Genetics and Molecular Research

Genetic diversity of *rhg1* and *Rhg4* loci in wild soybeans resistant to soybean cyst nematode race 3

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ABSTRACT. Over-utilization of germplasms that are resistant to the soybean cyst nematode (SCN) in soybean breeding programs can lead to genetic vulnerability in resistant cultivars. Resistant wild soybean (*Glycine soja*) is considered an invaluable gene source for increasing the genetic diversity of SCN resistance. In this study, we genotyped 23 *G. soja* accessions that are resistant to SCN race 3 for polymorphisms in the resistance genes, *rhg1*, *Rhg4*, and *SHMT*, and investigated their genetic relationship with eight *Glycine max* resistant cultivars. We identified 89 single nucleotide polymorphisms (SNPs) and 11 DNA insertion-deletions (InDels), of which 70 SNPs and 8 InDels were found

in *rhg1*, 9 SNPs were found in *Rhg4*, and 10 SNPs and 3 InDels were found in *SHMT*. Nucleotide diversity was $\pi = 0.00238$ and $\theta = 0.00235$, and haplotype diversity was 1.000. A phylogenetic tree comprising four clusters was constructed using sequence variations of the 23 *G. soja* and 8 *G. max* resistant accessions. Five *G. soja* accessions in subcluster A2, and four *G. soja* accessions in cluster B were genetically distant from *G. max* genotypes. Eight resistance-associated SNPs in the three resistance genes formed nine haplotypes in total. *G. soja* resistant accessions had different haplotypes (H2, H4, H5, H6, H7, and H8) compared with those of *G. max* (H1, H3, and H9). These results provide vital information on the use of wild soybeans for broadening the genetic base of SCN resistance.

Key words: Wild soybean; Genetic diversity; Soybean cyst nematode; Resistance gene

INTRODUCTION

The soybean cyst nematode (SCN) (*Heterodera glycines* Ichinohe) is one of the most devastating pests in soybean production worldwide. Annual economic losses caused by SCN have been estimated to be as high as $1.286 billion in the USA (Koenning and Wrather, 2010) and $120 million in China (Li et al., 2011).

Planting resistant cultivars is an effective method for controlling SCN; however, resistance genes of elite resistant cultivars have been traced to a few resistant genotypes in North America and China (Concibido et al., 2004; Yuan et al., 2009). Over utilization of these resistant genotypes in breeding programs can result in the genetic vulnerability of resistant cultivars. It has become increasingly important to incorporate novel genes into breeding programs to increase the development of resistant cultivars and to decrease the risk of virulent SCN populations.

Both *rhg1* and *Rhg4* are major SCN resistant genes. The *rhg1* gene (GenBank accession No. AF506516) is located on linkage group (LG) G [chromosome 18 (Chr 18)] (Concibido et al., 1994; Webb et al., 1995; Chang et al., 1997; Concibido et al., 1997; Yue et al., 2001; Guo et al., 2006; Jiao et al., 2015). Although Melito et al. (2010) reported no significant impact of the LRR-kinase gene on SCN resistance, nine *rhg1* haplotypes and five protein allotypes were identified from 112 resistant plant introductions (PIs) and 34 derived cultivars, and all PIs-bearing Peking type alleles were resistant to race 3 (Ruben et al., 2006). Moreover, six resistance-associated single nucleotide polymorphism (SNP) markers and one insertion-deletion (InDel) marker at the *rhg1* locus were developed (Li et al., 2009; Nan et al., 2009), and two SNPs (689 C/A and 757 C/T) were associated with SCN resistance. Association mapping also detected significant signals at the *rhg1* locus (Bao et al., 2014). *Rhg4* (GenBank accession No. AF506518) is located on LG A2 (Chromosome 8) (Concibido et al., 1994; Mahalingam and Skorupska, 1995; Webb et al., 1995; Chang et al., 1997; Heer et al., 1998; Guo et al., 2006). A total of 67 SNPs were identified in *Rhg4* (Yuan et al., 2012) and five SNP markers were developed (Yuan, 2007). Distribution of the five SNP sites between resistant and susceptible accessions indicated that *Rhg4* plays a role in soybean resistance to races 1, 3, and 4, and four SNPs were associated with resistance to SCN race 3. Liu et al. (2012) reported

that an SCN resistance gene, *SHMT,* was present at the *Rhg4* locus. *SHMT* encodes serine hydroxymethyltransferase, which is an enzyme responsible for interconversion of serine and glycine and is important for cellular one-carbon metabolism. Two genetic polymorphisms of 389 G/C and 1165 T/A, which result in the amino acid substitutions R130P and Y358N, respectively, were found to be correlated with SCN resistance (Liu et al., 2012).

Molecular genetic diversity among soybean germplasms could provide useful information for the identification of novel genes. Diers et al. (1997) estimated genetic diversity among 38 soybean PIs and investigated their genetic relationships with 138 genomic DNA clones, and found that one group of PIs potentially provided new genes for resistance to race 3. In another study conducted by Zhang et al. (1999), 102 soybean genomic probes representing five different restriction enzymes were used to analyze the genetic diversity and relationships between 56 soybean PIs. Chen et al. (2006) evaluated 122 resistant sources using 85 SSR markers and assigned 122 lines to 7 significantly different clusters; however, there were no significant differences among individuals within clusters.

Glycine soja is an invaluable genetic source for the genetic improvement of soybean, and likely possesses variations that are not present in domesticated soybean (Hyten et al., 2006; Yuan et al., 2008). Novel resistance loci have been found in some wild soybean sources, such as PI468916 (Wang et al., 2001) and PI464925B (Winter et al., 2007). Diers et al. (2005) successfully incorporated both resistance alleles from *G. soja* PI468916 into soybean germplasm LDX01-1-65. However, little information exists regarding the genetic diversity of *G. soja* resistant germplasms. In the present study, we genotyped 23 *G. soja* SCN race 3-resistant accessions for polymorphisms in *rhg1*, *Rhg4,* and *SHMT*, and compared their genetic diversity to *G. max* resistant cultivars that are frequently used in breeding programs. Our aim was to provide information for the rational use of wild soybean to increase the genetic diversity of SCN resistance.

MATERIAL AND METHODS

Plant materials

Twenty-three *G. soja* resistant accessions and eight *G. max* resistant cultivars were used in the present study. These materials were also used for genetic diversity analyses using simple sequence repeat (SSR) markers, and their origin and resistance level to SCN race 3 were reported (Yuan et al., 2014). Twenty-three *G. soja* accessions that are resistant to SCN race 3 were used to analyze genetic diversity. Among them 16 *G. soja* accessions were from Northeast China (Jilin, Heilongjiang and Liaoning province, and Inner Mongolia autonomous region), four were from North China (Hebei and Shanxi province), and three were from the Huang-Huai-Hai region of China (Henan and Shandong province). Eight *G. max* resistant cultivars that are frequently used in breeding programs and genetic mapping were provided by Zhangxiong Liu, Chinese Academy of Agricultural Sciences, and were used for genetic relationship and haplotype analyses.

DNA extraction

DNA was extracted from the leaves of 35-day-old seedlings from 3-5 plants using a DNA extraction kit (Tiangen Biotech, Beijing) according to the manufacturer protocols.

Primers design and polymerase chain reaction amplification

Four pairs of primers were designed using the sequence of the *G. max* receptor-like kinase RHG1 gene (*rhg1*) (GenBank accession No. AF506516) to produce overlapping fragments covering 4799 bp. Three pairs were designed from the sequence of the *G. max* receptor-like kinase RHG4 (*Rhg4*) gene (GenBank accession No. AF506518) to produce overlapping fragments covering 3276 bp. Two pairs were designed from *G. max* cultivar Forrest serine hydroxymethyltransferase gene (*SHMT*) (GenBank accession No. JQ714083) to produce overlapping fragments covering 2432 bp (Table 1 and Figure 1).

Table 1. Primers designed for PCR amplification and sequencing.

Primers	Sequence (5'→3')[c]	PCR product (bp)
1F (rhg1: 12U25[a])	CCGCCTGTCAACAAAAACAAGTATG	1176
1R (rhg1: 1164L26[a])	GCGGTCACCAGTGAAAAAGTTATGAT	
2F (rhg1: 902U24[a])	GCGTGCCCTTTGCTTCAGTCTCTT	1435
2R (rhg1: 2316L24[a])	GCGAGAGCAGCACACGAAGCTTAC	
3F (rhg1: 2293U24[a])	AAGTCTTGCTTCTTTCCTACATGG	1263
3R (rhg1: 3551L24[a])	CATCAAGCGCACTAGTCCAATCTC	
4F (rhg1: 3377U25[a])	CTCTTTCACGGCTGCTATCTTCTAT	1657
4R (rhg1: 4987L24[a])	TTTGTCGTATGTCAAGGTGACCTA	
5F (Rhg4: 1500U18[b])	TCGGGCTGGTCTGAAACA	1065
5R (Rhg4: 2540L25[b])	GGGATTGCCATTGAGAAACAAAGTC	
6F (Rhg4: 2420U27[b])	GCGGCGTGATGGTTGGAACTATGTTGT	1300
6R (Rhg4: 3695L25[b])	AACAGCTATAACCACCAAATACGAG	
7F (Rhg4: 3561U22)	GATGGGAAGTATTCTGTTGAGA	1338
7R (Rhg4: 4895L21	ATGCCCTTGGATAGTTGTCTC	
8F (SHMT: 2115U20)	TAAACCAGCACCAGGCGAAC	1470
8R (SHMT: 3564L21)	GGCCAAGGCACTGCTATCACC	
9F (SHMT: 3283U20)	CGAAGCAGGTTAAGGCGAAC	1361
9R (SHMT: 4624L20)	GGCGATAAACCCTATCACTC	

[a]Primers were originally designed by Li et al (2009). [b]Primers were originally designed by Yuan et al (2012). [c]Underlined characters denote added protective or mismatched nucleotide.

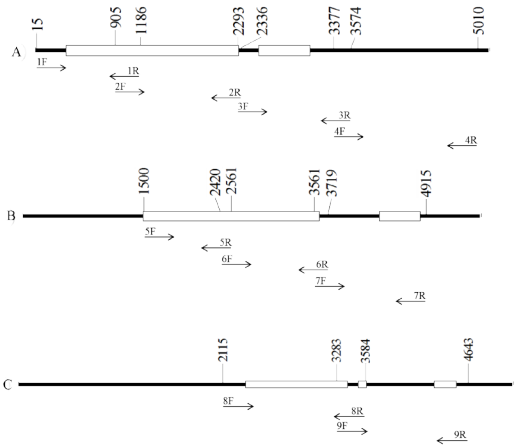

Figure 1. Schematic diagram showing nine sets of primers based on the reference sequence of the three resistance genes. Filled square represents uncoding regions, Open square represents exonic regions. **A.** Based on the *rhg1* reference sequence (GenBank accession No. AF506516), **B.** Based on the *Rhg4* reference sequence (GenBank accession No. AF506518), **C.** Based on the *SHMT* reference sequence (GenBank accession No. JQ714083).

Polymerase chain reaction (PCR) was performed in total volumes of 50 μL consisting of 150 ng DNA, 1X PCR TaKaRa Buffer (2 mM Mg^{2+}), 0.3 μM primers, 0.2 mM dNTP, and 1.25 U TaKaRa ExTaq polymerase. PCR was performed on a Biometra® thermal cycler using the following conditions: an initial 5 min denaturation at 94°C, then 30 s denaturation at 94°C, 30 s annealing at 58°C, 95 s extension at 72°C for 32 cycles, and a final 10 min extension at 72°C.

Sequencing and Sequence analysis

After collection and purification, the PCR products were sequenced with PCR primers (Table 1) by the Beijing Genomics Institute. Sequences were assembled with the SeqMan tool in DNAstar. Sequencing alignment was performed using ClustalX 1.8 with manual refinement. Two DNA polymorphism measures of nucleotide diversity (π and θ) and haplotype diversity were calculated using DnaSP v.5.10.01 software. A neighbor-joining (NJ) phylogenetic tree was constructed using the MEGA 5.0 software with a Kimura two-parameter model and 5000 bootstrap replicates.

RESULTS AND DISCUSSION

Sequence comparison with reference sequences of three resistance genes

Continuous sequences of 4799, 3276, and 2432 bp were generated through sequence assembly for *rhg1*, *Rhg4,* and *SHMT*, respectively. When aligned with reference sequences of the three resistance genes, 13 (two single-base insertions, two single-base deletions, and nine base changes) DNA polymorphic sites were found between the *rhg1* reference sequence (AF506516) and wild soybean, 40 (39 base changes and one 3-base insertion) between the *Rhg4* reference sequence (AF506518) and wild soybean, and none between the *SHMT* reference sequence (JQ714083) and wild soybean. Therefore, these sequences were presumed to be those of *rhg1*, *Rhg4,* and *SHMT* respectively.

DNA polymorphism among 23 *G. soja* resistant accessions

There were 10,507 nucleotide positions included in the sequence alignment of the three resistant genes of *rhg1*, *Rhg4*, and *SHMT* from 23 *G. soja* genotypes. A total of 89 SNPs and 11 InDels were identified, of which 70 SNPs and 8 InDels were found in *rhg1*, 9 SNPs in *Rhg4*, and 10 SNPs and 3 InDels in *SHMT* (Table 2). Of the 100 DNA variations, 76 were parsimony informative sites, and 24 were singletons. Of the 89 SNPs, 57 were involved in transitions and 32 were involved in transversions, with a transition:transversion ratio of 1.78:1. Forty-one SNPs occurred in coding regions, among which 17 were non-synonymous and 29 were synonymous. Nucleotide diversity in the 23 *G. soja* germplasms showed π = 0.00238 and θ = 0.00235, and haplotype diversity was 1.000.

Compared to the findings in *rhg1* reported by Li et al. (2009), who identified 37 SNPs and 5 InDels among 8 resistant *G. max* genotypes, we identified 70 SNPs and 8 InDels, among which 32 SNP loci were shared. For *Rhg4*, however, we only identified 9 SNPs, which was less than the 31 SNPs among 76 *G. max* genotypes found in the same sequence region by Yuan et al. (2012). Compared with the 19 SNPs in *SHMT* reported by Liu et al. (2012), 12 SNPs were found in the same region among the 23 *G. soja* accessions in our study, with six were

found in common with those reported by Liu et al. (2012). Therefore, these resistant *G. soja* accessions showed different patterns of sequence polymorphism at these resistance loci.

Table 2. Numbers of polymorphic sites among 23 *Glycine soja* resistant accessions.

		Insert-deletion	Single base change		Total
			Transition	Transversion	
rhg1	Noncoding region	8	28	16	52
	Coding region	0	21	5	26
	Total	8	49	21	78
Rhg4	Noncoding region	0	0	1	1
	Coding region	0	3	5	8
	Total	0	3	6	9
SHMT	Noncoding region	3	1	2	6
	Coding region	0	4	3	7
	Total	3	5	5	13

Genetic relationship among *G. soja* and *G. max* resistant accessions

A phylogenetic tree was constructed based on DNA sequences of the three resistance genes to investigate the genetic relationship among the 31 resistant germplasms (23 *G. soja* and 8 *G. max* resistant accessions). The 31 resistant resources were grouped in four clusters (Figure 2). Among the eight *G. max* resistant cultivars, six (Forrest, Franklin, Peking, PI437654, PI90763, and PI89772) were grouped in subcluster A1, whereas PI88788 was grouped in cluster C and was genetically distant from the six well-known resistance sources mentioned above. The genetic relationships among Peking PI437654, PI90763, PI89772, and PI88788 were consistent with those reported by Rao-Arelli and Anand (1988) and Myers and Anand (1991). The Chinese resistant landrace ZDD02315 was grouped in cluster D, and was genetically distant from the exotic resistant sources of Forrest, Franklin, Peking, PI437654, PI90763, PI89772, and PI88788, suggesting that Chinese *G. max* resistant germplasms had different alleles of the three resistance genes.

Six *G. soja* resistant accessions were grouped into subcluster A1 with six *G. max* resistant cultivars (Forrest, Franklin, Peking, PI437654, PI90763, and PI89772). Four *G. soja* resistant accessions were grouped into cluster C with PI88788, and four *G. soja* resistant accessions were grouped into cluster D with ZDD02315. The other nine *G. soja* resistant accessions were grouped into subcluster A2 (five *G. soja* accessions) and cluster B (four *G. soja* accessions), into which none of the eight *G. max* resistant cultivars was assigned, indicating that they possessed different resistance alleles that may have potential value for broadening the background of SCN resistance.

Haplotypes of *G. soja* accessions and *G. max* resistant accessions based on SCN-resistance associated SNPs

Two SNPs (689 C/A and 757 C/T) at *rhg1*, four SNPs (1724 C/A, 1760 C/T, 2120 T/A, and 2387 G/A) at *Rhg4*, and two SNPs (389 G/C and 1165 T/A) at *SHMT* were previously found to be associated with SCN resistance by Li et al (2009), Yuan (2007), and Liu et al. (2012), respectively. Haplotypes formed by these SNPs among 31 *G. soja* and *G. max* resistant accessions were investigated.

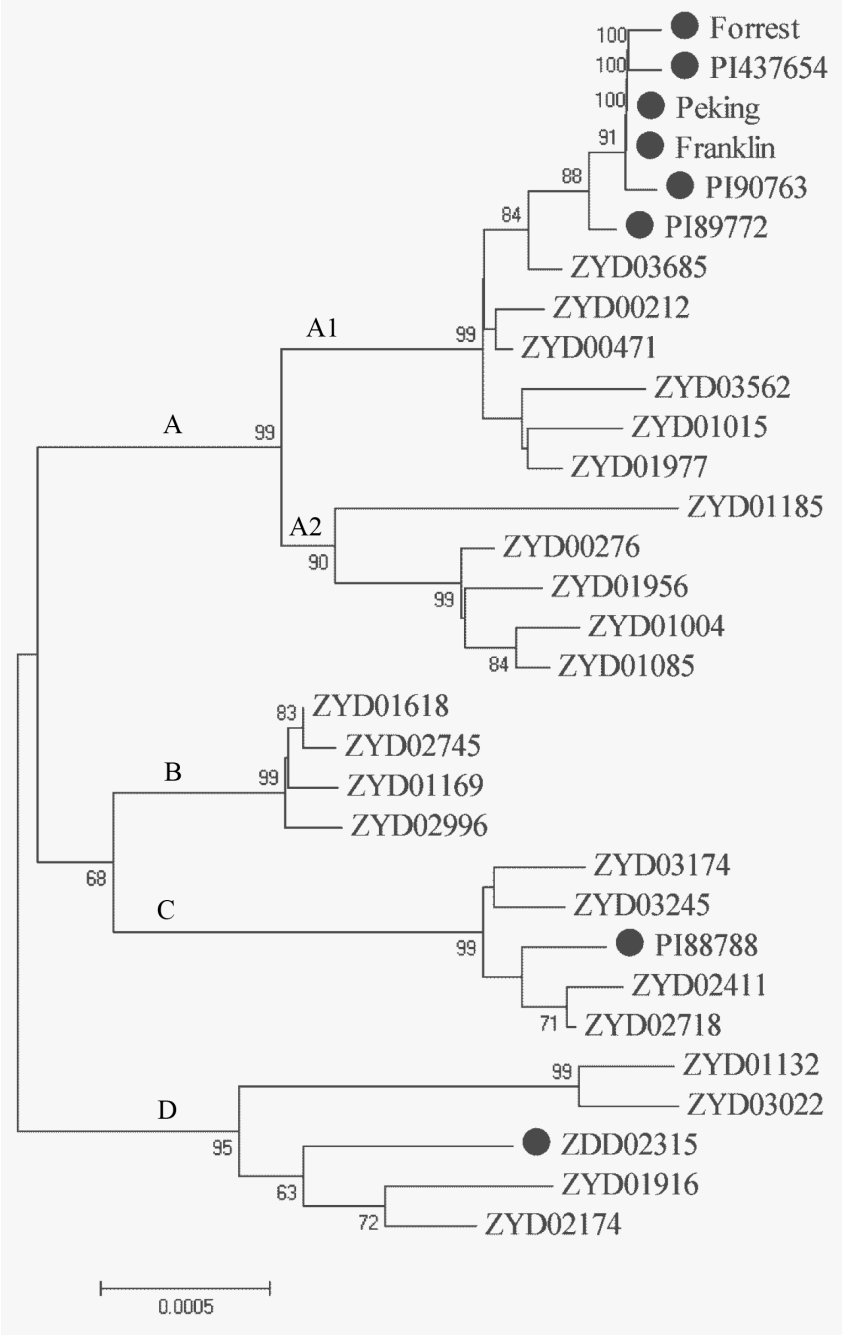

Figure 2. Neighbor-joining tree of the three resistance genes *rhg1*, *Rhg4,* and *SHMT* in 31 soybean resources including 23 *Glycine soja* and 8 *Glycine max* accessions. Bootstrap values of 60% or higher are shown on the branches. The phylogenetic tree comprised four clusters, cluster A (subcluster A1 and A2), B, C, and D. Filled circles: *Glycine max* resistant cultivars.

We found that *G. soja* resistant accessions and *G. max* resistant cultivars had different haplotypes. Nine haplotypes were identified in total, with three (H1, H3, and H9) found uniquely in the eight *G. max* resistant cultivars, and the other six haplotypes found in *G. soja* resistant accessions (Table 3). The H2, H6, and H8 haplotypes were common in the wild soybeans and were found in two or more samples; however, H4, H5, and H7 were found only in ZYD02718, ZYD03685, and ZYD01185, respectively.

Table 3. Haplotypes formed by SCN-resistance-associated SNPs among 31 resistant resources.

Haplotype	*rhg1*			*Rhg4*			*SHMT*		Resistant resource[a]	Cluster
	689 C/A	757 C/T	1724 C/ A	1760 C /T	2120 T /A	2387 G/A	389 G/C	1165 T/A/C		
H1	C	C	C	C	T	G	G	T	Forrest, Franklin, Peking, PI437654, PI90763	A1
									PI88788	C
H2	C	C	C	C	T	G	C	A	ZYD02411	C
									ZYD01132	D
H3	C	C	C	C	T	G	G	C	ZDD02315	D
H4	C	C	A	C	T	G	C	A	ZYD02718	C
H5	C	C	A	C	A	G	G	C	ZYD03685	A1
H6	C	C	A	C	A	G	C	A	ZYD00471, ZYD01015, ZYD01977, ZYD03562	A1
									ZYD00276, ZYD01956	A2
									ZYD01618, ZYD02745, ZYD02996	B
									ZYD03174	C
									ZYD03022	D
H7	A	T	A	C	A	G	C	A	ZYD01185	A2
H8	C	C	A	T	A	A	C	A	ZYD00212	A1
									ZYD01085, ZYD01004	A2
									ZYD01169	B
									ZYD03245	C
									ZYD01916, ZYD02174	D
H9	C	C	A	T	T	G	G	T	PI89772	A1

[a]*Glycine max* resistant cultivar is underlined.

Among domesticated soybean collections, the haplotypes 689 C-757 C in *rhg1* and 1724 C-1760 C-2120 T-2387 G in *Rhg4* were found to be predominant, and were considered to be SCN resistance-associated haplotypes (Yuan 2007; Li et al 2009). For wild soybeans, we found that, except for ZYD01185, 22 accessions possessed either one of the resistance-associated haplotypes or both. The 1724 A-1760 C-2120 A-2387 G haplotype in *Rhg4* was rare in domesticated soybeans (Yuan 2007); however, among *G. soja* resistant germplasms, this haplotype was predominant, and was distributed in 13 resistant accessions, indicating different haplotype frequencies between domesticated and wild soybeans.

The 1165 T/A SNP has been reported to alter a key regulatory property of the SHMT enzyme (Liu et al., 2012). We found a tri-allelic SNP at the 1165 position of *SHMT*, 1165 T/A/C, which resulted in the amino acid substitution 358 Y/N/H. Consequently, three haplotypes were identified, 389 G-1165 T, 389 C-1165 A, and 389 G-1165 C. The haplotype 389 G-1165 C was uniquely identified in Chinese *G. max* resistant landrace ZDD02315 and *G. soja* resistant accession ZYD03685, indicating that this novel allele may play a role in the resistance response. However, further analysis of allele function is required.

Conflicts of interest

The authors declare no conflict of interest.

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