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Mapping QTL tolerance to *Phytophthora* root rot in soybean using microsatellite and RAPD/SCAR derived markers

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Abstract Broad tolerance to *phytophthora* root rot (PRR) caused by *Phytophthora sojae* has become an important goal for the improvement of soybean (*Glycine max*) because of the rapid spread of races that defeat the available resistance genes. The aim of this research was to identify the location of quantitative trait loci (QTL) in 'Conrad', a soybean cultivar with broad tolerance to many races of *P. sojae*. A PRR susceptible breeding line 'OX760-6-1'was crossed with Conrad. Through single-seed-descent, 112, F_2 derived, F_7 recombinant inbred lines (RILs) were advanced. A total of 39 random amplified polymorphic DNA bands (RAPDs) and 89 type 1 microsatellite (simple sequence repeat; SSR) markers were used to construct a genetic linkage map. In the greenhouse, RILs were inoculated with four *P. sojae* isolates (three from China and one from Canada). Disease was measured as the percent of dead plants 20 days after germination in *P. sojae* inoculated vermiculite in the greenhouse. Three QTLs (QGP1, QGP2, QGP3) for PRR tolerance in the greenhouse were detected using WinQTLCart 2.0 with a log-likelihood (LOD) score 27.14 acquired through permutations (1,000 at $P \leq 0.05$). QGP1 (near Satt509) was located at linkage group F and explained 13.2%, 5.9%, and 6.7% of the phenotypic variance for tolerance to the JiXi, JianSanJiang and ShuangYaShan isolates, respectively. QGP2 (near Satt334) was located in a different interval on linkage group F and explained 5.1% and 2.4% of the phenotypic variance for JiXi and ShuangYaShan isolates, respectively. QGP3 was located on linkage group D1b + W (near $OPL18_{800}$/$SCL18_{659}$) and explained 10.2% of the phenotypic variance for Woodslee isolate. QGP1 and QGP2 appeared to be associated with PRR tolerance across a range of isolates but QGP3 was active only against the Woodslee isolate. At Woodslee and Weaver (in Ontario) in 2000, the interval associated with QGP3 explained 21.6% and 16.7% of phenotypic variance in resistance to PRR, respectively and was referred as QFP1. The identified QTLs would be beneficial for marker assistant selection of PRR tolerance varieties against both China and North America *P. sojae* races.

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Introduction

Phytophthora root rot (PRR) of soybean (*Glycine* max (L.) Merr.), caused by *Phytophthora sojae* was first noted as a soil-borne disease of unknown etiology in Northeast Indiana, USA in 1948 (Schmitthenner 1989). This disease was especially severe in low, poorly drained and clay soil, and has been found in most soybean-growing regions (Bernard et al. 1957; Kaufmann and Gerdemann 1958; Hildebrand 1959; Ryley et al. 1998; Jee et al. 1998 and Su and Yao 1993). During wet springs about 25% of damping-off of soybeans in Iowa is caused by PRR (Rizvi and Yang 1996). PRR causes significant yield loss worldwide (Wrather et al. 2001). PRR was identified in 22–25% of soybean-growing regions in the Heilongjiang Province of China, causing 50–80% yield losses in low temperature and high rainfall years (Xu et al. 2000).

Phytophthora sojae has a narrow host range; it is mainly a pathogen of soybean but has been reported to infect lupine species; *Lupinus angustifolius* L., *L. luteus* L., and *L. albus* L (Jones and Johnson 1969). In the pathogen population, new races continually develop by mutation or outcrossing (Bhat and Schmitthenner 1993; Irwin et al. 1995). Numerous races and virulence biotypes have been reported (Drenth et al. 1996; Abney et al. 1997; Leitz et al. 2000; Malvick and Grunden 2004).

In soybean, 14 dominant alleles conferring complete resistance to some races of *P. sojae* have been identified at eight loci (Burnham et al. 2003a); however, none of the single genes can control all of the races from this pathogen (Hartman et al. 1999). Moreover, single gene resistance has been suggested to follow a gene for gene relation, and intensive use of race-specific resistance for control has promoted selection for new races that are virulent on the current resistance genes (Drenth et al. 1996).

Tolerance to PRR is found in some soybean cultivars and appears to be a partial resistance largely mediated by reducing the rate of infection and subsequent growth of the fungus (Tooley and Grau 1982). Additional components of tolerance include growth stage dependent resistance, root re-growth after infection, and ultimately seed yield. The use of cultivars possessing quantitative trait loci (QTL) for tolerance to PRR may provide an alternative control method (Buzzel and Anderson 1982; Dorrance and St Martin 2000). A set of QTLs that reduced PRR to below the economic threshold in most environments but did not eliminate pathogen reproduction entirely might reduce the selection pressure for new virulent races of *P. sojae* (Schafer 1971; Thomison et al.1988).

Genetic control of PRR outbreaks with tolerance are complicated by significant interactions of genotype with the environmental conditions, especially temperature and moisture (Tooley and Grau 1984a, b; Walker and Schmitthenner 1984). In conventional breeding, the evaluation of tolerance to PRR in field requires multiple environments. The environments used were irreproducible, time consuming, and labor intensive. In contrast, the methodology of QTL detection and marker assistant selection provides the potential in developing broadly PRR tolerant soybeans from many environments, or a controlled environment in the greenhouse.

DNA-based markers like random amplified polymorphic DNA (RAPD) and microsatellites (SSR) have been used extensively for soybean germplasm analysis, genetic map and QTL map development (Song et al. 2004). The RAPD analysis is a straightforward technique that is quick to perform and requires only a small amount of DNA (Rafalski et al 1991). Large numbers of samples can be analyzed economically and quickly. SCAR analysis could improve the specificity and reproducibility of RAPD markers (Zheng et al. 2003). In comparison to RAPD, SSR markers are polymorphic, codominant, intelligible, and easily accessed by other laboratories via published primer sequences.

Several genes for complete race-specific resistance to PRR have been mapped to linkage groups (Diers et al. 1992; Lohnes et al. 1996; Weng et al. 2001; Demirbas et al. 2001; Sandhu et al. 2005). In some cases, genes for complete race-specific resistance that have already been defeated by new races of *P. sojae* may contribute to a partial resistance or the broad tolerance to PRR (Gebhardt and Valkonen 2001). Several QTLs have been mapped to linkage groups for partial resistance to PRR. Burnham et al. (2003b) used three recombinant inbred line (RIL) populations with the cultivar Conrad as the tolerant parent and

identified two putative QTLs on MLG F and D1b + W. The QTL on MLG F explained 34.4, 35.0 and 21.4% of the variability while the QTL on MLG D1b + W explained 10.6, 15.7 and 20.7% of the variability, for the three populations, respectively. These QTLs identified by Burnham et al. (2003b) were based on PRR field tolerance data from North America and the SSR marker system. There has been no research on QTL analyses for PRR tolerance in soybean using *P. sojae* isolates from both Northeastern China and North America.

The objective of the present study was to identify QTLs associated with PRR tolerance using Conrad x OX760-6-1 RILs inoculated with different *P. sojae* isolates from Northeastern China and an isolate from North American.

Materials and methods

Plant materials

The 112 $F_{2:7}$ recombinant inbred lines (RILs) were advanced by single-seed-descent from a cross between Conrad and OX760-6-1. Conrad (Fehr et al. 1989) was a soybean cultivar with tolerance to PRR. OX760-6-1 was highly susceptible to PRR. Seed were provided by the Greenhouse and Processing Crops Research Centre, Agriculture and Agri-food Canada, Harrow, Ontario.

Inoculation and disease susceptibility evaluation

The collection, isolation, identification, and purification of *P. sojae* followed the procedure of Meng et al (1999). Plant materials were inoculated with *P. sojae* isolates from three locations in Northeastern China (JiXi, ShuangYaShan, and JianSanJiang) and one location in Canada (Woodslee) in the greenhouse. Drinking cups (7 cm at radius) with a hole in the bottom were used to grow plants. A 9–10 cm layer of mixtures of vermiculite and sterile soil was put in each cup and drained sufficiently. *Phytophthora sojae* cultures in LBA solid medium (15% Frozen lima beans, 2% Agar) were placed on top of the soil mixtures, and covered with 1 cm of vermiculite. Five seeds were placed on top of the media and covered with 2 cm of additional vermiculite. The growth temperature was set at 25°C. Each RIL for each treatment provided 20 plants. The experiment was conducted in a complete randomized block design with three replicates (60 plants per genotype) and was repeated once so that 120 plants per genotype were scored across the experiments. The total number of plants that germinated was recorded daily. The number of dead plants was recorded at 20 days after sowing. The percentage of plant killed for each line was calculated as [(total plants −plants resistant to PRR)/(total plants)] × 100%.

SSR and RAPD/SCAR marker detection

Genomic DNA was isolated from leaf samples according to the procedures described by Yu et al. (1999). PCR amplifications were performed in 96-well micro titer plates using the PTC-100^{TM} politer thermal cycler. Oligonucleotide sequences were contributed by USDA-ARS Plant Genome Program, Cornell University and Iowa State University (http://129.186.26.94/ssr.html). SSR PCR reactions were made in 20 µl, containing 2 µl of genomic DNA (25 ng/µl), 1.5 µl $MgCl_2$ (25mM), 0.3 µl dNTP mixtures (10 mM), 2 µl of 10 × PCR buffer, 2 µl SSR primer (2 µM), 0.2 µl Taq polymerase enzyme (10 units/µl), and 12 µl of water. The amplification temperature profiles were modified from Hyten et al. (2004): 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 47°C, 30 s at 72°C, then 5 min at 72°C. After amplification, PCR products were mixed with loading buffer (2.5 mg/ml bromophenol blue, 2.5 mg/ml diphenylamine blue, 10 mM EDTA, 95% (v/v) formamide), denatured for 5 min at 94°C and kept on ice for 5 min. The PCR products were separated on 6% (w/v) denatured polyacrylamide gel and were detected by silver staining (Trigiano and Caetano-Anolles 1998).

The RAPD analysis used 1,200 random decamer primers obtained from Operon Technologies Inc., (Alameda, CA, USA). The 20 µl of reacting mixture was prepared for each sample, containing 2 µl of genomic DNA (15 ng/µl), 1.5 µl $MgCl_2$ (25 mM), 0.3 µl dNTP mixtures (10mM), 2 µl 10 × PCR buffer, 2 µl RAPD primer (2 µM), 0.2 µl Taq polymerase enzyme (10 units/µl) and 12 µl ddH_2O. The PCR program consisted of 2 min at 94°C, and 41 cycles of 1 min at 94°C, 1 min at 36°C, 1 min at 72°C. The final extension step of 10 min was carried out at 72°C. After PCR reaction, PCR products were separated on 1.5% (w/v) agarose gel.

Table 1 The mean percentage of plants killed by PRR for parents and RILs at the F_7 inoculated with different PRR isolates in the greenhouse

PRR[a] Isolates	Conrad	OX760-6-1	Mean percent of plants killed among RILs	Range of the percentage of plants killed among RILs
JX	22.2	54.6	47.3	0–79.3
JSJ	6.92	93.1	54.9	0–100
SYS	14.3	74.2	48.9	12.7–94.2
Woodslee	7.68	57.8	34.8	0–62.1
Average	12.7	69.9	46.5	3.2–83.9

[a] Isolates collected from JianSanJiang of Northeast China (JSJ); isolates collected from JiXi of Northeast China (JX); isolates collected from ShuangYaShan of Northeast China (SYS); isolates collected from Woodslee of North American (USA)

Clone and sequencing of SCAR analysis was according to the procedures described by Zheng et al. (2003). The forward primer of SCAR marker $SCL18_{659}$ was GCGGGGTAATTAGCAATCGTC AT. The reverse primer was designed from the $OPL18_{800}$ band sequence (GCGCACCCTAGCTATG CTATCCTAT). The SCAR amplification was performed in 20 µl reaction mix identical to the RAPD amplifications. Temperature cycling was performed using a thermal cycler programmed for an initial step of 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 65°C, 2 min at 72°C, and a final step of 10 min at 72°C. Agarose gel electrophoresis was as described for RAPD analysis.

Data analyses

Linkages among the markers were analyzed with Mapmaker3.0b (Lander et al. 1987), using the Kosambi mapping function. WinQTLCart2.1 (Zeng 1993) was used to detect QTL between marker intervals by permutations (1,000 at significance, $P \leq 0.05$). The genetic linkage map was constructed using Mapchart 2.1 (Voorrips 2002).

GT (Genotype by Trait) biplot methodology (Yan et al. 2001) was employed to analyze the interaction between genotypes and the different pathogen isolates in RIL, based on the formula: $T_{ij} - T_j/S_j = \lambda_1\zeta_{i1}\tau_{j1} + \lambda_2\zeta_{i2}\tau_{j2} + \varepsilon_{ij}$, where T_{ij} was the average value of line i for isolates j; T_j is the average value of isolates j over all lines; Sj is the standard deviation of isolates j among the line average; ζ_{i1} and ζ_{i2} are the PC1 (first principle component) and PC2 (second principle component) scores, respectively, for line i; τ_{j1} and τ_{j2} are the PC1 and PC2 scores, respectively, for isolate j; and, ε_{ij} is the residual of the model associated with the line i, challenged with isolate j.

Results

Analysis of plant death caused by PRR in the greenhouse

The mean percentage of plants killed by PRR across all three isolates was significantly different between the two parents, Conrad (12.7%) and OX760-6-1 (69.9%) (Table 1). Among the RILs, the numbers of plants killed were significantly different after inoculation with the isolates from JiXi (47.3%), JianSanJiang (54.9%), ShuangYaShan (48.9%) and Woodslee (34.8%) (Table 1). The range of the percentage of plants killed by the isolate from JianSanJiang was much wider than the other three isolates (Table 1). Shaprio-wilk tests showed that the frequency distribution of percentage plant kill was significantly deviated from a normal distribution ($W = 0.79$, $P < 0.0001$ for JiXi isolate; $W = 0.83$, $P < 0.0001$ for JianSanJiang isolate; $W = 0.75$, $P < 0.0001$ for ShuangYaShan isolate; $W = 0.80$, $P < 0.0001$ for Woodslee isolate). However, using SAS normal logarithm transformation resulted in normal distributions for the disease loss data (JiXi isolate, $W = 0.9$, $P = 0.2342$; JianSanJiang isolate, $W = 0.92$, $P = 0.1987$; ShuangYaShan isolate $W = 0.96$, $P = 0.3434$; Woodslee isolate: $W = 0.93$, $P = 0.2965$).

Linkage analysis

A total of 606 SSR markers were used to detect polymorphisms between the two parents. Eighty-nine of them (14.6%) were polymorphic among RILs. Of the 1,200 RAPD markers that were surveyed for polymorphisms between the two parents only 202 of them (16.8%) were polymorphic. Out of the 202 polymorphic RAPD markers 39 (3.25%) of them

Table 2 Markers associated with PRR based on the greenhouse and the field disease loss data

MLG	Marker	Interval	QTL	Locations of Isolates[a]	cM	R^2 (%)[b]	LOD[c]
F	Satt509	Satt509–Satt030	QGP1	JX	3.9	13.2	30.52
F	Satt509	Satt509–Satt030	QGP1	JSJ	2.3	5.9	28.99
F	Satt509	Satt509–Satt030	QGP1	SYS	8.6	6.7	34.77
F	Satt343	Satt343–$OPG16_{600}$	QGP2	JX	5.1	8.2	28.67
F	Satt343	Satt509–$OPG16_{600}$	QGP2	SYS	2.4	2.4	34.5
D1b+W	$OPL18_{800}$/$SCL18_{659}$	$OPL18_{800}$–Satt274	QGP3	Woodslee	10.2	9.6	30.56
D1b+W	$OPL18_{800}$/$SCL18_{659}$	$OPL18_{800}$–Satt274	QFP1	Woodslee 2000	10.63	21.55	34.23
D1b+W	$OPL18_{800}$/$SCL18_{659}$	$OPL18_{800}$–Satt274	QFP1	Weaver2000	2.35	16.71	29.77

[a] Isolates collected from JianSanJiang of Northeast China (JSJ); isolates collected from JiXi of Northeast China (JX); isolates collected from ShuangYaShan of Northeast China (SYS); isolates collected from Woodslee of North American (USA)

[b] R^2 is R-square or the proportion of the phenotypic data explained by the marker locus

[c] LOD is log of odd score

amplified clear segregating bands. SCAR amplifications showed $SCL18_{659}$ produced a band with the same molecular size and segregation phase as the RAPD. A total of 39 RAPD, 1 SCAR and 89 SSR markers were mapped to the integrated soybean linkage map designed by Cregan et al. (1999) and Song et al (2004).

QTL analyses based on PRR in the greenhouse

Three QTLs underlying PRR tolerance in the greenhouse were detected (Fig. 1). QGP1 (Satt509-Satt030) was associated with PRR tolerance for three isolates (JiXi, JianShangJiang and ShuangYaShan) and mapped to MLG F. QGP2 (Satt343-$OPG16_{500}$) was associated with PRR tolerance for two isolates (JiXi and ShuangYaShan) and mapped to MLG F. QGP3 ($OPL18_{800}$/$SCL18_{659}$-Satt274) was associated with PRR tolerance only for Woodslee isolate and mapped to MLG D1b + W. The phenotypic variation explained by the three QTLs across four isolates ranged from 2.4 to 13.2% (Table 2). QGP1 was most strongly associated with PRR tolerance, explaining 13.2%, 5.9% and 6.7% of the phenotypic variations for the isolates from JiXi, JianSanJiang, and ShuangYaShan, respectively (Table 2). QGP1, however, was not detected when the Woodslee isolate was used. QGP2 explained 8.2% and 2.4% of phenotypic variation for the isolates from JiXi and ShuangYaShan, and was not detected when the Woodslee isolate was used. QGP3 explained 9.6% of the phenotypic contribution for PRR tolerance only when the Woodslee isolate was used (Table 2).

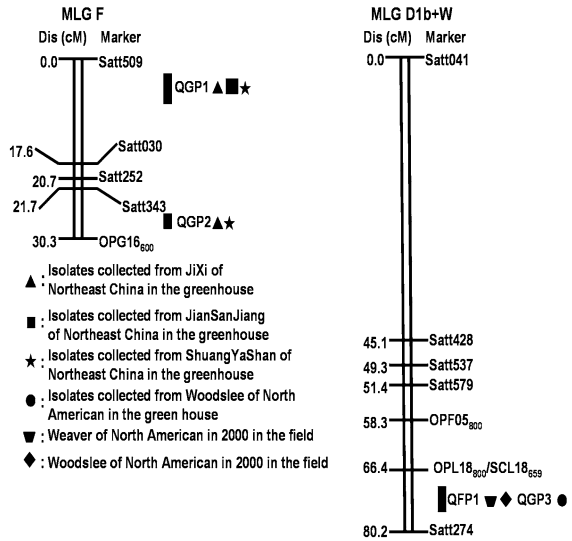

Fig. 1 Genomic locations of identified QTLs affecting *phytophthora* root rot tolerance of the Conrad/OX760-6-1 F_7 Recombinant inbred line population based on the greenhouse disease loss data and based on the field disease loss data. The map distances in cMs are shown on the left. The QTL locations are indicated on the right

The map distance between molecular marker and associated QTL was calculated by WinQTL2.1. The calculated distances between Satt509 and QGP1 were 3.9 cM for JiXi isolate, 2.35 cM for JianSanJiang isolate and 8.6 cM for ShuangYaShan isolate respec-

Table 3 Segregation of the QTL associated with PRR based on the greenhouse disease loss data in the F7 population (Conrad × OX760-6-1) consisting of 117 lines and tests for distortion using Chi-square tests

Locus	MLG	SSR marker genotype			X^2
		AA	BB	N	
Satt509	F	58	59	117	0.9263
Satt343	F	58	57	115	0.8362
$OPL18_{800}/SCL18_{659}$	D1b+W	57	57	114	0.7815

Note: Genotype AA is homozygous for OX760-6-1 allele, and Genotype BB is homozygous for Conrad allele. N is the total number of plants that had valid scores. X^2 is the Chi-square statistic, the expected ratio of AA: BB for each locus is 1:1

tively. The calculated distances between Satt343 and QGP2 were 5.1 cM for JiXi isolate and 2.4 cM for ShuangYaShan isolate. The calculated distance between $OPL18_{800}/SCL18_{659}$ and QGP3 was 10.2 cM for Woodslee isolate. Chi-square tests of marker segregation fit well the expected ratio of 1:1 for genotypes AA: BB (Table 3).

QTL analyses based on field data

Plant material in this study was evaluated for tolerance to PRR in the field (at Woodslee and Weaver during 2000). These disease tolerance data was used to detect QTLs in comparison to the map developed herein. Only one QTL was associated with PRR tolerance based on field disease data. Markers encompassing QFP1 ($OPL18_{800}/SCL18_{659}$-Satt274) were associated with PRR at Woodslee and Weaver in 2000 (Fig. 1). They explained 21.6% and 16.7% of phenotypic variance, with LOD scores of 34.2 and 29.8, respectively (Table 2). The distance between $OPL18_{800}/SCL18_{659}$ and the QFP1 peak was 10.63 cM and 2.35 cM for Woodslee and Weaver, respectively.

Co-analyses of genotypes and isolates

GT biplot analysis (Yan et al. 2001) for four different *P. sojae* isolates from four locations against 112 lines explained 82% the total variation of the standardized data. The largest variation explained by the biplot came from the inoculation result by the JianSanJiang isolate as indicated by the relative length of their vector. The correlation coefficient between any two

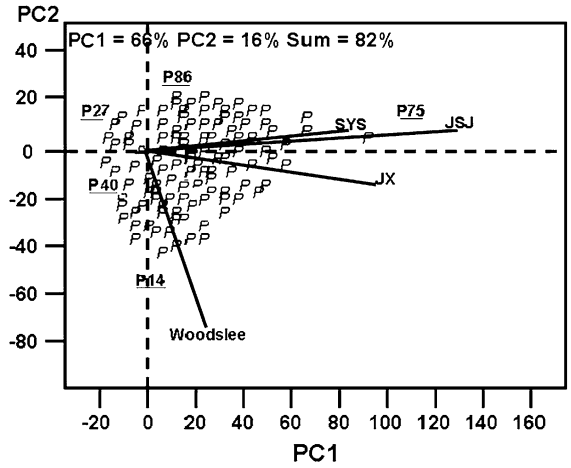

Fig. 2 GT biplot analysis for relationship among four different locations isolates. PC1: first principle component; PC2: second principle component; *p*: representing a genotype; isolates collected from JianSanJiang of Northeast China: JSJ, isolates collected from JiXi of Northeast China: JX, isolates collected from ShuangYaShan of Northeast China: SYS, isolates collected from Woodslee of North American: Woodslee

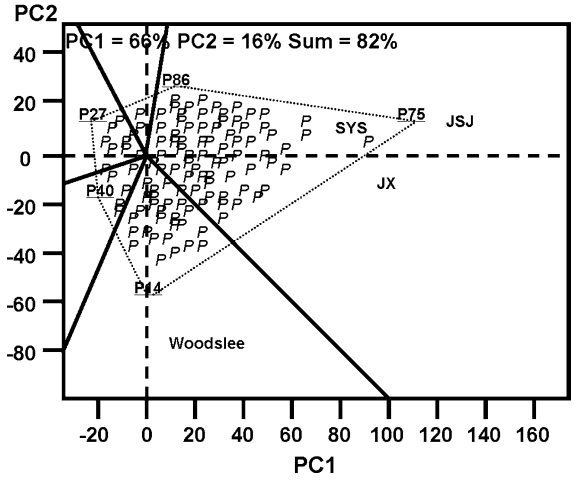

Fig. 3 GT biplot analysis for the relatedness of genotypes and isolates. PC1: first principle component; PC2: second principle component; *p*: representing a genotype; isolates collected from JianSanJiang of Northeast China: JSJ, isolates collected from JiXi of Northeast China: JX, isolates collected from ShuangYaShan of Northeast China: SYS, isolates collected from Woodslee of North American: Woodslee

isolates is approximated by the cosine of the angle between their vectors. Thus, cos180° = −1, cos0°= 1 and cos90° = 0. In Fig. 2, a stronger positive association between Jixi and JianSanJiang isolates existed

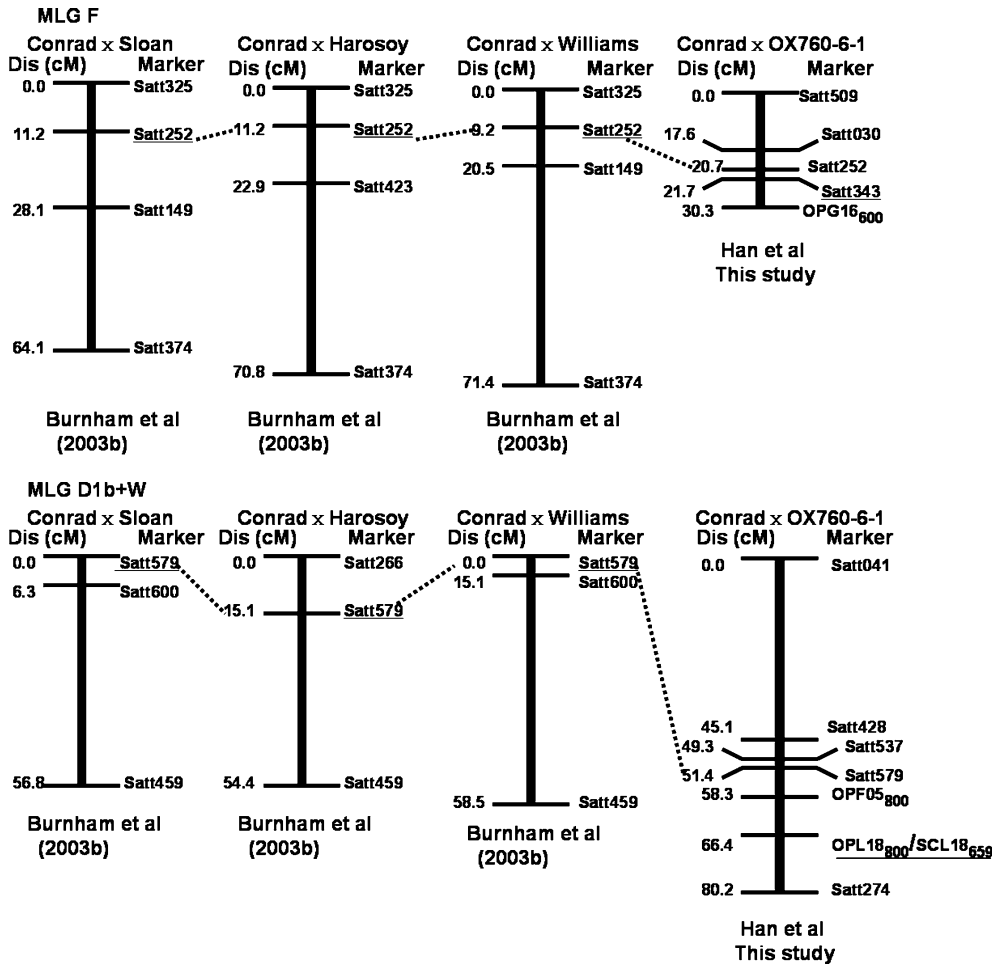

Fig. 4 QTL comparison between this study and results of Burnham et al (2003b). The map distances in cMs are shown on the left. The QTL locations are indicated on the right representing marker associated with PRR tolerance QTL

rather than other isolates. Furthermore, the performance of different RILs on each isolate was evaluated. With RILs P86, P27, P40, P14, P75 as the corner genotypes, three isolates from Northeastern China fell in the sector in which the genotypes, P75 and P86, were the best RILs for tolerance to these *P. sojae* isolates (Fig. 3). The genotype P14 was the best RIL for PRR tolerance inoculated with Woodslee isolate.

Discussion

In earlier studies, *P. sojae* was analyzed with 100 RAPD primer and EST-SSR primers and showed a good separation among four different locations (Zhu et al 2004). Though it was difficult to make comprehensive comparison of *P. sojae* from different locations, *P. sojae* from the same location often clustered in the same groups (Zhu et al 2004). The genetic distance between *P. sojae* isolates from Northeast China and *P. sojae* isolates from North America was difference (Wang et al. 2006). Therefore, it was no surprise that QTLs (QGP1 and QGP2) identified with isolates from Northeast China were different to QTL (QGP3) from Canada in the present study. QTLs identified with isolates from Northeast China (Satt509 in JiXi, ShuangYaShan and JianSanJiang; Satt343 in JiXi and ShuangYaShan) were across a range of isolates.

The variation explained by the QTLs in this study was comparable to earlier reports (Burnham et al. 2003b). QGP2 and QGP3 are located at chromosomal locations similar to those identified by Burnham et al. (2003b) using the same Conrad cultivar as the PRR

tolerant parent (Fig. 4) in greenhouse tests. Clearly, linkage group MLG F and D1b + W might be associated with PRR tolerance in Conrad, However, more closer QTLs were delimited in the present study.

The severity of PRR in infested fields are significantly influenced by temperature and moisture. Therefore, it was difficult to identified stable locations to evaluate disease resistance. The Woodslee site which has been used to evaluate tolerance of soybean cultivars in Ontario since 1975. A uniform disease development has been achieved for many years at this site. It was difficult to find a similarly infested location in Northeast China.

All RILs in this study was evaluated for disease resistance in the greenhouse with isolates from Woodslee. Although the usefulness of QTL identified in controlled environments has not been tested in the field, QTL with large effect should be useful. QGP3 found in the greenhouse was located in the similar chromosomal location as QFP1 found in the field (Fig. 1). Therefore, plant materials that were selected with *P. sojae* isolates from target location in the greenhouse were proven efficient. The QTL (QGP1, QGP2 and QGP3) that were relevant to SSR markers Satt509 and Satt343 in Northeast China and SCAR marker $OPL18_{800}$/$SCL18_{659}$ in North America might be beneficial for marker-assistant selection for PRR tolerant soybean, which could overcome the shortcomings of the field selection.

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