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Development of Molecular Markers for *Xanthomonas axonopodis* Resistance in Soybean

Ki-Seung Kim, Kyujung Van, Moon Young Kim, and Suk-Ha Lee†

School of Plant Science, Seoul National University, Seoul 151-921, Korea

ABSTRACT: A single recessive gene, *rxp*, controls the bacterial leaf pustule (BLP) resistance in soybean and in our previous article, it has been mapped on linkage group (LG) D2 of molecular genetic map of soybean. A total of 130 recombinant inbred lines (RILs) from a cross between BLP-resistant SS2-2 and BLP-susceptible Jangyeobkong were used to identify molecular markers linked to *rxp*. Fifteen simple sequence repeat (SSR) markers on LG D2 were screened to construct a genetic map of *rxp* locus. Only four SSR markers, Satt135, Satt372, Satt448, and Satt486, showed parental polymorphisms. Using these markers, genetic scaffold map was constructed covering 26.2 cM. Based on the single analysis of variance, Satt372 among these four SSR markers was the most significantly associated with the resistance to BLP. To develop new amplified fragment length polymorphism (AFLP) marker linked to the resistance gene, bulked segregant analysis (BSA) was employed. Resistance and susceptible bulks were made by pooling equal amount of genomic DNAs from ten of each in the segregating population. A total of 192 primer combinations were used to identify specific bands to the resistance, selecting three putative AFLP markers. These AFLP markers produced the fragment present in SS2-2 and the resistant bulk, and not in Jangyeobkong and the susceptible bulk. Linkage analysis revealed that MeetEact97 (P=0.0004, R^2=14.67%) was more significant than Satt372, previously reported as the most closely linked marker.

***Keywords*:** amplified fragment length polymorphism (AFLP), bacterial leaf pustule (BLP), bulked segregant analysis (BSA), *rxp*, simple sequence repeat (SSR), Soybean.

Xanthomonas axonopodis pv. *glycines*, a gram-negative bacterial plant pathogen, causes bacterial leaf pustule (BLP) in soybean. BLP is one of the prevalent bacterial diseases in the world and more common in July and August in Korea due to high relative humidity. Premature leaf drop caused by this disease are leaded to a yield loss. Seeds from BLP-infected soybean plants are much smaller and fewer than those from healthy plants (Weber *et al.*, 1996).

A soybean cultivar, CNS, has been known to be resistant to *Xanthomonas axonopodis* and rarely became infected under natural conditions in the field (Feaster, 1951). Hartwig & Lehman (1951) first observed a high degree of resistance in CNS and found that a single recessive gene conditioned its resistance. On the basis of the difference in degree of susceptibility of the parental lines and the range in degree of susceptibility among progenies, the dominant allele was thought to be affected by other genes, possibly determining the degree of susceptibility. This major gene was latter designated as *rxp* (Bernard & Weiss, 1973).

The *Rxp* locus was known to be linked to the malate dehydrogenase (*Mdh*) locus with a percentage recombination estimate of 15.18 ± 3.81% (Palmer, 1992). In the public genetic soybean map (Cregan *et al.*, 1999), the *Rxp* locus was located on linkage group (LG) D2/R and LG H where the *Mdh* locus was also located. Recently, the *Rxp* locus was mapped to the Satt372 locus on LG D2/R rather than any marker on LG H (Narvel *et al.*, 2000; Van *et al.*, 2004).

The first objective of this research was to confirm the resistance gene for BLP in the resistant mutant parent, SS2-2, and to construct a scaffold map for resistance locus using SSR markers. The second objective of this research was to develop the AFLP markers tightly linked to a resistance gene for BLP using BSA (Michelmore *et al.*, 1991).

Materials and Methods

Plant materials

A soybean population from a cross between BLP-resistant SS2-2 and BLP-susceptible Jangyeobkong was constructed to develop a RIL population. A total of 130 RILs were developed by direct descendants of the F_2 generation and used for constructing a genetic linkage map and evaluating the phenotypic traits.

Inoculation

Phenotypic evaluation for BLP resistance was conducted from spring to summer in 2002. The inoculum, 8ra, was prepared by culturing in potato sucrose agar (PSA) medium.

†Corresponding author (Phone) +82-2-880-4545 (E-mail) sukhalee@snu ac kr
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Bacterial cells on the culture plates were suspended in sterile distilled water and adjusted to 10^8 CFU/ml. Using an atomizer, the inoculum was sprayed onto the upper and lower leaf surfaces of one-month-old soybean plants.

DNA isolation

To obtain genomic DNA from soybean for the SSR and AFLP marker analyses, two or three young and healthy soybean leaves of each plant were harvested prior to full expansion in the greenhouse, placed in the paper bag on ice, and stored at 80°C until use. Genomic DNA was isolated using hexadecylatri methylammonium bromide (CTAB) method described by the procedure of Keim *et al.* (1988).

SSR marker analysis

A total of 15 SSR markers were selected on LG D2 from the molecular USDA genetic map of soybean (Cregan *et al.*, 1999). The 5-end of each primer was labeled with fluorescent tags, blue (6-Fam), green (Hex), or yellow (Ned) (Ziegle *et al.*, 1992). Genomic DNA was amplified by polymerase chain reaction (PCR) (Diwan & Cregan, 1997) as follows: 32 cycles of denaturation at 94 °C for 25 sec, annealing at 46 °C for 25 sec, and extension at 68 °C for 25 sec on a PTC100 programmable thermal Controller (MJ Research INC, Watertown, MA, USA). Amplified products from different SSR loci carrying the fluorescent label can be simultaneously analyzed in the same gel lane if allele size ranges do not overlap. The samples of combined PCR products were loaded and separated on a standard DNA sequencing gel containing 4 % polyacrylamide, 8 M urea, and 0.5X TBE at 1700 V constant power for 2 hours on an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Genescan and Genotyper 2.0 program (Applied Biosystems, Foster City, CA, USA) were used for gel image analysis and accurate characterization of the alleles and for automated data output Each primer was firstly used to screen for parental polymorphism between SS2-2 and Jangyeobkong and then polymorphic primers were used to genotype all the progenies in the mapping population.

AFLP marker analysis

The AFLP analysis was performed as described previously (Vos *et al.*, 1995). After two different restriction enzymes, *Mse*I and *Eco*RI, were used to digest the genomic DNA, DNA fragments were ligated with *Mse*I and *Eco*RI adapters.

Pre-selective amplification reaction was performed using two AFLP primers with a single selective nucleotide and AmpliTaq polymerase (Applied Biosystems, Foster city, CA, USA) in a PTC 100 programmable Thermal Controller (MJ Research Inc. Watertown, MA, USA). Cycling conditions consisted of a initial incubation at 72 °C for 2 min, followed by 30 sec of denaturation at 94 °C, 30 sec annealing at 56 °C, and 2 min of extension at 72 °C for 20 cycles, and 30 min of final incubation at 60 °C.

Selective amplification was conducted using two selective primers. In this study, selective *Eco*RI primers were end-labeled using florescent tages, blue (6-Fam), green (Hex), or yellow (Ned). For selective amplification, 76.6 nM fluorescent-labeled *Eco*RI primer was added into a 10 μl of PCR mixture containing 2.5 μl of the diluted pre-selective products. The selective amplification was performed for 36 cycles as follows: denaturing step 94 °C for 30 sec, annealing step at 65 °C for 30 sec, and extension step at 72°C for 2 min. The annealing temperature was reduced by 0.7C each cycle from starting 65 °C to 56 °C, and then remained at 56 °C for the remaining 23 cycles.

The reaction mixtures were loaded and separated on 7 % denaturing polyacrylamide gels containing 6 M urea at an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Genescan software and Genotyper software (Applied Biosystems, Foster City, CA, USA) were used for the same purpose as above.

Bulked segregant analysis (BSA)

Based on the SSR markers and phenotypic data, resistant and susceptible bulks were made by pooling separately with equal amount of 10 pre-selective amplified DNA obtained from resistant individuals and susceptible individuals in the population. The resistant and susceptible bulks were examined with 6 *EcoR*I and 32 *Mse*I primers, bringing up a total of 192 different primer combinations. Primer combinations yielding candidate markers were subsequently applied to all individuals of the population in order to confirm the linkage between the AFLP markers and a resistance locus.

Map construction

Marker data were obtained from each segregating progeny, which were used to construct a genetic map. The segregation ratio of each marker was calculated with a chi-square test under the hypothesis of the expected 1 : 1 ratio. Linkage analysis of the entire set of markers was performed using MAPMAKER version 3.0 (Lander *et al.*, 1987). A linkage map containing SSR loci and AFLP loci was constructed using the Kosambi map function (Kosambi, 1944). For combining markers into linkage groups, a minimum likelihood of odds (LOD) of 3.0 and maximum distance of 30 cM were

used. The primary linkage group was determined on the basis of public USDA map (Cregan *et al.*, 1999).

Statistical analysis

The general linear model (GLM) procedure of SAS was used for statistical analyses. Associations between marker genotypes and variations in phenotypic reaction were tested by single factor analysis of variance (SF-ANOVA) (Tanksley, 1989). Multiple linear regression within a single linkage group (SLG-Regr) was also applied to determine the most significant marker among those detected using SF-ANOVA. Two-factor analysis of variance was also used for checking all possible pairs of significant marker. To confirm the correct position of *rxp*, composite interval mapping was conducted using Windows QTL Cartographer 2.0 (Wang *et al.*, 2002).

RESULTS AND DISCUSSION

To evaluate disease severity, the parents and 130 RILs were inoculated with 8ra in the greenhouse. A number of small yellow-to-brown lesions with a raised pustule per leaflet were determined at 30 days after inoculation, which was changed into a scale of 0 to 7 = no lesions, 1 = 1 to 10 lesions, 2 = 11 to 20 lesions, 3 = 21 to 30 lesions, 4 = 31 to 40 lesions, 5 = 41 to 50 lesions, 6 = 51 to 60 lesions and 7 = more than 61 lesions). Wide variations (0 ~ 7) were observed in the reaction to 8ra among RIL progenies. The BLP-resistant female parent SS2-2 and BLP-susceptible male parent Jangyeobkong were scored as 3 and 7, respectively (Fig. 1). Since responses to BLP were observed in both resistant and susceptible plants, scoring disease severity of BLP had a very pivotal role in conducting the experiment.

The *rxp* locus was mapped near the Satt372 locus on LG D2 rather than any marker on LG H (Narvel *et al.* 2000; Van *et al.*, 2004). Each primer set was firstly used to screen for parental polymorphism between SS2-2 and Jangyeobkong. Of 15 SSR markers on LG D2 near *rxp* locus, only four markers showed parental polymorphism between SS2-2 and Jangyeobkong. These polymorphic SSR markers (Satt135, Satt372, Satt458, and Satt486) were used to construct a genetic linkage scaffold map of the LG D2. In a genetic linkage map, four markers were genetically linked, spanning about 26.2 cM.

To detect the presence of segregation distortion, Chi-square analysis was used to test a segregation ratio of 1 : 1. All polymorphic SSR markers showed the male-skewed segregation. Tsunematsu (1996) suggested that several genes such as gametophyte gene, low cross-ability gene, hybrid sterility gene or gametic lethal gene might be closely linked to the genomic regions exhibiting the segregation distortion. However, the reasons for this segregation are not still clear because those genes have not been mapped in the classical or molecular genetic maps of soybean.

Four SSR markers were used to identify the association between markers and disease symptoms. Based on single

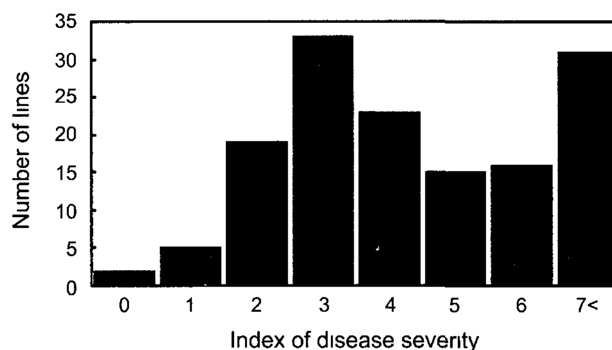

Fig. 1. Distributions of disease severity among 130 lines of the F_7 RIL soybean population one-month after inoculation with 8ra. BLP resistance segregated in the ratio of 1 (resistance): 1 (susceptible), confirming single gene inheritance.

Table 1. SF-ANOVA analysis and single regression for identification of SSR markers and AFLP markers associated with BLP resistance

Marker	LG	SF-ANOVA[a]		Alleic means		SLG-Rger[a]	
		P	R^2(%)	S/S[b]	J/J[b]	P	R^2(%)
Satt135	D2	0.8836	0	4 38	4 46		
Satt372	D2	0.0004	9.6	3 61	5 35		
Satt458	D2	0.0576	3 6	3 89	5 04		
Satt486	D2	0 0001	11 2	3.49	5.34		
McctEact97	D2	0.0009	8 39	3 92	5 44	0 0004	14 67
MaacEacc167	D2	0 0449	3.51	4 20	5.23		
MccaEaag122	D2	0.0067	5.63	3.96	5.24		

[a]SF-ANOVA : single factor analysis of variance SLG-Regr : multiple regression with markers on single linkage group
[b]S/S : homozygous SS2-2, J/J homozygous Jangyeobkong

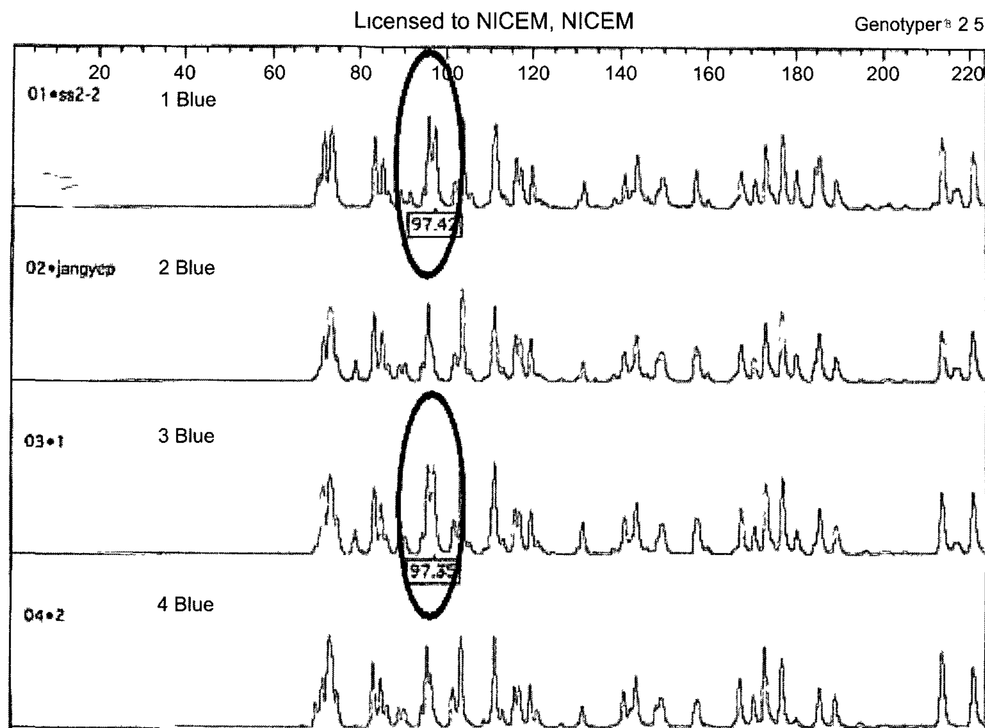

Fig. 2. Segregation of bulked segregant analysis (BSA) of 10 BLP-resistance and 10 BLP-susceptible lines using the AFLP technique. The circles indicate the potential marker (McctEact97) linked to the *rxp* Each line represents SS2-2, Jangyeobkong, the resistant bulk, and the susceptible bulk, from top to bottom.

factor ANOVA and single regression analysis, Satt372 was the most significantly associated marker to BLP resistance, locating near *rxp* based on the USDA map (Cregan *et al.*, 1999). This is in a good agreement with previous studies (Narvel *et al.*, 2000; Van *et al.*, 2004). Therefore, we concluded that *rxp* mainly controls the resistance to 8ra in this population.

BSA was used to identify AFLP markers tightly linked to the BLP resistant gene. A total of 192 primer combinations were used to identify markers specific to the resistant bulk and SS2-2. From this screening, three putative AFLP markers were identified. These AFLP markers produced fragments presented only in SS2-2 and the resistant bulk, not in Jangyoobkong and the susceptible bulk (Fig. 2). Three AFLP markers, McctEact97, MccaEaag122, and MaacEacc167 were fit to the expected 1 : 1 ratio and significantly ($P < 0.05$) associated with BLP resistance in the population. Regression analysis revealed that McctEact97 was the most significantly ($P = 0.0004$, $R^2 = 14.67\%$) associated with the BLP resistant gene (Table 1). McctEact97 was 11.9 cM apart from Satt372, which was known to tightly link to *rxp* on LG D2 (Fig. 3).

To confirm the correct position of *rxp*, composite interval mapping was conducted using Windows QTL Cartographer 2.0 (Wang *et al.,* 2002). Composite interval mapping revealed that a new AFLP marker, McctEact97, was more

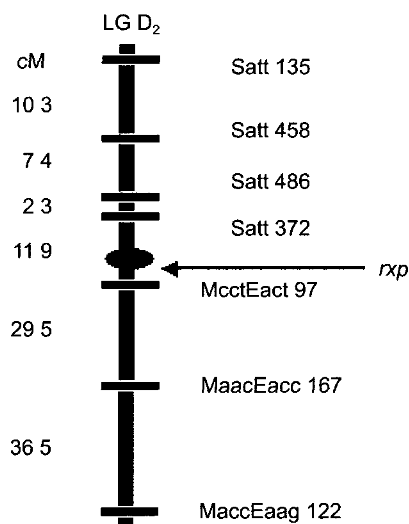

Fig. 3. Genetic linkage map of SSR and AFLP markers linked to the *rxp* in LG D2. The markers linked to the *rxp* were mapped with MAPMAKER 3 0 (Lander *et al* 1987) Genetic distances in centiMorgans were calculated using the Kosambi function (Kosambi, 1944) The marker positions are on the right-hand side, estimated map distance (cM) on the left-hand side and USDA linkage group designation (Cregan *et al.*1999) is on the top of each linkage group

closely linked to *rxp* than Satt372 and was apart 3 cM away from *rxp* (Fig. 3).

In this study, we applied BSA in conjunction with AFLP

marker technology and successfully identified AFLP markers tightly linked to the *rxp*. This newly identified AFLP marker may be helpful for marker-assisted selection (MAS) for BLP-resistant soybean lines in a segregating population.

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