

Marker-Assisted Foreground and Background Selection of Near Isogenic Lines for Bacterial Leaf Pustule Resistant Gene in Soybean

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Abstract

Bacterial leaf pustule (BLP) caused by *Xanthomonas axonopodis* pv. *glycines* is a serious disease to make pustule and chlorotic haloes in soybean [*Glycine max* (L.) Merr.]. While inheritance mode and map positions of the BLP resistance gene, *rxp* are known, no sequence information of the gene was reported. In this study, we made five near isogenic lines (NILs) from separate backcrosses (BCs) of BLP-susceptible Hwangkeumkong x BLP-resistant SS2-2 (HS) and BLP-susceptible Taekwangkong x SS2-2 (TS) through foreground and background selection based on the four-stage selection strategy. First, 15 BC individuals were selected through foreground selection using the simple sequence repeat (SSR) markers Satt486 and Satt372 flanking the *rxp* gene. Among them, 11 BC plants showed the BLP-resistant response. The HS and TS lines chosen in foreground selection were again screened by background selection using 118 and 90 SSR markers across all chromosomes, respectively. Eventually, five individuals showing greater than 90% recurrent parent genome content were selected in both HS and TS lines. These NILs will be a unique biological material to characterize the *rxp* gene.

Key words: bacterial leaf pustule, marker-assisted selection, near isogenic line, recurrent parent genome, *rxp* gene, *Xanthomonas axonopodis* pv. *glycines*

Introduction

Higher temperature and humidity by global warming has pre-disposed soybean [*Glycine max* (L.) Merr.] to more bacterial, fungal, and viral diseases during the growing season. Among several diseases, bacterial leaf pustule (BLP) caused by *Xanthomonas axonopodis* pv. *glycines* (*Xag*) is one of the most serious diseases of soybean in Korea. The typical symptom of BLP is a halo that generates a small yellow to brown lesion and a pustule, which cause premature defoliation resulting in a heavy

loss of soybean (Hartwig and Jonson 1953; Kennedy and Tachibana 1973). A resistance gene to BLP, supposedly recessive and designated as *rxp* (Hartwig and Johnson 1953; Palmer et al. 1992) was identified in CNS. The *rxp* locus has been found to be located on linkage group (LG) D2 using the phenotypic variation of the BLP reaction in segregation mapping populations including an F₂ (Narvel et al. 2001) or F₂-derived recombinant inbred line (RIL) populations (Kim et al. 2004a; Van et al. 2004) from a cross of distinct genotypes of the BLP resistance trait. The simple sequence repeat (SSR) markers, Satt372 and Satt014 were observed to flank the *rxp* locus, which was 3.9 cM away from Satt372 (Narvel et al. 2001). Furthermore, comparisons between phenotypic values for the BLP resistance and the SSR marker genotypes in the RIL populations suggested that

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Satt372 showed the most significant association with the trait variation (Kim et al. 2004b; Van et al. 2004). In the recent soybean consensus map (Song et al. 2004), Satt486 was mapped between those flanked markers.

Though several genetic studies and mapping of the *rxp* gene have been reported using such recombination frequency in the populations, no gene information such as nucleotide sequences is available. This may be due to the fact that resolutions of the markers surrounding *rxp* in the RIL populations are not high enough to isolate *rxp* by map-based cloning. The limited size of the mapping populations could not generate dense recombinant segments in the RIL populations.

Another type of plant genetic resources for gene mapping is near isogenic lines (NILs), which are developed through repeated backcrossing and extensive selection for a trait of interest. The NILs contain a single or a small number of genomic fragments from a donor parent with the target trait into a different homogeneous genetic background. By comparing such NILs containing the presence or absence of the target region, a large number of genes controlling the desirable traits such as pathogen resistance have been isolated through advanced molecular technologies because these pairs of NILs were useful and valuable for determining the target gene in many crops (Wight et al. 2004; Young et al. 1988). Furthermore, marker-assisted selection (MAS) can be applied for screening of individuals with not only the target gene but also the genomic regions of a recurrent parent in each backcrossing generation, which allows accelerating the development of NILs for the trait of interest (Matthias et al. 1999).

In the present study, the NILs with respect to the BLP resistance gene, *rxp* were developed by introducing the *rxp* gene into two Korean BLP-susceptible elite cultivars, Hwangkeumkong and Taekwangkong through consecutive backcrosses. These NILs were confirmed by foreground and background MAS, as well as phenotypic response to *Xag* inoculation.

Methods and materials

Plant Materials

For the construction of NILs for the BLP resistance in this study, Korean genotype SS2-2 showing BLP-resistant response to *Xag* was used as a donor parent. Hwangkeumkong and Taekwangkong were selected as a recurrent parent because they are Korean elite cultivars but BLP-susceptible (Fig. 1). We made two sets of NILs from the crosses Hwangkeumkong x

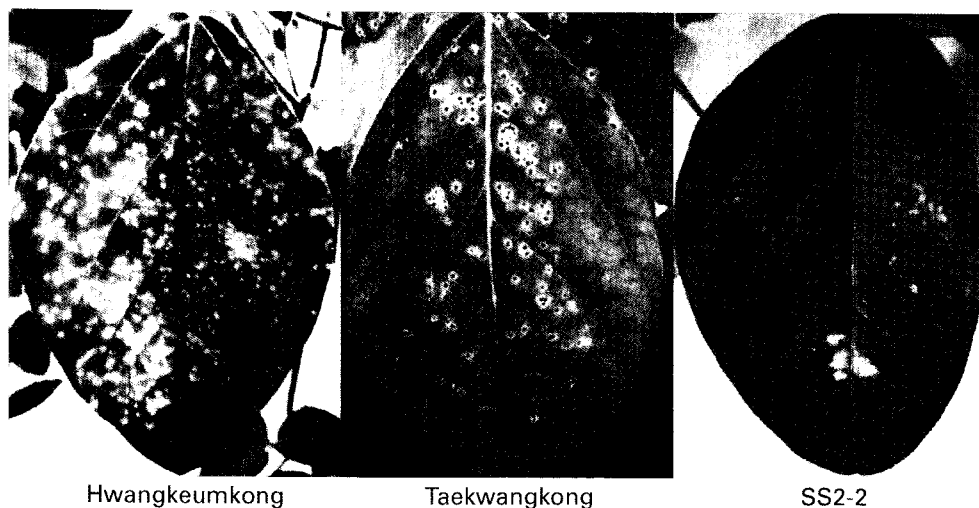


Fig. 1. The symptoms of inoculation with *Xanthomonas axonopodis* (strain 8ra) in Hwankeumkong (recurrent), Taekwangkong (recurrent), and SS2-2 (donor).

SS2-2 (HS) and Taekwangkong x SS2-2 (TS) by backcrossing three times in succession. Five BC₃F₃ and three BC₃F₂ lines were obtained from the HS and TS crosses, respectively (Fig. 2). For molecular marker genotyping and BLP phenotyping, five or eight seeds per BC lines were sown for growth of BC plants. However, one individual of HS3 lines, one individual of HS5 lines, one individual of TS1 lines, and two individuals of TS2 lines were not successfully grown (Table 1).

Bacteria Culture and Inoculation

The pathogen strain 8ra of *Xag* was cultured in peptone sucrose agar medium (Oh et al. 1999) at 28°C for 48 h. The culture was diluted with 10 mM MgCl₂ to make 1 × 10⁸ colony forming units (cfu) per milliliter under 0.1 ppm rifampicin antibiotics. The parents and four-week-old BC plants were inoculated by spraying the bacterial suspension to both sides of leaves using an atomizer. The infected plants were incubated in growth chambers under the conditions of 28°C, 100% relative humidity and 12 h illumination.

Table 1. Foreground selection of BC plants of HS and TS using SSR markers and phenotype of bacterial leaf pustule.

Cross	Line	No. of BC plants in each line ¹⁾	No. of selected BC plants by SSR markers ²⁾	No. of selected BC plants by BLP phenotype
Hwangkeumkong X SS2-2 (HS)	HS 1	5	4	3
	HS 2	5	1	1
	HS 3	4	1	1
	HS 4	5	1	0
	HS 5	4	2	2
Taekwangkong X SS2-2 (TS)	TS 1	7	2	1
	TS 2	6	1	0
	TS 3	8	3	3
Total	8	44	15	11

¹⁾ BC progenies of HS and TS used in this study were BC₃F₃ and BC₃F₂, respectively.

²⁾ Satt372 and Satt486 for HS and Satt486 for TS were used.

Foreground and Background Selection of NILs for the *rxp* Gene

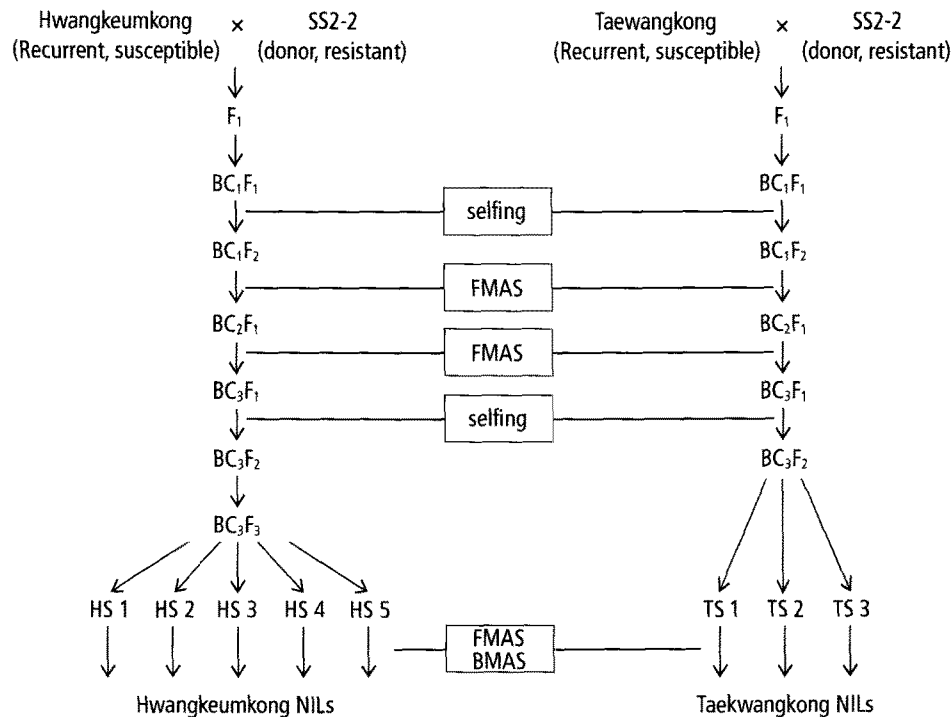


Fig. 2. Schematic diagram showing marker-assisted NIL selection through backcross to introduce the bacterial leaf pustule resistance gene. FMAS, foreground marker-assisted selection; BMAS, background marker-assisted selection

Genomic DNA extraction and SSR marker analysis

Total DNA was isolated from young leaves of Hwangkeumkong, Taewangkong, SS2-2, and all BC plants using the modified CTAB procedure of Keim et al. (1988). The HS and TS BC progenies were screened on the basis of foreground selection using SSR markers Satt372 and Satt486 flanking *rxp* gene on LG D2. Background selection was performed using the SSR markers on all chromosomes including the chromosome carrying the *rxp* gene to estimate the percentage of the recurrent parent genome (RPG) in the foreground-selected BC plants. Two steps of background selection were conducted for reduction of analysis cost. In the first background selection, a total of 26 and 21 non-carrier markers were only used in HS and TS lines respectively. For the second background selection, an additional 92 and 69 markers in HS and TS lines were used to obtain the BC plants with the highest RPG content.

All of forward SSR primers were labeled with different dyes, 6-FAM, HEX, or NED (Applied Biosystems, Foster City, CA, USA). Polymerase chain reaction (PCR) amplifications were performed in 5 μ l reactions containing 10 ng of template DNA, 1 x PCR buffer, 160 μ M dNTP mix, 0.5 μ M of the forward and reverse primers and 0.4 unit of *Taq* DNA polymerase (Vivagen, Sungnam, Korea) using a Tetrad Thermal Cycler (MJ Research Inc., Watertown, MA, USA). The PCR products were resolved on an ABI 3730xl automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA). Allelic differences of SSR markers were analyzed using a GeneMapper 3.0 program version 3.7 (Applied Biosystems, Foster City, CA, USA) to determine SSR genotypes of the BC plants.

Results and Discussion

To develop the NILs for BLP resistance gene, *rxp*, we carried out three cycles of backcrossing and advanced two more selfing generations (Fig. 2). Since the desirable allele of the BLP resistance gene is recessive, the recessive homozygote is needed. In general BC breeding programs, additional selfing process should be included to introgress a recessive gene, particularly in odd generations such as BC₁F₂, BC₂F₂, etc.

We selected the best BC individuals in the final generation on the BC breeding program based on the four-stage selection strategy (Fig. 2). The four-stage selection strategy is one of three selection strategies for marker-assisted backcrossing proposed

Table 2. Background selection of the foreground-selected 11 BC plants with SSR markers

Cross	Line	Selected individual	1st background selection (non-carrier)	2nd background selection		RPG content (%) ¹⁾
				non-carrier	carrier	
Hwangkeumkong X SS2-2 (HS)	HS 1	HS 1-1	20/26 (76.9%)	-	-	-
		HS 1-2	20/26 (76.9%)	-	-	-
	HS 2	HS 1-4	23/26 (88.4%)	78/85	6/7	107/118 (90.5%)
		HS 2-2	23/26 (88.4%)	76/85	6/7	105/118 (88.9%)
	HS 3	HS 3-1	22/26 (84.6%)	79/85	6/7	107/118 (90.5%)
HS 5	HS 5-1	23/26 (88.4%)	79/85	6/7	108/118 (91.5%)	
	HS 5-4	25/26 (96.1%)	66/85	6/7	97/118 (82.2%)	
Taewangkong X SS2-2 (TS)	TS 1	TS 1-1	13/21 (61.9%)	-	-	-
		TS 3	TS 3-2	20/21 (95.2%)	59/62	5/7
	TS 3-5	TS 3-5	19/21 (90.4%)	57/62	6/7	82/90 (91.1%)
		TS 3-8	19/21 (90.4%)	53/62	5/7	77/90 (85.5%)

¹⁾ RPG content (%), recurrent parent genome content

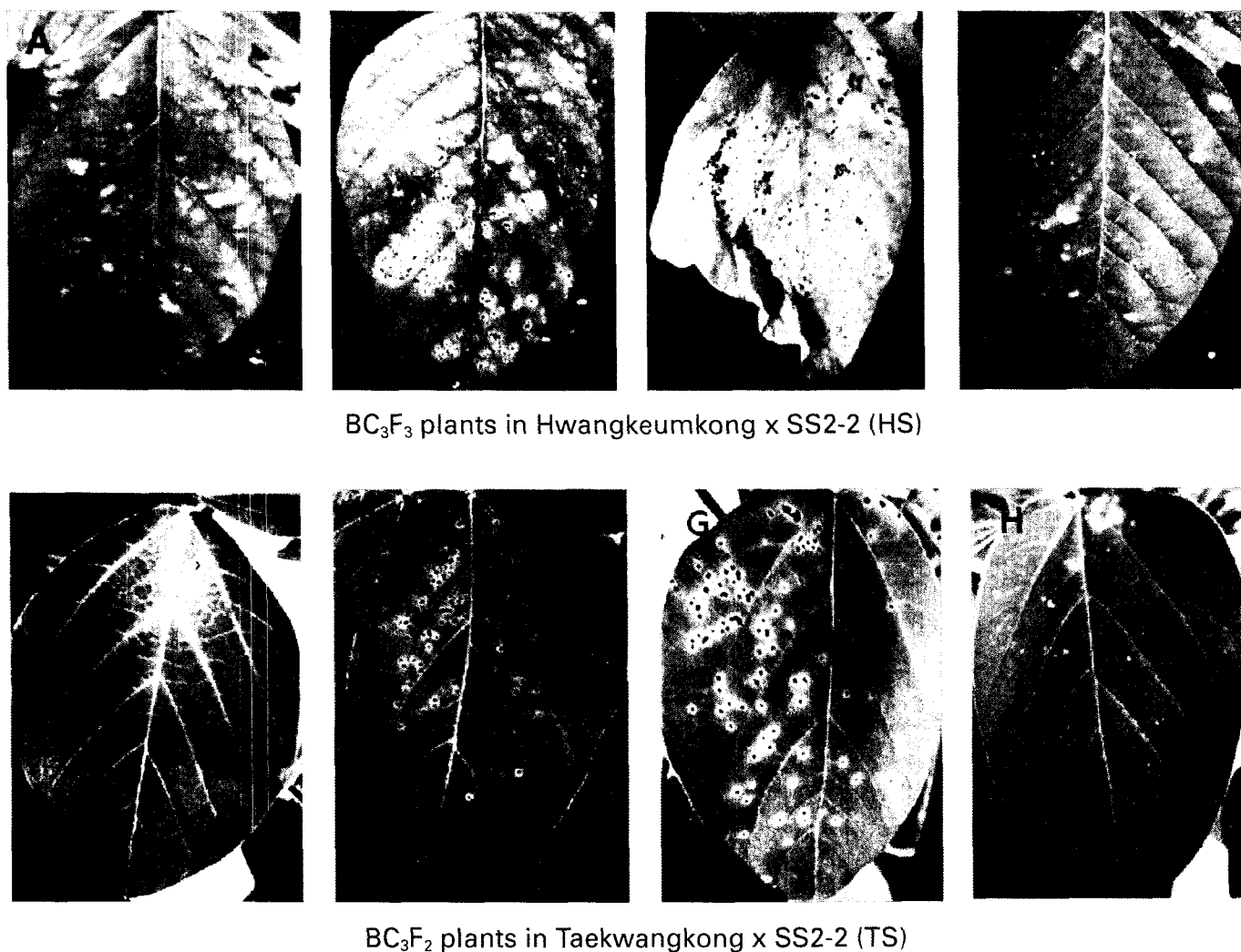


Fig. 3. The symptoms of inoculation with *Xanthomonas axonopodis* (strain 8ra) in BC_3F_3 plants in Hwangkeumkong x SS2-2(HS) and BC_3F_2 plants in Taekwangkong x SS2-2(TS).

by Matthias et al. (1999), which consists of four selection steps; i) select individuals having the target allele, ii) select homozygous individuals for recurrent parent allele at the flanked markers, iii) select homozygous individuals for recurrent parent allele at additional markers on the carrier chromosome, and iv) select one homozygous individual at the maximum number of markers across all chromosomes. Among these selection steps, the first and fourth steps are only carried out in the two-stage selection strategy, while the three-stage selection strategy skips the third step. Matthias et al. (1999) suggested that the more selection steps are progressed, the fewer markers are needed. In the present MAS program, the total of 44 plants from BC_3F_3 generation of HS and BC_3F_2 generation of TS were tested as the above selection steps (Tables 1 and 2).

First, to select individuals carrying the target gene *rxp*, the HS lines were screened using the flanked markers, Satt372 and Satt486, while the TS lines were only tested using Satt486 (Table 1). Nine and six individuals from 23 HS BC plants and 21 TS BC plants, respectively, showing donor parent genotypes were selected. After *Xag* strain 8ra was inoculated onto leaves of

the BC plants, 11 out of 15 individuals showing BLP-resistant responses were only selected one week later, shown as Fig. 3. The remaining four plants displayed severe lesion and pustules. Among these 11 BC plants, seven and four were selected from HS and TS lines, respectively. Shown as Table 1, in the HS population, 3 individuals in HS1 line, 1 in HS2 line, 1 in HS3 line and 2 in HS5 line displayed the consistent results of SSR genotyping and BLP phenotyping. From the TS population, the cosegregation of Satt486 and BLP resistance were observed in one individual in TS1 and three in the TS3 lines (Table 1).

Interestingly, the plants selected by the flanked markers were observed to be BLP-susceptible (Fig. 3B) and the plants phenotypically selected by BLP-resistant response did not show the donor parent genotypes at the SSR loci linked to *rxp* (Fig. 3H). These results are considered to be caused by recombination event or duplication of the *rxp* region. Palmer et al. (1992) reported that the *Rxp* locus was linked to the *Mdh* locus with recombination frequency of approximately 16% and Van et al. (2008) demonstrated that partially duplicated regions are located on LG A1 and LG D2. If the duplication or recombination

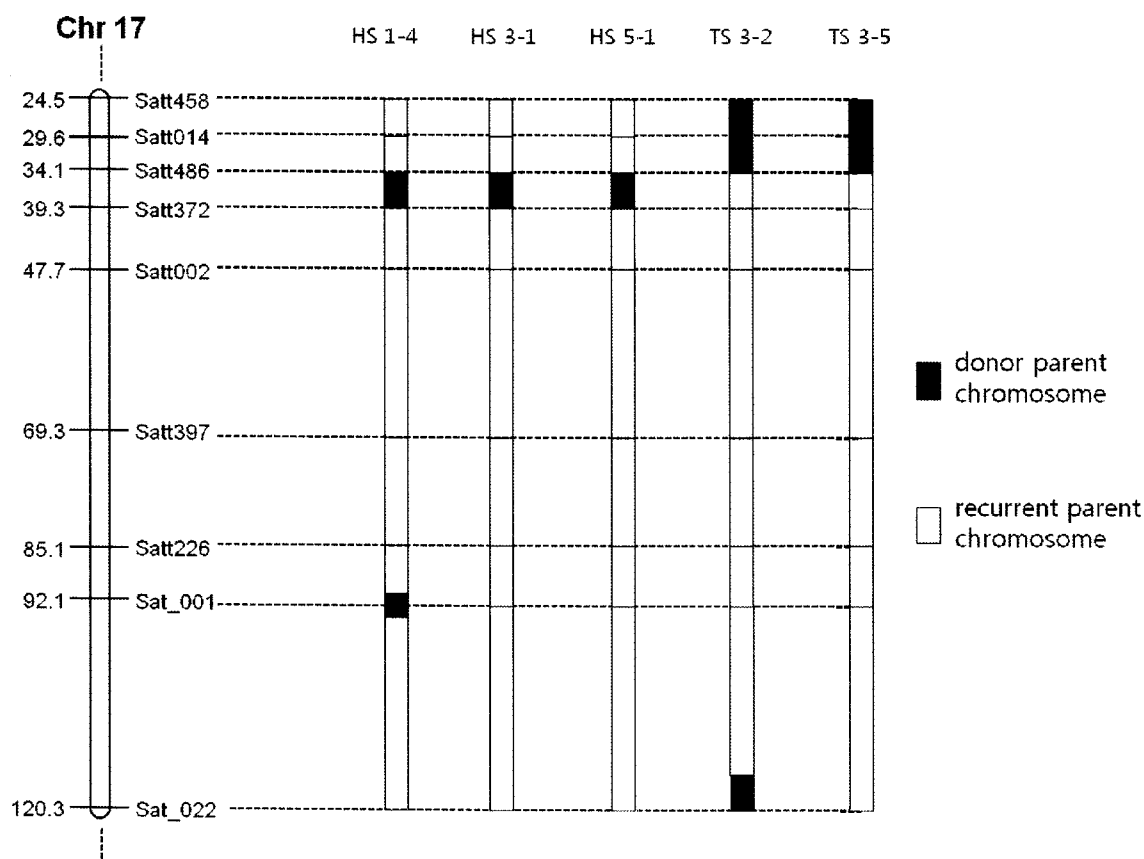


Fig. 4. Genetic composition of five NILs with greater than 90% recurrent parent genome content, near the *rxp* gene on Chr 17, as shown in Table 2. Genetic distances (cM) of seven SSR markers on Chr 17 were based on soybean consensus map (Song et al. 2004).

regions were known, we would find the region close to the target gene.

A total of 11 BC plants foreground-selected from HS and TS lines were screened through background selection using SSR markers on both non-carrier and carrier chromosomes. Two steps were conducted for our background selection. The first background selection was performed using 26 and 21 SSR markers on non-carrier chromosome in the HS and TS lines, respectively. After genotyping the SSR markers, HS 1-1, HS 1-2, and TS 1-1 were discarded because their RPG content was lower than 80% in the first step (Table 2). In the second background selection, additional 85 and 62 SSR markers on non-carrier chromosomes were utilized in HS and TS lines, respectively, and another seven SSR markers on carrier chromosome were used in both lines. Finally, five BC plants (HS1-4, HS3-1, HS5-1, TS3-2, and TS3-5) from the HS and TS lines showing greater than 90% RPG content were selected (Table 2).

To make the best BLP-resistant NILs containing the target gene, the genetic composition of selected individuals was investigated in the region harboring *rxp* on chromosome (Chr) 17 using seven carrier markers (Fig. 4). Genetic distance (cM) of seven SSR markers on Chr 17 was determined based on soybean consensus map (Song et al. 2004). In HS lines, three individuals (HS1-4, HS3-1, and HS5-1) were introgressed for the *rxp* gene to show donor parent genotype at the corresponding region.

However, HS1-4 still carried the genomic segment of donor parent at the SSR marker Sat_001 unlike HS3-1 and HS5-1 (Fig. 4). Two selected TS individuals (TS3-2 and TS3-5) also carried the *rxp* gene but genomic composition of donor parent was retained in wider range of Satt458 to Satt486 compared to HS lines. TS3-2 showed additional donor parent genotype at Sat_022 (Fig. 4). This phenomenon could be considered as linkage drag, which is defined as the genomic segments of donor parent near flanking target regions that are still maintained even after several backcrosses. Such linkage drag is the problem affecting other desirable traits in the backcrossing breeding program. Nevertheless, the linkage drag can be minimized by the application of MAS using more tightly linked markers and larger populations (Tanksley and Nelson 1996).

In conclusion, five NILs for the *rxp* gene were developed using MAS in this study. Our NILs would be the particularly powerful materials to isolate and characterize the *rxp* gene. Recent reports that new genes related to iron deficiency chlorosis in NILs were identified by microarray might support the importance of our NILs as biological materials (O'Rourke et al. 2007a, b) and Mao et al. (2008) also identified new genes in *Sinorhizobium meliloti* using GS-FLX. Advance in high-throughput DNA sequencing systems such as Roche's GS-FLX Genome Analyzer or Illumina's Solexa 1G sequencer makes it possible to conduct the whole genome shotgun sequencing as

well as whole transcriptome profiling. In the future, whole transcript analysis in the resistant and susceptible NILs infected with *Xag* using GS-FLX or Solexa should allow the *rxp* gene or candidate genes for BLP resistance to be characterized.

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References

- Hartwig EE, Johnson HW. 1953. Effect of the bacterial pustule on yield and chemical composition of soybean. *Agron. J.* 45: 22-23
- Keim P, Olson TC, Shoemaker RC. 1998. A rapid protocol for isolating soybean DNA. *Soybean Genet. Newsl.* 15: 150-154
- Kennedy BW, Tachibana H. 1973. Bacterial diseases. In: Caldwell BE (Ed.) *Soybeans: improvement, production, and uses.* American Society of Agronomy, Madison, WI, USA, pp 491-504
- Kim KS, Van K, Kim MY, Lee SH. 2004a. Development of molecular markers for *Xanthomonas axonopodis* resistance in soybean. *Kor. J. Crop Sci.* 49: 429-433
- Kim MY, Ha BK, Jun TH, Hwang EY, Van K, Kuk YI, Lee SH. 2004b. Single nucleotide polymorphism discovery and linkage mapping of lipoxygenase-2 gene (*Lx2*) in soybean. *Euphytica* 135: 169-177
- Mao C, Evans C, Jensen RV, Sobral BW. 2008. Identification of new genes in *Sinorhizobium meliloti* using the Genome Sequencer FLX system. *BMC Microbiol.* 8: 72
- Matthias F, Martin B, Melchinger AE. 1999. Comparison of selection strategies for marker-assisted backcrossing of gene. *Crop Sci.* 39: 1295-1301
- Narvel JM, Jakkula LR, Phillips DV, Wang T, Lee SH, Boerma HR. 2001. Molecular mapping of *Rxp* conditioning reaction to bacterial pustule in soybean. *J. Hered.* 92: 267-270
- Oh C, Heu S, Choi YC. 1999. Sensitive and pathovar-specific detection of *Xanthomonas campestris* pv. *glycines* by DNA hybridization and polymerase chain reaction analysis. *Plant Pathol. J.* 15: 57-61
- O'Rourke JA, Charlson DV, Gonzalez DO, Vodkin LO, Graham MA, Cianzio SR, Grusak MA, Shoemaker RC. 2007a. Microarray analysis of iron deficiency chlorosis in near-isogenic soybean lines. *BMC Genomics* 8: 476
- O'Rourke JA, Graham MA, Vodkin L, Gonzalez DO, Cianzio SR, Shoemaker RC. 2007b. Recovering from iron deficiency chlorosis in near-isogenic soybeans: A microarray study. *Plant Physiol. Biochem.* 45: 287-292
- Palmer RG, Lim SM, Hedges BR. 1992. Testing for linkage between the *rxp* locus and nine isozyme loci in soybean. *Crop Sci.* 32: 681-683
- Song QJ, Marek LF, Shoemaker RC, Lark KG, Concibido VC, Delannay X, Specht JE, Cregan PB. 2004. A new integrated genetic linkage map of the soybean. *Theor. Appl. Genet.* 109: 122-128
- Tanksley SD, Nelson JC. 1996. Advanced backcross QTL analysis: a method for the simultaneous discovery and transfer of valuable QTLs from unadapted germplasm into elite breeding lines. *Theor. Appl. Genet.* 92: 191-203
- Van K, Ha BK, Kim MY, Moon JK, Paek NC, Heu S, Lee SH. 2004. SSR mapping of genes conditioning soybean resistance to six isolates of *Xanthomonas axonopodis* pv. *glycines*. *Kor. J. Genet.* 26: 47-54
- Van K, Kim DH, Cai CM, Kim MY, Shin JH, Graham MA, Shoemaker RC, Choi BS, Yang TJ, Lee SH. 2008. Sequence level analysis of recently duplicated regions in soybean [*Glycine max* (L.) Merr.] genome. *DNA Res.* 15: 93-102
- Wight CP, O'Donoghue LS, Chong J, Tinker NA, Molnar SJ. 2004. Discovery, localization, and sequence characterization of molecular markers for the crown rust genes Pc 38, Pc 39, and Pc 48 in cultivated oat (*Avena sativa* L.). *Mol. Breed.* 14: 349-361
- Young ND, Zamir D, Ganai MW, Tanksley SD. 1988. Use of isogenic lines and simultaneous probing to identify DNA markers tightly linked to the *Tm-2a* gene in tomato. *Genetics* 120: 579-585