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Genetic analysis and identification of DNA markers linked to a novel *Phytophthora sojae* resistance gene in the Japanese soybean cultivar Waseshiroge

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Abstract The *Glycine max* (L.) Merr. cultivar Waseshiroge is highly resistant to several races of *Phytophthora sojae* in Japan. In order to determine which *Rps* gene might be present in Waseshiroge, 15 differential cultivars were challenged with 12 *P. sojae* isolates. None had a reaction pattern identical to that of Waseshiroge, indicating that Waseshiroge may contain a novel *Rps* gene. In order to characterize the inheritance of Waseshiroge resistance to *P. sojae* isolates, 98 F_2 progeny and 94 $F_{7:8}$ lines were produced from crosses between the susceptible cultivar Tanbakuro and Waseshiroge. Chi-square tests indicated that segregation fit a 3:1 ratio for resistance and susceptibility in two F_2 sub-populations of 42 and 56 seedlings. This and a 46.27:1.46:46.27 (or 63:2:63) ratio for resistance: segregation: susceptibility among the 94 $F_{7:8}$ lines indicated that resistance was controlled by a single dominant gene. DNA analyses were carried out on Tanbakuro, Waseshiroge and the 94 $F_{7:8}$ lines, and a linkage map was constructed with

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17 SSR markers and nine new primer pairs that amplify marker loci linked to *Rps1* on soybean chromosome 3 (linkage group N). The closest markers, Satt009 and T0003044871, map to locations 0.9 and 1.6 cM on each side of the estimated position of the *Rps* gene, respectively. The results showed that the *Rps* gene in Waseshiroge is either allelic to *Rps1*, or resides at a tightly linked locus in a gene cluster. A three-way-contingency table analysis indicated that marker-assisted selection with the two flanking markers could be used in the development of new resistant cultivars.

Keywords *Glycine max* · Marker-assisted selection · *Rps* gene · Soybean · SSR analysis

Introduction

Phytophthora stem rot disease, caused by the soilborne Oomycete *Phytophthora sojae* (Kaufmann and Gerdemann 1958), is one of the most serious and widespread diseases of soybean [*Glycine max* (L.) Merr.] (Schmitthenner 1999). When soybeans are infected by *P. sojae*, the stem of the plant appears water-soaked and turns brown, and the infection may result in wilting and the death of plants (Dorrance et al. 2003). The pathogen produces motile zoospores which initiate further cycles of disease (Schmitthenner 1985). Wrather and Koenning (2006) estimated that annual crop damage from this disease between 2003 and 2005 averaged about $251.6 million in the United States.

In Japan, Phytophthora stem rot disease was first observed in 1977 on the northern island of Hokkaidō (Tsuchiya et al. 1978), and it subsequently spread to several other regions of Japan (Sugimoto et al. 2006). Since 1987, this disease has been increasing in Hyogo, a prefecture north of Kōbe, on the island of Honshū. Hyogo is famous in Japan for growing the black-seeded soybean cultivar Tanbakuro, which fetches a market price of $30–60 kg^{-1} compared to only $2–3 kg^{-1} for soybeans with a yellow seed coat. Several studies have demonstrated that black soybeans contain abundant isoflavones and anthocyanins, as well as dietary fiber, minerals, and vitamins considered to have many positive effects on human health (Kohama et al. 2005; Takahashi et al. 2005). Tanbakuro is very susceptible to *P. sojae*, however (Sugimoto et al. 2006). Because of the high economic value of Tanbakuro seeds and their direct consumption by humans, it is essential to develop effective disease management strategies that do not rely on pesticides as quickly as possible.

Methods for managing economic losses to Phytophthora include fungicides (Anderson and Buzzell 1982), planting cultivars with race-specific or partial resistance (Dorrance et al. 2003), soil drainage (Schmitthenner 1985), tillage practices (Workneh et al. 1998), and calcium applications (Sugimoto et al. 2005, 2007, 2008b, 2009, 2010). Schmitthenner (1999) reported that the most effective method to reduce damage would be to plant resistant cultivars, and genes conditioning resistance to *P. sojae* (*Rps* genes) have been widely used in commercial soybean cultivars for controlling Phytophthora stem and root rot disease (Dorrance and Schmitthenner 2000). Cultivars combining the desirable characteristics of Tanbakuro with Phytophthora resistance would reduce crop losses from this disease and simultaneously allow producers to increase their incomes. Molecular markers linked to an *Rps* gene could be used for marker-assisted selection (MAS) to efficiently identify seedlings carrying the resistance gene.

Fifteen *Rps* genes have been reported at eight genomic loci designated *Rps1* through *Rps 8* (Sandhu et al. 2004). These loci have been mapped to molecular linkage groups (MLG) N (*Rps1* and *Rps7*), J (*Rps2*), F (*Rps3* and *Rps8*), and G (*Rps4*, *5* and *6*) (Cregan et al. 1999, 2003; Demirbas et al. 2001; Gordon et al. 2006; Lohnes and Schmitthenner 1997; Sandhu et al. 2005; Weng et al. 2001).

Phytophthora sojae exhibits race-specific pathogenicity towards soybeans, and 55 physiologic races of *P. sojae* have been identified in the USA (Grau et al. 2004). Sugimoto et al. (2006) studied the race distribution of *P. sojae* in Hyogo, Japan, and found that PI103091, a Chinese soybean land race carrying *Rps1*d (Buzzell and Anderson 1992), was resistant to all 51 of the *P. sojae* isolates, including eight pathotypes. The Japanese cultivar Waseshiroge, which was not known to carry any *Rps* genes, was resistant to six of the eight *P. sojae* pathotypes from Hyogo Prefecture (Sugimoto et al. 2006). These two lines were subsequently selected as sources of resistance for breeding new Phytophthora-resistant cultivars. Tsuchiya et al. (1990) had also reported that Waseshiroge was highly resistant to many *P. sojae* isolates from

Hokkaido and other locations in Japan. Simple sequence repeat (SSR) markers linked to *Rps1*d in PI103091 were identified by Sugimoto et al. (2008a), and these markers have been used for MAS to breed new resistant cultivars adapted to Hyogo (e.g., an advanced breeding line 262-1-1). However, there have been no published reports on the presence of *Rps* gene(s) in Waseshiroge, despite its specific resistance. Identification of DNA markers linked to the *Rps* gene(s) from Waseshiroge would facilitate the breeding of new resistant cultivars for Hyogo and other soybean production regions.

The objectives of this study were to evaluate the *Rps* gene(s) in the cultivar Waseshiroge and to identify SSR markers linked to the *Rps* gene(s) using information from SoyBase (http://soybeanbreederstoolbox.org/) and sequence data from the soybean genome (http://www.phytozome.net/soybean).

Materials and methods

Plant materials

Two F_1 soybean seeds were produced from a cross between the Japanese cultivars Tanbakuro (black seed coat) and Waseshiroge (yellow seed coat), which was obtained from the Hokkaido Prefectural Plant Genetic Resource Center in Hokkaido, Japan. The F_1 plants were self-pollinated to produce three separate populations consisting of 42 (WH-1-1), 56 (WH-1-2) and 94 F_2 plants (WH-2). The F_2 plants from WH-1-1 and WH-1-2 were used for the disease experiments described below. The F_2 plants from WH-2 were self-pollinated and threshed individually to obtain $F_{2:3}$ seeds. This population was then advanced to the F_7 generation by single-seed descent without selection, and $F_{7:8}$ plants were assayed as described below.

Pathogens used in this study

A total of 77 isolates of *P. sojae* were identified from 77 infected soybean plants collected from several soybean-producing fields in Hyogo between 2002 and 2006 using the method described by Sugimoto et al. (2006), and these isolates were tested for virulence towards Tanbakuro using the agar medium inoculation method described in that paper. Isolates PJ-H65, PJ-H30, PJ-H5M, PJ-H42, and PJ-H67 were selected for disease experiments because Sugimoto et al. (2006) had previously found these four isolates to be highly virulent on Tanbakuro (95–100%), and to induce different reactions on six Japanese differential cultivars ('Isuzu', 'Chusei-Hikarikuro', 'Kitamusume', 'Toyosuzu', 'Gedenshirazu-1', and 'Ohhojyu'). Seven additional isolates also were added for the virulence examinations: T-4 was obtained from Dr. H. Mukobata of the Toyama Prefectural Agricultural, Forestry and Fisheries Research Center; Shonai-3 was obtained from Dr. S. Koizumi of the National Agricultural Research Center for the Tohoku Region; and five isolates representing races 1 (R1), 4 (R4), 7 (R7), 17 (R17), 25 (R25) were obtained from Dr. A. Dorrance of the Department of Plant Pathology at The Ohio State University, USA.

Resistance profile of the Waseshiroge *Rps* gene relative to reactions conditioned by other known *Rps* genes

The seven Japanese isolates (PJ-H65, PJ-H30, PJ-H5M, PJ-H42, PJ-H67, T-4, and Shonai-3) and five American isolates of *P. sojae* (R1, R4, R7, R17, and R25) mentioned above were tested for their virulence on 15 differential lines and cultivars: L88-8470 (*Rps1*a), L77-1863 (*Rps1*b), L77-3735 (*Rps1*c), PI103091 (*Rps1*d), L77-1794 (*Rps1*k), L76-1988 (*Rps2*), L83-570 (*Rps3*a), L88-1479 (*Rps3*b), L92-7857 (*Rps3*c), L85-2352 (*Rps4*), L85-3059 (*Rps5*), L89-1581 (*Rps6*), L93-3258 (*Rps7*), PI399073 (*Rps8*), and 'Harosoy 63' (*Rps1*a +7). These lines were obtained from Dr. R. Nelson of the USDA-ARS in Urbana, Illinois, U.S.A. Tanbakuro and Williams (*rps*) were used as susceptible control plants during the virulence tests. The pathotypes of the 12 isolates were determined using the agar medium inoculation method described by Sugimoto et al. (2006, 2008a), which is a modification of the hypocotyl inoculation method of Laviolette and Athow (1981). Seedlings were evaluated for resistant or susceptible reactions according to the method of Sugimoto et al. (2006, 2008a). Briefly, after the first primary leaf appeared on a plant, the stem near ground level was covered with two 3-mm-diameter plugs of 20-day-old mycelium cultured on LBA (lima bean agar). Thereafter, the plants were incubated in a growth chamber at 23°C with a 16-h day length under fluorescent light (light intensity: 150 $\mu E\ m^{-2}s^{-1}$). About 10 days after inoculation,

the numbers of dead or infected surviving plants in each bottle were recorded, and cultivars or differential lines were rated as resistant or susceptible. Each cultivar or differential line was considered resistant if fewer than 20% of the plants were infected, and susceptible if more than 20% of the plants were infected (Sugimoto et al. 2006). In order to determine which *Rps* gene(s) might be present in Waseshiroge, reactions of plants with one of 14 *Rps* genes to the 12 *P. sojae* isolates were compared to the reactions of Waseshiroge. Virulence tests were repeated more than two times to confirm the results.

Phytophthora virulence tests in F_2 and F_7 populations

Isolates PJ-H42 and PJ-H67 were highly virulent on Tanbakuro, but induced a strong resistance response on Waseshiroge, so they were chosen from among the 12 *P. sojae* isolates mentioned above for phenotypic assays. Tanbakuro and Williams (*rps*) were used as susceptible control plants during the virulence tests. Marker data indicated that both cultivars were homozygous recessive at the locus where the *Rps* gene from Waseshiroge had been mapped. Each of the F_2 plants in the WH-1-1 and WH-1-2 population and more than 20 individual F_8 seedlings per $F_{7:8}$ line in the WH-2 population were inoculated with PJ-H42 and screened for virulence in the tests described below to determine the number of resistant genes in Waseshiroge. The F_2 and $F_{7:8}$ phenotypes were determined according to previously reported methods (Sugimoto et al. 2006, 2008a). Approximately 10 days after inoculation, the numbers of dead or surviving plants were recorded. Each F_2 plant was scored as resistant (R + Rs) [homozygous-resistant (R) + segregating (Rs)] or susceptible (S) according to the method of Sugimoto et al. (2008a). A F_2 plant in the WH-1-1 or WH-1-2 population was considered resistant if it was alive and devoid of disease symptoms caused by *P. sojae,* and susceptible if the plant was dead or had disease symptoms characteristic of Phytophthora stem rot. Each $F_{7:8}$ line was scored as R, Rs, or S, according to the method of Sugimoto et al. (2008a). A WH-2 line was considered R if fewer than 20% of the plants were infected, Rs if 20–79% plants were infected, and S if more than 80% of the plants were infected (Sugimoto et al. 2008a). If all of the seedlings in a particular $F_{7:8}$ line were considered to be homozygous resistant, an additional 20 seedlings from the same line were inoculated to confirm the virulence results. Bioassays were repeated two or three times, depending on the number of seeds available from F_7 plants. The segregation ratios of the WH-1-1, WH-1-2, and WH-2 populations were evaluated using the chi-square goodness of fit test for the appropriate genetic models; a 3:1 ratio in the F_2 generation (WH-1-1 and WH-1-2), and a 63:2:63 ratio in the F_7 generation (WH-2). An additional 94 $F_{7:8}$ lines with more than 20 seeds per line were inoculated with PJ-H67 to confirm the phenotypic results with PJ-H42. This scoring was used to determine the F_7 plant genotype.

DNA samples

DNA was extracted using the CTAB method of Rowland and Nguyen (1993) scaled down for the isolation of DNA from 100 mg fresh weight of plant tissue (Sugimoto et al. 2008a). Leaf tissue was sampled in the field or greenhouse from each parent and all 94 F_7 plants, and juvenile, pre-expanded leaves were ground into a fine powder with liquid nitrogen. The powder was transferred to a 1.5 ml Eppendorf tube, and 500 µl of buffer was added and mixed at 60°C for 25 min. The extraction buffer consisted of 75 mM Tris–HCl (pH 8.0), 15 mM EDTA, 1.05 mM NaCl, 0.75% polyvinyl pyrrolidone (Katayama Chemicals, Osaka, Japan), 1.5% CTAB, and 1.5% mercaptoethanol. Chloroform (800 µl) was added and the aqueous and organic phases were mixed at room temperature for 15–20 min, after which the tubes were centrifuged at 15000×*g* for 20 min. The DNA pellet precipitated from the aqueous phase was dissolved in 50 µl TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), and later diluted to a final concentration of 10 ng μl^{-1} for PCR analysis.

Mapping of the *Rps* gene in Waseshiroge

SSR markers were chosen from the SoyBase web site (http://soybeanbreederstoolbox.org/) on the basis of the estimated location of the *Rps* gene in Waseshiroge. Additional SSR markers near the *Rps* gene in Waseshiroge were developed using three different recources: (1) the sequence information for the *Rps1*k gene (Bhattacharyya et al. 2005), (2) the *G. max* sequence information released by the Soybean Genome Project, Department of Energy, Joint Genome Institute

(http://www.phytozome.net/soybean) (Schmutz et al. 2010), and (3) the batchprimer3 utility (http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi), which was used according to the instruction manuals, and with *Rps1*k or soybean genome sequence information. The physical locations and nucleotide positions of the SSR markers were determined using a 'BLAST genome' search of their primer sequences in the Phytozome soybean genome database.

PCR amplifications were performed as previously reported by Sugimoto et al. (2008a), with small modifications. The 10 μl reaction mixtures contained 10 ng genomic DNA, 1× AmpliTaq Gold PCR buffer (Applied Biosystems, Foster City, CA, USA), 2.0 mM $MgCl_2$, 200 μM each of the four dNTPs, 0.5 units of AmpliTaq Gold, and 1.5 pmol of each primer. The reaction was performed by pre-denaturing the DNA at 95°C for 9 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 47°C for 30 s, extension at 67°C for 1 min, and a final extension at 72°C for 10 min. Amplification products were mixed with 5 μl of loading dye (95% formamide, 10 mM NaOH, 2.5 mg/ml bromophenol blue and 2.5 mg/ml xylene cyanol), heated for 5 min at 95°C, and chilled on ice for 5 min. The PCR amplicons were separated by electrophoresis on either an agarose gel containing 3.5% TBE (2.5 mM Tris–HCl, 2.5 mM boric acid, 0.05 mM EDTA, pH 8.0) and stained with ethidium bromide, or on a polyacrylamide gel with 8.0% TBE that was run at 150 V/cm for 3 h and stained with SYBR Green II gel stain (BioWhittaker Molecular Applications, Rockland, ME, USA), according to instructions.

The numbers of polymorphic DNA fragments between cvs. Tanbakuro and Waseshiroge were counted. SSR markers were scored as AA (homozygous for the Waseshiroge allele), AB (heterozygous) or BB (homozygous for the Tanbakuro allele) in 30 of the 94 $F_{7:8}$ lines. These consisted of 15 homozygous phytophthora-resistant and 15 homozygous phytophthora-susceptible lines. The segregation patterns of each of the selected SSR markers in the 94 $F_{7:8}$ lines were then tested for goodness-of-fit to expected segregation ratios using a χ^2 test. Linkage analysis was carried out using MAPMAKER/EXP, version 3.0b (Lincoln et al. 1993). The error detection probability level was set at 5%, and recombination frequencies were converted to map distances using the Kosambi mapping function (Kosambi 1944). A three-way contingency table was used to examine relationships between the genotypes at two markers flanking the *Rps* gene in Waseshiroge and plant phenotypes (Cheng et al. 2006).

Sequence homology search

The candidate genome region containing the *Rps* gene in Waseshiroge was estimated by conducting a BLAST search using the sequence data of the flanking markers and the soybean genome database mentioned above. Homology searches for similarities between *Rps1*k sequences (*Rps1*k-1 and *Rps1*k-2; Bhattacharyya et al. 2005) and the candidate genomic loci were done using GENETYX-MAC (versus 13) software (GENETYX Co. Ltd, Tokyo, Japan).

Results

Resistance profile of the Waseshiroge *Rps* gene relative to reactions conditioned by other known *Rps* genes

Reactions of Waseshiroge and 15 differential cultivars (containing 14 *Rps* genes) to several *P. sojae* isolates are listed in Table 1. When seven Japanese isolates were used for the virulence tests, Waseshiroge had the same reaction pattern as L77-3735 (*Rps1*c). However, the reaction pattern of Waseshiroge to five *P. sojae* isolates from the United States did not correspond to the reaction patterns of any of the 15 differential lines. This indicates that Waseshiroge may contain a novel *Rps* gene.

Phytophthora virulence results in F_2 and F_7 populations

The PJ-H42 (vir cvs. Isuzu, Kitamusume) and PJ-H67 (vir cv. Isuzu) isolates were highly virulent towards Tanbakuro and Williams (*rps*), while Waseshiroge was highly resistant to both isolates (Fig. 1; Table 2). The virulence tests of PJ-H42 on the 42 F_2 plants from WH-1-1 and 56 F_2 plants from WH-1-2 identified 30 or 41 resistant [(R + Rs); homozygous-resistant (R) + segregating (Rs)], respectively, and 12 or 15 susceptible (S) plants. These numbers fit a 3:1 segregation ratio ($\chi^2 = 0.28$ or 0.10, $P = 0.59$ or 0.76). Among the 94 $F_{7:8}$ lines, 51 were R, two were

Table 1 Reactions of Washeshiroge and a panel of differential lines with 14 *Rps* genes to 12 different *P. sojae* isolates

Isolate	Origin	Race[a]	Test lines with different *Rps* genes[b]															
			Waseshiroge	*rps*	*1*a	*1*b	*1*c	*1*d	*1*k	2	*3*a	*3*b	*3*c	*4*	*5*	*6*	*7*	*8*	*1*a + *7*
PJ-H65	Hyogo, Japan	39	S	S	S	S	S	R	S	R	S	S	S	S	S	S	S	S	S
PJ-H30	Hyogo, Japan	ND	S	S	S	S	S	R	S	S	S	S	S	S	S	S	R	S	R
PJ-H5M	Hyogo, Japan	ND	S	S	R	S	S	R	R	S	S	S	S	S	S	S	R	S	R
PJ-H42	Hyogo, Japan	ND	R	S	R	S	R	R	R	S	S	S	S	S	S	S	S	S	R
PJ-H67	Hyogo, Japan	ND	R	S	R	S	R	R	R	R	S	S	S	S	S	S	S	S	R
T4	Toyama, Japan	37	S	S	S	R	S	R	R	S	S	R	S	S	S	S	S	R	S
Shounai-3	Yamagata, Japan	ND	S	S	S	S	S	R	R	R	R	R	R	S	S	S	S	R	S
R1	Ohio, USA	1	R	S	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R
R4	Ohio, USA	4	R	S	S	R	S	R	R	R	R	R	R	R	R	R	S	R	S
R7	Ohio, USA	7	S	S	S	R	R	R	R	S	S	R	S	S	S	S	S	R	S
R17	Ohio, USA	17	R	S	R	S	R	S	R	S	S	S	S	S	S	S	S	R	R
R25	Ohio, USA	25	R	S	S	S	S	R	S	R	R	R	R	R	R	R	S	R	S

Race 37 and 39 were determined based on the virulence pathotypes reported by Grau et al. (2004)

Isolates of races R1, R4, R7, R17, and R25 were obtained from A. Dorrance of the Department of Plant Pathology at The Ohio State University

R resistant, *S* susceptible

ND (not determined), Five *P. sojae* isolates derived from Japan (PJ-H30, PJ-H42, PJ-H5M, PJ-H67, and Shounai-3) which elicited different virulence reactions on six Japanese differential cultivars (Isuzu, Chusei-Hikarikuro, Kitamusume, Toyosuzu, Gedenshirazu-1, and Ohhojyu), (Sugimoto et al. 2006), were not identical to any of 55 races reported in the USA (Grau et al. 2004)

[a] Race of each isolate was determined by the reactions of standard sets of eight of the 14 *Rps* differentials, including lines with *Rps1*a, *Rps1*b, *Rps1*c, *Rps1*d, *Rps1*k, *Rps3*a, *Rps6,* or *Rps7* (Grau et al. 2004)

[b] The pathotype of each isolate was examined on the following 15 differentials; L88-8470 (*Rps1*a), L77-1863 (*Rps1*b), L75-3735 (*Rps1*c), PI103091 (*Rps1*d), L77-1794 (*Rps1*k), L76-1988 (*Rps2*), L83-570 (*Rps3*a), L91-8347 (*Rps3*b), L92-7857 (*Rps3c*), L85-2352 (*Rps4*), L85-3059 (*Rps5*), L89-1581 (*Rps6*), L93-3258 (*Rps7*), PI399073 (*Rps8*), Harosoy 63 (*Rps1*a +7). Williams (*rps*) were used as susceptible control plants during the virulence tests

Rs, and 41 were S, thus fitting a 46.27:1.46:46.27 (i.e., 63:2:63) segregation ratio ($\chi^2 = 1.28$, $P = 0.53$); most of the inbred lines would be homozygous in this generation, but a few would still be heterozygous at the locus. These results suggest that Phytophthora resistance in Waseshiroge is controlled by a single dominant gene (Table 2). The phenotypic data for the responses of the 94 $F_{7:8}$ lines to PJ-H67 were similar to those for PJ-H42.

Location of the *Rps* gene in Waseshiroge

Twenty-four out of 41 SSR primer pairs for marker loci on chromosome 3 (MLG N) (Song et al. 2004) produced 27 scoreable polymorphic fragments between Tanbakuro and Waseshiroge. SSR genotyping of the 94 $F_{7:8}$ lines using 17 of the 24 polymorphic SSR markers linked to the *Rps1* locus revealed a marker segregation pattern that almost fit a 46.27:1.46:46.27 (or 63:2:63) ratio (Table 3). Four markers (Sct_195, Sct_379, Sat_186, and Sat_266) did not produce heterozygous DNA banding patterns. This may be due to that fact that SSR loci with dinucleotide repeats often to not amplify as well or as consistently as SSR markers with trinucleotide repeats, in our experience. In any case, however, the segregation patterns of plant SSR genotype (AA:BB) and phenotype (i.e., resistant:susceptible) fit the expected 47.73:46.27 (or 65:63) ratio (Table 3). These data were used to construct a linkage map that was 75.0 cM long, as shown in Fig. 2a. Sct_195, Sct_379, Satt631, Sat_186, and Satt009 were located on one side of the estimated position of the gene, while the other 12 markers (Satt530, Satt683, Satt675, Satt624, Sat_084, Satt584, Satt485, Sat_208, Sat_166, Sat_280, Sat_275, and Sat_266) were mapped to the other side. Satt683 had the same banding patterns as Satt675, Satt624, Sat_084, and Satt584. The closest marker,

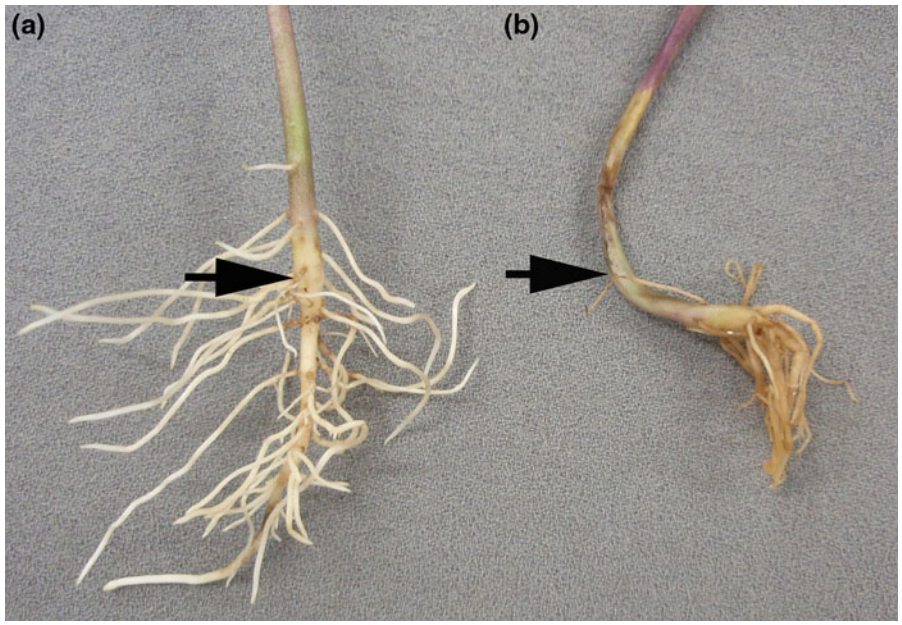

Fig. 1 Phytophthora virulence tests with *P. sojae* (PJ-H42). **a** Waseshiroge showing strong resistance to *P. sojae* isolate. **b** Takubakuro showing disease symptoms characteristic of Phytophthora stem rot. The stem of the plant appeared water-soaked and brown, and infection resulted in wilting and plant death. Ten days after inoculation, dead and surviving plants were scored. *Arrows* indicate the inoculation sites

Table 2 Genetic segregation of resistance to *P. sojae* isolate PJ-H42 in the F_2 and F_7 soybean populations (Tanbakuro/Waseshiroge) consisting of 94 lines

Parental type or cultivars	Generation	Segregation of F_2 or F_7 population[a]				Test and fit[b]		
		R	Rs	S	Total	R + Rs:S or R:Rs:S	χ^2	*P*
Waseshiroge × Tanbakuro (WH-1-1)	F_2	30		12	42	3:1	0.28	0.59
Waseshiroge × Tanbakuro (WH-1-2)	F_2	41		15	56	3: 1	0.10	0.76
Waseshiroge × Tanbakuro (WH-2)	F_7	51	2	41	94	63:2:63	1.28	0.53
Tanbakuro	–	0	–	121	121	0:1	–	–
Williams	–	0	–	111	111	0:1	–	–
Waseshiroge	–	154	–	0	154	1:0	–	–

PJ-H67 phenotypic data were identical to those of PJ-H42

[a] Each F_2 plant in WH-1-1 and WH-1-2 was scored as resistant (R + Rs) [homozygous-resistant (R) + segregating (Rs)] or susceptible (S) according to the method of Sugimoto et al. (2008a). Each $F_{7:8}$ line in WH-2 was scored as homozygous-resistant (R), segregating (Rs), or homozygous-susceptible (S), according to the method of Sugimoto et al. (2008a)

[b] The segregation ratios of the WH-1-1, WH-1-2, and WH-2 populations were evaluated using the chi-square goodness of fit test for the appropriate genetic models; a 3:1 ratio in the F_2 generation (WH-1-1 and WH-1-2), and a 63:2:63(or 46.27:1.46:46.27) ratio in the F_7 generation (WH-2)

Satt009, was mapped to a position only 0.9 cM from the estimated location of the gene locus, and Satt530 is about 12.1 cM from the gene. The banding pattern of Satt009, which showed a 98.9% correlation with resistance, is shown in Fig. 3.

Since Satt530 was not tightly linked to the *Rps* gene in Waseshiroge, primer pairs were designed that amplified eight marker loci (Raso1_Satt009OPT, Raso1_Satt009, T000303809 l, s026000076, T000303939 s, T000304754 l, T000304487 l, and T000304488 l) on

Table 3 Segregation of 25 selected SSR markers among 94 $F_{7:8}$ families

Markers[a]	Fragment size (bp)		Plant DNA genotype			Expected ratio (63:2:63 or 65:63)		Marker				Expected ratio		Recombination frequencies
								AA + AB		BB				
			AA	AB	BB	X^2	*P* value	R + Rs	S	R + RS	S	χ^2	*P*	
Sct_195	160		48	0	46	0.003	0.96	37	11	16	30	0.00	0.96	0.287
Sat_379	260		51	0	43	0.45	0.50	40	11	13	30	0.45	0.50	0.255
Satt631	110	150	51	3	40	2.93	0.23	49	5	4	36	1.67	0.20	0.096
Satt009	210		50	2	42	0.89	0.64	52	0	1	41	0.77	0.38	0.011
Sat_186	220	260	53	0	41	1.18	0.28	52	1	1	40	1.18	0.28	0.021
Satt530	200		53	2	39	2.31	0.31	49	6	4	35	2.25	0.13	0.106
Satt683	210		50	2	42	0.89	0.64	46	6	7	35	0.77	0.38	0.138
Satt675	130		50	2	42	0.89	0.64	46	6	7	35	0.77	0.38	0.138
Satt624	150		50	2	42	0.89	0.64	46	6	7	35	0.77	0.38	0.138
Sat_084	100		50	2	42	0.89	0.64	46	6	7	35	0.77	0.38	0.138
Satt584	100		50	2	42	0.89	0.64	46	6	7	35	0.77	0.38	0.138
Satt485	180		49	2	43	0.58	0.75	45	6	8	35	0.45	0.50	0.149
Sat_166	280		46	2	46	0.20	0.91	41	7	12	34	0.00	0.96	0.202
Sat_208	250		49	2	43	0.58	0.75	45	6	8	35	0.45	0.50	0.149
Sat_275	100		47	2	45	0.24	0.89	41	8	12	33	0.07	0.79	0.213
Sat_280	210	230	45	1	48	0.25	0.88	39	7	14	34	0.13	0.72	0.223
Sat_266	210		51	0	43	0.45	0.50	41	10	12	31	0.45	0.50	0.234
Rasol_Satt009OPT	280		50	3	41	2.50	0.29	52	1	1	40	1.18	0.28	0.021
Rasol_Satt009	280		50	3	41	2.50	0.29	52	1	1	40	1.18	0.28	0.021
T0003038091	293		53	3	40	2.93	0.23	52	2	1	39	1.67	0.20	0.032
s026000076	580		51	3	41	2.50	0.29	52	1	1	40	1.18	0.28	0.021
T000303939s	183		50	3	41	2.50	0.29	52	1	1	40	1.18	0.28	0.021
T0003047541	427		50	3	38	4.05	0.13	51	5	2	36	2.91	0.09	0.074
T0003044871	251		53	3	41	2.50	0.29	52	1	1	40	1.18	0.28	0.021
T0003044881	355		50	3	41	2.50	0.29	52	1	1	40	1.18	0.28	0.021

AA homozygous for the Waseshiroge allele, *AB* heterozygous, *BB* homozygous for the Tanbakuro allele, *R* resistant, *Rs* segregating, *S* susceptible

[a] Markers Sct195, Sat_379, Satt631, Satt009, Sat_186, Satt530, Satt530, Satt675, Satt683, Sstt624, Sat_084, Satt584, Satt485, Sat_166, Sat_208, Sat_275, Sat_280, and Sat_266 were named according to the SoyBase internet site (http://soybeanbreederstoolbox.org/). Markers Raso1_Satt009OPT, Raso1_Satt009, T0003038091, s026000076, T000303939s, T0003047541, T0003044871, and T0003044881 were developed using the phytozome database (http://www.phytozome.net/soybean)

chromosome 3 that were polymorphic between Tanbakuro and Waseshiroge (Table 4). DNA analysis of the 94 $F_{7:8}$ lines using these eight markers revealed a marker segregation pattern that almost fit a 46.27:1.46:46.27 (or 63:2:63) ratio (Table 3). Another linkage map 22.4 cM in length was constructed with the eight markers and three SSR markers (Satt631, Satt009, and Satt530) linked to the *Rps* gene in Waseshiroge (Fig. 2b). Raso1_Satt009OPT, Raso1_Satt009, s026000076, and T000303939 s had the same banding patterns. T0003044871 and T0003044881 were mapped to locations 1.6 cM from the estimated position of the gene, on the same side as Satt530. A χ^2 contingency table analysis indicated that the SSR marker genotypes of Satt009 and T0003044871 were significantly associated with plant phenotype (Table 5), suggesting that the two markers were closely linked to the novel *Rps* gene in Waseshiroge. This *Rps* gene mapped to the same position when the PJ-H67 phenotypic data were used.

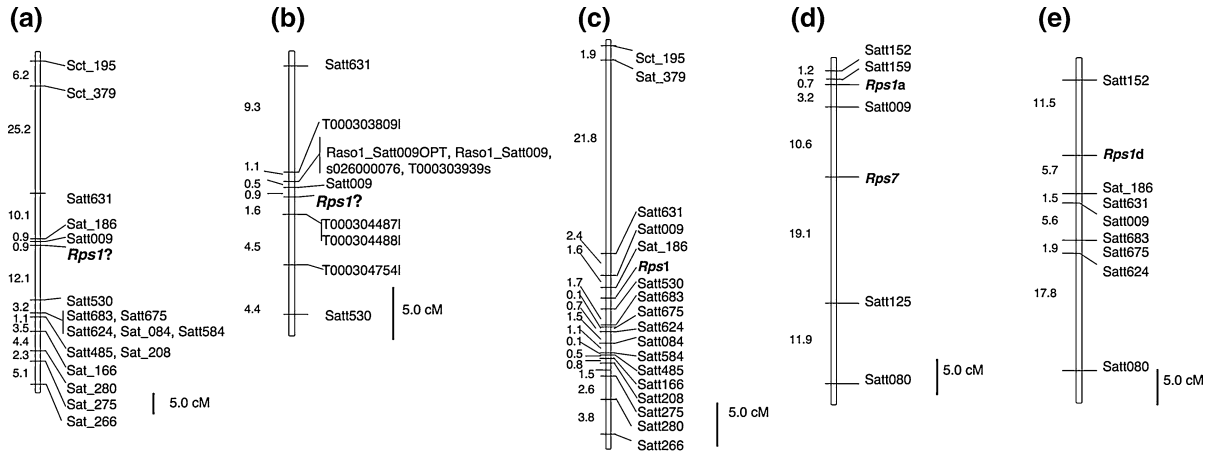

Fig. 2 Simple sequence repeat (SSR)-based genetic linkage map of the *Rps1* region. **a** Genetic linkage map developed using 17 SSR markers on MLG N and 94 $F_{7:8}$ lines from a cross between *Glycine max* cultivars Tanbakuro and Waseshiroge. **b** Genetic linkage map of three SSR markers on MLG N and eight new SSR markers segregating in 94 $F_{7:8}$ lines produced from the cross between Tanbakuro and Waseshiroge. **c** Genetic linkage map of the *Rps1* region with the map distances reported by Cregan (2003). **d** Genetic linkage map from Weng et al. (2001). **e** Genetic linkage map from Sugimoto et al. (2008a). Marker names and distances are on the right and left of the linkage map, respectively. Map distances are reported in Kosambi units

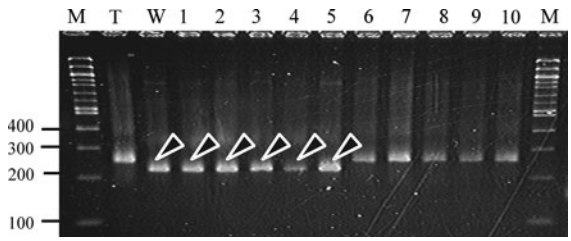

Fig. 3 Simple sequence repeat (SSR) analysis of the cultivar Tanbakuro, Waseshiroge and ten $F_{7:8}$ lines using SSR primers Satt009. *M* Molecular weight marker (100 bp ladder marker) *T* Tanbakuro, *W* Waseshiroge, *Lanes 1–5* homozygous-resistant $F_{7:8}$ lines, *Lanes 6–10* homozygous-susceptible $F_{7:8}$ lines. *Diamonds* indicate the Waseshiroge-specific SSR fragment of Satt009

Homologous genes in the mapped region

A BLAST search showed that Satt009 and T0003044871, which flank the *Rps* gene in Waseshiroge, are at nucleotide positions 3,919,203 and 4,486,048, respectively (Supplementary Table S1, online source), indicating that the *Rps* gene may reside in this region. Current gene annotation for this 567 kb region on the Phytozome database indicates the presence of eight loci with genes that share sequence homology with *Rps1*k (Supplementary Table S1, online source). *Rps1*k and the *Rps* gene from Waseshiroge were considered to possibly be allelic with *Rps1* (Bhattacharyya et al. 2005). The eight genomic loci showed 57.0–95.0% identity to *Rps1*k-1 and *Rps1*k-2, respectively.

Discussion

In this study, 98 F_2 plants from two related populations and 94 $F_{7:8}$ lines from crosses between the cultivars Tanbakuro (*rps/rps*) and Waseshiroge were used to determine the number of *Rps* genes conditioning resistance in Waseshiroge and to identify DNA markers tightly linked to the *Rps* gene(s). In previous assays, Waseshiroge had been found to be highly resistant to many *P. sojae* isolates from Japan (Sugimoto et al. 2006; Tsuchiya et al. 1990), and it was therefore selected as a donor parent to breed new lines resistant to *P. sojae* populations in Hyogo Prefecture (Sugimoto et al. 2006).

The PJ-H42 isolate (virulent against plants with the *Rps1*b, *Rps2*, *Rps3*a, *Rps3*b, *Rps3*c, *Rps4*, *Rps5*, *Rps6*, *Rps7*, and *Rps8* genes) was used for most of the disease resistance tests because this pathotype of *P. sojae* was the second most prevalent in the Hyogo region between 2002 and 2004 (Sugimoto et al. 2006). According to virulence tests and SSR analysis of the

Table 4 Eight SSR markers that were developed based on the soybean genome sequence from the Phytozome database

Marker name	Forward	Reverse	Position[a]
Raso1_Satt009OPT	AACCAACTTGAAATTACTAGAGAAATTAG	CCAACGTACAAGTAGTAAATAACTG	3,910,201
Raso1_Satt009	TGCTTAAAAATAAAAATGTTACG	ACTAGCGTATTAACCCTTGG	3,910,153
T0003038091	TCAAATAATCCTCGAATGTTATGG	GAAATAACATATTTGGAGTCACCTG	3,808,119
s026000076	CTAGGAGGACATGACTCGTGTTG	TTATATTTGATGCGACAATGGAA	3,912,394
T000303939s	AATTGTGCGTGATACTTTGGAGT	TCAATTATTGTAATGGAAAGGCG	3,938,892
T0003047541	AATAGTTGGTGAGTGGGTCAGTG	CAGTTGTTCGGACAAATCCAA	4,753,742
T0003044871	ATTAAGCATGCTACACAAGCAGC	TTCATGTAGGGACTAAATTGATGG	4,486,048
T0003044881	GAAGTGAAGGGTAACCATAACCA	TCCATTTCATCTACTCCTGCACT	4,487,418

[a] The nucleotide positions of the SSR markers were determined by a 'BLAST genome' search utility of their primer sequences on the Phytozome soybean genome database (http://www.phytozome.net/soybean)

Table 5 Contingency table for association of SSR plant genotype with plant phenotype in the F_7 soybean population (Tanbakuro/Waseshiroge) consisting of 94 lines

SSR genotype		Phenotype			
Satt009	T0003044871	R	Rs	S	Total
AA	AA	49	0	0	49
	AB	0	0	0	0
	BB	0	1	0	1
AA	Total	49	1	0	50
χ^2	*P*	50.0	$P < 0.001$		
AB	AA	0	0	0	0
	AB	0	2	0	2
	BB	0	0	0	0
AB	Total	0	2	0	2
χ^2	*P*	–	–		
BB	AA	1	0	0	1
	AB	0	0	1	1
	BB	0	0	40	40
BB		1	0	41	42
χ^2	*P*	42.0	$P < 0.001$		
Sum (AA, AB, and BB)	AA	50	0	0	50
Sum (AA, AB, and BB)	AB	0	2	1	3
Sum (AA, AB, and BB)	BB	0	1	40	41
Sum	Total	50	3	41	94
χ^2	P	132.8	$P < 0.001$		

AA homozygous for the Waseshiroge allele, *AB* heterozygous, *BB* homozygous for the Tanbakuro allele Phenotype, *R* resistant, *Rs* segregating, *S* susceptible

94 $F_{7:8}$ lines, reactions to the PJ-H42 isolate and the patterns of 13 of the 17 markers segregated in a 46.27:1.46:46.27 (or 63:2:63) ratio for resistant : segregating : susceptible plants and AA:AB:BB SSR marker genotypes. The segregation patterns of the remaining four markers (Sct195, Sct379, Sat186, and Sat_266) fit a 47.73:46.27 (or 65:63) ratio, and were considered dominant markers. These data therefore support the hypothesis of a single dominant gene inheritance model. The phytophthora stem rot disease resistance gene of Waseshiroge was subsequently characterized using 12 *P. sojae* isolates and 15 differential cultivars. The virulence results indicated that Waseshiroge may carry a novel single dominant gene.

The apparently novel *Rps* gene in cv. Waseshiroge was mapped to a position on MLG N, 0.9 cM "below" Satt009 and 12.1 cM "above" Satt530, markers closely linked to the *Rps1* locus (Song et al. 2004). This indicates that the *Rps* gene in Waseshiroge is likely to reside either at the *Rps1* locus or at a tightly linked locus. Although the sizes of the populations used were somewhat small for mapping, the data unambiguously show that a gene in the vicinity of the *Rps1* locus was associated with resistance in the plants and lines assayed. The *Rps1* locus has been reported to have at least five functional alleles (*Rps1*a, *Rps1*b, *Rps1*c, *Rps1*d, and *Rps1*k) (Demirbas et al. 2001). SSR markers linked to *Rps1* (Cregan et al. 1999), *Rps1*a (Weng et al. 2001), *Rps1*b (Demirbas et al. 2001), *Rps1*c (Demirbas et al. 2001), *Rps1*d (Sugimoto et al. 2008a) and *Rps1*k (Bhattacharyya et al. 2005; Kasuga et al. 1997) have been mapped near the 28-cM map position on MLG N (chromosome 3). Because of the

complexity of the *Rps1* locus, it is difficult to determine whether the reported resistance genes are allelic, or reside at closely linked loci.

According to our data, the *Rps* gene in Waseshiroge is not at the exact location as *Rps1* that Cregan (2003) reported as being flanked by Sat_186 (1.4 cM) and Satt530 (1.4 cM) (Fig. 2c). In the present study, Satt530 was 12.1 cM from estimated location of the gene, but the relatively small population sizes used could account for the observed discrepancies. Weng et al. (2001) stated that *Rps1*a was located "above" Satt009, and was flanked by Satt159 (0.7 cM) and Satt009 (3.2 cM), as shown in Fig. 2d. In our previous report, *Rps1*d was flanked by Sat_186 (5.7 cM) on one side and Satt152 (11.5 cM) on the other side (Sugimoto et al. 2008a). Satt152 and Satt159 might therefore be closely linked to the *Rps* gene in Waseshiroge, but these two markers were unfortunately not polymorphic between the two parents.

On the basis of our data, the distance between Sct_195 and Sat_266 was 75.0 cM (Fig. 1a, c), whereas the distance reported by Cregan (2003) was only 44.8 cM. The order of the SSR markers was also different from that reported by Cregan (2003), with Sat_208 and Sat_280 located distal to Sat_166 and Sat_275, respectively, whereas Cregan (2003) estimated that Sat_208 and Sat_280 were "below" Sat_166 and Sat_275. These discrepancies might be caused by (1) the use of different mapping populations and/or population sizes; or (2) the relatively small mapping population sizes (both <100 individuals) used by Cregan (2003), which might have reduced the precision of estimated map distances and the ability to precisely estimate the location of the gene relative to polymorphic markers. The differences in the orders of the SSR markers can also be seen in previous studies (Cregan et al. 1999; Sandhu et al. 2005; Sugimoto et al. 2008a; Weng et al. 2001).

To obtain additional closely linked markers on the Satt530 side of the *Rps* gene, eight primer pairs were developed using the JGI genome sequence data for the region between Satt009 (3,910,203) and Satt530 (5,813,486) on chromosome 3. Marker T0003044871 mapped only 1.6 cM from the gene, on the same side as Satt530. Satt009 and T0003044871 could therefore be used for marker-assisted selection (MAS) of progeny carrying the *Rps* gene from Waseshiroge. The accuracy of genotyping was estimated to be 98.94–97.87% for MAS with Satt009 and T0003044871, respectively. It was theoretically estimated to be 99.98% for MAS using both markers. Three-way contingency table analysis suggests that the selection of Phytophthora-resistant progenies with homozygous types [49 homozygous (R)] should be possible by selecting for the AA/AA SSR genotype at Satt009 and T0003044871. Selection using these two flanking markers should be more efficient and reliable than selection using one marker alone.

A BLAST analysis identified eight homologous sites with homology to the *Rps1*k gene between Satt009 (start position, 3,919,203) and T0003044871 (start position, 4,486,048), but the *Rps* gene from Waseshiroge was considered to have the highest level of homology with *Rps1* or *Rps1*k. Bhattacharyya et al. (2005) and Gao et al. (2005) reported that a larger cluster of highly polymorphic loci paralogous to the *Rps1*k sequence was located in the genomic regions adjacent to *Rps1*k. Plant disease resistance (R) genes often occur in clusters (Richly et al. 2002), which may facilitate the expansion of R gene loci and the generation of new R gene specificities through recombination and positive selection (Michelmore and Meyers 1998).

The *Rps* gene was mapped 0.9 cM from Satt009, indicating that the *Rps* gene from Waseshiroge may be located close to nucleotide position 4,123,267 on Williams 82. However, none of the potential candidates for the *Rps* gene listed in Supplementary Table S1 (online source) were found within the region between Satt009 and T0003044871, so a much higher mapping resolution and larger mapping populations will be required to obtain a more precise estimate of the location of *Rps* gene from Waseshiroge, since T0003044871 (1.6 cM) is still not closely linked to the gene. Another reason may be that this estimation is based on the sequence data of Williams 82.

To find additional markers near the *Rps* gene in Waseshiroge, we need to identify individuals with recombinations near the *Rps* gene(s), or develop backcross-derived isolines that differ for the presence of the *Rps* gene(s). In this study, recombinant line No. 94 was resistant to PJ-H42 (PJ-H67), but did not contain both Satt009 and T0003044871. Two other segregating lines, which have Satt009 and T0003044871 alleles, were detected and could be used to develop larger mapping populations that could be used to construct a high-density linkage map.

*Rps1*k and *Rps1* are in a region that contains very tightly linked clustered genes and repetitive sequences

(Bhattacharyya et al. 2005; Demirbas et al. 2001; Gao et al. 2005; Gao and Bhattacharyya 2008). Sequence information for the *Rps1*k and BAC clones (GS_18J19, GS_43D, or GS_99I16) (Bhattacharyya et al. 2005) could be useful for developing additional markers flanking the *Rps* gene in Waseshiroge. With this information, it might be possible to isolate/identify the *Rps* gene in Waseshiroge by comparing the sequences of coding regions in Waseshiroge with those of 'Kingwa' and 'Williams 82', which carry the *Rps1*k gene.

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