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ORIGINAL PAPER

Fine mapping and identification of candidate genes controlling the resistance to southern root-knot nematode in PI 96354

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Abstract *Meloidogyne incognita* (Kofoid and White) Chitwood (Mi) is the most economically damaging species of the root-knot nematode to soybean and other crops in the southern USA. PI 96354 was identified to carry a high level of resistance to galling and Mi egg production. Two Quantitative Trait Locus (QTLs) were found to condition the resistance in PI 96354 including a major QTL and a minor QTL on chromosome 10 and chromosome 18, respectively. To fine map the major QTL on chromosome 10, $F_{5:6}$ recombinant inbred lines from the cross between PI 96354 and susceptible genotype Bossier were genotyped with Simple Sequence Repeats (SSR) markers to identify recombinational events. Analysis of lines carrying key recombination events placed the Mi-resistant allele on chromosome 10 to a 235-kb region of the 'Williams 82' genome sequence with 30 annotated genes. Candidate gene analysis identified four genes with cell wall modification function that have several mutations in promoter, exon, 5′, and 3′UTR regions. qPCR analysis showed significant difference in expression levels of these four genes in Bossier compared to PI 96354 in the presence of Mi. Thirty Mi-resistant soybean lines were found to have same SNPs in these 4 candidate genes as PI 96354 while 12 Mi-susceptible lines possess the 'Bossier' genotype. The mutant SNPs were used to develop KASP assays to detect the resistant allele on chromosome 10. The four candidate genes identified in this study can be used in further studies to investigate the role of cell wall modification genes in conferring Mi resistance in PI 96354.

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Introduction

The root-knot nematode (RKN) *Meloidogyne* species are economically important nematodes in soybean in the USA. From 1974 to 2007, it was recorded that *Meloidogyne* species caused yield loss every year in soybean where yield loss varied from a trace amount to 5 %, resulting in yield reduction up to 2.1 million metric tons (Wrather et al. 1995; Wrather and Koenning 2009). Economic loss of soybean due to RKN was estimated to be nearly $80 million in USA in 1994 (Pratt and Wrather 1998). Specially, in southern states such as Kentucky, Alabama, Georgia, Florida, and Louisiana, RKN is the most damaging nematode in soybean that caused economic loss of $30 million annually (Sciumbato 1993; Wrather and Koenning 2006).

Common management practices to prevent yield loss due to RKNs are crop rotation, nematicides, and host plant resistance. However, the banning of important fumigant nematicides including 1,2-dibromo-3-chloropropane (DBCP) and ethylene dibromide (EDB), the existence of few profitable

rotation crops, as well as the broad range host of *Meloidogyne spp.* made nematicides and crop rotation less effective and placed the emphasis on the usage of resistant cultivars (Kinloch et al. 1985; Ruckelshaus 1983). Soybean cultivars with resistance to RKN and good agronomic performance have been developed and have demonstrated great economic benefit in highly infested fields (Kinloch et al. 1985). Not only does the utilization of resistant cultivars offer great economic profits, it also reduces environmental impacts of using toxic chemicals and impedes disease complexes associated with the nematode (Boerma and Hussey 1992).

Among *Meloidogyne* species, *Meloidogyne incognita* (Kofoid and White) Chitwood (Mi) is the most common and destructive species (Taylor and Sasser 1987; Walters and Barker 1994). Screening of commercial soybean cultivars and plant introductions (PI) from USDA soybean germplasm collection from 1983 to 2003 identified a total of 27 US cultivars and 20 PIs with modest to high levels of resistance to Mi (Harris et al. 2003; Hussey et al. 1991; Luzzi et al. 1987). In addition, genetic and quantitative trait locus (QTLs) mapping studies helped to discover genomic regions responsible for the trait. A genetic study on a population developed from the cross between Mi-resistant cultivar, Forest and Mi-susceptible cultivar, Bossier indicated that a single additive gene *Rmi1* is responsible for the resistance to Mi in Forest (Luzzi et al. 1994). Among the identified resistant sources, an accession from Japan, PI 96354, possessed the highest level of resistance with fewer galls and egg masses (Luzzi et al. 1987; Moura et al. 1993). The resistance in PI 96354 was shown to be controlled by 2 QTLs with a major QTL on chromosome 10 and a minor QTL on chromosome 18 that explained 56 and 17 % of the variation in gall number, respectively (Li et al. 2001; Tamulonis et al. 1997). Based on the mapping results from the population of Forrest × PI 96354, Tamulonis et al. (1997) indicated that *Rmi1* gene was located in the QTL region on chromosome 10. Ha et al. (2004) demonstrated that all of soybean cultivars from maturity groups V, VI, VII, and VIII with Mi resistance possess the *Rmi1* gene, which was inherited from the ancestral cultivar 'Palmetto'. Based on the association of Mi gall number and soybean pubescence color in two breeding populations which trace their resistance to PI 96354, Shearin et al. (2009) reported the linkage between the *T* locus on chromosome 6 in soybean and resistance to Mi. In their study, soybean lines with gray pubescence color in both populations had an average of 75 % fewer Mi eggs per plant than soybean lines with tawny pubescence color. Recently, a study by Fourie et al. (2008) showed that resistance to Mi race 2 which is found dominantly in South Africa was controlled by a major QTL on chromosome 7 and a minor QTL on chromosome 10 that was reported previously. However, simple sequence repeats (SSR) marker data in this study showed that although PI 96354 does not have a resistant allele on chromosome 7, it was still reported as resistant to Mi race 2 in addition to race 3 which is found in southern USA. Although two major and two minor QTL that confer the resistance to Mi in soybean were reported in the literature, the gene(s) that was mapped to chromosome 10 is probably the most important one which conditions the Mi resistance in majority of southern US elite soybean cultivars.

Although molecular markers are available to aid the introgression of the resistance allele on chromosome 10 from PI 96354 into the elite soybean cultivars, the gene(s) underlying the Mi resistance remains unknown. With the advance of technology and availability of the Williams 82 sequence data, 210,990 new soybean SSR markers were discovered from which primers for 33,065 markers were generated (Song et al. 2010). This enables us to add more markers into the previously mapped 9.1-cM interval on chromosome 10 to fine map the region and identify candidate genes responsible for the Mi resistance. In addition, the availability of microarray data for the genes that have differential expression in soybean roots infested with Mi provides information on promising candidate genes for us to unveil the resistance to Mi in soybean using a reverse genetic approach (Ibrahim et al. 2011). The objectives of this study were to: (1) fine map the 9.1-cM interval on chromosome 10 containing the *Rmi1* gene, (2) predict candidate genes by using available soybean genomic data and examine their expression level in the presence of Mi, and (3) develop functional SNP markers for effective marker-assisted selection in soybean for Mi resistance.

Materials and methods

RIL population development

A cross between cultivar Bossier and PI 96354 was made during the summer of 2007 at the University of Georgia Plant Sciences Farm in Watkinsville, GA. PI 96354 is a Japanese landrace with a high level of resistance to Mi for both gall formation and nematode reproduction, while Bossier is highly susceptible in both terms (Luzzi et al. 1987). An F_2 population was developed from a single F_1 plant and was grown at the Plant Sciences Farm in 2008, and then subsequently was advanced to a population consisting of 269 $F_{5:6}$ recombinant inbred lines (RILs) by the single seed decent method (Poehlman and Sleper 1995). The F_3 and F_4 populations were grown in the USDA-ARS Winter Nursery near Isabella Puerto Rico in the winter of 2008–2009, and F_5 population was planted at Plant Sciences Farm in the summer of 2009. Of the 269 $F_{5:6}$ RILs, 188 were randomly chosen for this study.

Root-knot nematode phenotyping of the RIL population in the greenhouse

The 188 RILs and their parents, PI 96354 and Bossier, were arranged in a randomized complete block design (RCBD) with eight replications and evaluated for Mi galling in the greenhouse facility at the University of Georgia, Athens, GA in February 2010. Three seeds of each genotype were planted in Ray Leach Cone-tainers (20.6 cm long cones) filled with fumigated sandy loam soil. Four extra Cone-tainers of each Bossier and PI 96354 were included in each of the eight replications. Forty-nine Cone-tainers were placed in a RL-98 tray, filling every other row of the tray. The trays were placed on a greenhouse bench under supplemental lighting provided by 400-watt metal halide lamps and under an automatic irrigation system. Ten days after planting, the plants were thinned to one seedling per Cone-tainer and inoculated with 3,000 RKN eggs (Tamulonis et al. 1997). Forty days after inoculation the first four replications were uprooted, the roots were washed free of soil, and the Mi galls counted. Remaining replications were uprooted and the galls counted at 50 days after inoculation.

Genotyping the RIL population using SSR markers

Two flanking SSR markers Satt358 and Satt492 on chromosome 10 were chosen based on previous studies (Li et al. 2001). Young trifoliolate leaves from three plants of each line were sampled for DNA extraction. DNA was extracted using Hexadecyltrimethylammonium bromide (CTAB) procedure modified from Keim et al. (1988). The PCR reaction was conducted on 384-well GeneAmp PCR System 9700 (PE-ABI, Foster City, CA) using fluorescent dyes labeled primers by the Diwan and Cregan 1997 protocol. The PCR product was mixed with Rox 500 ladder (Life Technologies, Carlsbad, CA) and assayed with 3730 DNA Analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA) at Georgia Genomic Facility using the procedure described by Abdel-Haleem et al. (2011). The marker fragments were analyzed and scored with Genemarker software (SoftGenetics, State College, PA).

Identifying and genotyping recombinant events

Of the 188 RILs genotyped with Satt358 and Satt492, 17 RILs possessed recombination break points (i.e. having Bossier's allele for Satt358, but PI 96354's allele for Satt492 or vice versa). Twenty-eight SSR markers were selected from the interval of Satt358 and Satt492 based on their location in the Williams 82 sequence for screening polymorphism between two parents (Song et al. 2010). Out of the 28, seven markers were found polymorphic and subsequently used for genotyping the 17 lines with recombination events (Supplement S2). The PCR was performed in 20 µl reactions containing 50–100 ng DNA template, 0.5 µM final concentration of primers, Flexi buffer, 3.5 mM $MgCl_2$, 200 µM dNTPs, and 0.2X Flexi Promega *Taq* polymerase (Promega Corporation, Madison, WI). PCR products were separated using capillary gel electrophoresis in Advance FS96 (Advanced Analytical Technologies Inc., Ames, IA). Genotypic classes were assigned by visual comparison of PCR products obtained from the RILs to those obtained from parental lines using PRO size data analysis software (Advanced Analytical Technologies Inc., Ames, IA).

Candidate gene discovery and SNP assays development for the detection of the resistance allele

Based on previous data for resistance reaction to Mi, 30 soybean accessions and cultivars were selected for sequencing of four candidate genes (Glyma10g02090, Glyma10g02100, Glyma10g02140, and Glyma10g02160). In addition, 12 Mi-susceptible soybean PIs and cultivars, also determined from previous studies, were selected for sequencing as controls (Supplement S3).

DNA of 42 soybean cultivars and PIs was isolated from seeds using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), and 5–50 ng DNA were used in PCR with (Promega) gene specific primers (Supplement S2) under the following conditions: 95 °C for 5 min, followed by 34 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min per 1 kbp of predicted product size. After PCR, products were examined on a 1.5 % agarose gel by electrophoresis and then sequenced at the Georgia genomics facility. Sequence traces were assembled and manually evaluated for polymorphisms using Geneious version 5.5.7 (Drummond et al. 2001). Putative polymorphisms were verified by a second, independent PCR and sequencing reaction.

To test the association of these SNPs with the Mi resistance phenotype and develop robust SNP assay for marker-assisted selection, Kompetitive Allele Specific PCR (KASP) assays (KBioscience, Herts, England) were developed for one SNP from each of the four candidate genes except for Pectin esterase 2 (Glyma10g02160). On chromosome 10, for Glyma10g02090 (extensin 1 gene), a SNP in the promoter region (T > A) that is expected to give good amplification in a PCR reaction was selected for KASP assay. For Glyma10g02100 (extensin 2 gene), the SNP that causes amino acid change $Q^{158}L$ was chosen. For the Glyma10g02140 (Pectin esterase 2) gene, the SNP that causes the change of Q^{190} to stop codon was used. On chromosome 18, gene Glyma18g48730 homologous to the Glyma10g02080 (EREBP transcriptional factor) was sequenced and a SNP ($G^{283} > T$) that results in amino acid change of Q^{72} to P was used to design

the KASP assay. Primer sequences are listed in Supplement S2. KASP reactions were run in 4 µl reactions including 2 µl of 2× premade KASP master mix, 0.106 µl of primers mix (both from KBiosciences, Herts, England), and 2 µl of 10–20 ng/µl genomic DNA. PCR and fluorescent endpoint readings were carried out using the LightCycler® 480 Real-Time PCR System (Roche, Germany). A panel of 158 cultivars and germplasm lines with known reaction to Mi as well as 78 PIs which were not previously evaluated for Mi resistance were genotyped using these KASP assays (Supplement S5).

Gene expression study using qPCR assay

The Mi phenotyping was conducted in a greenhouse at the University of Georgia in Athens GA using procedures described by Luzzi et al. (1994) with some modifications. Briefly, there were two set of trays, each containing four trays representing four replications. The first set was inoculated with Mi eggs while the second set was inoculated with only water. Within each tray, eight Ray Leach Cone-tainers, four for each of the parents (Bossier and PI 96354), were randomized using the RCBD. The randomizations were the same for each replication in either Mi inoculated tray or water-inoculated tray. For the inoculated set, the inoculum concentration for tray 1 and 2 was 3,000 eggs and tray 3 and 4 was 10,000 eggs. The reason of having two inoculation rates was to test if the expression of the candidate genes correlates with the number of Mi eggs. Three seeds of each parent were planted in each cone. Seven days after planting, the plants were thinned to one seedling per Cone-tainer and inoculated with corresponding amount of Mi eggs or distilled water. At 6, 12, 18 and 32 days after inoculation (dai), root tissues from one plant of each genotype were washed with distilled water, excised from the stem and kept in 15-ml tubes (Sarstedt Inc., Newton, NC) temporarily in liquid nitrogen before storing permanently in a −80 °C freezer.

qPCR analysis of four candidate genes and cDNA cloning for Pectin esterase 1

qRT-PCR was carried out using the LightCycler® 480 Real-Time PCR System (Roche, Germany). Specific primers for each candidate gene were designed using Primer 3 program (Untergasser et al. 2007). PCR products amplified from these primers were sequenced to determine if the correct genes of interest were amplified. Only primer pairs that give a single amplicon were selected for qRT-PCR. Primer efficiency was determined using serial dilutions of five folds using DNA of soybean cultivar 'Tokyo'. RNA was extracted using TRIZOL RNA extraction reagent by following the procedure from the manufacture (Life Technologies, Carlsbad, CA). The qRT-PCR reactions were conducted with three technical replications in 10 µl reaction for each biological replicate using Qiagen QuantiFast SYBR Green RT-PCR Kit (Qiagen, Valencia CA). Following the reverse transcriptase reaction, amplification was conducted at 95 °C for 15 min, then 35 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. Soybean gene cons7 was used as internal control (Libault et al. 2008). The quantification of gene expression was performed using the relative ΔΔCT method. cDNA cloning and sequencing was conducted using QIAGEN OneStep RT-PCR Kit (Qiagen, Valencia, CA) and specific primers.

Results

Identification of recombinants within the delimited *R* region on chromosome 10

The mean number of Mi galls for eight replications of 188 RILs was 15.6 with the mean gall number for RILs ranging from 0 to 52.9. The mean number of Mi galls for resistant parent, PI 96354, and susceptible parent, Bossier, were 0.45 and 53.2, respectively. Based on the range of gall numbers, RILs with less than 10 galls were considered as resistant and RILs with greater than 20 galls were considered as susceptible (data not shown).

Based on genotyping results using the two flanking markers Satt358 and Satt492 on chromosome 10, 17 of 188 RILs were identified to possess recombinant events. Of these 17 RILs, 10 had Bossier's allele for Satt358 and PI 96354's allele for Satt492 while seven had the reverse allelic configuration. Seven polymorphic SSR markers (BARCSOYSSR 10_0082, BARCSOYSSR 10_0090, BARCSOYSSR 10_0095, BARCSOYSSR 10_0098, BARCSOYSSR 10_0103, BARCSOYSSR 10_0105 and BARCSOYSSR 10_0111) were used to genotype these 17 individual RILs and 5 recombinant break points were identified among these 17 RILs. The representative RILs with each recombinant break point are shown in Fig. 1. According to the recombinant break point, the location of the Mi resistance gene(s) is predicted to be in the 235-kbps region flanked by BARCSOYSSR 10-0090 and BARCSOYSSR 10-0105 (Fig. 1). This region contains 30 annotated genes with high confidence (http://soybase.org).

Identification of four genes as candidates for the Mi resistance

The expression data of the 30 genes in the 235-kbps region were obtained from Soyseg on Soybase (http://soybase.org/soyseq/) (Severin et al. 2010). These 30 genes were compared to the lists of genes reported to have differential

A

850 kbps

Mi reaction	Average gall number	RIL ID	Satt358	BARCSOYSSR 10-0082	BARCSOYSSR 10-0090	BARCSOYSSR 10-0095	BARCSOYSSR 10-0098	BARCSOYSSR 10-0103	BARCSOYSSR 10-0105	BARCSOYSSR 10-0111	Satt492
Resistance	3	6841	3	3	3	3	3	3	3	3	3
Resistance	0.3	6920	3	3	3	3	3	3	3	3	3
Resistance	0.8	6999	1	3	3	3	3	3	3	3	3
Resistance	3.5	6845	1	1	3	3	3	3	3	3	3
Susceptible	24	7003	1	1	1	1	1	1	3	3	3
Susceptible	22	7070	1	1	1	1	1	1	3	3	3
Susceptible	33	6954	3	1	1	1	1	1	1	1	1
Susceptible	23	7074	3	3	3	1	1	1	1	1	1
Susceptible	53.2	Bossier	1	1	1	1	1	1	1	1	1
Resistance	0.45	PI96354	3	3	3	3	3	3	3	3	3

B

235 kb
30 annotated genes

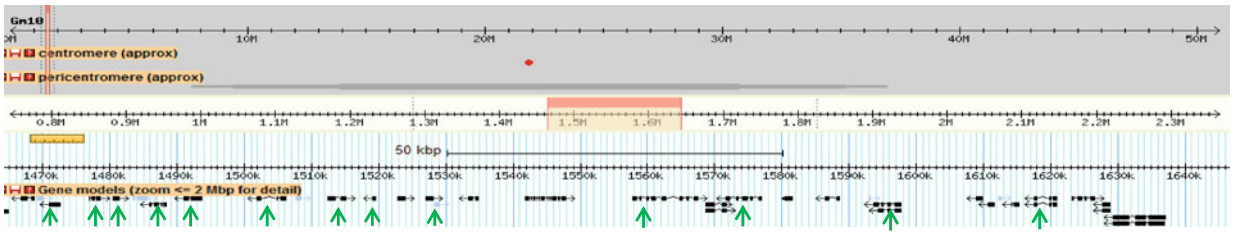

Fig. 1 SSR alleles and average gall number caused by root-knot nematode *Meloidogyne incognita* for eight selected $F_{5:6}$ RILs based on recombinations. **a** The average gall number and SSR alleles for selected $F_{5:6}$ RIL lines used to fine map the locus responsible for resistance to *Mi* in PI 96354. **b** Screen shot of Williams 82 genome (assembly version 1.0) in SoyBase.org, displaying the 235 kb region on chromosome 10 which contained the locus responsible for the Mi resistance in PI 96354. *Arrows* indicate the candidate genes selected for sequencing

expression levels in *Arabidopsis*, soybean, tomato, and cowpea when infested with nematodes (Bar-Or et al. 2005; Das et al. 2010; Gal et al. 2006; Gheysen and Fenoll 2002; Ibrahim et al. 2011). Based on the data, 13 candidate genes were selected as they either have a root-specific expression pattern on Soyseg or were reported to have an altered expression level in plants infected with nematodes when compared to the mock control (Table 1). Of them, seven of these candidate genes encode cell wall-related proteins including extensin, pectin esterase, and gamma tubulin complex proteins. One gene is an ethylene responsive transcriptional factor (EREBP) and five are genes related to different biological processes.

The exons, introns, and 1-kb promoter region of 10 out of 13 candidate genes were sequenced in Bossier and PI 96354. For the remaining three genes including Glyma10g02210, Glyma10g02250, and Glyma10g02340, only exon regions and 100 bps in the intronic regions upstream and downstream adjacent to the exons were sequenced due to the long intron regions of these genes. In addition to using soybase's gene model, we also used the programs Fgenesh (http://linux1.softberry.com) and Expasy (http://web.expasy.org/cgi-bin/translate/dna_aa) to predict gene structure and protein sequence. Except for Glyma10g02140, all of the other genes have the same model as predicted in Soybase. The obtained sequences of the 13 candidate genes in the PI 96354 and Bossier were aligned and compared to the sequences of the Williams 82 assembly version 1.01. A total of 93 changes in genomic sequences (SNPs, indels, insertion) were identified in PI 96354 and 15 were found in Bossier when compared with Williams 82 in the 13 sequenced genes covering 36-kbps (Table 1). No SNP was identified in either PI 96354 or Bossier in the genes of Glyma10g02170, Glyma10g02170 (both are pectin esterase inhibitors), and Glyma10g02030 (O-glycosyl hydrolase). Five genes which have root-specific expression in Soybase including Glyma10g02120, Glyma10g02080, Glyma10g02210, Glyma10g02250, and Glyma10g02230 have either silent mutations or few SNPs in intron regions in either PI 96354 or Bossier. The root-specific gene Glyma10g02340 (universal stress protein) had a CGT^{10-12} deletion in the exon region resulting in the deletion of the fourth residue in protein sequence. Sequencing 22 RILs with lowest (14 RILs) and highest (8 RILs) gall numbers revealed that there was no association of this deletion with gall number variation.

Among 93 changes in DNA sequence of 13 candidate genes identified in PI 96354, 84 events (90 %) were identified from the four genes that encode proteins with cell wall-related functions (Table 1). In the Glyma10g02090

Table 1 SNPs identified from candidate genes selected from the 235 kb fine-mapped region containing major QTL on chromosome 10 by comparing to Williams 82 genome (version 1.0)

Gene ID	Leaf	Flower	Pod[a]	Seed[b]	Root	Nodule	Annotated gene	SNP in PI 96354	SNP in Bossier
Glyma10g02030	1[c]	3	2	2	7	1	O-glycosyl hydrolase	No	No
Glyma10g02080	0	1	1	6	23	70	EREBP transcriptional factor	Exon: $A^{462}G$ (silent), intron: −/TT	Intron: −/T
Glyma10g02090	0	2	0	0	13	1	Extensin1	Promoter: $T^{-250} > G$, $-/A^{-420}$, $T^{-680} > A$; intron: G > T, C > T, T > A; 3′UTR: T/−; TT/−	3′UTR: TTT/−
Glyma10g02100	0	34	0	0	5	0	Extensin2	Promoter: $C^{-371>}T$, $G^{-431} > T$; exon: $C^{53}T$ ($A^{18}V$), $G^{196}T$ ($A^{66}S$), $A^{473}T$($Q^{158}L$); 3′UTR: T/A,−/A, C/T, C/−, G/A	No
Glyma10g02120	0	0	0	0	0	0	O-glycosyl hydrolase	Intron: G > A, A > T, A > T Exon: $T^{122} > G$ (silent)	Intron: G > A, A > T, A > T Exon: $T^{122} > G$ (silent)
Glyma10g02140	0	0	0	0	0	0	Pectin esterase 1	Promoter: deletion of 41 bps from −172 to −213 Exon: 12 SNPs: 6 silent, 6 nonsense including $V^{93}I$, $D^{120}Y$, $L^{149}R$, $C^{167}Y$, $E^{214}D$, Q^{242}Stop Intron: 23 SNPs, 9 indels, 1 insertion	No
Glyma10g02160	0	0	0	0	0	0	Pectin esterase 2	Promoter: $A^{-610}/-$, indel of 6 bps at −598 to −592 Exon: $A^{105}T$, $T^{396}C$, $A^{429}C$, (silent), $G^{653}C$ ($R^{218}T$) Intron: 13 SNPs and 1 deletion of 11 sequential bps	No
Glyma10g02170	0	8	0	0	0	0	Plant pectin methylesterase inhibitor	No	No
Glyma10g02210	1	7	1	3	78	73	Late embryogenesis abundant protein	Exon: no SNPs Intron: A > C	No
Glyma10g02230	30	37	27	16	36	32	Gamma tubulin complex protein	No	Intron: 2 SNPs (T > C, T > G)
Glyma10g02250	1	2	2	0	3	24	Ca2+/Na+ exchanger	$A^{1296}G$, $C^{1375}A$ (silent)	Exon: $A^{17}G$,$C^{504}T$(silent) Introns: 7 SNPs
Glyma10g02300	0	0	0	0	0	0	Plant pectin methylesterase inhibitor	No	G > C in introns
Glyma10g02340	4	13	8	3	41	265	Universal stress protein family	No	Deletion of CGT in the 1st exon resulting in deletion of a S^4

[a] Average data of pod 1 cm, pod-shell 10 days after flowering (DAF) and pod-shell 14-DAF

[b] Average data of seed 10-DAF, seed 14-DAF, seed 21-DAF, seed 25-DAF, seed 28-DAF, seed 35-DAF and seed 42-DAF

[c] Normalized data processed from raw digital gene expression counts using a variation of the reads/Kb/Million (RPKM) method (Severin et al. 2010)

gene model encoding extensin 1 (Ext1), there were three SNPs in the promoter region, three in the first intron, and two in 3′UTR region (Table 1; Supplement S1). In the Glyma10g02100 gene model encoding extensin 2 (Ext2), there were two SNPs in the promoter regions, three missense mutations in the exons that cause three amino acid changes including $C^{53}T$ ($A^{18}V$), $G^{196}T$ ($A^{66}S$), $A^{473}T(Q^{158}L)$, and five SNPs in the 3′UTR region (Table 1; Supplement S1). Blasting the mRNA sequences from each of the two extensin genes against the expressed sequence tag (EST) database of the National Center for Biotechnology Information (NCBI) resulted in a 100 % similar EST sequence from soybean roots. Therefore, no attempt on cDNA cloning was made for these two genes in this study. In the Glyma10g02140 gene model encoding pectin esterase 1(PE1), there were 12 SNPs with 6 silent and 6 missense mutations resulting amino acid changes: $V^{93}I$, $D^{120}Y$, $L^{149}R$, $C^{167}Y$, $E^{214}D$ and Q^{242}Stop. In the promoter, there was a deletion of 41 bp from −172 to −213 upstream of the first codon. In addition, 23 SNPs, nine deletions (Indel), and one insertion were found in the intronic sequences (Table 1; Supplement S1). Among the Indels and insertion in the intronic region, there were two deletions with 227 and 48 bps, respectively, one insertion of seven bps, and the rest were single nucleotide deletions. The cDNA cloning indicated that the protein sequence of PE1 contains only 399 amino acid (aa), which is 123 aa shorter than the model in Soybase (Supplement S1). However, the amino acid changes caused by the SNP found in PI 96354 were not different in both models. In the Glyma10g02160 gene model encoding pectin esterase 2 (PE2), there are four SNPs in the first exon one of which resulted in an amino acid change in protein sequence ($R^{218}T$), and 13 SNPs and one deletion of 11 bps in the only intron of the gene. The amino acid changes in Ext 2, PE1, and PE2's proteins may impact the proteins' functions, especially the stop codon in PE1 which results in a truncated and premature protein. In addition, the SNPs and deletions/insertions in the promoter and intronic regions of these four cell wall-related genes may influence their expression level.

Two online databases were used to predict whether the SNPs in the promoters of the cell wall candidate genes lie in potential binding sites of transcriptional factors. One website was Plant cis-acting regulatory DNA elements (PLACE) developed by Higo et al. (1999), and the other is Plant Promoter Analysis Navigator (PlantPan) by Chang et al. (2008) which offers search for soybean transcriptional factors and their binding sites. For both databases, none of the SNPs in extensin 1 was found in any potential binding site. For extensin 2, $C^{-371>}T$ was detected to be in the binding site of RYREPEATBNNAPA (the complex containing the two RY repeats and the G-box) of *Brassica napa*. None of the three SNPs in promoter of extensin 2 were lying within any potential binding site for known soybean transcriptional factors. For Pectinesterase 1, the deletion of 41 bps in the promoter was found potentially to cover five different binding sites including TATABOX5, MARTBOX, POLASIG1, ATHB5, and SEF4MOTIF-GM7S (soybean embryo factor 4 motif). For pectin esterase 2, the indel of 6 bps at −598 to −592 was found to be potentially the ROOTMOTIFTAPOX1 (motif found both in promoters of rolD) or SEF1MOTIF (soybean embryo factor 1 motif). In summary, the sequencing analysis of the 13 candidate genes indicated that the four cell wall-related genes of the two families extensin and pectin esterase contain mutations in DNA sequence that may change expression level and affect function of these genes leading to the Mi resistance in PI 96354.

Based on gall number, 14 Mi-resistant and 8 Mi-susceptible RILs were selected and the four cell wall candidates were sequenced. Given that all of the 84 SNPs found in four candidate genes in PI 96354 constitutes the PI 96354 haplotype and all in Bossier creates the Bossier haplotype, our data indicated that the 14 Mi-resistant RILs all had the PI 96354 haplotype, while the eight Mi-susceptible RILs had the Bossier haplotype.

Expression analysis of four candidate genes revealed difference between PI 96354 and Bossier

As described above, 51 SNPs and 13 indels/insertions were discovered in promoter and intronic regions of four candidate genes. In addition, PE1 shared 54 % similarity with a pectin esterase gene that was shown to be highly up-regulated in William 82 only when infected with Mi (Ibrahim et al. 2011). Therefore, we carried out an expression experiment with four time points including 6, 12, 18 and 30 dai. Ibrahim et al. (2011) showed that 12 dai is the critical time for gall formation coinciding with the initiation of nematode feeding. The time-point 32 dai was selected because that was usually the time when the difference in gall number can be visually differentiated and counted. Although each replication showed the same trend and pattern of four genes in PI 96354 and Bossier across the four time points, the magnitude of the relative expression levels (RELs) for each gene in each replication were different, causing the average REL from all replications of each genotype to be not significantly different from each other. Therefore, one replication with the highest magnitude is presented in Fig. 2, and the data of four replications are presented in Supplement S4.

In all replications, the expression levels of Glyma10g02090 (Ext1) in Mi-infested PI 96354 plants was 3×

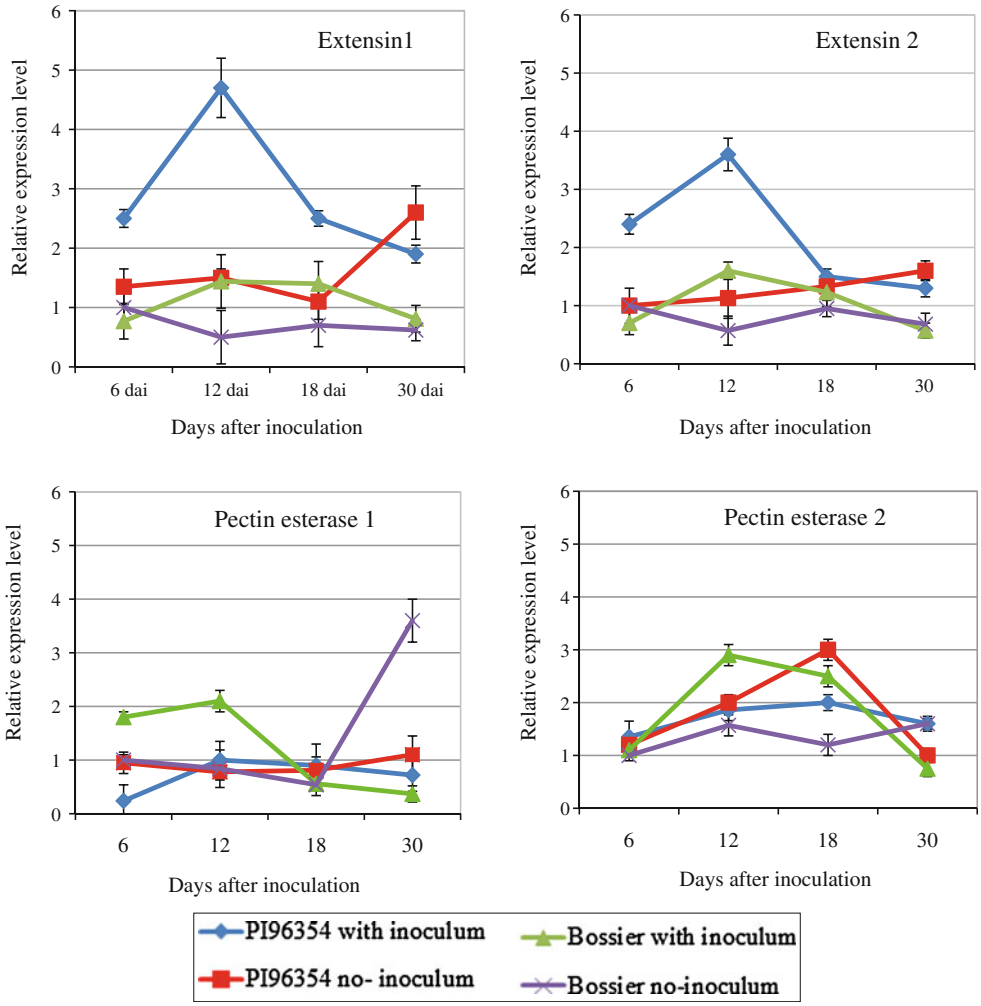

Fig. 2 Relative expression level of four cell wall candidate genes in PI 96354 and Bossier in the presence or absence of root-knot nematode *Meloidogyne incognita*

higher than those of Mi-infested Bossier plants at the four tested time points (Fig. 2). In mock control (no inoculum), the expression level of Ext 1 in PI 96354 was similar to that of this gene in Bossier. The expression level of Ext1 in PI 96354 in the presence of Mi was 2–3× higher than that in PI 96354 in the absence of Mi at 6 and 12 dai, but there was no difference observed at either 18 and 30 dai. For plants that were inoculated with Mi, in replication 1 and 3 there was no difference in expression of Ext2 between PI 96354 and Bossier at the four time points (Fig. 2; Supplement S4).

In replication 2 and 4, the Ext2 gene was expressed in PI 96354 2 to 5× higher than in Bossier at 6 and 12 dai. At 18 and 30 dai, there was no difference in expression level of this gene between PI 96354 and Bossier. This is surprising because replication 1 and 2 have the same inoculation level of 3,000 eggs while replication 3 and 4 have the same inoculation level of 10,000 eggs. Compared to the mock control, PI 96354 plants that were inoculated with Mi have 2× higher expression level of Ext2 at 6 dai, but at 12 dai the gene in Mi-infected plant decreased in expression level to be 3–5× lower than mock control. At 18 and 30 dai, the expression level of Ext2 in mock and infected PI 96354 plants showed no difference (Fig. 2; Supplement S4).

In the mock control, both PI 96354 and Bossier have very low expression of PE1, and it is only at 30 dai in Bossier that PE1 has an increased expression level (up to 3×) compared to other time points. The expression of PE1 in Bossier inoculated with Mi increased 4× compared to that of PI 96354 at 6, 12, and 18 dai, and decreased to the level of PI 96354 at 30 dai (Fig. 2; Supplement S4).

In the mock control, PE2 had the same low expression level in PI 96354 and Bossier at 6, 12, and 30 dai. At 18 dai, PE2 was 2.5× higher in PI 96354 than in Bossier. When inoculated with Mi, PI 96354 still has the same expression levels of PE2 at 6, 12, 30 dai compared to those in the mock control and is down-regulated at 18 dai. In contrast, PE2 in Mi-infected Bossier was up-regulated 2× at 12 and 18 dai compared to mock control (Fig. 2; Supplement S4).

Analysis of four candidate genes in Mi-resistant PIs for potential alternative alleles of pectin esterase and extensin genes

In order to test the hypothesis that the identified mutations in the 4 cell wall candidate genes on chromosome 10 are mainly responsible for the resistance to RKN in PI 96354, these genes were sequenced in additional 20 soybean PIs and 10 soybean cultivars that were reported to have high resistance to Mi based on gall number (Harris et al. 2003; Hussey et al. 1991; Luzzi et al. 1987). These soybean PIs were collected from Japan (10 PIs), China (6 PIs), Korea (3 PIs), and India (1 PI) from 1947 to 1996. We also sequenced these candidate genes in six soybean PIs and six soybean cultivars with high level of susceptibility to Mi for comparison. These soybean cultivars and PIs are listed in Supplement S3. Strikingly, we found that all of the 30 Mi-resistant PIs and cultivars possess the PI 96354 haplotype, while all of Mi-susceptible PIs and cultivars have the Bossier haplotype with the exception of PI 374170, a Mi-resistant line from India. In the Ext 1 gene, PI 374170 has only two SNPs in 3′UTR region, one was an addition of two thymine (T) bases compared to the addition of one T in PI 96354 and the other SNP was a deletion of two T bases that is similar to PI 96354. In extensin 2, PI 374170 also has only two SNPs, one is $A^{473}T$ ($Q^{158}L$) in the exon and one in the 3′UTR region (C>T) (Supplement S1). Both of them are the same as those identified in PI 96354. In PE2, this PI has only two SNPs in the exon region ($A^{105}T$, $T^{396}C$) that are similar to PI 96354, but these are silent mutations. The PE1 gene in this PI has the same SNPs as those found in PI 96354. Beside this PI, there was no alternative allele identified for the four candidate genes in 20 Mi-resistant PIs.

KASP assay development for SNP genotyping

Based on the SNPs from Ext1, Ext2, and PE1 on chromosome 10, 3 KASP assays were developed for genotyping the population of 188 $F_{5:6}$ RILs derived from the cross Bossier × PI96354. A KASP assay for a SNP found in Glyma18g48730 on chromosome 18 (described in "Materials and methods") was also designed. The four KASP assays for Ext1 (Glyma10g02090), Ext2 (Glyma10g02100), PE1 (Glyma10g02140), and EREBP transcriptional factor (Glyma18g48730) were encoded as GSM0038, GSM0039, GSM0040, GSM0041. KASP assays were chosen for detection of these SNPs due to their simplicity and high-throughput for marker-assisted selection.

Overall, these four KASP assays showed capacity to effectively distinguish mutant and wild type alleles in this RILs population (Fig. 3). The SNP alleles detected with KASP assays for each RIL are the same on chromosome 10, which explained 50 % ($P < 0.0001$) of the phenotypic variation while the SNP on chromosome 18 explained 5 % of the phenotypic variation ($P < 0.1$). In addition, when a panel of 236 soybean PIs and germplasm lines were genotyped with the three KASP SNP assays of Ext1, Ext2, and PE1, a complete association was observed between SNP alleles and reaction to Mi recorded for the 158 soybean cultivar and germplasm lines except for N05-7462 and R02-3065 (success rate is 99 %) (Fig. 3; Supplement S5). These two soybean lines were evaluated for Mi reaction in the USDA Regional Uniform Test in 2005 and 2008 and rated as susceptible, however, the KASP assays indicated they have the PI 96354 haplotype. When these two soybean lines were re-screened with Mi using the same protocol, they were found to be resistant to Mi. For the set of 78 PIs, three KASP SNP assays for three genes on chromosome 10 identified five PIs from Japan (PI 227687, PI 416826A, PI 417128, PI 507008, PI 594149), one from Malaysia (PI 197182), and one from Vietnam (PI 605791A) that also had the PI 96354's haplotype for four candidate genes while the rest have Bossier's haplotype (Supplement S5). These PIs were then sequenced using primers of the four candidate genes and were confirmed to possess the same mutant alleles for the four candidate genes present in PI 96354. When these seven PIs were inoculated with Mi, only PI 197182, PI 416826A and PI 507008 had moderate resistance to Mi, the other four PIs had as many gall number in roots as Bossier. The KASP assay for the SNP on chromosome 18 indicated that most of the soybean germplasm lines in the panel have wild type alleles.

Discussion

Using plant resistance is the most economical way to prevent yield loss caused by the Mi in soybean and most other crops. Finding candidate gene(s) responsible for the resistance not only provides knowledge about the mechanism of the resistance, but also allows for the development of functional diagnostic markers for effective marker-assisted selection to incorporate the trait into elite soybean cultivars. In this study, we were able to narrow the genomic region of 850 kbps between two flanking markers, Satt358 and Satt492, on chromosome 10 to a 235 kbps region flanked by two SSR markers, BARCSOYSSR 10_0090 and BARCSOYSSR 10_0105. Notably, among 30 annotated genes found in this genomic region, there is no gene encoding for plant resistance proteins with characteristic nucleotide-binding-site-leucine-rich repeat (NBS-LRR). However, we identified four candidate genes belonging to two families (pectin esterase and extensin) in PI 96354 that contained 84 SNPs and indels/insertions, of which many caused amino acid changes or protein truncation.

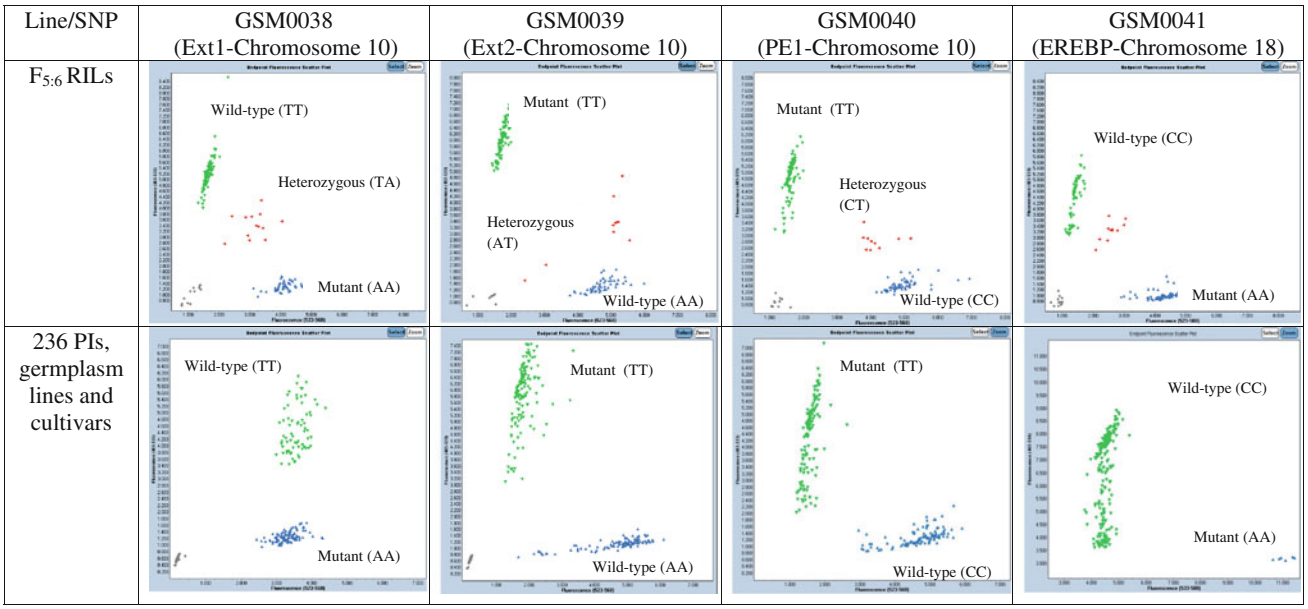

Fig. 3 SNP graphs of KASPar assays for the $F_{5:6}$ RIL population of Bossier x PI 96354 and 236 soybean PIs, germplasms lines and cultivars

Genotyping results using KASP assays developed from these SNPs demonstrated a significant association of the gall number and SNP alleles in the RIL population. The expression levels of these genes in PI 96354 were shown to be different from those of Bossier in the presence of Mi, especially before 12 dai. Sequencing these 4 genes in 20 PIs with Mi resistance from Japan, China, India, and Korea showed that all of the mutant SNP alleles found in these genes in PI 96354 are also present in the 20 Mi-resistant PIs, but not in 12 Mi-susceptible PIs. All of the evidence above indicated that the four cell wall modification genes encoding for pectin esterase and extensin proteins are promising candidate genes in conferring the resistance to Mi in PI 96354.

Entensin genes encode hydroxyproline-rich glycoproteins (HRGPs) that have a pivotal role in cell wall structure, cell shape, and self-defense to both biotic and abiotic stresses (Hall and Cannon 2002; Lamport et al. 2011). In dicots, extensin proteins consist of pentapeptide Ser-$(Hyp)_4$ repeats and a profusion of repetitive motifs composed of Tyr, Lys, His, and Val residues (Lamport et al. 2011). The expression of extensin was demonstrated in many studies to be highly induced in response to wounding, pathogen attack, and elicitors (Corbin et al. 1987; García-Muniz et al. 1998; Guzzardi et al. 2004; Hirsinger et al. 1997; Memelink et al. 1993; Merkouropoulos and Shirsat 2003). Esquerré-Tugayé et al. (1979) first reported the accumulation of extensin mRNA induced by a pathogen, which was anticipated as mechanism of disease resistance (Esquerré-Tugayé et al. 1979). Corbin et al. (1987) supported this hypothesis with evidence that when French common bean was inoculated with the fungi that causes anthracnose (*Colletotrichum lindemuthianum*), the candidate extensin genes in resistant plants had an early accumulation and higher level of mRNA than those in susceptible plants to anthracnose disease. A study on sunflower (*Helianthus annuus L.*) showed that infection with *Sclerotinia sclerotiorum* (Lib.) resulted in the accumulation of extensin proteins within 2 days in a tolerant line but only after 3 days in a susceptible line (Mouly et al. 1992). In soybean, extensin genes were found to be one of the cell wall genes that have the highest level of up/down regulation in response to Mi (Ibrahim et al. 2011). Although neither extensin 1 nor 2 in this study was found to be homologous with the reported extensin gene in literature, the expression pattern of these extensin genes complied with the reported pattern for this gene family in plants in response to pathogens. Comparing extensin 1 and 2, extensin 1 is specifically expressed only in roots under normal conditions, while extensin 2 is expressed in both flowers and roots (Severin et al. 2010). Higher accumulation of extensin 1 protein is predicted to create cell wall rigidification or lignification to prevent the nematode from loosening cell walls to create giant cells, penetrating into the root cells, and moving from cell to cell. An overexpression of cell wall components was suggested to help resist the diffusion of toxins or elicitors of the nematode (Almagro et al. 2009). Another hypothesis was that extensins may immobilize pathogens by binding to their surfaces or form adhesive polymers because HRGPs were demonstrated to be the primary constituent of most plant gum exudates (Anderson et al. 1986; Mazau et al. 1987; Wei and Shirsat 2006). The amino acid change in protein sequence of extensin 2 from Ala to Val which is one of the

residue constitutes of the core repeat of extensin protein may create a harder-to-degrade cell wall type for the nematode by enhancing the intermolecular cross-linking (Schnabelrauch et al. 1996).

In addition, the changes in both mRNA and protein sequences of both PE1 and PE2 proteins may also help limit the nematode's invasion in PI 96354. Pectin esterase is an enzyme involved in the process of cell wall break down. Pectin esterase catalyzes the breaking down of methyl ester linkages of the galacturonan backbone of pectic elements to produce acidic pectins and methanol (Cosgrove 1997). Among all the cell wall-related genes reported to be up-regulated in the presence of a nematode, pectin esterase is the most frequently reported gene (Barcala et al. 2010; Favery et al. 1998; Williamson and Hussey 1996). A pectin methylesterase protein 3 gene (PME3) in *Arabidopsis* was strongly expressed and specifically interacts with a cellulose-binding protein secreted by sugar beet cyst nematode *Heterodera schachtii* (Hewezi et al. 2008). Moreover, it was shown that the pme3 knock-out mutant plants have shorter root length and higher resistance to the sugar beet cyst nematode while overexpression of the PME3 gene increases root length and susceptibility. The pectin esterase gene CF808202 was found to be up-regulated 24× and 45× in the Mi-susceptible 'Williams 82' cultivar at 12 dai and 12 weeks after inoculation, respectively, compared to the mock control (Ibrahim et al. 2011). In the present study, both pectin esterase genes were up-regulated in Bossier in the presence of Mi, but suppressed in PI 96354. This may be related to the deletion of 41 and 6 bps in the promoter of PE1 and PE2, respectively, that cover binding sites for many transcriptional factors. Further study using gene overexpression and complementation testing to verify the true role of the four candidate genes to the resistance to Mi in PI 96354 is underway.

The overexpression of extensin proteins and the suppression/truncation of pectin esterase enzymes in PI 96354 suggested a dual effective mechanism to completely hamper nematode from using cell wall-degrading enzymes of either plant or nematode origin to loosen plant cell wall. It was reported that nematodes secret their own enzymes to soften cell walls in root cells to penetrate through the root epidermis as well as migrate within root tissues (Huang et al. 2003, 2005; Popeijus et al. 2000; Rosso et al. 1999; Smant et al. 1998). When a β-1,4 endoglucanase gene, which functions in cell wall digestion, was silenced by RNA interference, J2 infectivity of potato cyst nematode *Globodera rostochiensis* was reduced (Chen et al. 2005). Sequencing the genome of Mi revealed that Mi has 81 cell wall-degrading enzymes that were absent from all other metazoans studied to date. Among these 81 enzymes, 30 were pectin esterase and 20 were expansin, demonstrating the importance of these cell wall-degrading enzymes in the parasitism mechanism of Mi (Abad et al. 2008). In addition to using their own enzymes, nematodes were also shown to manipulate the plant genes to do the same work. Many cell wall-degrading genes in plants were up-regulated upon the penetration of the nematode and pectin esterase and expansin are some of the most frequently reported (Barcala et al. 2010; Favery et al. 1998; Jammes et al. 2005; Williamson and Hussey 1996). Specifically for Mi it was shown that in Mi-infected *Arabidopsis* and soybean plants, pectin esterase and extensin are two genes that have the highest degree of altered expression level (Ibrahim et al. 2011; Jammes et al. 2005). The presence of all of the SNPs in both extensin and pectin esterase gene families in PI 96354 and 20 other PIs with resistance to Mi, but not in Mi-susceptible soybean lines, seems to support the hypothesis that Mi-resistant PIs may employ both gene families to create a barrier preventing Mi from entering the cell. The malfunctioning pectin esterase 2 and truncated enzyme pectin esterase 1 will disable the nematode from exploiting this plant enzyme to degrade cell walls, while the enrichment of extensin 1 and modification of extensin 2 will create a more rigid and harder-to-break cell wall type for cell wall-degrading enzymes of Mi to act on and prevent diffusion of any toxin or elicitors. Interestingly, the resistance to Mi in PI 96354 was found to be caused by the differential emigration of second-stage juveniles (Herman et al. 1991). In this study, it was shown that although more second-stage juveniles (J2) had penetrated roots of PI 96354 than those of Forrest and Bossier by 2 dai, fewer J2 were present in roots of PI 96354 at 4 dai and nearly 70 % of the J2 penetrating the roots of PI 96354 emerged within 5 dai compared to 4 % in Bossier. This is supported by the evidence from the real-time PCR data that the difference in expression level of all of the four candidate genes between PI 96354 and Bossier generally can be seen at 6 and 12 dai.

Currently, six root-knot nematode resistance genes in plants have been characterized and five of them fall into the class of disease resistance proteins with characteristic NBS-LRR structure encoded by R genes (Caromel and Gebhardt 2011). Obviously, the candidate genes identified as being responsible for Mi resistance in the present study do not belong to the canonical class of the R proteins used by other plants to defend against pathogens. However, there is evidence that resistance to nematode in soybean is not always controlled by conventional R genes. The *rgh1* and *Rhg4* loci controlling the resistance to soybean cyst nematode were extensively studied and a leucine-rich repeat receptor-like kinase (LRR_RLK) was patented for both genes (Hauge et al. 2001; Lightfoot and Meksem 2002; Ruben et al. 2006). However, a recent study using artificial microRNA showed that silencing this LRR-RLK gene at the *rhg1* locus does not change the reaction of soybean from resistance to susceptible to soybean cyst

nematode (Melito et al. 2010). In fact, the *rhg1-b* locus was thoroughly studied and the resistance to soybean cyst nematode controlled by *rhg1-b* is demonstrated to be contributed by three genes encoding an amino acid transporter, an α-SNAP protein, and a wound-inducible domain (WI12) protein (Cook et al. 2012). Similarly, using TILLING to identify and phenotype a soybean line possessing a mutant allele of the LRR-RLK gene that was previously identified by map-based cloning strategy at the *Rhg 4* locus, Liu et al. (2011) showed that the LRR-RLK gene is not responsible for the resistance to cyst nematode in soybean (Liu et al. 2011). A serine hydroxymethyl transferase gene was evaluated and proved to be responsible for the resistance gene at the *Rhg 4* locus (Liu et al. 2012). The modifications of cell wall-related genes in PI 96354 in the presence of Mi found in this study again showed that soybean deployed various mechanisms in defense against nematode.

After identifying SNPs from all extensin and pectin esterase genes, we sequenced these genes in 20 Mi-resistant PIs in order to identify other mutant alleles of these genes and try to rule out whether extensin or pectin esterase truly caused underlying Mi resistance in the PIs. However, the result was surprising as these 20 PIs all contain the same mutant alleles for these four genes as PI 96354 except for PI 374170 from India. Ten Mi-resistant landraces from Japan were not bred for resistance to Mi and were collected from seven regions from 1952 to 1977. Six Mi-resistant PIs from China are cultivated soybean lines collected from six different Chinese provinces, but there is the possibility that they may be derived from a common ancestor. Recently, the resistance to soybean cyst nematode conditioned by *Rhg1* locus was demonstrated to be controlled by all of three disparate but tightly linked genes in a 31.2 kb genome section (Cook et al. 2012). Therefore, the presence of the same SNPs in four candidate genes in all of the Mi-resistant PIs found in this study may indicate a similar disease resistance mechanism. The KASP assays and sequencing data also identified an additional seven PIs from three countries Japan, Malaysia, and Vietnam that possess the same SNP alleles as those identified in PI 96354 in the four candidate genes. However, only three of these PIs were found to be resistant to Mi. The susceptibility to Mi found in the other four PIs may need further validation and investigation. If they are truly susceptible to Mi, then it is possible that these PIs may contain some elements that may be negative regulators of the cell wall genes. It is inclusive from this study how all the Mi-resistant PIs collected from different countries in Asia have a high conservation of mutations in the extensin and pectin esterase genes. However, the data suggested that the resistance genes to Mi in soybean PIs are not highly diverse and further study needs to be conducted to determine if the identified Mi-resistant PIs from Asia possess different QTLs from those already discovered. A soybean cultivar was found to carry a major resistance QTL on chromosome 7 to Mi race 2 in South Africa in addition to a minor QTL on chromosome 10 (Fourie et al. 2008). In the same study, PI 96354 was found resistant to the Mi race 2. Although no incidence of a new Mi race that can overcome resistance in a Mi-resistant soybean cultivar has been reported, continuous use of the same resistant soybean cultivars will certainly select for virulent races of Mi. In tomatoes, resistance-breaking nematode races have been demonstrated to develop within 1–3 years. Trials in resistant tomatoes in growth chamber have shown that there are *Meloidogyne* races from Florida that can invade and develop well in resistant tomato cultivars (Brito et al. 2007; Huang et al. 2004). Therefore, using different sources of resistance alleles or pyramiding resistance genes are of importance to create a highly stable resistance to *Meloidogyne incognita* in soybean.

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