Indian J. Genet., 80(3) 267-274 (2020)
DOI: 10.31742/IJGPB.80.3.5

Identification of novel haplotype of a cyst nematode resistance gene, GmSNAP18 in soybean [*Glycine max* (L.) Merr.]

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(Received: February 2020; Revised: July 2020; Accepted: August 2020)

Abstract

Soybean cyst nematode (SCN) is one of the most damaging pest of soybean. Discovery and characterization of the genes involved in SCN resistance are important in soybean breeding. Soluble NSF attachment protein (SNAP) genes are related to SCN resistance in soybean. SNAP genes include five gene families, and 2 haplotypes of exons 6 and 9 of SNAP18 are considered resistant to the SCN. In present study the haplotypes of *GmSNAP18* were surveyed and chacterized in a total of 60 diverse soybean genotypes including Korean cultivars, landraces, and wild-types. The target region of exons 6 and 9 in *GmSNAP18* region was amplified and sequenced to examine nucleotide variation. Characterization of 5 haplotypes identified in present study for the *GmSNAP18* gene revealed two haplotypes as resistant, 1 as susceptible and two as novel. A total of twelve genotypes showed resistant haplotypes, and 45 cultivars were found susceptible. Interestingly, the two novel haplotypes were present in 3 soybean lines. The information provided here about the haplotypic variation of *GmSNAP18* gene can be further explored for soybean breeding to develop resistant varieties.

Key words: Soybean cyst nematode, resistance, single nucleotide polymorphism, alignment, haplotypes

Introduction

Soybean [*Glycine max* (L.) Merr.] is one of the most important economic leguminous crops cultivated for human and animal consumption worldwide. Various abiotic and biotic factors limit the production of soybean plants, including the soybean cyst nematode (SCN; *Heterodera glycines*), which is the most harmful species to soybean. The SCN is responsible for substantial losses to soybean production in many countries. Pest and diseases are one of the factors that limits the profitability and success of soybean production (Juliatti et al. 2005). Soybean cyst nematodes invade fields in large numbers, causing extensive losses, amounting to more than $1 billion annually in the United States (Koenning et al. 2010). The SCN was first discovered in the USA and confirmed in northern California in 1954 (Winstead et al. 1955). In Korea, the SCN was first reported in by Yokoo (1936). Additionally, Choi and Choi conducted field and research experiments on soybean parasitic nematodes in Korea with soil samples and identified different SCN races (Choi and Choi 1983).

With limited methods of elimination, the SCN has become one of the most detrimental pathogens of soybean, causing considerable losses in different soybean-producing countries worldwide (Niblack et al. 2006). By successive cell wall dissolution and the combination of hundreds of adjacent cells, the nematode penetrates the root and moves towards the vascular tissue, which results in the formation of a feeding site (Endo 1964). Currently, there are no methods, including pesticides, with which to control the SCN in soybean fields. Planting SCN-resistant soybean cultivars and crop rotation with non-host crops are effective means of managing the SCN in infested fields (Niblack 1999). One of the foremost approaches to protecting against SCN damage is the cultivation of resistant cultivars (Mitchum 2016). Thus, genetic breeding and research on genes related to SCN resistance have been performed to overcome nematode virulence.

Caldwell et al. (1960) identified the major SCN

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Published by the Indian Society of Genetics & Plant Breeding, A-Block, F2, First Floor, NASC Complex, IARI P.O., Pusa Campus, New Delhi 110 012; Online management by www.isgpb.org; indianjournals.com

resistance gene called *rhg1*. Most of the SCN-resistant cultivars are from three plant sources: Peking (PI54802), PI88788 and PI437654 (Meksem et al. 2001; Concibido et al. 2004). Recently, many findings regarding the molecular nature and genetics of SCN resistance have been reported. Numerous papers and studies have focused on the identification and mapping of quantitative trait loci (QTLs) conferring resistance to the SCN in soybean from different plant sources (Liu et al. 2017). Two major QTLs have been discovered that support SCN resistance: *rhg1* on chromosome 18 and *Rhg4* on chromosome 8 (Concibido et al. 2004). Three genes underlie the *rhg1* QTL, with copy number of variations that confer resistance: An acid transporter, an N-ethylmaleimide-sensitive factor attachment protein (SNAP) and a wound-inducible protein (WI12) (Cook et al. 2012). The gene responsible for *Rhg4* conferring resistance to SCN was found to be a serine hydroxymethyltransferase (SHMT) (Liu et al. 2012).

A study of single nucleotide polymorphism and insertion/deletions revealed GmSNAP18, found in the *rgh1* locus, to be one of the strongest candidate genes conferring resistance to the SCN and to be required for Peking-type resistance (Liu et al. 2017). Peking-type soybean cultivars require *rgh1* and *Rhg4* alleles for resistance to the SCN (Meksem et al. 2001). The *rhg1* of PI88788-type cultivars is sufficient for resistance to the SCN (Concibido et al. 2004). By combining genetic complementation analysis with an integrated set of genomic and genetic approaches, GmSNAP18 was identified as the *rgh1-a* gene, which primarily confers to the devastating pathogen called the soybean cyst nematode (Liu et al. 2017).

In plants, SNAPs have been studied extensively, and α-SNAPs are linked to disease resistance in plants and can also be used marker(s) to identify genetic purity in varieties (Lakhssassi et al. 2017; Bodanapu et al. 2019). Therefore the present study was conducted to know the SNAP gene family and haplotypes of the target gene GmSNAP18 in 60 germplasms, including cultivars and landraces.

Materials and methods

Plant materials and DNA isolation

Sixty soybean lines were used to identify the haplotypes of *GmSNAP18* genes. The plants consisted of wild types, landraces, local cultivars and collection types (Table 1). Collection are those genotypes collected from different provinces of Korea. The leaf tissues from all plants were individually collected, frozen immediately in liquid nitrogen and stored at –80°C for DNA isolation. All samples were ground into a fine powder with liquid nitrogen by crushing in Eppendorf tubes. Approximately 100 mg of the crushed samples was used for genomic DNA isolation. The DNA was isolated using a DNeasy kit according to the manufacturer's protocol (Qiagen, Inc., Hilden, Germany). The results were checked on a 1% agarose gel by observing visual bands under UV radiation with a GelDoc system.

SNP position and resistance gene information

Information on the resistance gene GmSNAP18 was collected from Liu et al. (2017). We downloaded the gene sequence from the NCBI website (www. https://www.ncbi.nlm.nih.gov/) and studied the sequence. The position of the SNP was found to be associated with resistance to the soybean cyst nematode. The data for the soybean SNAP gene family was down loaded from the NCBI website (https blast.ncbi.nlm.nih.gov/Blast.cgi). The variation of sequence in the target site of the GmSNAP18 gene was examined by comparing the DNA sequence among the resistant samples, namely, Peking (KX 147329.1) and PI88788 (KX 147330.1), and the susceptible sample, namely, Essex (KX 147331.1). GmSNAP18 consists of nine exons and eight introns, for a total length of 4223 bp. The SNPs were found in exon 6 and exon 9, representing the haplotypes related to SCN resistance.

Primer design and validation

Primers targeting exon 6 and exon 9 were designed using NCBI primer blast tools (https://www.ncbi.nlm.nih.gov/tools/primerblast). We performed primer blast of the GmSNAP18 sequence and selected one primer set for amplification. The target sites were amplified with the following cycling parameters: 95^{o} for 2 minutes, 95^{o} for 30 s, 55^{o} for 30 s (repeated for 35 cycles) and 72^{o} for 30 s, with a 7 minutes extension at 72^{o}. The PCR products were checked on a 2% agarose gel by observing visual bands under UV radiation with a GelDoc system.

Purification and sequencing

The PCR products of the samples were purified with the GeneAllExpin^{TM} PCR purification kit (GeneAll Biotechnology Co. Ltd., Seoul, South Korea). The purified PCR products were sequenced using theABI3730 sequencing platform (Applied Biosystem LLC, San Francisco, US) according to the manufacturer's protocol at the NICEM (National Instrumentation Center for Environmental

Table 1. A list of soybean genotypes used to study haplotypes based on the SNP in exon 6 and exon 9 of GmSNAP18 gene

S.No.	Seed #	Name	Type
1	KB000001	KWS19	Landrace
2	KB000002	IT191199	Weedy type*
3	KB000004	KWS79	Landrace
4	KB000005	IT191202	Weedy type*
5	KB000007	Peking	Cultivar
6	KB000008	IT182936	Weedy type*
7	KB000057	Williams82	Cultivar
8	KB000058	daewonkong	Cultivar
9	KB000059	jwinunisujib	Cultivar
10	KB000061	Collection_1	Landrace
11	KB000062	Gichankong	Cultivar
12	KB000063	Cheongakong	Cultivar
13	KB000067	Collection_2	Landrace
14	KB000070	Pungsannamulkong	Cultivar
15	KB000083	Haessalkong	Cultivar
16	KB000084	Collection_3	Landrace
17	KB000089	Ru-4	Cultivar
18	KB000090	Arksoy	Cultivar
19	KB000091	Gwangankong	Cultivar
20	KB000093	Manlikong	Cultivar
21	KB000095	Bangsakong	Cultivar
22	KB000096	Collection_4	Landrace
23	KB000098	Suwon-115	Cultivar
24	KB000099	Suwon-182	Cultivar
25	KB000100	Sinbuseogtae	Cultivar
26	KB000102	Yesanseolitae	Cultivar
27	KB000103	Wonhyeon	Cultivar
28	KB000104	Eunhakong	Cultivar
29	KB000106	Jinpum 1ho	Cultivar
30	KB000107	Jinpum 2ho	Cultivar
31	KB000108	Jinpumkong	Cultivar
32	KB000109	Cheongja	Cultivar
33	KB000110	Taegwang	Cultivar
34	KB000112	Wonyul	Cultivar
35	KB000113	Heugcheong	Cultivar
36	KB000114	Collection_5	Landrace
37	KB000115	Cheongmiwon	Cultivar
38	KB000116	Paldalkong	Cultivar
39	KB000117	Hwanggeumkong	Cultivar
40	KB000118	Ilmikong	Cultivar
41	KB000119	Baegunkong	Cultivar
42	KB000120	Daepungkong	Cultivar
43	KB000121	Danbaegkong	Cultivar
44	KB000122	Collection_6	Landrace
45	KB000123	Collection_7	Landrace
46	KB000124	Collection_8	Landrace
47	KB000125	Collection_9	Landrace
48	KB000126	Collection_10	Landrace
49	KB000064.1	Gangilkong	Cultivar
50	KB000065.1	Collection_11	Landrace
51	KB000052.1	Sinhwakong	Cultivar
52	KB000546	Gwangankong	Cultivar
53	KB000544	Collection_12	Landrace
54	KB000545	Collection_13	Landrace
55	KB000546	Socheongja	Cultivar
56	KB000547	Yagseonkong	Cultivar
57	KB000548	Collection_14	Landrace
58	KB000549	Cheongja-3ho	Cultivar
59	KB000552	Collection_15	Landrace
60	KB000553	Taegwangkong	Cultivar

* = *G. soja*; all other genotypes are *G. max*

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Characterization of the GmSNAP18 haplotypes

The quality of all sequences was checked with Chromas DNA sequencing software (v. 2.6.6, https://technelysium.com.au/wp/). Multiple sequence alignment to discover DNA variation was performed using MAFFT software version 7 (https://mafft.cbrc.jp/alignment/software/). The sequences of Peking, PI88788, a resistant cultivar and Essex, a susceptible cultivar, were used as reference sequences for our study. PI88788 was used as a reference cultivar for assessing the DNA variation among the germplasms.

Results

DNA isolation, PCR and sequencing

DNA was successfully isolated and later normalized to 20 ng/µl with a NanoDrop. The results were observed by 1% gel electrophoresis. A PCR based SNP marker was used for determining the presence of SNP of resistant and susceptible cultivars. The primers was designed in exon 6 with forward primer 5'-ACTTCTAGTTAGAGCATGAACTGC-3' and reverse primer, 5'-TGACCTACCGCCAACAATCT-3'. In exon 9 the forward primer, 5'- GTTCACAGTGCAATTTATT-

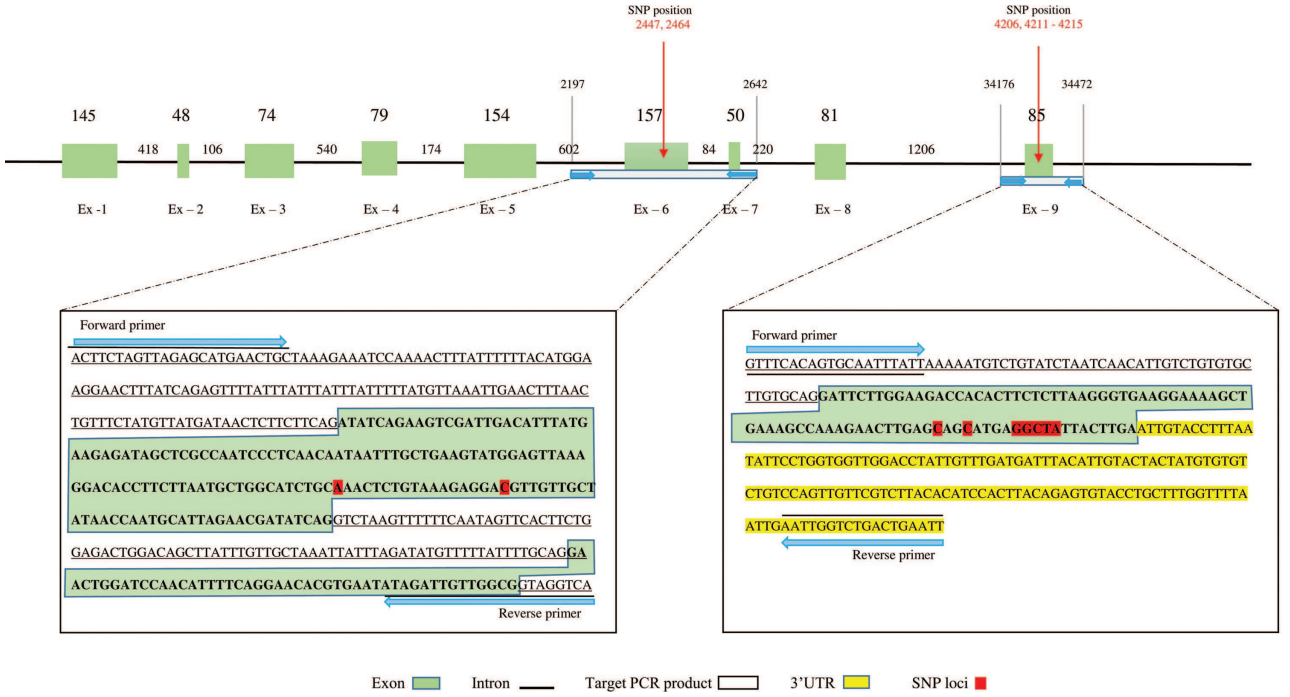

Fig. 1. The structure of GmSNAP18 protein sequence of PI88788 and primers to amplify exon 6 and exon 9

3' and reverse primer, 5'- AATTCAGTCAGACCAATT was used for amplification of DNA (Fig. 1).

The primers were validated by PCR, and the products were viewed in a gel, which revealed that they were of sufficient quality for sequencing. The PCR product size of exon 6 was 445bp and of exon 9 was 292 bp (Fig. 2). The FASTA file of all the sequenced

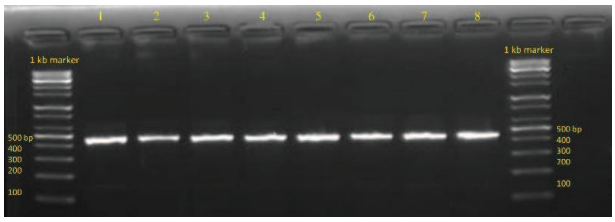

Fig. 2. Bands observed on the 2% agarose gel-electrophoresis of PCR products of exon 6. The samples of 1~8 are: KB000001, KB000002, KB000004, KB000005, KB000007, KB000008, KB000057 and KB000058

samples was received from the NICEM, and the quality of all the samples was checked with Chromas software.

Comparison of soybean SNAP genes

We studied the family of SNAP genes in soybean, which comprises five members, i.e., GmSNAP18, GmSNAP11, GmSNAP14, GmSNAP02, and GmSNAP09 (Table 2). Among these genes, GmSNAP18 is considered a potent gene for resistance to the soybean cyst nematode (Lakhssassi et al. 2017). We downloaded all five genes and studied their location, length, mRNA size and coding sequence (CDS), which allowed us to study the genes individually.

The function of all the genes in conferring resistance to the SCN was studied. A phylogenetic tree for the SNAP family was constructed which revealed that GmSNAP09 did not cluster with the remaining members, forming single cluster (Fig. 3). GmSNAP18 is most closely related to ancestral SNAPs, gave rise to GmSNAP11 and GmSNAP14 (Lakhssassi et al. 2017) and is considered superior to others.

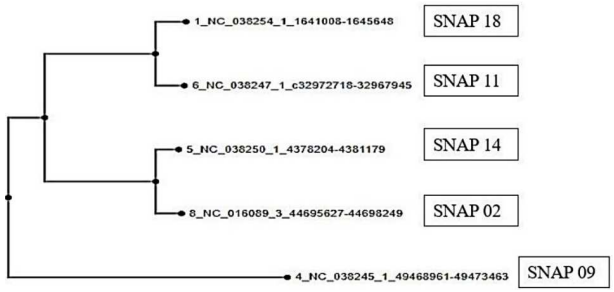

Fig. 3. Phylogenetic tree of SNAP gene family members of soybean. The bootstrap value is n=100

Table 2. Gene family of soluble NSF attachment protein concern to resist soybean cyst nematode in soybean.

Gene Name	Chr	Location	Gene length	mRNA ID	mRNA size	CDS length
SNAP18	18	1641008-1645648	4641 bp	NM_001255130	1290 bp	870 nt
SNAP 11	11	32967945-32972718	4774 bp	NM_001357417	1312 bp	870 nt
SNAP14	14	4378204-4381179	2976 bp	XM_006595549	1172 bp	741 nt
				NM_001255626	1244 bp	870 nt
SNAP02	2	44695627-44698249	2623 bp	XM_003519364	1239 bp	870 nt
SANP09	9	49468961-49473463	4503 bp	XM_014762465	1409 bp	882 nt
				NM_001357420	1305 bp	873 nt

SNP identification

The PI88788 cultivar was used as a reference to find the target position. The GmSNAP18 gene of PI88788 and Peking was downloaded from the NCBI. Based on Liu et al. (2017) and the position of a SNP that enables the protein-coding gene to confer nematode resistance was studied. The nucleotide bases that differed among Peking, PI88788 and Essex and their respective positions were also determined. The target site of exon 6 and exon 9 of the GmSNAP18 gene was successfully sequenced to characterize SNP haplotypes. The sequence of GmSNP18 located in the *rhg1* locus of chromosome 18 was identified (based on the published sequences from the NCBI). Two SNPs were found at positions 2447 and 2464 in exon 6. Six SNP positions were found in exon 9 with some deletions, the positions of which were 4203, 4206, 4211, 4212, 4213 and 4215 (Fig. 4). To evaluate SNPs that correlate with those in known cultivars, multiple alignment was performed using MAFFT 7 (https://mafft.cbrc.jp/alignment/server/).

Haplotype analysis and classification

Haplotypes were examined with multiple alignment on the BOXSHADE website (https://embnet.vital-it.ch/software/BOX_form.html). The SNPs found in exon 6 and exon 9 were combined to form haplotypes. The SNPs found in exons 6 and 9 were also studied in a sample cultivar. The haplotypes were identified in three known cultivars, namely, Peking, PI88788 and Essex. Peking and PI88788 carry resistant haplotypes while Essex carries a susceptible haplotype. In present analysis, the samples that carried the resistant haplotype were categorized as resistant, and those with the susceptible haplotype were classified as susceptible. According to the SNP haplotypes found in the target position in exons 6 and 9, the samples were also classified as resistant and susceptible. The focus was on other SNP positions to identify new haplotypes.

In present study haplotypes based on exon 6 and exon 9 of the GmSNAP18 gene were discovered in a total of 60 soybean lines. The haplotypes were classified based on the resistant and susceptible haplotypes reported by Liu et al. (2017). The present study identified 2 resistant haplotypes and one susceptible haplotype (Table 3). Therefore, all the samples were checked for haplotypes for resistance and suscepitibility. Among the 60 soybean lines, 9 matched the PI88788 haplotype of ACCCGGCA and 3 matched the Peking haplotype of CGGTGGTA at positions 2447, 2464, 2403, 4206, 4211, 4212, 4213, and 4215, respectively. Additionally, 45 genotypes matched the susceptible haplotype (Essex type) of CCGG - - - C at positions 2447, 2464, 2403, 4206, 4211, 4212, 4213, and 4215 of GmSNP18, respectively. Interestingly, we discovered 2 new haplotypes consisting of CCCCGGCA (2 genotypes) and ACGG - - - C (1 genotype), which were new combinations of exon 6 and exon 9 of the GmSNAP18 gene. Each new haplotype is sharing one common exon 6 of PI88788 and Essex (Fig. 4). From the

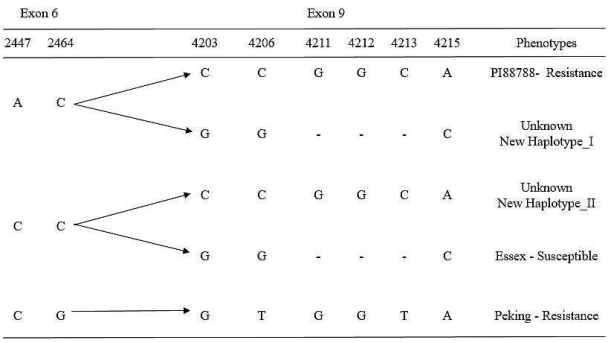

Fig. 4. The structure of SNP haplotypes with combination of exon 6 and exon 9

Table 3. The SNP haplotypes based on the exon 6 and exon 9 of GmSNAP18 in the 60 sample

Phenotypes	Representative haplotype	SNP Haplotypes		No. of samples
		SNP loci in Exon 6	SNP loci in Exon 9	
		2447, 2464	4203, 4206, 4211, 4212, 4213, 4215	
Resistant	PI88788	AC	CCGGCA	9
Resistant	Peking	CG	GTGGTA	3
Susceptible	Essex	CC	GG - - - C	45
Unknown	New haplotype 1	CC	CCGGCA	2
Unknown	New haplotype 2	AC	GG - - - C	1

Given known haplotype is referred by Liu S et al. 2017.

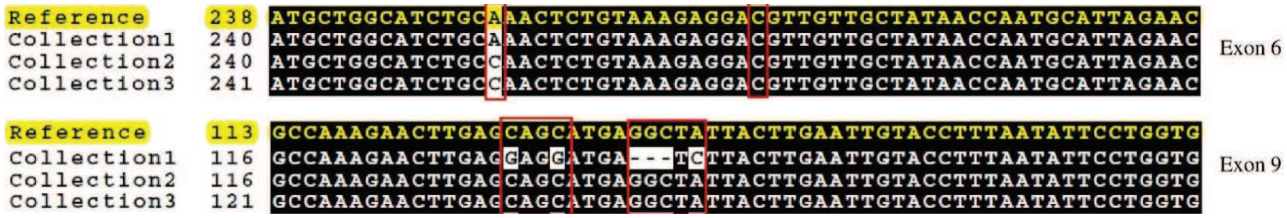

Fig. 5. Multiple alignment structure of new haplotypes with reference within exon 6 and 9. Alignments are generated using MAFT7 software

sequence alignment of three samples of two new haplotypes with reference using MAFT7 software also provides an evidence for confirmation of new haplotypes with the combination of exon 6 and 9 (Fig. 5).

The seed weight and seed coat colour among the 5 haplotypes was examined. There was no correlation between seed weight and haplotype. However, the proportion of resistant lines differed based on the seed coat colour. Specifically, 20% of the lines with a black seed coat colour had a resistant haplotype, and 70% of those with a yellow seed coat colour had a susceptible haplotype. The two new haplotypes occurred in the genotypes with a black seed coat colour and one genotype with a yellow seed coat colour (Table 4). We speculate that one of the cultivars may be resistant. Additionally, three cultivars had new SNPs in the target position that did not match any of those in the other cultivars. We checked the sequence quality and amino acids coded, which were different from those of the other examined cultivars.

Table 4. The statistics summary of seed weight and seed coat color based on the resistant and susceptible haplotypes

Phenotypes	SNP haplotypes		Av. seed weight	No. of Samples (seed coat color %)				Total
	SNP loci in Exon 6	SNP loci in Exon 9						
	2447, 2464	4203, 4206, 4211, 4212, 4213, 4215	(g/100 seeds)	Black (%)	Yellow (%)	Brown (%)	Mix (%0)	
Resistant	AC	CCGGCA	15.9	4 (16%)	5 (19%)	9		
Resistant	CG	GTGGTA	11.83	1 (4%)	2 (7%)			3
Susceptible	CC	GG - - - C	19.32	18 (72%)	19 (70%)	2 (100%)	6 (100%)	45
Unknown	CC	CCGGCA	13.84	2 (8%)				2
Unknown	AC	GG - - - C	25.6		1 (4%)			1

Discussion

The results of present study revealed two new haplotypes in three samples. These groups of alleles may be inherited and show a new genetic makeup. Three haplotypes were previously reported by Liu et al. (2107), and the present findings add two new haplotypes. The sequence quality from the NICEM, genotyping data and haplotype analysis showed good results, which helped confirm the findings of our research. Studying the phenotype of the new haplotypes will be an objective of our next research which may be expected to reveal important new findings. In this study, the Korean cultivars were distinguished under resistant and susceptible category. We isolated the GmSNAP18 gene from the respective plant samples was isolated which is considered as *rhg1* region. This region is the most important that plays a vital role to provide the resistance in plants against the cyst nematode.

The region of *rhg1,* which consists of three genes, is responsible for SCN resistance (Liu et al., 2017). The position of the SNP in the new haplotype was determined. The haplotype sequences found in exons 6 and 9 are responsible for the translation of a protein that confers resistance. Peking-type resistance requires both the *rhg1* and *Rhg4* alleles, whereas PI88788 requires only *rhg1* for SCN resistance (Concibido et al. 2004; Meksem et al. 2001). Here, we sequenced alleles of only *rhg1*, which is also called GmSNAP18. As Peking requires both alleles (*rhg1* and *Rhg4*), the present study sequenced and studied the *rhg1* and it was expected that both the alleles are present. The genotype, PI88788 requires only *rhg1*, so we classified the sample cultivars according to *Rhg1* haplotype. After the completion of the life cycle of the SCN, cyst remain as a cluster of eggs in the cyst wall for up to 9 years (Inagake and Tsutsumi 1971). GmSNAP18 is the most important candidate gene that confers resistance to the SCN. For resistance to all SCN races, the *rhg1* locus on chromosome 18 is needed (Kazi et al. 2010). There seems quick response for resistance to SCN in Peking and slow response in PI88788, ultimately resulting in disintegration of syncytium (Kim et al. 2012). Among the five members of the SNAP gene family, GmSNAP18 is the major contributor to SCN resistance in Peking, PI88788 and Essex. According to Lakhssassi et al. (2017) GmSNAP11 also plays a minor role in Peking-type SCN resistance. In breeding programs PCR-based molecular markers are one of the best method for the selection of desired alleles (Kumar 1999). Identification of new haplotypes has been done in soybean related to salt tolerance. Forty different haplotypes with three known haplotypes were identified in soybean conferring salt tolerance (Lee et al. 2018). Ibba et al. (2018) used PCR based haplotype specific molecular marker to identify and differentiate low molecular weight glutenin subunits genes present in wheat variety. Development of PCR based marker specific for the haplotypes are efficient and easy methods for allele discrimination. This study of haplotypes of SNPs found in the target region helps differentiate resistant and susceptible cultivars. Additionally, this research will be helpful in identifying new haplotypes and will aid in molecular genetic breeding to develop SCN-resistant cultivars.

Authors' contribution

Conceptualization of research (IYC); Designing of the experiments (IYC); Contribution of experimental materials (PB); Execution of field/lab experiments and data collection (PB); Analysis of data and interpretation (HY, NSR, RR, KCP); Preparation of manuscript (PB).

Declaration

The authors declare no conflict of interest.

Acknowledgement

This work was supported by the National Research Foundation of Korea (NRF) grant (No. 2017R1AB4011198) and Rural Development Administration (RDA) grant (No. PJ015326022020) funded by the Korea government.

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