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ORIGINAL PAPER

Genetic characterization and fine mapping of the novel Phytophthora resistance gene in a Chinese soybean cultivar

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Abstract Phytophthora root rot (PRR), caused by *Phytophthora sojae* Kaufmann & Gerdemann, is one of the most destructive diseases of soybean [*Glycine max* (L.) Merr.]. Deployment of resistance genes is the most economical and effective way of controlling the disease. The soybean cultivar 'Yudou 29' is resistant to many *P. sojae* isolates in China. The genetic basis of the resistance in 'Yudou 29' was elucidated through an inheritance study and molecular mapping. In response to 25 *P. sojae* isolates, 'Yudou 29' displayed a new resistance reaction pattern distinct from those of differentials carrying known *Rps* genes. A population of 214 $F_{2:3}$ families from a cross between 'Jikedou 2' (PRR susceptible) and 'Yudou 29' was used for *Rps* gene mapping. The segregation fit a ratio of 1:2:1 for resistance:segregation:susceptibility within this population, indicating that resistance in 'Yudou 29' is controlled by a single dominant gene. This gene was temporarily named *RpsYD29* and mapped on soybean chromosome 03 (molecular linkage group N; MLG N) flanked by SSR markers SattWM82-50 and Satt1k4b at a genetic distance of 0.5 and 0.2 cM, respectively. Two nucleotide binding site-leucine rich repeat (NBS-LRR) type genes were detected in the 204.8 kb region between SattWM82-50 and Satt1k4b. These two genes showed high similarity to *Rps1*k in amino acid sequence and could be candidate genes for PRR resistance. Based on the phenotype reactions and the physical position on soybean chromosome 03, *RpsYD29* might be a novel allele at, or a novel gene tightly linked to, the *Rps1* locus.

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Introduction

Phytophthora root rot (PRR), caused by *Phytophthora sojae* Kaufmann & Gerdemann, is one of the most destructive diseases of soybean [*Glycine max* (L.) Merr.] (Bernard et al. 1957; Kuan and Erwin 1980). This disease has been reported in most soybean-production regions of the world, since it was first noted in America in 1948 (Tyler 2007; Wrather et al. 2001). In China, PRR was first observed in 1991 in Heilongjiang Province (Shen and Su 1991). Since then, the disease has become widespread in Heilongjiang Province, and the annual infected area is estimated at over 150,000 ha (Zhang et al. 2010). It has now also spread to Fujian Province (Chen et al. 2004).

PRR can be effectively controlled through the use of complete or partial resistance genes (Sugimoto et al. 2012). Partial resistance to *P. sojae* can be ineffective when plants are subjected to high disease pressure (Dorrance et al. 2003), so the deployment of race-specific, complete resistance genes in soybean cultivars has been the primary method used to control PRR (Schmitthenner 1999). To date, 14 genes conferring resistance to *P. sojae* (*Rps*) have been identified at 8 loci, *Rps1* to *Rps8*, which were mapped to molecular linkage groups (MLG) N, J, F, G, G, G, N and F, respectively (Sandhu et al. 2005). Five functional genes *Rps1*a, *Rps1*b, *Rps1*c, *Rps1*d and *Rps1*k have been mapped at the *Rps1*

locus (Bernard et al. 1957; Buzzell and Anderson 1992; Mueller et al. 1978), and three alleles *Rps3*a, *Rps3*b and *Rps3*c have been mapped at the *Rps3* locus (Mueller et al. 1978; Ploper et al. 1985). Additionally, five *Rps* genes, *RpsYB30*, *RpsYD25* (*Rpsyu25*), *RpsZS18*, *RpsSN10* and the *Rps* gene in soybean cultivar 'Waseshiroge', were mapped to MLG L, N, D1b, F and N, respectively (Fan et al. 2009; Sugimoto et al. 2011; Sun et al. 2011; Yao et al. 2010; Yu et al. 2010; Zhu et al. 2007).

Among these *Rps* genes, *Rps1*k is of great interest because it confers stable, broad-spectrum resistance in the USA (Schmitthenner et al. 1994), although new *Rps1*k-virulent *P. sojae* isolates have been reported in some areas (Dorrance et al. 2003; Schmitthenner 1999). This gene was first identified in cultivar 'Kingwa' (Bernard and Cremeens 1981) and has been transferred to soybean cultivars in the USA (Slaminko et al. 2010). The BAC-cloned *Rps1*k region contains two classes of functional coiled coil-nucleotide binding-leucine rich repeat genes (*Rps1*k-1 and *Rps1*k-2) and repetitive sequences (Gao et al. 2005; Gao and Bhattacharyya 2008). In addition to *Rps1*k, the *Rps1*c and *Rps1*a genes are also frequently introduced into commercial cultivars in the USA (Slaminko et al. 2010). However, the continuous use of the *Rps* genes in soybean cultivars has created selection pressure for the evolution of new pathogenic *P. sojae* isolates that can overcome the resistance conferred by these genes.

Since 1955, at least 55 *P. sojae* races with virulence pathotype varying from 7 to 1a, 1b, 1c, 1d, 1k, 3a, 6 and 7 were identified in the USA (Grau et al. 2004). *P. sojae* also presents similarly abundant virulence diversity in China (Zhang et al. 2010; Zhu et al. 2003). So far, 12 *P. sojae* races (1, 3, 4, 5, 9, 11, 13, 17, 21, 24, 44 and 54) were found in Heilongjiang Province, China (Zhang et al. 2010). In addition, other virulence pathotypes that differ from the 55 *P. sojae* races in the USA were also found in China (Zhu et al. 2003). Most of the soybean cultivars or germplasms containing the known *Rps* genes, except *Rps1*a, *Rps1*c and *Rps1*k, are not effective at controlling PRR in the major soybean-production regions (Cui et al. 2010; Tang et al. 2010; Zhang et al. 2010; Zhu et al. 2003). Although new races of *P. sojae* appear with the release of resistant cultivars, the use of genetic resistance still remains the most effective strategy to reduce losses caused by the pathogen. Thus, it is necessary to identify new *Rps* genes and develop molecular markers to genes of interest for marker-assisted selection (MAS).

In our previous study, we found that the cultivar 'Yudou 29' had a broad-spectrum resistance (Chen et al. 2008). The objectives of this study were to further characterize the inheritance of the *Rps* gene(s) in the cultivar 'Yudou 29', identify SSR markers for fine mapping and predict the candidate gene(s).

Materials and methods

Plant materials

'Yudou 29' is a PRR-resistant cultivar, and 'Jikedou 2' is a PRR susceptible cultivar. A population of 214 families derived from a cross between 'Yudou 29' and 'Jikedou 2' was used for gene mapping. The F_1 plants from the cross were self-pollinated to produce a population of F_2 plants. Each F_2 plant was self-pollinated and threshed individually to yield seeds of the $F_{2:3}$ families for both genotype and phenotype evaluations.

To determine which *Rps* gene or *Rps* gene combination was present in 'Yudou 29', phenotype analysis was performed using 15 differentials carrying a single *Rps* gene: Harlon (*Rps1*a), Harosoy13XX (*Rps1*b), Williams79 (*Rps1*c), PI103091 (*Rps1*d), Williams82 (*Rps1*k), L76-988 (*Rps2*), L83-570 (*Rps3*a), PRX146-36 (*Rps3*b), PRX145-48 (*Rps3*c), L85-2352 (*Rps4*), L85-3059 (*Rps5*), Harosoy62XX (*Rps6*), Harosoy (*Rps7*), PI399073 (*Rps8*) and Yudou25 (*RpsYD25*). Williams (*rps*) was used as the susceptible check to indicate successful inoculation.

Phytophthora sojae inoculation and PRR evaluation

A total of 25 *P. sojae* isolates with different virulence were used in the phenotype test (Table 1). For each isolate inoculation, 12 seeds of each differential were planted per replication in a pot with a diameter of 10 cm, and all differentials were tested in 3 replications using the hypocotyl-inoculation technique (Haas and Buzzell 1976). To test the phenotypes of the population, the isolate PsMC1 was used to inoculate 'Yudou 29', 'Jikedou 2', and 30 individual seedlings of each $F_{2:3}$ family using the same technique as above.

After inoculation, the plants were placed in a mist room with relative humidity 100 % and an average temperature of 25 °C for 2 days. They were then placed in a greenhouse with an average temperature of 25 °C. Reactions were recorded as the percentage of dead seedlings in each $F_{2:3}$ family at 6 days post-inoculation (DPI). In accordance with Gordon et al. (2006), families with 0–20 % dead seedlings were scored as homozygous resistant (R), families with 80–100 % dead seedlings were considered homozygous susceptible (S), and families with 21–79 % of dead seedlings was scored as segregating (Rs).

DNA preparation and pooling for bulk segregation analysis

DNA was extracted from soybean leaf tissues collected from 15–30 seedlings of each family using the CTAB method with minor modifications (Allen et al. 2006). Resistant and susceptible bulks for the bulk segregation analysis (BSA)

Table 1 Virulence pathotype of 25 *P. sojae* isolates from China classified using 15 differentials carrying the *Rps1*a, *Rps1*b, *Rps1*c, *Rps1*d, *Rps1*k, *Rps2*, *Rps3*a, *Rps3*b, *Rps3*c, *Rps4*, *Rps5*, *Rps6*, *Rps7*, *Rps8* and *RpsYD25* gene, respectively

Isolate	Virulence pathotype	Isolate	Virulence pathotype
PsJL1-1	1a, 1c	PsTA3	1b, 3a, 3c, 4, 5, 6, 7, 8
PsJMS3	1a, 2	PsJS9	1d, 3a, 3b, 3c, 4, 5, 6, 8
PsHJL1	3c, 7, YD25	Ps41-1	1a, 1d, 2, 3b, 3c, 5, 7, 8
PsAH4	2, 3a, 4, 5	PsJS7	1a, 1d, 3a, 3b, 3c, 4, 5, 6
PsJL4-1	1a, 1b, 1c, 1k	PsJL5	1a, 1b, 1c, 1d, 2, 3b, 3c, 7, 8
PsJL3-2	1a, 1b, 1c, 1k, 7	PsJS8	1b, 1d, 3a, 3b, 3c, 4, 5, 6, 7, 8
PsFJ	1b, 4, 5, 6, 7, YD25	Ps52	1b, 1d, 3c, 4, 5, 6, 7, 8, YD25
PsSX1	1a, 1b, 1c, 1d, 1k, 7	Ps53	1a, 1b, 1d, 2, 3b, 3c, 5, 7, YD25
PsAH3	1a, 1k, 3a, 4, 5, 6, 7	PsMC1	1a, 1c, 1k, 2, 3b, 3c, 4, 5, 6, 7, 8
PsXJ,	1a, 1d, 2, 3b, 3c, 7, 8	PsJN4	1b, 1c, 1d, 2, 3b, 3c, 4, 5, 6, 7, 8
PsAH1	1b, 1c, 2, 3a, 4, 5, 6, 8	PsAH5	1a, 1b, 1d, 2, 3a, 3c, 4, 5, 6, 7, 8, YD25
PsBr1	1a, 1b, 4, 5, 6, 8, YD25	PsHY33-1	1a, 1k, 2, 3a, 3b, 3c, 4, 5, 6, 7, 8, YD25
PsAM1	1b, 1c, 1d, 1k, 2, 6, 7, YD25		

were prepared from DNA samples of either ten homozygous-resistant or ten homozygous-susceptible $F_{2:3}$ families (Michelmore et al. 1991). DNA bulks were prepared by pooling 1 μg DNA of each selected family. The final concentration of the DNA bulks was adjusted to 50 ng/μl.

SSR marker development and analysis

The *Rps* gene in 'Yudou 29' was first mapped with the SSR markers described in Soybase (http://soybase.org). Since the *G. max* sequences (http://www.phytozome.net/soybean) have been released (Schmutz et al. 2010), new markers could be developed to finely map the gene. Thus, new SSR markers between Sat_186 and Satt530 were designed with Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) with default parameters according to the sequence downloaded from Phytozome (http://www.phytozome.net/soybean), in which the simple repeat sequences were identified by SSR Hunter 1.3 (http://en.bio-soft.net/dna/SSRHunter). Because *Rps1*k is located at the *Rps1* locus on MLG N (chromosome 03) and has been sequenced, new SSR markers were also designed from the contig containing the *Rps1*k-1, *Rps1*k-2 and *Rps1*k-3 (GenBank accession no. EU450800).

PCR reactions were performed on a thermal cycler (Biometra, USA) in 20 μl reaction volumes containing 50 ng genomic DNA, 2.5 μl 10× PCR reaction buffer (2.0 mM $MgCl_2$), 0.2 mM each dNTP, 1.0 U of Taq DNA polymerase and 0.2 μM of each primer. The PCR amplification consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 49–52 °C for 45 s and extension at 72 °C for 45 s, and final extension at 72 °C for 10 min. PCR products were mixed with 4 μl of 6× loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 40 % sugar) and were separated by 6 % polyacrylamide sequencing gel. The BSA polymorphic SSR markers were further tested for the entire $F_{2:3}$ mapping population.

Data analysis and linkage map construction

Resistant (R) and intermediate (I) phenotypes to the isolates (resistant reactions) were recorded as "1" for presence, and susceptible phenotypes were recorded as "0". Cluster analysis was performed with different modules of the NTSYS-PC software, version 2.11L (Rohlf 2000).

The segregation patterns of phenotypes and selected SSR markers in the mapping population were tested for goodness-of-fit to Mendelian segregation ratio with Chi-square (χ^2) analysis. A genetic linkage map of *RpsYD29* was constructed with the Joinmap 4.0 linkage analysis software (Van Ooijen 2006). Linkage groups were determined using a log-likelihood (LOD) threshold of 3.0.

Results

Phenotype reaction of 'Yudou 29' to *P. sojae* isolates

'Yudou 29' was resistant to 19 of the 25 isolates tested, while the 15 differentials carrying a single known *Rps* gene were resistant to 8–18 isolates. The reactions of 'Yudou 29' differed from those of Harlon, Harosoy13XX, Williams79, PI103091 and Williams82 to 17, 10, 10, 11 and 13 isolates, respectively. The reaction pattern of 'Yudou 29' to the 25 *P. sojae* isolates did not resemble any of the 15 differentials. The genetic diversity and phenotypic relationships between 'Yudou 29' and the 15 differentials in this study were depicted by cluster analysis (Fig. 1). Cutting at the coefficient 0.726, 'Yudou 29' comprised a single subgroup, which suggested that it might contain a novel *Rps* gene.

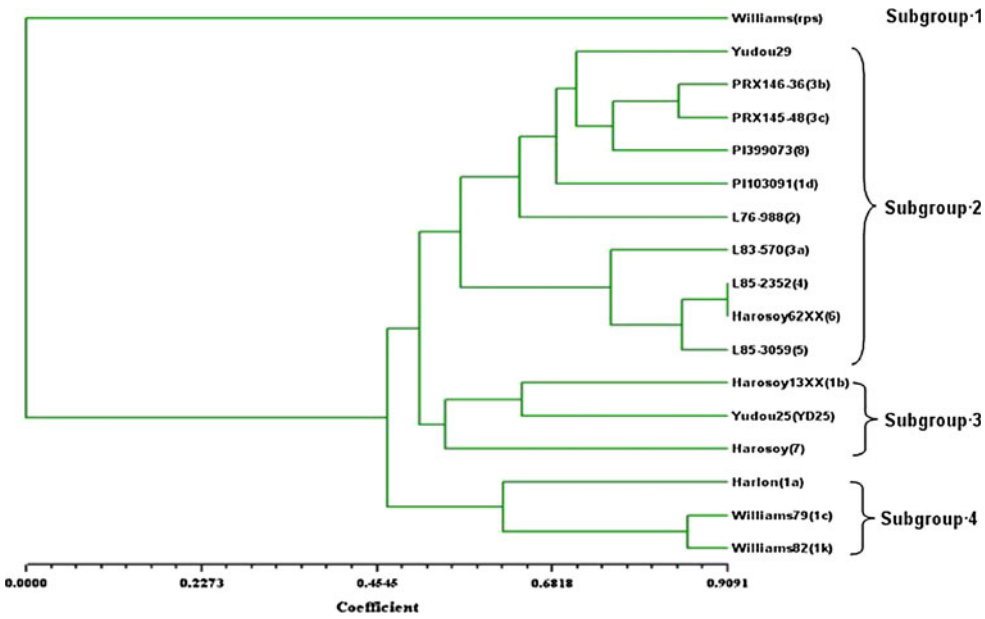

Fig. 1 Dendrogram revealed by UPGMA cluster analysis of the Phytophthora resistance among 'Yudou 29', 15 differentials carrying a single *Rps* gene: Harlon (*Rps1*a), Harosoy13XX (*Rps1*b), Williams79 (*Rps1*c), PI103091 (*Rps1*d), Williams82 (*Rps1*k), L76-988 (*Rps2*), L83-570 (*Rps3*a), PRX146-36 (*Rps3*b), PRX145-48 (*Rps3*c), L85-2352 (*Rps4*), L85-3059 (*Rps5*), Harosoy62XX (*Rps6*), Harosoy (*Rps7*), PI399073 (*Rps8*) and Yudou25 (*RpsYD25*) and the susceptible cultivar Williams

Phenotype analysis for the mapping population

'Jikedou 2' plants inoculated with PsMC1 displayed severe rot at the inoculation site and all plants were dead at 6 DPI. 'Yudou 29' plants showed no symptoms at 6 DPI, and thus were resistant to the isolate PsMC1.

Among the 214 $F_{2:3}$ families of the mapping population, 51 were homozygous resistant (R), 60 were homozygous susceptible (S) and 103 were segregating (Rs) to the isolate PsMC1 (Table 2). A segregation ratio of 51:103:60 in the $F_{2:3}$ population fits well with the genetic model ratio 1:2:1 ($\chi^2 = 1.06$, $p = 0.62$). This result suggested that Phytophthora resistance in 'Yudou 29' was controlled by a single dominant gene, which was temporarily named *RpsYD29*.

Molecular mapping of the *RpsYD29* gene

Using the BSA method, five SSR markers, Satt152, Satt159, Sat_186, Satt631 and Satt530 on chromosome 03 (MLG N), showed polymorphisms between 'Yudou 29' and 'Jikedou 2', as well as between the resistant bulk and the susceptible bulk. Linkage analysis further revealed that *RpsYD29* was linked to these five SSR markers and located between Sat_186 and Satt530 (Fig. 2).

A 2.34 Mb DNA segment of soybean chromosome 03 between Sat_186 and Satt530 was identified using a "BLAST genome" search in the Phytozome soybean genome database. Within this region, 218 SSR loci were identified by SSR hunter. A total of 156 SSR loci with 15 bp or greater repeat motifs were selected to design primers for fine mapping of *RpsYD29*. Three SSR markers, SattWM82-32, SattWM82-39 and SattWM82-50, showed polymorphisms between 'Yudou 29' and 'Jikedou 2' (Supplementary Table 1).

In addition, 11 SSR markers were designed from the 184 kb contig containing the *Rps1*k region (GenBank accession no. EU450800), three of which (Satt1k2a, Satt1k3 and Satt1k4b) were polymorphic between 'Yudou 29' and 'Jikedou 2' (Supplementary Table 1).

Molecular analysis of the 214 $F_{2:3}$ families using these 11 polymorphic markers revealed that their segregation

Table 2 Genetic segregation of resistance to *P. sojae* isolate PsMC1 in the 214 $F_{2:3}$ families derived from the cross between Yudou 29 and Jikedou 2

Cultivar and the cross	Generation	Amount	Observed number			Expected ratio and goodness of fit		
			R	Rs	S	(R:Rs:S)	χ^2	*p*
Yudou 29	P_1	20	20	–	–			
Jikedou 2	P_2	20	–	–	20			
Jikedou 2 × Yudou 29	$F_{2:3}$	214	51	103	60	1:2:1	1.06	0.62

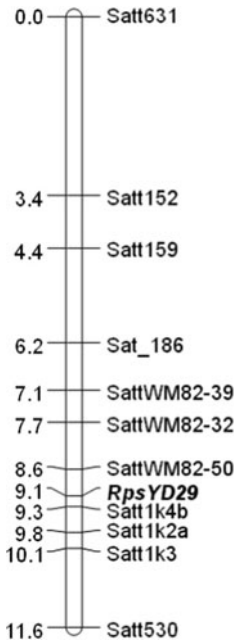

Fig. 2 Simple sequence repeat (SSR)-based genetic linkage map of the *RpsYD29* region. The map was deduced from segregation analysis of 214 $F_{2:3}$ families from a cross between *Glycine max* cultivars 'Yudou 29' and 'Jikedou 2'. The map was generated in Joinmap using Kosambi's mapping function. Marker names are on the *right* and distances (cM) on the *left*

pattern fits the 1:2:1 ratio (Supplementary Table 2). *RpsYD29* was closely linked to the SSR markers SattWM82-50 and Satt1k4b at a distance of 0.5 and 0.2 cM, respectively, and a genetic map consisting of the 11 SSR markers and *RpsYD29* was constructed (Fig. 2).

Candidate gene prediction

A BLAST search showed that the physical distance of the region between markers SattWM82-50 and Satt1k4b, which are at nucleotide positions 3,857,715 and 4,062,474, respectively, is approximately 204.8 kb. According to the Glyma1.0 annotations, a total of 11 genes have been identified in this region, of which 9 are supported by ESTs and two are putative (Supplementary Table 3).

Nucleotide binding site-leucine rich repeat (NBS-LRR) type resistance genes have been implicated in the resistance of *Rps1*k (Gao and Bhattacharyya 2008). Therefore, the identified 204.8 kb region containing *RpsYD29* was strictly evaluated for this motif, and two full-length NBS-LRR type genes, Glyma03g04030.1 and Glyma03g04080.1, were found to share amino acid sequence identities of 62–75 % with Rps1k-1 and Rps1k-2.

Discussion

The soybean originated in China and large quantities of Chinese germplasms have been screened for PRR resistance (Tang et al. 2010; Xia et al. 2011; Zhu et al. 2006). All identified *Rps* genes (except *Rps8*) and *Rps* gene combinations are present in germplasms from China (Kyle et al. 1998; Lohnes et al. 1994, 1996) and many germplasms are postulated to carry novel *Rps* genes (Xia et al. 2011; Zhu et al. 2006).

In this study, we investigated the resistance phenotype and genomic location of the *RpsYD29* gene in 'Yudou 29'. The results confirm that *RpsYD29* is a strong, broad-spectrum resistance gene that is likely a novel allele at the *Rps1* locus, or a novel gene at another locus tightly linked to *Rps1* locus.

In addition to *RpsYD29*, eight known *Rps* genes were previously mapped to MLG N, including five alleles at the *Rps1* locus (Bernard et al. 1957; Buzzell and Anderson 1992; Mueller et al. 1978), *Rps7* (Weng et al. 2001), *RpsYD25* (*Rpsyu25*) (Fan et al. 2009; Sun et al. 2011) and an *Rps* gene in cv. Waseshiroge (Sugimoto et al. 2011). In our cluster analysis based on the Phytophthora resistance to 25 *P. sojae* isolates, 'Yudou 29' and the differentials carrying *Rps1* alleles (*1*a, *1*b, *1*c, *1*d, *1*k), *Rps7* and *RpsYD25* fell into different subgroups cutting at the coefficient 0.726, and 'Yudou 29' was clustered in a subgroup different from all these genes (except *Rps1*d) on MLG N even cutting at 0.518. Sugimoto et al. (2008) stated that *Rps1*d was located "above" Sat_186, and was flanked by Satt152 (11.5 cM) and Sat_186 (5.7 cM). In the present study, *RpsYD29* was located "below" Sat_186, and was flanked by Sat_186 (2.9 cM) and Satt530 (2.5 cM). These indicated that *RpsYD29* is a distinct gene from the *Rps1* alleles, *Rps7* and *RpsYD25*. In addition, the *Rps* gene in Waseshiroge was located between Satt009 and T003044871 and may reside in the nucleotide region between 3,919,203 and 4,486,048 of the chromosome 03 (MLG N) (Sugimoto et al. 2011). *RpsYD29* is not at the same location as the *Rps* gene in Waseshiroge and thus is distinct from this gene as well.

Carrying the novel resistance gene, *RpsYD29*, conveying broad-spectrum resistance to *P. sojae* isolates, 'Yudou 29' could be used as a new source of resistance for breeding Phytophthora-resistant cultivars. The markers identified in this study will facilitate the tracking of *RpsYD29* in progenies or cultivars for reliable MAS in breeding programs. The SSR markers SattWM82-50 and Satt1k4b positioned *RpsYD29* within very tight genetic distances of 0.5 and 0.2 cM, respectively. The accuracy of genotyping was theoretically estimated to be 99.60 and 99.80 % for MAS with SattWM82-50 and Satt1k4b, respectively. The selection accuracy would be as high as 99.99 % for MAS using both markers.

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