was made by the cross with the resistant parent line was segregated in a ratio 51:3 which was the expected ratio of 1(R):0(S) and this result implied that the resistant parent line had a single dominant gene (Table 1). In the F_1 and F_2 populations, clubroot resistance also showed single gene inheritance to the SSI race 4 as we expected with a segregation ratio 1(R):0(S) and 3(R):1(S), respectively (Table 1).

Table 1. Segregation analysis of clubroot disease resistance in BC_1P_1, BC_1P_2, F_1, and F_2 populations in Chinese cabbage using single spore isolates (race 4 by Williams differential host).

Population	Phenotype			Expected ratio (R:S)	χ^2	*P-value*
	Total	Resistance (R)	Susceptible (S)			
BC_1P_1	53	39	14	1:1	0.0006	0.98
BC_1P_2	54	51	3	1:0	-	-
F_1	31	31	0	1:0	-	-
F_2	203	154	49	3:1	0.78	0.38

P_1: Susceptible parent, P_2: Resistant parent, F_1: CR saerona (Resistant variety)

(A) (B)

Fig. 2. Phenotype of infected and healthy roots of Chinese cabbage after soil inoculation method with single spore isolates (race 4) of *Plasmodiophor brassicae* after 35 DAI (days after innoculation). A: Susceptible individual plants, B: Resistant individual plants.

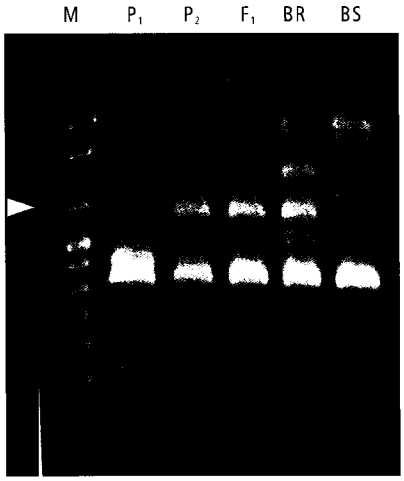

Fig. 3. Bulked Segregant Analysis-RAPD profile generated by the primer OPJ from genomic DNA of Chinese cabbage. Triangle indicated polymorphic band (OPJ_{1100}) between resistant and susceptible plants. P_1; Susceptible parent, P_2; Resistant parent, F_1; CR Saerona, BR; Resistant bulk (10 plants were bulked), BS; Susceptibility bulk (10 plants were bulked)

However, in the susceptible parent backcross population, the segregation was not significant in a single gene inheritance. In the present study, we used the same SSI that Piao et al. (2004) used and they reported that CR-Shinki carried a single dominant gene, *Crb*.

Development of RAPD marker linked to clubroot resistance gene

To develop RAPD markers linked to clubroot resistance genes in CR Saerona, each resistance and susceptible plant in the F_2 plant was used in bulked segregant analysis. A total of 300 arbitrary primer (OPERON OPA, OPB, OPC, OPD, OPJ, OPK, OPQ, OPR, OPS, OPT, OPU, OPW, OPV, OPX, and OPZ) was applied in parental lines, F_1 plants, BR and BS,. A RAPD marker, OPJ_{1100} which originated from resistant parents (930WG) was identified in BSA-RAPD analysis. This maker was uniquely amplified in the resistance parent line, F_1 plants, and BR (Fig. 2). To identify the genetic distance from clubroot

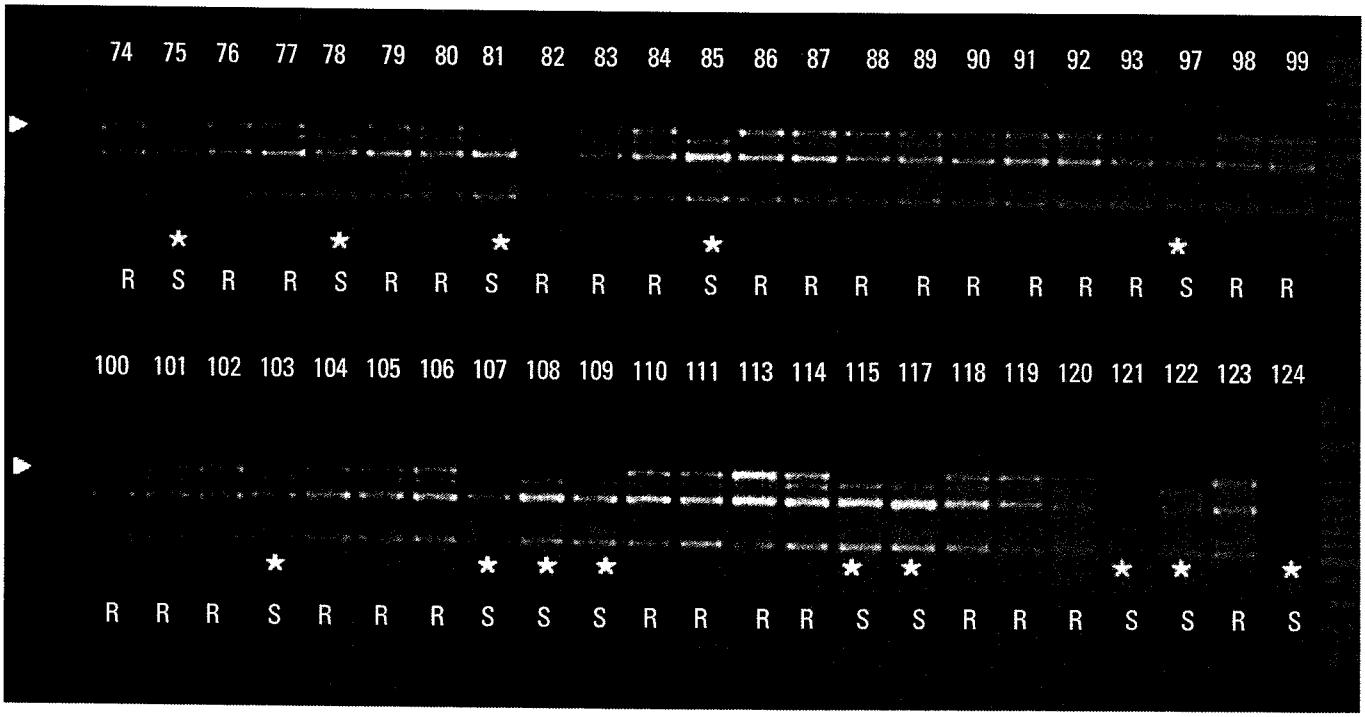

Fig. 4. Genotyping of F_2 progeny with OPJ_{1100} marker on 185 individual plants. R, Resistant plants; S, Susceptible plants. Triangles indicate polymorphic band (1.1kb).

Table 2. Segregation of resistance to clubroot disease and OPJ_{1100} marker in F_2 population.

Phenotype	No. of plants	Segregation of OPJ_{1100} marker		Recombination ratio (cM)
		Present[z]	Absent[y]	
Resistant	139[x]	131	8	3.1
Susceptible	46[w]	1	45	

[z,y] OPJ_{1100} band was present and absent after agarose gel electrophoresis, respectively. [x], 15 resistant plants identified in seedling test were missed in segregation analysis in OPJ_{1100} marker linked to clubroot disease resistance. [w], 3 susceptible plants identified in seedling test were missed in segregation analysis in OPJ_{1100} marker linked to clubroot disease resistance.

resistance gene, 185 plants from the F_2 population were analyzed with OPJ_{1100} marker (Fig. 3). Linkage analysis showed that this marker was positioned in 3.1cM from clubroot resistance gene in CR Saerona (Table 2).

Discussion

The genetic resistance gene against clubroot disease has been reported as a single major gene in *B. rapa* (Hirai et al. 2004; Kuginuki et al. 1997; Mastumoto et al. 1998; Piao et al. 2004; Suwabe et al. 2003) and QTL (Suwabe et al. 2006). In our data, the clubroot resistance gene in CR Saerona was thought to be a single dominant gene when the BC_1 (backcross to resistance parent line), F_1, and F_2 populations were used for genetic analysis (Table 1) against SSI (race 4) of *P. brassicae*. But in the backcross populations that were crossed to the susceptible parent line, it was segregated as double recessive genes; data are shown in Table 1. This discrepancy between backcross populations might be an effect of environment or imply the existence of minor genes in the susceptible parent line. We used SSI as inoculums that were maintained in susceptible cultivars and this might change the virulence of *P. brassicae* which has a high mutation rate (Kuginuki et al. 1999). Suwabe et al. (2006) reported two QTL, *Crr 1* and *Crr 4*, had isolate specific resistance to the pathogen in *B. rapa* and Rocherieux et al. (2004) also indicated *B. oleracea* had five QTLs with minor effects to several isolates. For elucidating the interaction between *P. brassicae* race and plant, this analysis of resistance to SSI will be useful.

Most genetic resources of resistance in *B. rapa* originated from European turnips, Gelria R, Siloga, Debra, and Milan White (Hirai 2006) because any Brassica lines originating from East Asia did not show resistance to *P. brassicae* (Yoshikawa 1981). Although the detailed history in the breeding of CR Chinese cabbage in Korea was not disclosed, its genetic resource was assumed as the same origin from Japan (Hirai et al. 2004) and this might affect on rapid breakdown of resistance in CR hybrid cultivar (Cho et al. 2003). CR cultivars bred by seed companies have recently become susceptible to clubroot in some cultivation areas (Cho et al. 2003). Therefore, there is a

need to breed more resistant CR cultivars using accumulation of various resistance sources (Kuginuki et al. 1997).

Breeding resistance to clubroot disease can be accelerated by using molecular markers such as RAPDs, SSRs, AFLPs, and SCARs (Hirai et al. 2004; Kuginuki et al. 1997; Nomura et al. 2005; Piao et al. 2004; Suwabe et al. 2006). And more sustainable resistance will be succeeded by gene pyramiding of more than one CR gene in one cultivar using DNA markers (Hirai 2006).

We identified one RAPD marker linked to a clubroot resistance gene using BSA method and this marker was 3.1 cM from the CR gene (Table 2). CR Saerona was bred by selfing some CR cultivars after several the selections in seedling tests (personal communication with breeder). Despite the unclearness of the origin of resistance, it might have a different genetic background. However, further identification of the origin is needed using common anchoring markers, SSRs, or RFLPs.

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