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Genomic-assisted phylogenetic analysis and marker development for next generation soybean cyst nematode resistance breeding

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ABSTRACT

Soybean cyst nematode (SCN, *Heterodera glycines* Ichinohe) is a serious soybean pest. The use of resistant cultivars is an effective approach for preventing yield loss. In this study, 19,652 publicly available soybean accessions that were previously genotyped with the SoySNP50K iSelect BeadChip were used to evaluate the phylogenetic diversity of SCN resistance genes *Rhg1* and *Rhg4* in an attempt to identify novel sources of resistance. The sequence information of soybean lines was utilized to develop KASPar (KBioscience Competitive Allele-Specific PCR) assays from single nucleotide polymorphisms (SNPs) of *Rhg1*, *Rhg4*, and other novel quantitative trait loci (QTL). These markers were used to genotype a diverse set of 95 soybean germplasm lines and three recombinant inbred line (RIL) populations. SNP markers from the *Rhg1* gene were able to differentiate copy number variation (CNV), such as resistant-high copy (PI 88788-type), low copy (Peking-type), and susceptible-single copy (Williams 82) numbers. Similarly, markers for the *Rhg4* gene were able to detect Peking-type (resistance) genotypes. The phylogenetic information of SCN resistance loci from a large set of soybean accessions and the gene/QTL specific markers that were developed in this study will accelerate SCN resistance breeding programs.

1. Introduction

Soybean cyst nematode (SCN, *Heterodera glycines* Ichinohe) is the most economically important soybean [*Glycine max* (L.) Merr.] pathogen in the United States because it causes more yield loss than any other disease [1]. Management of this pest is limited to crop rotation and the use of resistant cultivars. Soybean resistance against SCN is complex because of the involvement of more than one gene and the structural changes resulting from copy number variation (CNV). To date, two major genes, *Rhg1* and *Rhg4*, and many quantitative trait loci (QTL) harboring minor genes have been identified for SCN resistance [2–7]. Three copy number classes of the *Rhg1* gene have been observed in soybean; these are generally categorized as the Plant Introduction (PI) 88788-type, carrying the highest number of copies (>6 copies); Peking-type (2 to 4 copies); and Williams 82-type (single copy) [8]. The highest magnitude of resistance has been observed within PI 88788, which has approximately nine copies [2]. In the case of *Rhg4*, the highest level of resistance is observed for the Peking-type allele because of non-synonymous variation [3]. These major genes are valuable sources of resistance and have been widely employed for the development of soybean cultivars. However, major gene resistance is not often durable, and there is an immense need for the effective utilization of more horizontal (quantitative) resistance that is derived from minor genes or QTL. Recently, Vuong et al. [4] identified a novel QTL on chromosome (Chr.) 10 (qSCN10) from an exotic accession, PI 567516C [9]. Interestingly, PI 567516C is SCN resistant and lacks the two major genes, *Rhg1* and *Rhg4* [4]. There are several other minor QTL that have been reported for SCN resistance. Among these, QTL on Chr. 11 (qSCN11) has been consistently identified from PI 437654, PI 90763, and PI 404198B [5,10].

A total of 19,652 cultivated soybean (*Glycine max*) and wild soybean (*Glycine soja* Sieb. and Zucc) accessions have been characterized using the SoySNP50K iSelect BeadChip [11]. These valuable

Abbreviations: SCN, Soybean cyst nematode; QTL, quantitative trait loci; CNV, copy number variation; SNP, single nucleotide polymorphism.

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resources can be used to find existing genetic variations in soybean germplasm. Lee et al. [12] analyzed the soybean population using SoySNP50K for the 1.5-Mb region that is centered on *Rhg1*. The identification of molecular markers that are associated with disease resistance would be helpful for developing resistant varieties because phenotyping soybean for SCN resistance is time-consuming and costly. Among the molecular markers, single nucleotide polymorphisms (SNPs) present several advantages over other genetic marker types [13]. At present, only a few simple sequence repeat (SSR) markers have been reported for the identification of the *Rhg1* gene. For instance, the marker Satt309 has been widely employed in several soybean breeding programs [14,15]. However, it has limited use because the Satt309 marker is able to detect only three copies of the *Rhg1* gene. SSR markers are not practical for a high level of multiplexing. High-throughput genotyping and the development of multiplex marker panels are more feasible with SNP markers. Some studies have reported SNP markers for the *Rhg1* and *Rhg4* genes [16–18]. Several cost effective platforms are available for SNP identification and subsequent genotyping [19,20]. Recently, next-generation KASPar assays (KBiosience, Hoddesdon, UK) have become the new SNP genotyping method because they have high-throughput, low error rates, and are cost-effective [21].

Cook et al. [2] showed that the soybean cultivar Fayette, that was developed from the *Rhg1* resistant source PI 88788 has ten copies of the *Rhg1* gene compared to nine copies in PI 88788. Recent medical science reports highlighted how CNV can be enumerated using digital polymerase chain reaction (dPCR) [22,23]. The determination of CNV with dPCR is costly and time-consuming and is not suitable for plant breeding applications; however, it is feasible to identify a haplotype that represents a particular CNV that can then be utilized for marker development. Such an assay will help breeders to select for high copy numbers of the *Rhg1* gene.

In this study, over 19,000 soybean accessions from the USDA Germplasm Collection were utilized for phylogenetic analysis of the major SCN resistant loci (*Rhg1* and *Rhg4*) using the SoySNP50K molecular marker data. The development of a panel of breeder-friendly genetic markers representing the major genes (*Rhg1* and *Rhg4*) and QTL (qSCN10 and qSCN11) is also reported. The phylogenetic information of SCN resistance loci in a large set of soybean accessions and gene/QTL specific marker resources that were developed in this study will be helpful in accelerating SCN resistance breeding in soybean.

2. Materials and methods

2.1. Phylogenetic tree of soybean accessions

A complete data set of 19,652 *G. max* and *G. soja* accessions that were genotyped with 52,041 SNPs was downloaded from the Soybase website [11, www.soybase.org]. The SNP information from the 0.5-Mb region flanking the *Rhg1* and *Rhg4* loci was selected to analyze phylogenetic diversity. The 0.5-Mb region flanking the genes was selected for three reasons: (1) to ensure a sufficient number of SNPs for phylogenetic analysis because there is no SNP present in the 50K data set located in the *Rhg4* or *Rhg1* genes; (2) Sonah et al. [24] and Lam et al. [25] suggested that there is up to a 1 Mb linkage disequilibrium decay in soybean; and (3) as reported in many previous studies that in the evolution major genes always carry the linked flanking region. To develop the phylogenetic tree, a single representative line from each haplotype was considered. The maximum likelihood (ML) model implemented in the MEGA6 (MEGA Inc., Englewood, NJ) program was used to construct the phylogenetic tree [26].

2.2. Plant materials

Several recombinant inbred line (RIL) populations, parental lines, and diverse PI lines, including known sources of SCN resistance, were used to identify and validate genetic markers. A subset of 95 diverse germplasm lines (64 soybean PIs, eight cultivars, and 23 elite breeding lines) and three RIL populations (Pana x PI 567690, Essex x PI 567690, Essex x PI 437654, and Magellan x PI 567305) were used for marker validation. Among the RIL populations, a first subset of 92 RILs that were derived from a Pana x PI 567690 cross, was used to validate markers linked to the *Rhg1* gene. A second subset of 44 RILs, from a Essex x PI 437654 cross, was used to test *Rhg1*, *Rhg4* and the minor QTL qSCN11 [5]. A third subset of 92 RILs, from a Magellan x PI 567305 cross, was used to validate markers linked to the novel QTL qSCN10.

2.3. Phenotyping

A subset of 95 soybean lines and three different RIL populations (Pana x PI 567690, Essex x PI 567690, Essex x PI 437654, and Magellan x PI 567305) were evaluated for SCN resistance following a well-established greenhouse bioassay [27,28] at the University of Missouri, Columbia, Missouri. Briefly, five plants from each test line, indicator lines for HG Type test (PI 548402, PI 88788, PI 90763, PI 437654, PI 209332, PI 89772, PI 548316), and susceptible checks (cv. Lee 74 and cv. Hutcheson) [29] were arranged in a randomized complete block design. Two days after transplantation, seedlings were inoculated with 2000 ± 25 eggs from near-homogenous SCN isolates (HG Types 2.5.7 (PA1), 1.2.5.7 (PA2), 0 (PA3), 2.5.7 (PA5), or 1.3.6.7 (PA14)). The experiments were maintained at $27 \pm 1\,^{\circ}C$ and were watered daily. Thirty days post-inoculation, nematode cysts were washed from the roots of each plant and were counted using a fluorescence-based imaging system [30]. The Female Index (FI) was calculated to evaluate the response of each line to SCN using the following formula: FI = (average number of female cyst nematodes on a test soybean line/average number of female nematodes on the susceptible check) $\times$ 100.

2.4. SNP identification and development of KASPar genotyping assays

For developing a KASPar assay, sequence information of *Rhg1*, *Rhg4*, the novel QTL qSCN10, and the minor QTL qSCN11 was retrieved from the available sources for diverse soybean lines [8,31]. The SNPs identified at these loci using GATK (Genome Analysis Toolkit, www.broadinstitute.org) software [32] were reconfirmed by examining read alignments in the Integrative Genomics Viewer (IGV) tool [33]. Four SNPs of *Rhg1* and three SNPs of *Rhg4* were selected. Similarly, four SNPs were selected from each of the qSCN10 and qSCN11 QTL regions. The selected SNP set was targeted for the development of KASPar assays (Table 1). Two allele-specific forward primers, along with tail sequences and one common reverse primer, were synthesized for SNP genotyping assays. The reaction mixture was prepared according to the protocol described by KBiosciences (Herts, UK) (http://www.ksre.ksu.edu/igenomics). The following cycling conditions were used: 15 min at 95 °C, followed by 10 touchdown cycles of 20 s at 94 °C, 1 min at 65–57 °C (dropping 0.8 °C per cycle) and then 23 cycles of 20 s at 94 °C, 1 min at 57 °C. The fluorescent end-point genotyping method was carried out using a Roche LightCycler (LC) 480 instrument (Roche Applied Sciences, Indianapolis, IN, USA).

2.5. Development of a Taqman assay and CNV determination

The conserved sequences of the *Rhg1* gene were selected to develop a Taqman assay to run on dPCR (Table 2). The FAM™ dye-

Table 1
Primer sequences of the KASPar assays of the SNP markers linked to *Rhg1*, *Rhg4*, novel QTL on Chr. 10, and minor QTL on Chr. 11 for SCN resistance.

Marker name	Chr. no.	Position (bp)	Allele specific 1	Allele specific 2	Common primer
Rhg1-2	18	1643225	TCTAATGCATTGGTTATAGCAACAACG	TCTAATGCATTGGTTATAGCAACAACC	TGCTGGCATCTGCCAACTCTGTAAA
Rhg1-5	18	1644968	GAAAGCCAAAGAACTTGAGGAGC	GAAAGCCAAAGAACTTGAGGAGG	CCAACCACCAGGAATATTAAAGGTACAAT
Rhg4-3	8	8357600	TCGTTGTGTGATTGTTTTGCAGGGA	TCGTTGTGTGATTGTTTTGCAGGGT	CAGAGATCACAGAGTTTCTCCACCTT
Rhg4-5	8	8356824	GAGGTGGCCGCCGGAGG	GAGGTGGCCGCCGGAGC	CGACCGCATCATGGGGCTAGAT
O-8	10	42245205	CCAAAATCAGCAGGGAATAGCTTG	CCCAAAATCAGCAGGGAATAGCTTA	GAAAAATGTCAGCAATACTCTCTCTCTCTT
B1-7	11	36999475	GGCGGAGGCAGTGGCGG	AGGCGGAGGCAGTGGCGT	TTGCACCCAATGTGGTCATGGTCAT

based assay was designed to detect the gene of interest, and the VIC® dye-based assay was used for the reference gene, lectin. Both target and reference assays were run in duplex. Genomic DNA was extracted using the CTAB method [34], and 2.5 ng of DNA was added to the dPCR reaction mix. Each dPCR chip was loaded with 14 μl of dPCR reaction mixture and then sealed. The dPCR chips were loaded into a GeneAmp® PCR System 9700 with the following conditions 96 °C for 10 min, 60 °C for 2 min, and 98 °C for 30 s for 39 cycles, then 60 °C for 2 min and then held at 10 °C. The chips were read on a QuantStudio™ 3D dPCR System (Life Technologies, Carlsbad, CA), and the data was processed with the QuantStudio™ 3D Analysis Suite™, which is available at https://apps.lifetechnologies.com/quantstudio3d/. The same Taqman assay was run on the ABI 7900HT qRT-PCR (Applied Biosystems, Foster City, CA) instrument to validate the *Rhg1* gene. The reaction mixture containing a 2X Taqman environment master mix, 10X target gene (FAM) and reference gene (VIC) primer mix, and 5 ng of genomic DNA was run on the ABI 7900HT qRT-PCR system. The program was set to hold at 50 °C for 2 min, hold at 95 °C for 15 s, and then to 95 °C for 15 s for 40 cycles, and 60 °C for 1 min.

2.6. Statistical analysis

The segregation ratios of SNPs selected for the *Rhg1*, *Rhg4*, novel QTL, and minor QTL regions for SCN resistance were tested in three RIL populations based on resistant and susceptible reactions to SCN for goodness-of-fit with Chi-squared tests.

3. Results

3.1. Phylogenetic analysis

The flanking regions (0.5 Mb both sides) of the *Rhg1* and *Rhg4* genes, that were retrieved from the SoySNP50K information for 19,652 soybean accessions possessed 104 and 64 SNPs, respectively. Haplotype grouping (based on 100% similarity) revealed 5451 and 4654 groups of lines for the *Rhg1* and *Rhg4* loci, respectively (Supplements S1 and S2). Phylogenetic analysis of these PIs, including known SCN resistant lines, showed high and low copies of the *Rhg1* gene in separate clusters (Fig. 1, Supplements S3 and S4). Indicator lines for the HG type test clustered separately with PI 88788, PI 209332, and PI 548316 in the group with high copy numbers of the *Rhg1* gene and with PI 548402, PI 90763, PI 89772, and PI 437654 in the group with low copy numbers of the *Rhg1* gene (Fig. 1).

Table 2
Taqman primer/probe sequences for the Digital PCR and ABI 7900HT instruments to detect the copy number variation (CNV) of the *Rhg1* gene.

Gene name	Primer sequences	Probe sequence
Rhg1	Fwd: GTTATTACTTCAATCGAC-GAGTGTGTTG Rev: AAATATTTTCCAGTAAAATCA-GATTAAAACTATACTTCA	FAM: TCGGACACCTCAAAACT

The lines that were known to be resistant with the *Rhg4* gene were clustered together, except for PI 438489B (Fig. 2). PI 437654 was sub-clustered from PI 548402 (Peking) along with PI 404198B, PI 303602, and PI 468915, predicting that it may have different alleles from cv. Peking. As expected, the non-*Rhg4* resistant lines formed separate clusters in the phylogenetic tree (Fig. 2).

3.2. Informative SNP markers associated with SCN resistance genes/QTL

The available genome sequence information was used to identify SNPs from the selected genes and QTL regions [8,31]. The SNP identified in this study matched with the previously known SNP information of the *Rhg1* gene using the whole genome sequence information of six SCN resistant lines: Peking (PI 548402), PI 90763, PI 437654, PI 209332, PI 89772, and Cloud (PI 548316) [8]. Among the four SNPs selected from the *Rhg1* gene (*Glyma18g02590*) to develop KASPar assays, two markers, Rhg1-2 and Rhg1-5 (Table 1), were successfully validated in known indicator lines for SCN resistance. The other two assays, Rhg1-1 and Rhg1-3, were not able to differentiate genotypes between resistance and susceptible cultivars.

In the case of the *Rhg4* gene (*Glyma08g11490*), three KASPar assays were designed using sequencing information from soybean lines [8]. Two assays, named Rhg4-3 and Rhg4-5, were able to differentiate the *Rhg4* resistant and susceptible alleles; however, the third assay, Rhg4-1, failed to differentiate the genotype clusters.

SNPs were selected from the QTL region sequence of resistance genotypes PI 567516C [4] and PI 567305 (Nguyen lab, unpublished data), which are known to carry the novel QTL qSCN10. Four SNPs from the candidate genes, *Glyma10g34061,* present at the QTL qSCN10 were selected to develop the KASPar assays (Supplement S5). Three assays, named O-6, O-7, and O-8, were able to differentiate genotypes in separate clusters as expected (Fig. 3E), but the fourth assay, O-5, was unable to develop the separate genotype clusters on the Roche LC 480 platform. Furthermore, the SNP assay O-8 was validated in the germplasm set of 95 lines and a subset of RILs (Supplement S6 and S9).

A similar approach was used to select and develop SNP genotyping assays for QTL qSCN11. The SNP information was retrieved from the sequencing information for SCN resistant accessions, PI 437654, PI 90763, and PI 404198B, which have been reported to carry qSCN11 [5,6]. Four SNPs were selected for the development of KASPar assays. Among these, three markers were able to differentiate genotypes; these markers clustered separately on the Roche LC 480 system (Fig. 3F). The KASPar assay, B1-7, developed from the SNP present in the *Glyma11g35320* gene, was validated in the germplasm set of 95 lines and a second subset of RILs (Table 1 and Supplement S5).

3.3. Allelic discrimination of SCN resistant genes/QTL using SNP assay

Two SNP markers specific to the low and high copy numbers of the *Rhg1* gene were validated. The first marker, Rhg1-2,

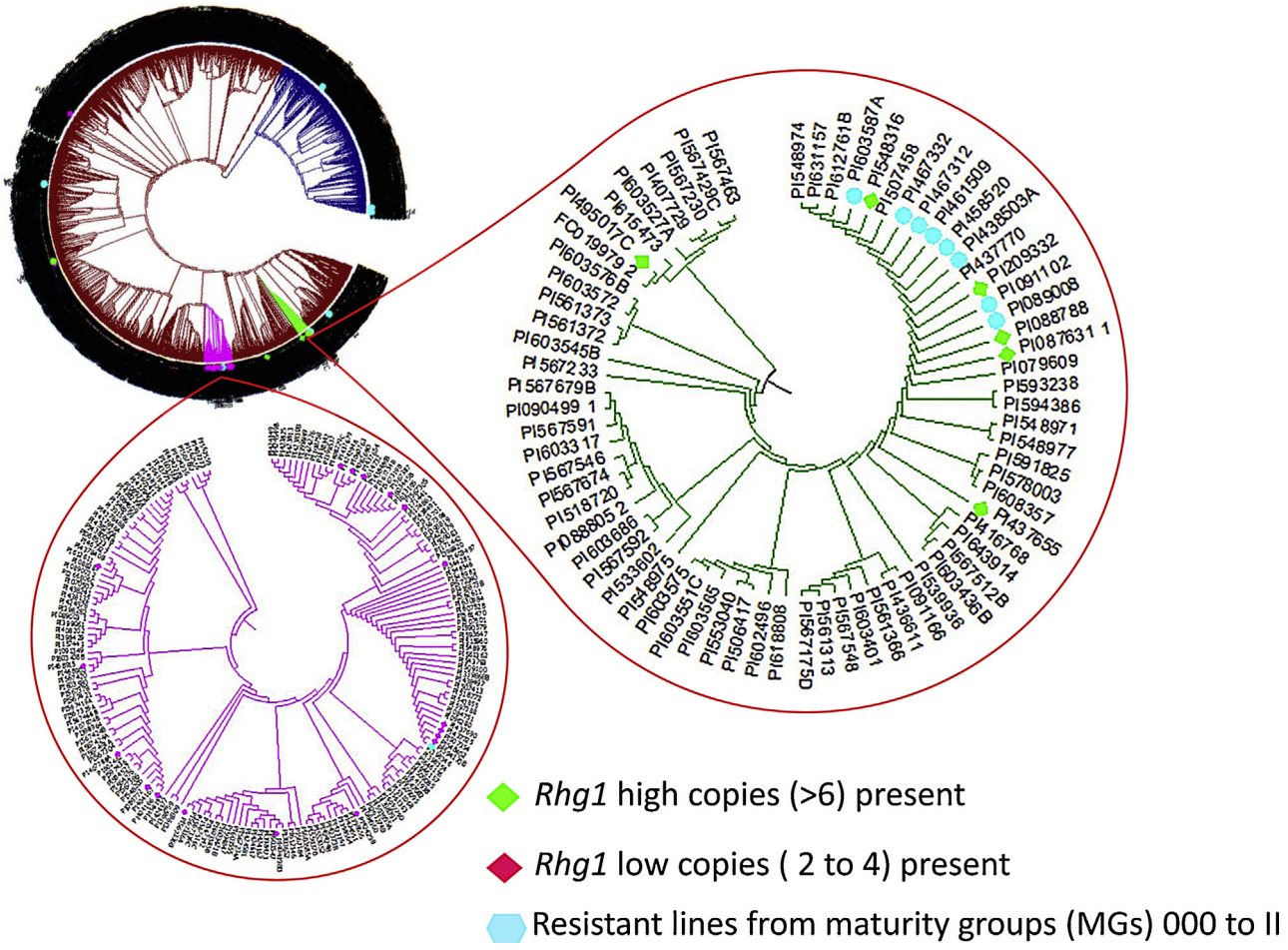

Fig. 1. Phylogenetic tree of the *Rhg1* locus constructed on the basis of 5451 haplotypes using 19,652 accessions and the SoySNP50K. Green diamond shaped bullets showthe high copies of the *Rhg1* allele present in the known soybean lines from maturity groups III to V; pink diamond shaped bullets show the low copies of the *Rhg1* allele present in the known soybean lines from maturity groups III to V; and light blue hexagon shaped bullets showing the resistant lines (on the basis of phenotyping in our lab) from maturity groups 000 to II. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

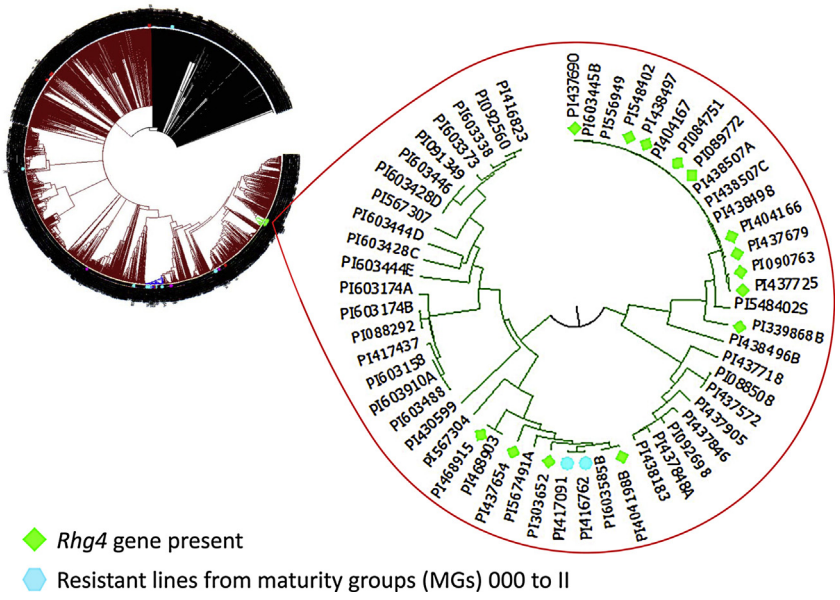

Fig. 2. Phylogenetic tree of the *Rhg4* locus constructed on the basis of 4654 haplotypes using 19,652 accessions and the SoySNP50K. Green diamond shaped bullets showing the *Rhg4* gene present in the known soybean lines from maturity groups III to V; and light blue hexagon shaped bullets showing the resistant lines (on the basis of phenotyping in our lab) from maturity groups 000 to II. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

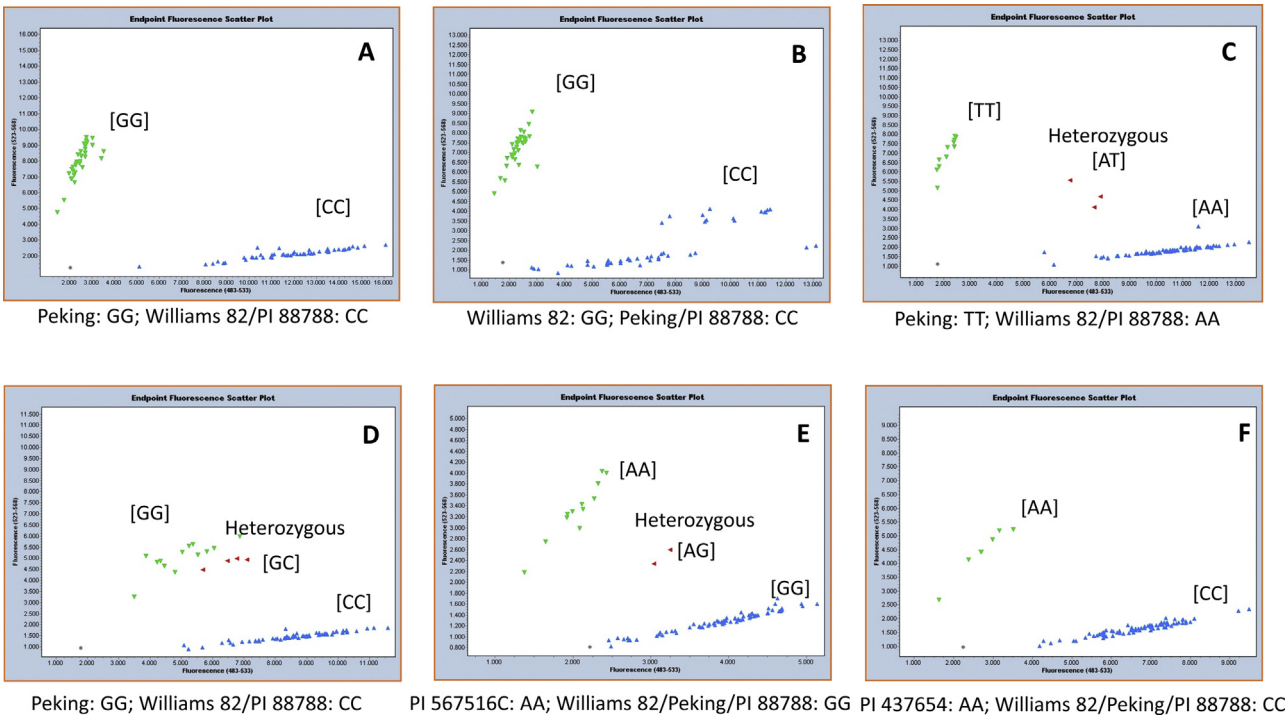

Fig. 3. Endpoint fluorescence scatter plot of the KASPar assays: (A) Rhg1-2; (B) Rhg1-5; (C) Rhg4-3; (D) Rhg4-5; (E) O-8; and (F) B1-7 tested in a set of 95 soybean germplasm lines. Allele specific primer 1 was reported by FAM (blue), allele-specific primer 2 was reported by HEX (green), heterozygous lines appeared as red, and black data points are the no template controls. *X* axis- florescence of FAM at 523 nm to 568 nm and *Y* axis-fluorescence of HEX at 483 nm to 533 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

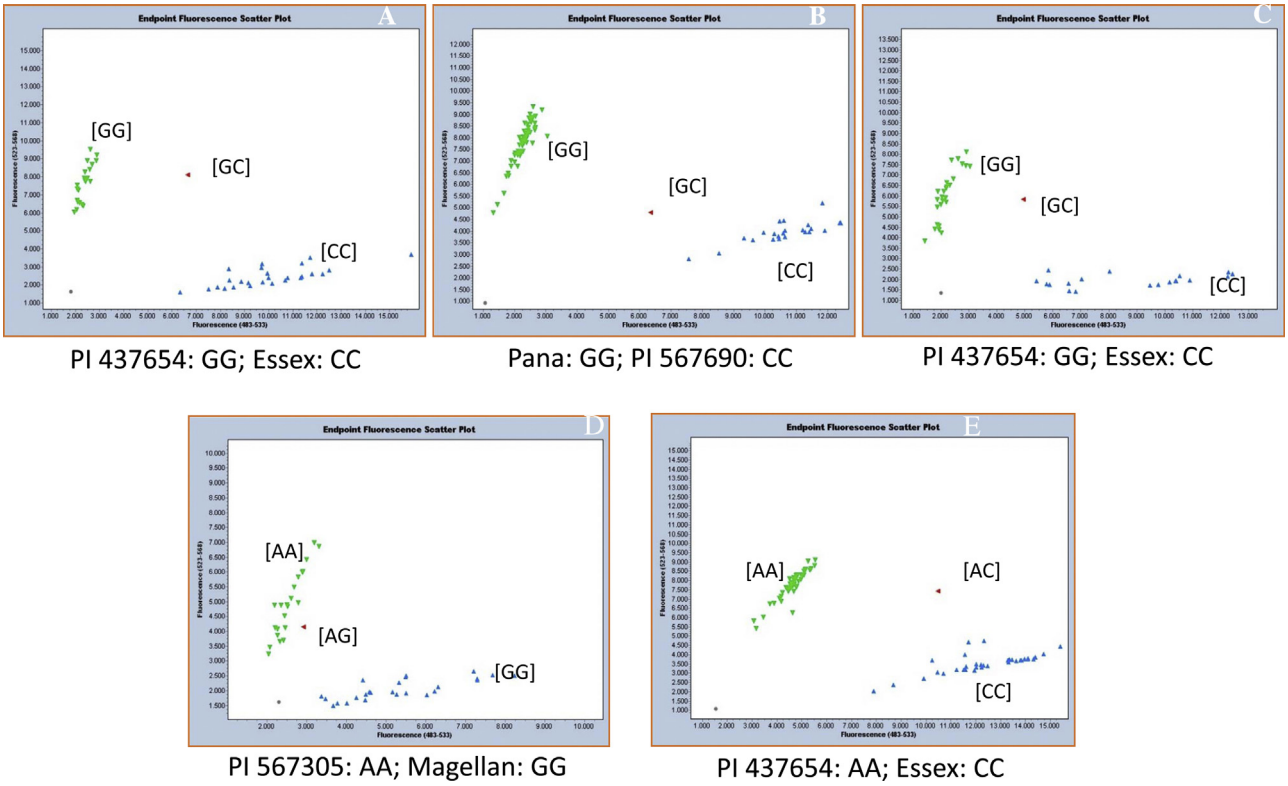

Fig. 4. Endpoint fluorescence scatter plot of the KASPar assays: (A) Rhg1-2 marker on a subset of 44 RILs from the Essex x PI 437654 population; (B) Rhg1-5 marker on a subset of 94 RILs with parents from the Pana x PI 567,690 population; (C) Rhg4-5 marker on a subset of 46 RILs with parents from the Essex x PI 437,654 population; (D) O-8 marker on a subset of 94 RILs with parents from the Magellan x PI 567,305 population; (E) B1-7 marker on a subset of 44 RILs from the Essex x PI 437,654 population. KASPar data points are color-coded based on cluster membership: Allele specific primer 1 was reported by FAM (blue), allele-specific primer 2 was reported by HEX (green), and heterozygous lines appeared as red, and black data points are no template controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was able to distinguish the separate genotypes of allele G and C, which represents the low copy and high copy along with a single copy of *Rhg1* (Fig. 3A and Supplement S6). The genotyping data that was generated using the Rhg1-2 marker supported the CNV that was reported in 13 soybean lines out of the set of 95 germplasm lines (Supplement S6) [7,8]. A significant association was observed between the Rhg1-2 SNP allele and the reaction to SCN that was recorded for the set of 95 soybean germplasm lines, except for PI 567230, with a success rate of 95% (Fig. 3A and Supplement S6). PI 567230 is susceptible to all races, even though it has allele G (Peking-type), as determined using the Rhg1-2 marker (Supplement S6). Subsequently, a second subset of the Essex x PI 437, PI 437654 population was used to validate the Rhg1-2 marker (Fig. 4A). Chi-square analysis indicated that marker segregation followed a single-gene inheritance pattern and did not significantly deviate from an expected 1:1 (resistant:susceptible) ratio (Table 3). Genotype vs. phenotype was completely matched for the Rhg1-2 allele and the subset of RILs (Supplement S7).

The second marker, Rhg1-5, was utilized to determine soybean lines with high-copy numbers of the *Rhg1* gene. The KASPar assay of the Rhg1-5 marker successfully differentiated genotypes of allele G for the single copy gene from allele C, which represents low and high copy numbers of the *Rhg1* genotypes (Fig. 3B). The Rhg1-5 marker was used to select the *Rhg1* resistance allele, and the Rhg1-2 marker was employed to differentiate Peking and PI 88788-type resistance. A significant association was observed for the Rhg1-5 marker and phenotypes for SCN resistance, except for PI 603154. Interestingly, PI 603154 was susceptible to SCN even though it has an allele C of the *Rhg1* gene (Supplement S6). Moreover, this PI was not grouped with low-copy and high-copy numbers of *Rhg1* in the phylogenetic tree (Supplement S6 and Fig. 1). The validation of the Rhg1-5 allele was performed using the subset of RILs from the Pana x PI 567, 690 population (Fig. 4B). The cultivar Pana has a high copy number of the *Rhg1* gene, like PI 88788 (allele C), and is resistant to SCN [35]. PI 567690 has a single copy of the *Rhg1* gene (allele G) and is susceptible to SCN (Supplement S6). Segregation analysis indicated that this population deviates significantly from a 1:1 (resistant:susceptible) ratio, which was unexpected for a single gene (Table 3). Genotype vs. phenotype completely matched for the Rhg1-5 allele on the first subset of RILs population Pana x PI 567, 690 (Supplement S8). A significant association was observed between the genotype and phenotype in the set of germplasm and cultivars, except for PI 567230 and PI 603154, as explained above, and PI 549031, which has susceptible alleles for both *Rhg1* markers, but is moderately resistant to SCN (Supplement S6). Three other accessions, PI 548317, PI 407729, and PI 548349, showing the dissimilarity of the genotype and phenotype association, were moderately resistant to SCN, but had susceptible alleles of the *Rhg1* gene. The involvement of other genes may be responsible for SCN resistance in these soybean lines.

Two markers were tested to confirm the *Rhg4* gene. The first marker, Rhg4-3, was able to differentiate resistant lines carrying the *Rhg4* (allele T) from the non-*Rhg4* (allele A), except PI 438489B (Fig. 3C and Supplement S6). The second assay, Rhg4-5, was able to detect all of the resistant lines with *Rhg4* (allele G), including PI 438489B (Fig. 3D and Supplement S6). The Rhg4-3 marker showed heterozygous genotypes of A/T in three resistant lines, PI 437654, PI 404198B, and PI084751, but these lines were homozygous in the Rhg4-5 assay (Supplement S6). In the phylogenetic analysis, PI 438489B was grouped separately from *Rhg4* resistant cultivars (Fig. 2). A significant association was observed between the genotypic data that was generated using these markers and their phenotype in a set of soybean germplasm and cultivars (Supplement S6). Further validation of the Rhg4-3 marker was carried out in a second subset of RILs derived from the Essex x PI 437,654 population (Fig. 4C). The Chi-square test of this marker indicated that the populations did not deviate significantly from the 1:1 (resistant:susceptible) ratio, as expected for a single gene (Table 3). The genotype vs. phenotype was perfectly correlated for the Rhg4-3 allele on the second subset of Essex x PI 437,654 RILs (Supplement S7).

The KASPar assay for the O-8 marker from the candidate gene *Glyma10g34061* that is present in QTL qSCN10 was able to separate genotypes for SCN resistant lines with allele A (Fig. 3E and Supplement S5). A total of six PI lines, PI 209332, PI 438503A, PI 548317, PI 548349, PI 567343, and PI 567336B, and the two known lines, PI 567516C and PI567305, were identified with resistant allele A (Supplement S6). This assay showed a significant association with the phenotypic data in a set of germplasms and cultivars. A third subset of RILs derived from the Magellan x PI 567305 population was screened for O-8 marker validation. Additionally, the O-8 marker was able to confirm heterozygous genotypes in this population (Fig. 4D). The test of genotypic segregation indicated that the alleles in this RIL population did not significantly deviate from the 1:1 (resistant:susceptible) ratio as expected for a single gene pattern (Table 3). Genotype vs. phenotype was significantly correlated for the O-8 allele on a subset of the RIL population (matched 87 out of 92 RILs) (Supplement S9).

A minor QTL for SCN resistance, qSCN11, is important for broad-based resistance to SCN HG Type 1.2.5.7 and HG Type 2.5.7 [5]. The KASPar assays were developed for four SNPs in this QTL region. Of these, three assays were able to separate genotypes for qSCN11 carrier lines. The B-7 marker, that was designed from the candidate gene *Glyma11g35320,* was validated in a set of germplasm lines and a third subset of RILs (Figs. 3 F and 4 E). The qSCN11 allele was present in 13 genotypes of the set of 95 germplasm lines (Fig. 3F and Supplement 6), including PI 437654, PI 90763, and PI 404198B; known to carry qSCN11. The resistant allele (A) showed a significant association with the phenotypic data (Supplement S6). A second subset of RILs from the Essex x PI 437,654 population was screened with the B1-7 marker (Fig. 4E and Supplement S7). Segregation analysis of the B1-7 marker indicated that the populations did not significantly deviate from a 1:1 (resistant:susceptible) ratio as expected for a single gene pattern (Table 3).

3.4. Copy number variation using Digital PCR and ABI 7900HT

A Taqman assay was developed from the conserved region of the *Rhg1* gene to run on dPCR (Table 2). Three replications of the known genotypes, PI 88788, PI 548402, PI 437654, and cv. Williams 82, for high, low, and single copy numbers were validated using dPCR amplification as well as on the ABI 7900HT instrument. The aim of this study was to use new platforms for the identification and validation of copy number variation. The results obtained from the assays tested by dPCR and the ABI 7900HT platforms showed similar results for the CNV of the *Rhg1* locus, as identified earlier using genome resequencing (Fig. 5).

4. Discussion

4.1. Effect of phylogenetic diversity on breeding strategies

Similar to other important crop plants, soybean has undergone continuous selection by humans, involving domestication, intensive breeding, and probable founding events [36,37]. These selection activities likely decreased the genetic diversity [38,39], changed the allelic frequencies, and eliminated rare alleles. Cultivated soybean (*G. max*) was domesticated from wild soybean (*G. soja*) in China approximately 5000 years ago [40]; the domestication immediately resulted in *G. max* landraces [41]. This differently affected phylogenetic diversity at loci regulating domestication

Table 3
Genotypic segregation tests of the allele-specific markers linked to SCN resistance genes in different subsets of recombinant inbred lines selected from three mapping populations.

Marker	Population	Seed Gen.	Total	Allele *X*	Allele *Y*	Chi-square	*P* value
Rhg1-5	Pana × PI 567690	F8	92	67	25	19.174	0.0001
Rhg1-2	Essex × PI 437654	F8	44	23	21	0.091	0.763
Rhg4-5	Essex × PI 437654	F8	44	17	27	2.273	0.132
B1-7	Essex × PI 437654	F8	44	26	18	1.455	0.228
O-8	Magellan × PI 567305	F8	92	38	54	2.783	0.095

related traits (e.g., seed size, quality, and yield related traits) and disease resistance traits. In this study, a very high level of phylogenetic diversity was observed in two major SCN resistance genes, the *Rhg1* and *Rhg4* loci, in accessions of both the *G. max* and *G. soja* species. Cook et al. [2] has shown that the *Rhg1* gene repeats variations not only between haplotype classes but also within the high-copy number class and between lines with a recently shared ancestry.

Interestingly, in the phylogenetic analysis, two known SCN resistant cultivars, Fayette (PI 518674) and LD00-3309 (PI 639740), which have a high copy number (approximately 10 copies) of the *Rhg1* gene [8], were grouped differently from PI 88788 (Fig. 1 and Supplement S3). It was possible that the *Rhg1* gene sequence was similar in these PIs; however, the sequence of the *Rhg1* gene flanking regions up to 0.5 cM was likely different from PI 88788. The results suggested that breeders had been able to select the linkage breakpoint at the *Rhg1* gene to develop new cultivars from PI 88788. Recently, Lee et al. [12] classified the accessions of soybean that likely carry alleles of the *Rhg1* locus using information from the 10 linked SNPs from the SoySNP50K data. Having accessed the phenotypic data from a separate study of soybean germplasm accessions belonging to MGs 000 to II (Nguyen lab, unpublished data), we found that several SCN resistant PI lines were grouped in the same cluster for high copy numbers of the *Rhg1* gene. Based on the phylogenetic tree (Fig. 1), PI 603587A, PI 467332, PI 467312, PI 461509, PI 458520, PI 438503A, PI 91102, and PI 89008, were predicted to have a high copy number of the *Rhg1* gene, while PI 416762 and PI 417091 were grouped together with PI 437654, suggesting that these lines may possess the *Rhg4* gene, similar to PI 437654.

In contrast, there was a different scenario for the *Rhg4* gene, in which SCN resistant PI 518674 (cv. Forrest), derived from PI 548402 (cv. Peking), was found in the Williams 82 clusters. It is possible that the SoySNP50K data was not adequate to cluster the *Rhg4* gene carrier lines because the *Rhg4* gene-based SNPs were not present in the SoySNP50K set. Additionally, only 64 SNPs were present in the 0.5-Mb flanking region of *Rhg4* compared to 104 SNPs in the *Rhg1* flanking region. Apparently, the SNP information that was generated from the SoySNP50K iSelect BeadChip was valuable and helpful for the haplotype analysis of some genes, but was not sufficient for a comprehensive investigation of all of the genes in the soybean genome. Recently, it has been reported that the cost of next-generation sequencing technology has been significantly reduced and has therefore become reasonable. This suggests that whole-genome resequencing can be an alternative approach for the generation of high-density SNP information of soybean accessions, by which rare alleles of several genes can be efficiently detected, leading to the successful differentiation of soybean accessions.

4.2. Next-generation markers for SCN resistant breeding

The SSR marker Satt309, which is linked to a low copy number of the *Rhg1* gene, was the first marker to be used for SCN resistance breeding [15]. In next-generation breeding, SSR markers cannot be used because high-throughput genotyping technologies are needed by breeders to conduct their breeding programs for a number of traits [42]. There is a need to develop several genetic markers in conjunction with cost-effective and high-throughput molecular genotyping assays. In this study, we successfully identified and validated several SNP marker-based KASPar assays for SCN resistance using sequencing information. The development of markers for SCN resistance genes has presented considerable challenges because of the copy number variation of the major gene *Rhg1*.

The KASPar assays were designed for the novel QTL region, qSCN10, from the gene *Glyma10g34061*. This gene belongs to the defense response gene family (CC NBS LRR class), which has been identified in *Arabidopsis* [43] and rice [44,45]. The KASPar assays for the minor QTL, qSCN11, were designed from the gene *Glyma11g35320*, which has a sequence-specific DNA binding transcription factor.

The SNP alleles for *Rhg1* high copy numbers showed resistant phenotypes in the set of 95 germplasm lines, except for PI 603154 (Supplement S6). PI 603154 also had the resistant allele, similar to PI 88788, but was susceptible to SCN. It is possible that the genetic interaction of this gene with other genes might show differential regulation at the transcriptional level. Another accession, PI 567230, has allele G of the marker Rhg1-2, similar to Peking, for the *Rhg1* gene without any minor or novel QTL; this accession is susceptible to SCN. This suggests that low copies of *Rhg1* are not sufficient to confer resistance to SCN. PI 567336B has allele G of the marker Rhg1-2 of the *Rhg1* gene and qSCN10, as does PI 567516C and PI 567305, suggesting the same type of resistance in these soybean accessions. In contrast, PI 548317 and PI 548349 have a susceptible allele C of the marker Rhg1-2, such as a single copy of the *Rhg1* gene and the novel QTL allele (qSCN10) with the minor QTL allele (qSCN11). These lines may have alleles that are different from those of PI 567516C and PI 567305. Other soybean lines, PI 407729, PI 548415, and PI 549031, with moderate resistance to SCN, have only one minor QTL allele (qSCN11) without any major gene/QTL, suggesting that this minor QTL alone could provide resistance. PI 567387 has an allele of the *Rhg4* gene different from cv. Peking (PI 548402), PI 437654, and PI 438489B (Supplement S6). Interestingly, seven elite breeding lines in the germplasm set carrying the *Rhg1* allele, such as cv. Peking (low copy), which does not have the *Rhg4* gene, were resistant to SCN, suggesting that resistance in these lines is because of other major or minor genes. As expected, none of the elite breeding lines in a germplasm set carries either QTL qSCN10 or qSCN11.

A Taqman assay was developed and tested on the dPCR system to identify copies of the *Rhg1* gene. The same assay was also validated on the ABI 7900HT platform. The results of both techniques matched with the genome sequence information. Similarly, Lee et al. [12] also demonstrated the use of genomic qPCR assays to confirm the results that were obtained through whole-genome sequencing for the CNV in *Rhg1* gene. The ABI 7900HT platform was cost-effective, with a cost of $0.50 per sample compared to the dPCR platform with a cost of $10 per sample.

KASPar assays emerged as a powerful tool because of their locus specificity, co-dominant inheritance, simple documentation, high-throughput analysis with relatively low genotyping error rates, and low assay cost. Moreover, KASPar assays have many genetic

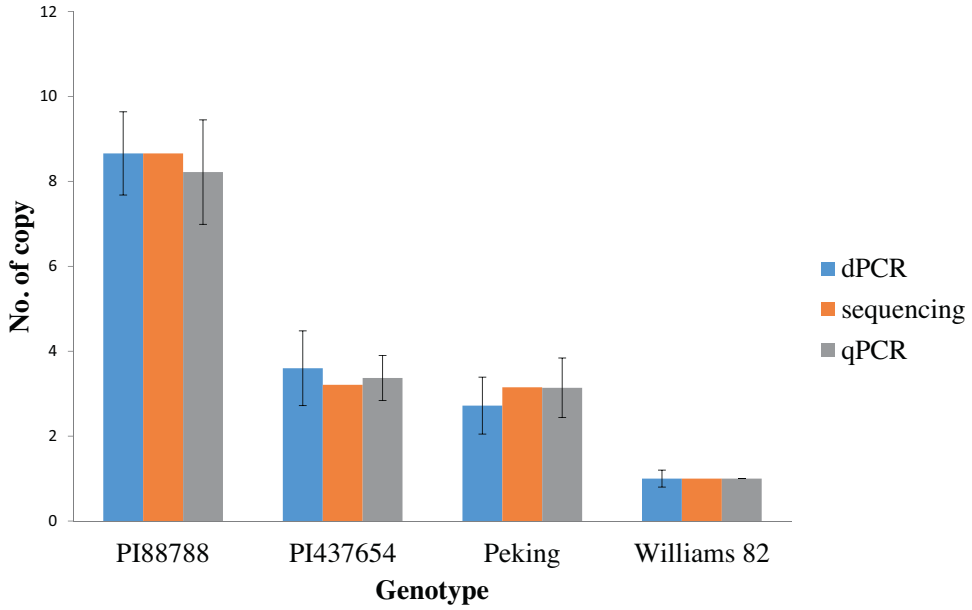

Fig. 5. Copy number estimated using three different genotyping techniques: Taqman-based dPCR assay: qPCR; along with available sequencing information. Standard error of dPCR and qPCR is denoted by the black vertical bar with cap.

applications, including germplasm characterization, allele mining, marker-assisted backcrossing (MABC), and marker-assisted recurrent selection (MARS). The functional SNP markers developed in this study will be a useful resource for breeding SCN resistance in soybean as well as for gene discovery programs for different traits in soybean.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

SK: Designed the study, performed the data analysis, and wrote the manuscript; TDV: designed the study and wrote the manuscript; CGM: carried out SCN bio-assays; DQ, VB and GP: discussed the resequencing information; RD: participated in the data analysis; SK, LS and JW: performed digital PCR and ABI 7900 for CNV; JGS and AMS: maintained the seed inventory of the soybean accessions; and HTN: designed and supervised the research. All of the authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.08.015.

References

[1] S.R. Koenning, J.A. Wrather, Suppression of soybean yield potential in the continental United States from plant diseases from 2006 to 2009, Plant Health Prog. (2010), http://dx.doi.org/10.1094/PHP-2010-1122-01-RS.
[2] D.E. Cook, T.G. Lee, X. Guo, S. Melito, K. Wang, A.M. Bayless, J. Wang, T.J. Hughes, D.K. Willis, T.E. Clemente, Copy number variation of multiple genes at Rhg1 mediates nematode resistance in soybean, Science 338 (2012) 1206–1209.
[3] S. Liu, P.K. Kandoth, S.D. Warren, G. Yeckel, R. Heinz, J. Alden, C. Yang, A. Jamai, T. El-Mellouki, P.S. Juvale, A soybean cyst nematode resistance gene points to a new mechanism of plant resistance to pathogens, Nature 492 (2012) 256–260.
[4] T.D. Vuong, D.A. Sleper, J.G. Shannon, H.T. Nguyen, Novel quantitative trait loci for broad-based resistance to soybean cyst nematode (*Heterodera glycines* Ichinohe) in soybean PI 567516C, Theor. Appl. Genet. 121 (2010) 1253–1266.
[5] X. Wu, S. Blake, D.A. Sleper, J.G. Shannon, P. Cregan, H.T. Nguyen, QTL, additive and epistatic effects for SCN resistance in PI 437654, Theor. Appl. Genet. 118 (2009) 1093–1105.
[6] V.C. Concibido, B.W. Diers, P.R. Arelli, A decade of QTL mapping for cyst nematode resistance in soybean, Crop Sci. 44 (2004) 1121–1131.
[7] Y. Jiao, T.D. Vuong, Y. Liu, C. Meinhardt, Y. Liu, T. Joshi, P.B. Cregan, D. Xu, J.G. Shannon, H.T. Nguyen, Identification and evaluation of quantitative trait loci underlying resistance to multiple HG types of soybean cyst nematode in soybean PI 437655, Theor. Appl. Genet. 128 (2015) 15–23.
[8] D.E. Cook, A.M. Bayless, K. Wang, X. Guo, Q. Song, J. Jiang, A.F. Bent, Distinct copy number, coding sequence, and locus methylation patterns underlie Rhg1-mediated soybean resistance to soybean cyst nematode, Plant Physiol. 165 (2014) 630–647.
[9] P.R. Arelli, V.C. Concibido, L.D. Young, QTLs associated with resistance in soybean PI567516C to synthetic nematode population infecting cv. Hartwig, J. Crop Sci. Biotechnol. 13 (2010) 163–167.
[10] B. Guo, D. Sleper, P. Arelli, J. Shannon, H. Nguyen, Identification of QTLs associated with resistance to soybean cyst nematode races 2, 3 and 5 in soybean PI 90763, Theor. Appl. Genet. 111 (2005) 965–971.
[11] Q. Song, D.L. Hyten, G. Jia, C.V. Quigley, E.W. Fickus, R.L. Nelson, P.B. Cregan, Development and evaluation of SoySNP50K, a high-density genotyping array for soybean, PLoS One 8 (2013) e54985.
[12] T.G. Lee, I. Kumar, B.W. Diers, M.E. Hudson, Evolution and selection of Rhg1, a copy-number variant nematode-resistance locus, Mol. Ecol. 24 (2015) 1774–1791.
[13] X. Wu, C. Ren, T. Joshi, T. Vuong, D. Xu, H.T. Nguyen, SNP discovery by high-throughput sequencing in soybean, BMC Genomics 11 (2010) 469.
[14] P. Cregan, J. Mudge, E. Fickus, D. Danesh, R. Denny, N. Young, Two simple sequence repeat markers to select for soybean cyst nematode resistance coditioned by the rhg1 locus, Theor. Appl. Genet. 99 (1999) 811–818.
[15] M.F.d. Silva, I. Schuster, J.F.V.d. Silva, A. Ferreira, E.G.d. Barros, M.A. Moreira, Validation of microsatellite markers for assisted selection of soybean resistance to cyst nematode races 3 and 14, Pesquisa Agropecuária Brasileira 42 (2007) 1143–1150.
[16] C.-P. Yuan, Y.-H. Li, Z.-X. Liu, R.-X. Guan, R.-Z. Chang, L.-J. Qiu, DNA sequence polymorphism of the Rhg4 candidate gene conferring resistance to soybean cyst nematode in Chinese domesticated and wild soybeans, Mol. Breed. 30 (2012) 1155–1162.

[17] Y. Bao, T. Vuong, C. Meinhardt, P. Tiffin, R. Denny, S. Chen, H.T. Nguyen, J.H. Orf, N.D. Young, Potential of association mapping and genomic selection to explore pi 88788 derived soybean cyst nematode resistance, Plant Genome 7 (2014) 1–13.
[18] Z. Shi, S. Liu, J. Noe, P. Arelli, K. Meksem, Z. Li, SNP identification and marker assay development for high-throughput selection of soybean cyst nematode resistance, BMC Genomics 16 (2015), 314.
[19] R.K. Deshmukh, W. Chen, S. Prince, G. Patil, R. Mutava, H. Sonah, B. Valliyodan, T. Vuong, H. Nguyen, Integrating omic approaches for abiotic stress tolerance in soybean, Front. Plant Sci. (2014), http://dx.doi.org/10.3389/fpls.2014.00244.
[20] A.A. Deokar, L. Ramsay, A.G. Sharpe, M. Diapari, A. Sindhu, K. Bett, T.D. Warkentin, B. Tar, Genome wide SNP identification in chickpea for use in development of a high density genetic map and improvement of chickpea reference genome assembly, BMC Genomics 15 (2014) 708.
[21] M.L. Rosso, S.A. Burleson, L.M. Maupin, K.M. Rainey, Development of breeder-friendly markers for selection of MIPS1 mutations in soybean, Mol. Breed. 28 (2011) 127–132.
[22] A.S. Whale, J.F. Huggett, S. Cowen, V. Speirs, J. Shaw, S. Ellison, C.A. Foy, D.J. Scott, Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation, Nucleic Acids Res. 40 (2012) e82.
[23] C.H. Roberts, W. Jiang, J. Jayaraman, J. Trowsdale, M.J. Holland, J.A. Traherne, Killer-cell Immunoglobulin-like Receptor gene linkage and copy number variation analysis by droplet digital PCR, Genome Med. 6 (2014) 20.
[24] H. Sonah, L. O'Donoughue, E. Cober, I. Rajcan, F. Belzile, Identification of loci governing eight agronomic traits using a GBS-GWAS approach and validation by QTL mapping in soya bean, Plant Biotechnol. J. 13 (2015) 211–221.
[25] H.-M. Lam, X. Xu, X. Liu, W. Chen, G. Yang, F.-L. Wong, M.-W. Li, W. He, N. Qin, B. Wang, Resequencing of 31 wild and cultivated soybean genomes identifies patterns of genetic diversity and selection, Nat. Genet. 42 (2010) 1053–1059.
[26] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6. 0, Mol. Biol. Evol. 30 (2013) 2725–2729.
[27] P.R. Arelli, D.A. Sleper, P. Yue, J.A. Wilcox, Soybean reaction to Races 1 and 2 of *Heterodera glycines*, Crop Sci. 40 (2000) 824–826.
[28] T.D. Vuong, Y. Jiao, J.G. Shannon, H.T. Nguyen, Nematode resistance in soybean Translational Genomics for Crop Breeding: Biotic Stress, vol. 1, 2013, pp. 95–124.
[29] T. Niblack, P. Arelli, G. Noel, C. Opperman, J. Orf, D. Schmitt, J. Shannon, G. Tylka, A revised classification scheme for genetically diverse populations of *Heterodera glycines*, J. Nematol. 34 (2002) 279.
[30] S. Brown, G. Yeckel, R. Heinz, K. Clark, D. Sleper, M.G. Mitchum, A high-throughput automated technique for counting females of *Heterodera glycines* using a fluorescence-based imaging system, J. Nematol. 42 (2010) 201.
[31] Z. Zhou, Y. Jiang, Z. Wang, Z. Gou, J. Lyu, W. Li, Y. Yu, L. Shu, Y. Zhao, Y. Ma, Resequencing 302 wild and cultivated accessions identifies genes related to domestication and improvement in soybean, Nat. Biotechnol. 33 (2015) 408–414.
[32] A. McKenna, M. Hanna, E. Banks, A. Sivachenko, K. Cibulskis, A. Kernytsky, K. Garimella, D. Altshuler, S. Gabriel, M. Daly, The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data, Genome Res. 20 (2010) 1297–1303.
[33] B.L. Robberson, G.J. Cote, S.M. Berget, Exon definition may facilitate splice site selection in RNAs with multiple exons, Mol. Cell. Biol. 10 (1990) 84–94.
[34] S.L. Dellaporta, J. Wood, J.B. Hicks, A plant DNA minipreparation: version II, Plant Mol. Biol. Rep. 1 (1983) 19–21.
[35] G.L. Tylka, M. Mullaney, Soybean cyst nematode-resistant soybean varieties for Iowa, Iowa State University, University Extension, 2002.
[36] G. Chung, R.J. Singh, Broadening the genetic base of soybean: a multidisciplinary approach, Crit. Rev. Plant Sci. 27 (2008) 295–341.
[37] D.L. Hyten, Q. Song, Y. Zhu, I.-Y. Choi, R.L. Nelson, J.M. Costa, J.E. Specht, R.C. Shoemaker, P.B. Cregan, Impacts of genetic bottlenecks on soybean genome diversity, Proc. Natl. Acad. Sci. 103 (2006) 16666–16671.
[38] M.I. Tenaillon, M.C. Sawkins, A.D. Long, R.L. Gaut, J.F. Doebley, B.S. Gaut, Patterns of DNA sequence polymorphism along chromosome 1 of maize (*Zea mays* ssp. *mays* L.), Proc. Natl. Acad. Sci. 98 (2001) 9161–9166.
[39] Q. Zhu, X. Zheng, J. Luo, B.S. Gaut, S. Ge, Multilocus analysis of nucleotide variation of Oryza sativa and its wild relatives: severe bottleneck during domestication of rice, Mol. Biol. Evol. 24 (2007) 875–888.
[40] T. Hymowitz, C. Newell, Taxonomy of the genusGlycine, domestication and uses of soybeans, Econ. Bot. 35 (1981) 272–288.
[41] D.L. Hyten, I.-Y. Choi, Q. Song, R.C. Shoemaker, R.L. Nelson, J.M. Costa, J.E. Specht, P.B. Cregan, Highly variable patterns of linkage disequilibrium in multiple soybean populations, Genetics 175 (2007) 1937–1944.
[42] Y. Xu, Y. Lu, C. Xie, S. Gao, J. Wan, B.M. Prasanna, Whole-genome strategies for marker-assisted plant breeding, Mol. Breed. 29 (2012) 833–854.
[43] B.C. Meyers, M. Morgante, R.W. Michelmore, TIR-X and TIR-NBS proteins: two new families related to disease resistance TIR-NBS-LRR proteins encoded in Arabidopsis and other plant genomes, Plant J. 32 (2002) 77–92.
[44] B. Yuan, C. Zhai, W. Wang, X. Zeng, X. Xu, H. Hu, F. Lin, L. Wang, Q. Pan, The Pik-p resistance to Magnaporthe oryzae in rice is mediated by a pair of closely linked CC-NBS-LRR genes, Theor. Appl. Genet. 122 (2011) 1017–1028.
[45] J. Ma, C. Lei, X. Xu, K. Hao, J. Wang, Z. Cheng, X. Ma, J. Ma, K. Zhou, X. Zhang, Pi64, encoding a novel CC-NBS-LRR protein, confers resistance to leaf and neck blast in rice, Mol. Plant Microbe Interact. 28 (5) (2015) 558–568, http://dx.doi.org/10.1094/MPMI-11-14-0367-R.