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Discovery of two tightly linked soybean genes at the *qSCN10* (O) locus conferring broad-spectrum resistance to soybean cyst nematode

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Soybean cyst nematode (SCN, *Heterodera glycine* Ichinohe) is a major threat to global soybean yield. Resistance genes at the *rhg1* locus from PI 88788 are majorly utilized in 95% of the U.S. breeding programs. Continuous use of this resistance source leads to a shift in the virulence of SCN populations and overcomes host resistance. Therefore, it is necessary to identify alternative SCN resistance sources to combat this ever-changing pest. Previously, we identified an exotic soybean line, PI 567516C, which carries a novel *qSCN10* (O) locus for SCN resistance demonstrating different resistance responses compared to the known *rhg1* and *Rhg4* loci. Here, we narrowed the *qSCN10* QTL region to 142-kb (containing 20 genes). Based on gene expression, gene ontology, *in-silico* analysis, and QTL-based haplotyping, two genes were identified for functional characterization. Overexpression of the transcription factor TGA1-related and Shugoshin C-terminus in the SCN-susceptible Williams 82 reduced the cyst number by 6.4-fold (84.6%) and 5.3-fold (81.2%), respectively. *GmTGA1-10* and *GmSCT-10* Tilling mutants showed high cyst numbers. The two genes associated with the *qSCN10* QTL have significant potential to reduce the SCN population. They also offer an alternative source of durable SCN resistance that is independent of *rhg1* and *Rhg4*.

Soybean cyst nematode (SCN, *Heterodera glycine* Ichinohe) is a microscopic roundworm that feeds on the roots of soybean and was first found in the U.S. in North Carolina in 1954[1], and since then, it has spread to all soybean production areas[2]. Syncytium, a physiological sink that takes energy from the host by competing for water and nutrient uptake, is formed when the nematode sends a signal to the initial feeding cell. The effector proteins secreted by nematodes are the key to unraveling the complete mechanism of parasitism in general, and the effectiveness of different strains with a wide range of virulence[3]. Effector proteins cause suppression or reprogramming of host defense in susceptible soybeans. The host resistance mechanism plays a crucial role during its interaction with the nematode's effector proteins[4,5]. Identification and functional characterization of host resistance genes and their interactions with effector proteins and understanding their corresponding transcription factors that regulate effector-triggered immunity and hormonal signaling networks will advance our understanding of parasitism suppression in resistant soybeans[6,7]. Molecular mechanisms of host defense during SCN invasion are still poorly understood. Most of the identified effector proteins physically interact with and modulate the functions of host proteins involved in various defense mechanisms.

Nematode management strategies require a holistic approach that includes nematicide seed treatment, crop rotation, and the use of host plant resistance[8,9]. Because there are many nematode species in soil, identifying the correct species is essential for determining the most effective control measure. Genetic resistance plays a significant role in controlling SCN populations and offers a positive return on investment. Over the last decade, farmers planted SCN-resistant cultivars as the most efficient management

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strategy to control SCN. However, over 95% of resistant cultivars carry the same source of resistance derived from plant introduction (PI) 88788 that carries SCN resistance genes harbored at the *rhg1-b* locus and provides protection to SCN races 3 (HG type 0) and 14 (HG type 1.3.5.6.7)[9]. Although PI 88788-based cultivars are still more effective than susceptible lines in reducing the number of SCN eggs in plants[10], planting varieties with a single source of resistance causes a tremendous risk of increasing nematode virulence associated with evolutionary changes and natural fitness between the pathogen and its host[9,11]. The continuous use of PI 88788- type resistance caused nematode population shifts, also referred to as "race shift", by decreasing SCN races 3 and 14 and increasing the prevalence of other races that can reproduce on resistant cultivars and the subsequent loss of genetic effectiveness over the last ten years[11–13]. Therefore, SCN management strategies used in the past are no longer efficient due to limited genetic diversity in available soybean breeding programs. Many soybean farmers do not realize their fields offer a buffet for nematodes, despite the use of "resistant" soybean varieties. Non-effective varieties grown on fields with increasing SCN population densities could be compared to a ticking time bomb, and field management is the most challenging[9]. Finding new resistant sources seems to provide real solutions to cope with nematode virulence and pathotype specialization against host resistance genes.

Currently, hundreds of SCN-resistant soybean varieties are derived from either PI 88788 (95% of modern varieties) or Peking (3%), and the same sources of resistance are used in most soybean breeding programs[14]. This limited genetic diversity has created a bottleneck in the development of new soybean varieties carrying novel SCN resistance genes, essential for effectively countering this constantly evolving pest. It is more critical than ever to identify and characterize additional sources of SCN resistance for more effective and durable control of this ever-changing pathogen. Utilizing a global germplasm pool and advanced molecular breeding tools has a tremendous opportunity to develop new varieties with improved resistance targeting additional SCN races.

Soybean accession PI 567516C displays different reactions to multiple SCN races, which are different from known sources of resistance PI 88788 and Peking[15]. PI 88788 and Peking carry known genes at the *rhg1* and *Rhg4* loci, which are race-specific and cause complete resistance to nematode infection[16–20]. Recently, the molecular mechanisms of these genes involving the GmSNAP18 (*rhg1-a*), the GmSHMT08 (*Rhg4*), and a novel pathogenesis-related protein has been revealed[21,22]. Under SCN infection, *GmSNAP18*, *GmSHMT08*, and *GmPR10-08* transcripts were induced in the SCN-resistant line (cv. Forrest), showing a physical interaction at the molecular level which triggers an extreme reaction in the host that prevents cyst formation and limiting cyst structure at a very minimum level[21,22]. Additionally, few other genes involved in SCN resistance have been discovered during the last decade, including the *GmSNAP11*, *t-SNARE-syntaxins* (chrs. 12 and 16), and *GmSYP31A* (chr. 02) in Peking type of resistance and the *rhg1-b* (AAT, α- SNAP, WI12), *NSF*, and *γ-SNAP* (chr. 07) in PI 88788 type of resistance[23]. Unlike other known sources of SCN resistance, PI 567516C shows broad-spectrum resistance to multiple SCN races[15]. This phenomenon suggests a different mode of action as a race-nonspecific type of resistance compared with the known *Rhg* genes. Few nematodes are capable of infecting and reproducing on PI 567516C roots[24]. The broad-spectrum resistance caused by novel genes could be explained by the production of proteins compatible with conserve nematode effector proteins common to many SCN virulent populations. Another explanation could involve restricting nematode in water and nutrients or activating the plant dead signaling pathway. Here, we describe the identification of two novel genes at the *qSCN10* locus that contribute to SCN resistance in PI 567516C. A *transcription factor TGA1-related* was initially reported to regulate effector-triggered immunity and hormonal signaling networks[25]. The second gene, *Shugoshin C-terminus*, was previously described to be required with SGO2 for complete protection of centromeric cohesion during anaphase I mitosis, guiding chromosome cohesion during cell division, and also may function as a stress regulator by binding to and regulating RNA Polymerase (RNAP) II and regulating the transcription of heat shock protein genes[26]. However, to our knowledge, no previous study has shown their involvement in plant pathogen resistance nor for broad-spectrum resistance to SCN.

Results

Progeny test of additional recombinants and high-throughput SCN phenotyping

We identified about 18 individual recombinants as cross-recombinants in the target *qSCN10* region by two SNP markers: O1_Gm10_42597820 and O15_Gm10_42898898. To further genotype these recombinants, four additional SNP markers (O7_Gm10_42756962, O9_Gm10_42780065, O11_Gm10_42817297, and O12_Gm10_42819414) were designed between the two SNP markers to dissect crossover points (Table S1). Six recombinants (W1300, W1500, W1101, W299, W661, and W1783) were selected for SCN phenotyping because the QTL region contains distinct crossover sites (Fig. 1). The progeny tests were carried out for these six recombinants against SCN HG type 1.3.5.6.7 type in the greenhouse, as previously reported[24], in which HG Type 1.3.5.6.7 has been shown the most significant reduction in the FIs in NILs for the *qSCN10* locus[24].

As expected, Magellan (recurrent parent) showed susceptibility with a FI count of 131, while PI 567516C (donor parent & source of the *qSCN10* resistant locus) showed resistance with FI count of 14 against HG Type 1.3.5.6.7 (Table S2). Most of the recombinants containing genomic fragments between the markers O7_Gm10_42756962 and O15_Gm10_4298898 from PI567516C showed significant reductions in FIs according to Tukey's HSD test ($p < 0.001$), ranging from 54% to 73%, in W1783, W299 and W1101 lines compared to Magellan. We did not find any significant differences in the other two recombinants: W1300 and W1500. These genotypic and phenotypic results narrowed the *qSCN10* locus to a 142- kb interval between markers O7_Gm10_42756962 and O15_Gm10_4298898 (Fig. 1). We developed additional KASP markers (Table S3) from the fine-mapped region that can be used to genotype these recombinants; however, these markers are unable to further decrease the target interval.

Haplotype analysis of the *qSCN10* QTL using the 106 diverse soybean panel

Previously, we conducted haplotype analysis to pinpoint the causal variation of several traits, including salt tolerance[27] and SCN resistance[28]. Haplotyping analysis using 106 different soybean accessions provided specific features at the promoter region and coding sequence of the *rhg1* and *Rhg4* genes that correlate with resistance to SCN. Using the same approach, we revealed the lack of functional redundancy within the rest of the *GmSHMT* gene family members in resistance to soybean cyst nematode, including all four classes (cytosol, nucleus, mitochondrion, and chloroplast)[20]. While several haplotypes at the *GmSHMT08c* are resistant to SCN, haplotyping analysis of the 106 lines showed that none of the *GmSHMT* gene family members presented specific *GmSHMT* haplotypes that correlate with SCN resistance. The possible role of the *GmPR08-Bet VI* in resistance to SCN, newly cloned genes that confer SCN resistance, was also explored using the natural variations within the gene in different soybean germplasms. In fact, soybean lines belonging to the *GmPR08-Bet VI-c* haplotype showed resistance to moderate resistance reaction to SCN. However, soybean lines associated with the *GmPR08-Bet VI-a* and *GmPR08-Bet VI-b* haplotypes exhibited a susceptible to moderate susceptible resistance reaction against SCN. Thus, haplotype clustering analysis using the whole genome resequencing data from a collection of 106 diverse soybean germplams (15X) was crucial to identify allelic variants and haplotypes within several SCN-resistant candidate genes.

Similarly, in this study, we used the WGRS data from the 106 diverse soybean accessions comprising wild, landraces, and elite lines[29] and conducted QTL-based haplotype analysis[27] to identify allelic variation at the *qSCN10* QTL (Fig. 2). Briefly, SNP haplotypes were analyzed by generating map and genotype data files, and clustering results for the *qSCN10* QTL genomic regions were depicted using FLAPJACK[30]. SNPs from each line

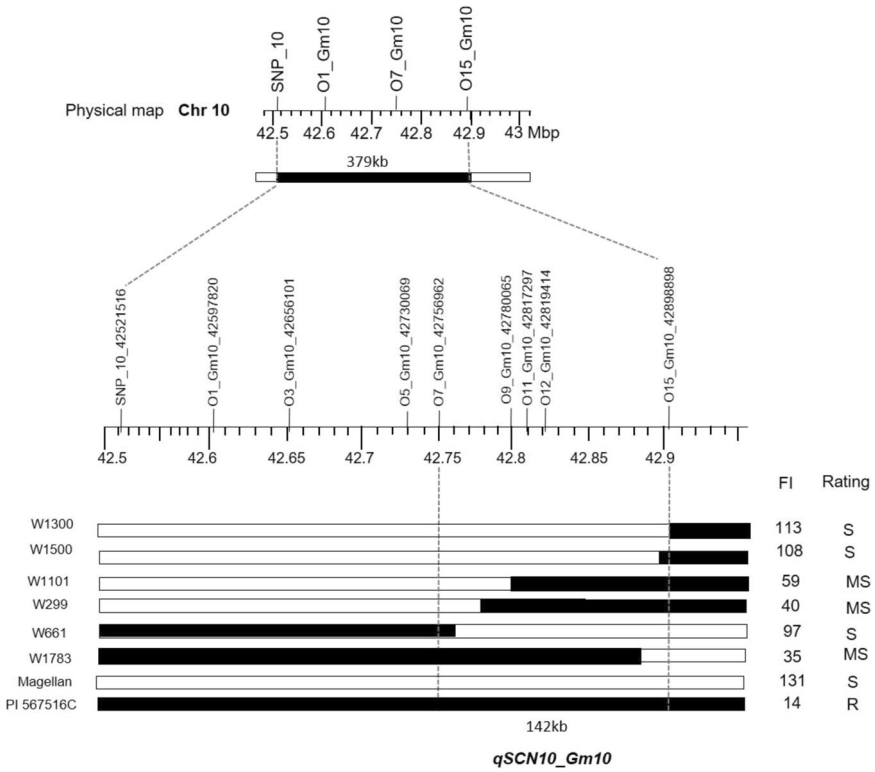


Fig. 1 | Fine-mapping of the *qSCN10* QTL. The target region of *qSCN10* was narrowed down to a 142-kb interval between O7_Gm10_42756962 and O15_Gm10_4298898 in the soybean genome using 6 key near-isogeneic lines (NIL) recombinants. Here, black bars indicate PI 567516C derived sequences, whereas white bars indicate Magellan derived sequences. The SCN phenotypes were demonstrated on the right as resistant (R) and susceptible (S) based on SCN phenotyping assays. The female index is shown as mean ±SE (n > 10), and the Tukey's HSD test determined significant differences.

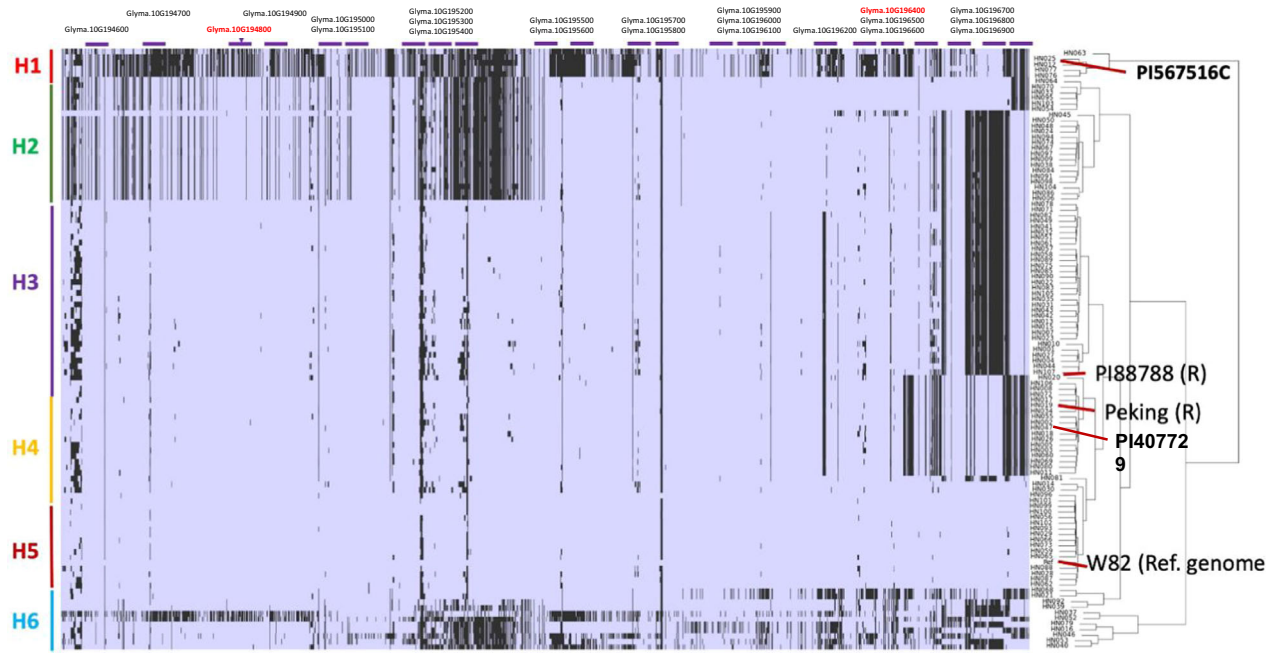
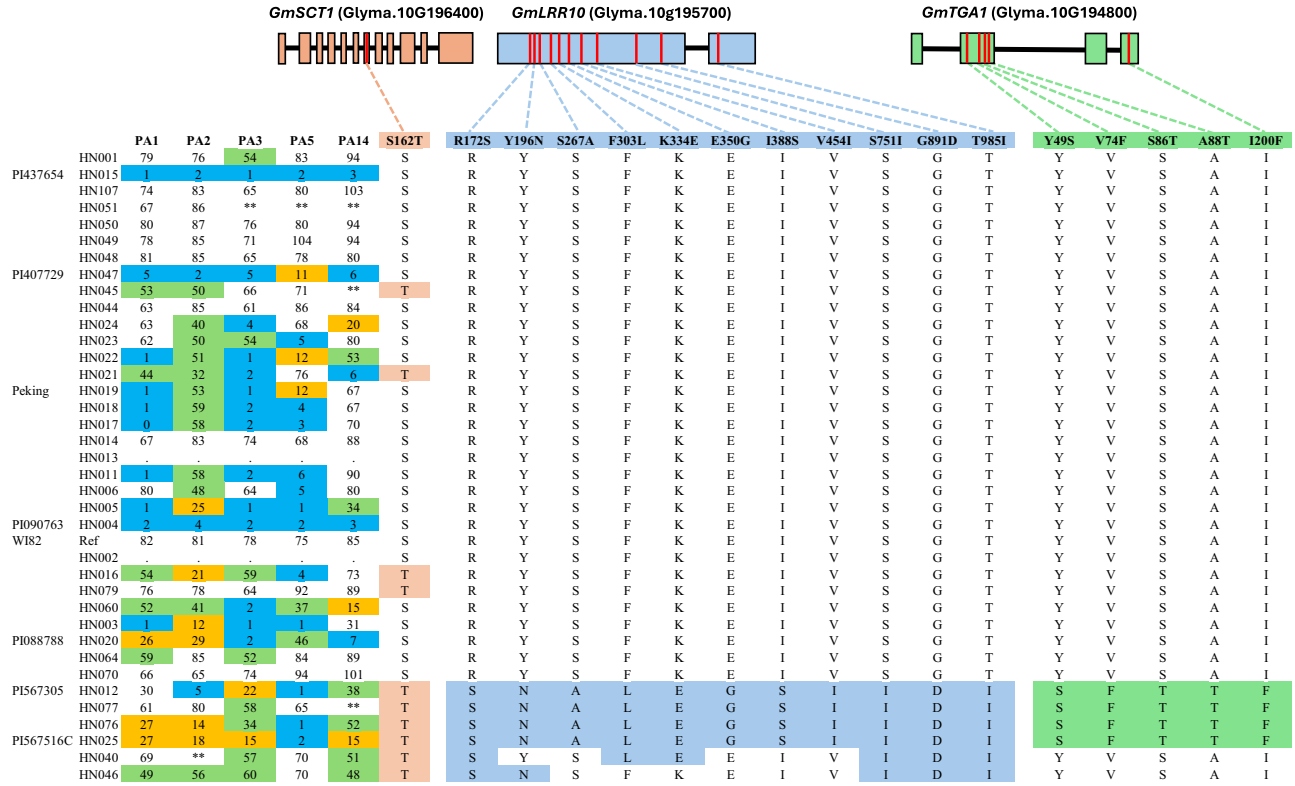


Fig. 2 | Haplotype analysis of qSCN10 QTL using WGRS data suggests unique allelic variation in PI 567516C as compared to known SCN resistant (PI 88788 (*rhg1*) and Peking (*Rhg4*)) lines. *GmTGA1-10* gene is noted in red text. SNPs were positioned relative to the genomic position in the genome version W82.a2. The SNPs in the black background are different from the reference genome ('Williams 82').

were grouped by using a neighbor-joining (NJ) tree and further assessed for synonymous or non-synonymous differences through amino acid sequence translation. SNP diversity metrics, including average pairwise variation within populations (θπ), Watterson's estimator (θw), and Fst, were calculated using methods outlined earlier[29]. We identified six major haplotypes (H1– H6) based on SNP variations. PI 567516C was represented by H1 which carried a unique set of SNPs as compared to known resistant lines with the *rhg1* and *Rhg4* loci.

We performed in-depth haplotype analysis in all the genes in *qSCN10* locus among all 106 soybean germplasms and found multiple SNPs that cause an altered amino acid sequence (non- synonymous) in several genes belonging to H1 haplotype group harboring PI 567516C (Fig. 3, Supplementary Data 1). For instance, *GmTGA1* (Glyma.10G194800) gene has 5 non- synonymous SNPs (Y49S, V74F, S86T, A88T and I200F), *GmNUP1* (Glyma.10G194700) has 10 non-synonymous SNPs (R126G, P173S, H538N, R587G, C911S, F927L, R977S, R1043S, M1063L, and S1143A)



		PA1	PA2	PA3	PA5	PA14	S162T	R172S	Y196N	S267A	F303L	K334E	E350G	I388S	V454I	S751I	G891D	T985I	Y49S	V74F	S86T	A88T	I200F
	HN001	79	76	54	83	94	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
PI437654	HN015	1	2	1	2	3	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN107	74	83	65	80	103	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN051	67	86	**	**	**	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN050	80	87	76	80	94	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN049	78	85	71	104	94	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN048	81	85	65	78	80	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
PI407729	HN047	5	2	5	11	6	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN045	53	50	66	71	**	T	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN044	63	85	61	86	84	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN024	63	40	4	68	20	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN023	62	50	54	5	80	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN022	1	51	1	12	53	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN021	44	32	2	76	6	T	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
Peking	HN019	1	53	1	12	67	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN018	1	59	2	4	67	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN017	0	58	2	3	70	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN014	67	83	74	68	88	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN013	.	.	.	.	.	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN011	1	58	2	6	90	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN006	80	48	64	5	80	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN005	1	25	1	1	34	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
PI090763	HN004	2	4	2	2	3	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
WI82	Ref	82	81	78	75	85	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN002	.	.	.	.	.	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN016	54	21	59	4	73	T	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN079	76	78	64	92	89	T	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN060	52	41	2	37	15	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN003	1	12	1	1	31	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
PI088788	HN020	26	29	2	46	7	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN064	59	85	52	84	89	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
	HN070	66	65	74	94	101	S	R	Y	S	F	K	E	I	V	S	G	T	Y	V	S	A	I
PI567305	HN012	30	5	22	1	38	T	S	N	A	L	E	G	S	I	I	D	I	S	F	T	T	F
	HN077	61	80	58	65	**	T	S	N	A	L	E	G	S	I	I	D	I	S	F	T	T	F
	HN076	27	14	34	1	52	T	S	N	A	L	E	G	S	I	I	D	I	S	F	T	T	F
PI567516C	HN025	27	18	15	2	15	T	S	N	A	L	E	G	S	I	I	D	I	S	F	T	T	F
	HN040	69	**	57	70	51	T	S	Y	S	L	E	E	I	V	I	D	I	Y	V	S	A	I
	HN046	49	56	60	70	48	T	S	N	S	F	K	E	I	V	I	D	I	Y	V	S	A	I

Fig. 3 | Haplotype clustering of qSCN10 locus harboring *GmSCT1* (Glyma.10G196400), *GmLRR10* (Glyma.10g195700), and *GmTGA1* (Glyma.10G194800) genes in different soybean lines. Schematic graphs depict the position of amino acid change (non-synonymous SNP/indel) for *GmSCT1*, *GmLRR10*, and *GmTGA1* genes. The SNPs in orange (*GmSCT1*), blue (*GmLRR10*) and green (*GmTGA1*) background in H1 haplotype group containing PI 567516C are different from the reference genome ('Williams 82'). In the gene model figure (top of the figure), the box denotes exons, the black bar represents introns, and the red lines represent SNP/ changes. SNPs/ haplotypes were located relative to the genomic position in the genome version W82.a4.

(Figure S1), *GmLLR1-PK-10* (Glyma.10g195700) has 11 non- synonymous SNPs (R172S, Y196N, S267A, F303L, K334E, E350G, I388S, V144I, S431N G891D, and T675I), and the *Shugoshin-1* (Glyma.10G196400) has 1 non-synonymous SNP in PI 567516C in H1 haplotype group at the *qSCN10* locus that highly correlate with SCN resistance (Fig. 3, Supplementary Data 1). This analysis confirmed that PI 567516C has a unique SNP variation in *qSCN10* locus, leading to an amino acid change. This analysis helped narrow down the candidate genes and focus on the H1 haplotype (Supplementary Data 1).

SNPs in promoter regions and UTRs can alter gene function and expression levels as shown earlier[31,32]. Several researchers have successfully shown that alteration in promoter sequences harboring SNPs leads to variation in phenotypes such as enhanced disease tolerance and increased yield[33,34]. Here, we performed haplotype analysis for promoters of three genes from the *qSCN10* QTL namely *GmTGA1-10* (Glyma.10g194800), *GmSCT-10* (Glyma.10G196400) and *GmLRR- PK-10* (Glyma.10g195700). These genes harbor unique SNPs and Indels in their promoter regions in resistant line PI 567516C (HN025) than susceptible WI82 (Supplementary Data 2). This analysis indicates that SNPs/Indels detected in *GmTGA1-10, GmSCT-10*, and *GmLRR-PK- 10* promoters may be contributing to altered transcript abundance and thereby SCN resistance in PI 567516C.

Identification of potential candidate genes for resistance to SCN at the *qSCN10* locus in PI 567516C

The 142-kb interval in the *qSCN10* locus contains 20 candidate genes for SCN resistance (Table 1). After narrowing the QTL from 379-kb to a 142-kb region, we followed a three-step procedure to select promising candidate genes for further functional characterization.

The presence of non-synonymous SNPs was crucial to identify several genes for resistance to SCN, including the two major genes in Peking-type resistance; the *rhg1-a* (*GmSNAP18*) and the *Rhg4-a* (*GmSHMT08*). The GmSHMT08 showed the presence of two non-synonymous SNPs (R130P and Y358N)[17] and the GmSNAP18 showed 8 non-synonymous SNPs (Q203K, D208E, E285Q, D286Y, D286H, D287E, −288A, −288V and L289I)[16] between the SCN-resistant and SCN-susceptible soybean varieties[28]. Therefore, as the first selection criterion, we compared non-synonymous SNPs within these 20 candidate genes between Magellan and PI 567516C parents. Out of the 20 candidate genes, only seven genes contained non-synonymous SNPs. Nine non-synonymous SNPs were present in the *GmNUP1-10*, five non-synonymous SNPs in *GmTGA1-10*, and one non-synonymous SNPs in Shugoshin-1 (*GmSCT-10), leucine-rich repeat receptor-like protein kinase* (*GmLLR1-PK*), Myb family transcription factor (*GmMYB-TF*), *K4BPD4 MATE efflux family protein*, and the unknown protein (Glyma.10G195100) (Table 2, Fig. 4). While the rest of the genes (13 genes) did not show any non-synonymous SNPs (Fig. 4). Therefore, we selected these 7 candidate genes containing non-synonymous SNPs, and discarded the rest of the 13 candidate genes at the *qSCN10* locus since they presented similar coding sequences between the SCN-susceptible line Magellan and the SCN-resistant PI 567516C.

Next, as a second criterium to narrow further the list of candidate genes that might play a role in SCN resistance, we compiled gene expression under SCN infections in PI 567516C of the selected seven candidate genes[35]. Soybean breeding to target and functionally characterize essential genes to improve important agronomic traits (i.e. oil, protein, yield, resistance to biotic and abiotic stresses) is challenging due to the presence of two duplication events that occurred in the soybean genome about 13 and 59 million years ago, resulting in a highly duplicated genome with nearly 75% of the genes present in multiple copies[36]. The use of gene expression analysis was very useful to select top candidate genes that are involved in several quantitative traits[19,20,37–42]. Previously, we performed expression analysis of soybean root tissue from SCN-infected and non-infected using the two soybean lines; Magellan and PI 567516C[35]. Gene expression analysis showed

Table 1 | Functional annotation of the genes identified within the 142-kb fine-mapped region of *qSCN10* locus

Gene candidate	Start	End	Gene Annotation
Glyma.10g194700	42670924	42678035	FI19922P1-related
Glyma.10g194800	42686749	42691742	Transcription Factor TGA1-Related
Glyma.10g194900	42694105	42699129	N-terminal C2 in EEIG1 and EHBP1 proteins (NT-C2)
Glyma.10g195000	42702305	42705748	7-methylguanosine nucleotidase/N(7)-methylguanylate 5'-phosphatase
Glyma.10g195100	42705870	42706336	7-methylguanosine phosphate-specific 5'-nucleotidase
Glyma.10g195200	42708491	42710238	Glutaredoxin family protein
Glyma.10g195300	42711052	42712419	Plastocyanin-like domain (Cu_bind_like)
Glyma.10g195400	42713356	42713802	Ring-H2 finger protein ATL70
Glyma.10g195500	42720899	42721369	Ring-H2 finger protein ATL70
Glyma.10g195600	42722946	42727022	SF17 - SNF7 - RELATED
Glyma.10g195700	42731233	42735014	leucine-rich repeat receptor-like protein kinase
Glyma.10g195800	42735927	42737795	Mannosylfructose-phosphate synthase / MFPS
Glyma.10g195900	42743520	42746720	Dinucleosidetetraphosphatase (asymmetrical)
Glyma.10g196000	42749400	42751583	Uncharacterized protein
Glyma.10g196100	42752699	42761978	Exocyst subunit EXO70 Family Protein A3-RELATED
Glyma.10g196200	42765160	42768645	Nuclear transport factor 2 (NTF2) FAMILY PROTEIN
Glyma.10g196300	42778690	42782165	ATP-dependent CLP protease
Glyma.10g196400	42788040	42790903	Shugoshin C-terminus
Glyma.10g196500	42797101	42798670	Protein of unknown function PUF
Glyma.10g196600	42802270	42805638	MYB Family Transcription Factor

Table 2 | List of Non-synonymous SNPs and effect on the amino acid change of the candidate genes at the *qSCN10* locus in PI 567516C with reference to Magellan

Gene id	Position (W82 v4)	Ref_allele	Alt_allele	SNP effect annotations of amino acid	Mag	PI516
Glyma.10G194700	42758112	A	G	Arg126Gly	Ref	Alt
Glyma.10G194700	42758944	C	T	Pro173Ser	Ref	Alt
Glyma.10G194700	42760303	C	A	His538Asn	Ref	Alt
Glyma.10G194700	42760698	A	G	Arg587Gly	Ref	Alt
Glyma.10G194700	42762400	T	A	Cys911Ser	Ref	Alt
Glyma.10G194700	42762450	T	A	Phe927Leu	Ref	Alt
Glyma.10G194700	42762798	G	T	Arg1043Ser	Ref	Alt
Glyma.10G194700	42762856	A	T	Met1063Leu	Ref	Alt
Glyma.10G194700	42763096	T	G	Ser1143Ala	Ref	Alt
Glyma.10G194800	42773840	A	C	Tyr49Ser	Ref	Alt
Glyma.10G194800	42773914	G	T	Val74Phe	Ref	Alt
Glyma.10G194800	42773950	T	A	Ser86Thr	Ref	Alt
Glyma.10G194800	42773956	G	A	Ala88Thr	Ref	Alt
Glyma.10G194800	42777325	A	T	Ile200Phe	Ref	Alt
Glyma.10G194900	42782615	A	G	Ile112Val	Ref	Alt
Glyma.10G195100	42792115	A	C	Leu33Val /Arg172Ser	Ref	Alt
Glyma.10G195700	42817961	A	C	Arg172Ser	Ref	Alt
Glyma.10G196400	42875484	T	A	Ser162Thr	Ref	Alt
Glyma.10G196600	42891281	G	C	Ser324Thr	Ref	Alt

that out of the seven candidate genes at the *qSCN10* locus showing non-synonymous SNPs, three genes showed higher transcript abundance under SCN infection in PI 567516C including the *Shugoshin C-terminus* (*GmSCT-10; Glyma.10G196400*), the *leucine-rich repeat receptor-like protein kinase* (*GmLRR-PK-10*; *Glyma.10g195700*), and the *transcription factor TGA1-related* (*GmTGA1-10*; *Glyma10g194800*). *GmSCT-10* and *GmTGA1-10* were induced at an early stage (2 days after SCN infection) in the resistant soybean line PI 567516C, while *GmLRR-PK-10* transcripts were induced at the late stage of infection (10 days after SCN infection). *GmSCT-10* transcripts were induced up to 3.5-fold under SCN infection, *GmTGA1-10* transcripts were 3.6-fold induced, while *GmLRR-PK-10* transcripts were 2-fold induced under SCN infections in roots (Fig. 4). However, the rest of the other four genes showed either moderate gene expression, barely detected, or non-detected transcripts. Thus, due to the low or absence of

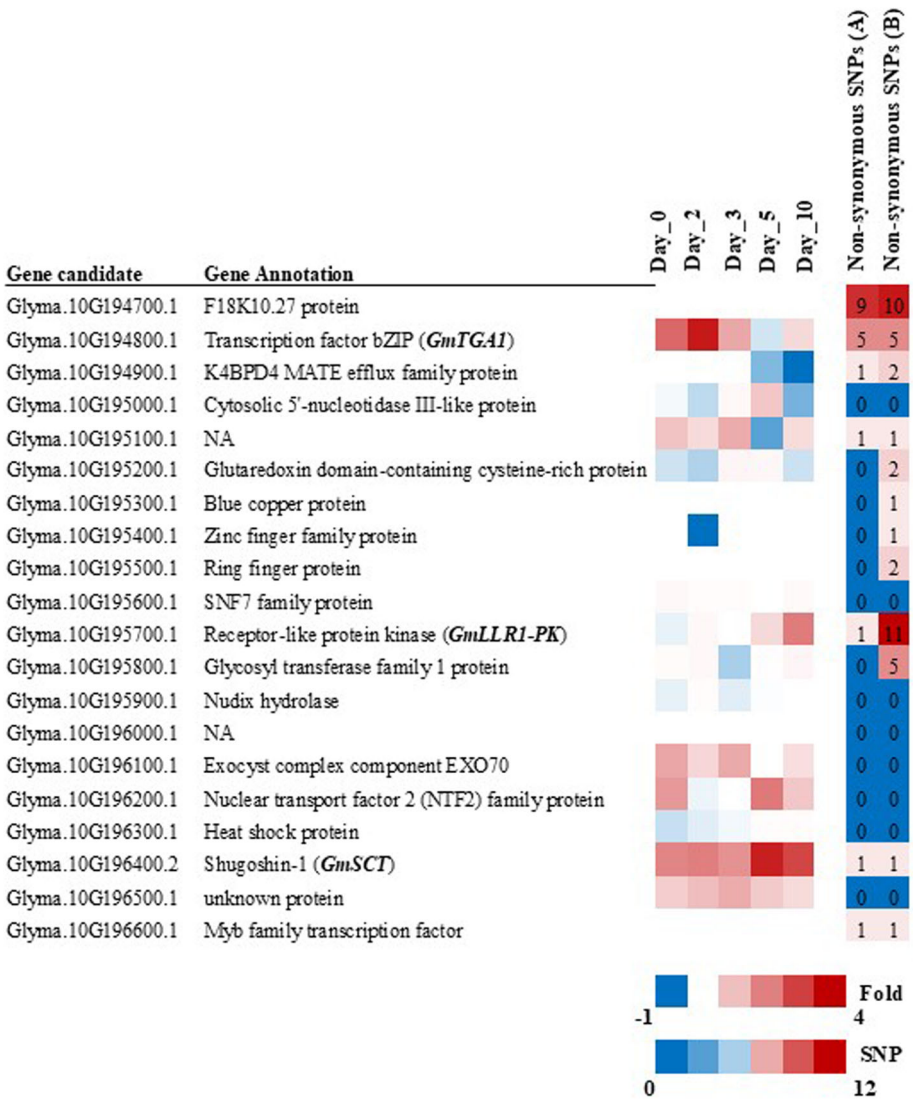


Fig. 4 | RNAseq expression analysis of the 20 candidate genes at the *qSCN10* locus in SCN-infected PI 567516C roots at 0, 2-, 3-, 5-, and 10-days post-inoculation. No expression data were available for Glyma.10G195400, Glyma.10G195500, and Glyma.10G196000 genes. Non- synonymous SNPs (A) represents the number of Non-synonymous SNPs present at the 20 candidate genes at the *qSCN10* locus between the 2 parents only (Magellan and PI567516C). Non- synonymous SNPs (B) represents the number of Non-synonymous SNPs present at the 20 candidate genes at the *qSCN10* locus among all the 106 soybean germplasms analyzed.

transcripts induction/expression under SCN infection in the resistant PI 567516C soybean roots (Fig. 4), these four genes were not selected for further functional characterization. Considering the previous haplotyping analysis (gene indicating the presence of non-synonymous SNPs) (Figs. 2, 3) and gene expression analysis (genes displaying increased gene expression in the root of PI 567516C in comparison to Magellan) (Fig. 4, S2), only three genes (*GmSCT-10*, *GmTGA1-10*, and *GmLRR-PK-10*) were chosen for further characterization and deemed as potential candidates for SCN resistance.

The third criterium consisted of the analysis of putative *cis-elements* in the promoter region (−2 Kb) upstream of the translation start codon of the three selected genes; *GmSCT-10, GmTGA1- 10*, and *GmLRR-PK-10*. The analysis showed several losses and gains in *cis-elements* between PI 567516C & Magellan (Supplementary Data 3–5). In the case of *GmTGA1-10*, while 86 matrix families were conserved between PI 567516C and Magellan, five matrix families were specifically present in PI 567516C and eight in Magellan promoter (Fig. 5A, Supplementary Data 6-A). For the *GmSCT-10*, while 80 matrix families were conserved between PI 567516C and Magellan, two matrix families were specifically present in PI 567516C and two were present in the Magellan promoter (Fig. 5B, Supplementary Data 6-B). For the *GmLRR-PK-10* gene, no differences were observed between the resistant and susceptible lines since all 92 matrix families were conserved between PI 567516C and Magellan (Fig. 5C, Supplementary Data 6-C).

Among all the *cis-elements* identified, the following five *cis-elements* FORC, TCPF, HOCT, TRIH, and E2FF are specifically present at the *GmTGA1-10* promoter (Supplementary Data 3), and the following two *cis-elements* SCAP and BBZF are only present at the *GmSCT-10* promoter (Fig. 4, Supplementary Data 4) in the resistant PI 567516C. The exclusive five *cis-elements* in *GmTGA1-10* are majorly involved in fungal and oomycete pathogen response (FORC), cell cycle regulators (E2FF), histone modifications (HOCT) indicating their crucial involvement in plant immune response, and cell cycle regulations (Supplementary Data 3). While the other two *GmSCT-10* unique *cis-elements* play a key role in protein-protein interactions along with biotic stress (BBZF) and cell cycle regulation (SCAP) (Supplementary Data 4). suggesting the importance of this gene in conferring SCN resistance in PI 567516C.

Moreover, we investigated if the three *GmSCT-10*, *GmTGA1-10* and *GmLRR-PK10* genes have any common unique *cis-elements* in their promoters among the two parents. While none of the *GmSCT-10*, *GmTGA1-10*, and *GmLRR-PK10* carried a common *cis element* at the PI 567516C promoter (Fig. 5E, Supplementary Data 6-K), the "NAC domain-containing protein 87" was found to be commonly present in the promoter of *GmSCT-10* and *GmTGA1-10* genes (while absent in *GmLRR-PK10*) in Magellan, but not in PI 567516C (Fig. 5F, Supplementary Data 6-L). The presence of the "NAC domain-containing protein 87" *cis-elements* in both *GmSCT-10* and *GmTGA1-10* genes in the SCN susceptible soybean Magellan, but not in the

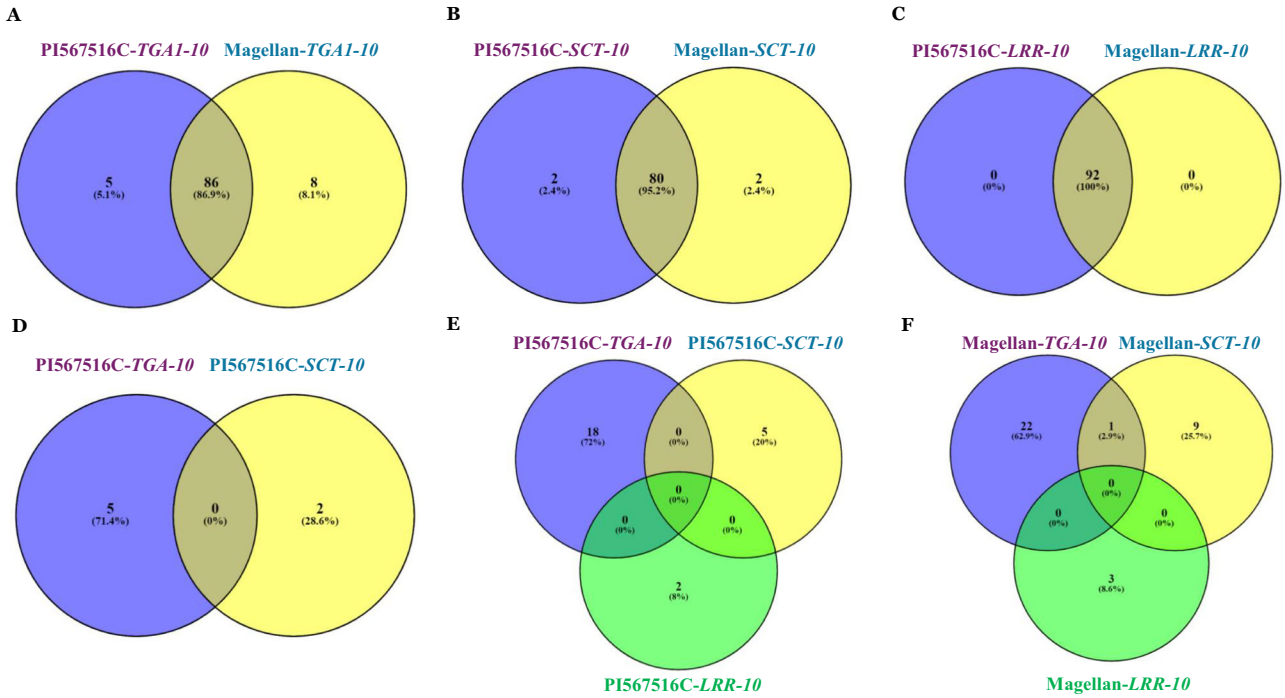


Fig. 5 | Venn diagrams revealing all common, non-common, and unique *cis-elements* that are present among the *GmSCT-10*, *GmTGA1-10*, and *GmLRR/RLK-10* gene's promoter in PI 567516C and Magellan. A–C Venn diagram showing common and non-common *cis-elements* within the three-candidate gene's promoter (*GmSCT-10, GmTGA1-10*, and *GmLRR/RLK-10*) in PI 567516C and Magellan. **D** Among all the *cis-element* identified, five *cis-elements* (FORC, TCPF, HOCT, TRIH, and E2FF) are specifically present at the *GmTGA1-10* promoter (Supplementary Data 3), and 2 *cis- elements* (SCAP and BBZF) are only present at the *GmSCT-10* promoter (Supplementary Data 4) in the resistant PI 567516C. **E, F** Only *cis-elements* that were uniquely present either in PI 567516C or in Magellan were compared. The analysis showed the presence of a common element, NAC domain- containing protein 87, between *GmSCT-10* and *GmTGA1-10* genes (but not in *GmLRR-10*) in the Magellan promoter.

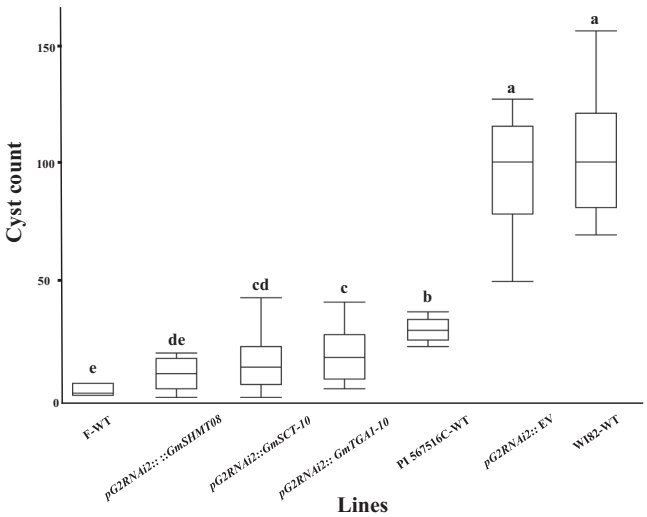


Fig. 6 | Overexpression analysis in transgenic WI82 composite roots transformed by *pG2RNAi2::GmSCT-10* and *pG2RNAi2::GmTGA1-10* and SCN phenotype of the different soybean lines (wild type and transgenics) using HG type 0 (race 3). The data shown represent the averages from all three biological repeats (n = 53 for *pG2RNAi2*::*GmSCT-10* and n = 42 for *pG2RNAi2*::*GmTGA1-10*). F-WT: Forrest wild-type (n = 19), PI 567516C-WT: PI 567516C wild- type (n = 10), and WI82-WT: Williams 82 wild-type (n = 9) (Supplementary Data 8). *GmSHMT08* (*Rhg4*) was used as a positive control (n = 14). *pG2RNAi2*::EV (Empty Vector) was used as negative control (n = 10). Connecting letters indicate significant differences between the tested lines as determined by ANOVA (P <0.0001). The experiments were repeated three times, and similar results were obtained.

SCN-resistant PI 567516C indicated that this *cis-element* might play a role in SCN resistance.

Thus, promoter analysis demonstrated that both *GmSCT-10* and *GmTGA1-10* genes showed several important *cis-elements* including "NAC domain-containing protein" that was commonly present in the promoter regions of *GmSCT-10* and *GmTGA1-10* genes, while absent in *GmLRR-PK10* promoter. Therefore, promoter analysis pointed to the possible involvement of *GmSCT-10* and *GmTGA1-10* genes in SCN resistance, but not *GmLRR-PK10*, and thus were selected for further functional characterization. Taken together, and considering all previous three-step procedures described above, both *GmSCT-10* and *GmTGA1-10* were selected for functional characterization including gene overexpression using hairy root composite plant transformation in SCN-susceptible Williams 82 soybean lines, in addition to mutational analysis using TILLING-by-sequencing^{+} technology.

Functional characterization of *GmTGA1-10* and *GmSCT-10* using hairy root composite plant transformation

To test the effect of the *GmSCT-10* and *GmTGA1-10* genes on SCN resistance, we overexpressed their corresponding coding sequences in susceptible genotype Williams 82. To conduct the *GmSCT-10* and *GmTGA1-10* overexpression analysis, the nucleotide coding sequence of the *GmSCT-10* and *GmTGA1-10* genes, in addition to the *GmSHMT08 (Rhg4-a)* (positive control) and *WI82::EV* (empty vector, negative control) were overexpressed under the control of a soybean ubiquitin promoter using a transgenic hairy root system. As expected, WI82-WT and *pG2RNAi2::EV* showed a susceptible reaction to SCN infection (HG type 0) with 102 and 96 cyst numbers (CN) on average, respectively (Fig. 6). Overexpression of the positive control *pG2RNAi2::GmSHMT08* in transgenic hairy roots

decreased the CN to 10% showing a resistant reaction to SCN (HG type 0) when compared to WI82-WT and *pG2RNAi2::EV*. Most importantly, overexpression of *pG2RNAi2::GmSCT-10* and *pG2RNAi2::GmTGA1-10* genes reduced the CN from 96 contained in *pG2RNAi2::EV* to 15 and 18, respectively (Fig. 6). Thus, overexpression of both *GmSCT-10* and *GmTGA1-10* genes in susceptible Williams 82 transgenic roots reduced cyst by 6.4-fold (84.6%) ($P < 0.0001$) and 5.3-fold (81.2%) ($P < 0.0001$) when compared to the control (*pG2RNAi2::EV*) (Fig. S3).

Investigating the role of *GmTGA1-10* and *GmSCT-10* in a closely related source of SCN resistance using TbyS$^+$

Hairy root transformation brought evidence that the two *GmTGA1-10* and *GmSCT-10* genes play a role in SCN resistance in PI 567516C. While we could not isolate any EMS TILLING mutants in PI 567516C for the *GmTGA1-10* and *GmSCT-10* genes, we decided to investigate the role of both *GmTGA1-10* and *GmSCT-10* genes using other closely related sources of SCN resistance in soybeans such as PI 407729. PI 407729 is genetically and phenotypically similar to PI 567516C[24,28]. Both PI 407729 and PI 567516C carry the *rhg4-b* coding sequence susceptible haplotype (Williams 82-like). Additionally, PI 407729 and PI 567516C share the same *Rhg4-a* promoter (Peking-like)[28]. PI 407729 is known to have broad resistance to SCN HG types (2.5.7, 1.2.5.7, 0, 2.5.7, 1.3.5.6.7) [races 1, 2, 3, 5, 14], which is similar to PI 567516C, and does not require the typical SCN resistance *Rhg4-a* haplotype[24,28]. Altogether, SCN resistance offered by both PI 407729 and PI 567516C are independent of the resistant *rhg1* and *Rhg4* loci.

To investigate the role of the *GmSCT-10* and *GmTGA1-10* genes in closely related sources of SCN resistance, we screened our soybean EMS mutant collection using a reverse genetic approach. We developed earlier a high-throughput TILLING-by-Sequencing$^+$ (TbyS+) technology, along with universal bioinformatics tools, to identify mutations across soybean populations[37,39,42]. Based on seed quality and availability, we selected three *Shugoshin C- terminus* and three *transcription factor TGA1-related* EMS TILLING mutants. At least ten plants of each of the TILLING mutants were screened against HG type 0 and HG type 2.5.7 SCN races. The identified *GmTGA1-10* $_{E213K}$ and *GmSCT-10*$_{M417I}$ PI 407729 background mutants have been next mapped on their corresponding protein homology models. *GmTGA1-10*$_{E213K}$was mapped at the DOG1 conserved (controlling seed dormancy) (Fig. 7A), while *GmSCT-10*$_{M417I}$ was mapped at the Shugoshin C-terminus (protects Rec8 at centromeres during meiosis) (Fig. 7B).

To elucidate the impact of the isolated TILLING mutations, both *GmTGA1-10*$_{E213K}$ and *GmSCT-10*$_{M417I}$ mutants were screened for their reaction under SCN infections. Under HG type 0 SCN infection, the PI 407729 wild-type presented a moderate resistant reaction to SCN (21 CN on average) (Fig. 7C). The *transcription factor GmTGA1-10*$_{E213K}$ EMS mutant showed increased CN to 53 in average (max of 61 CN) (P <0.0001) when

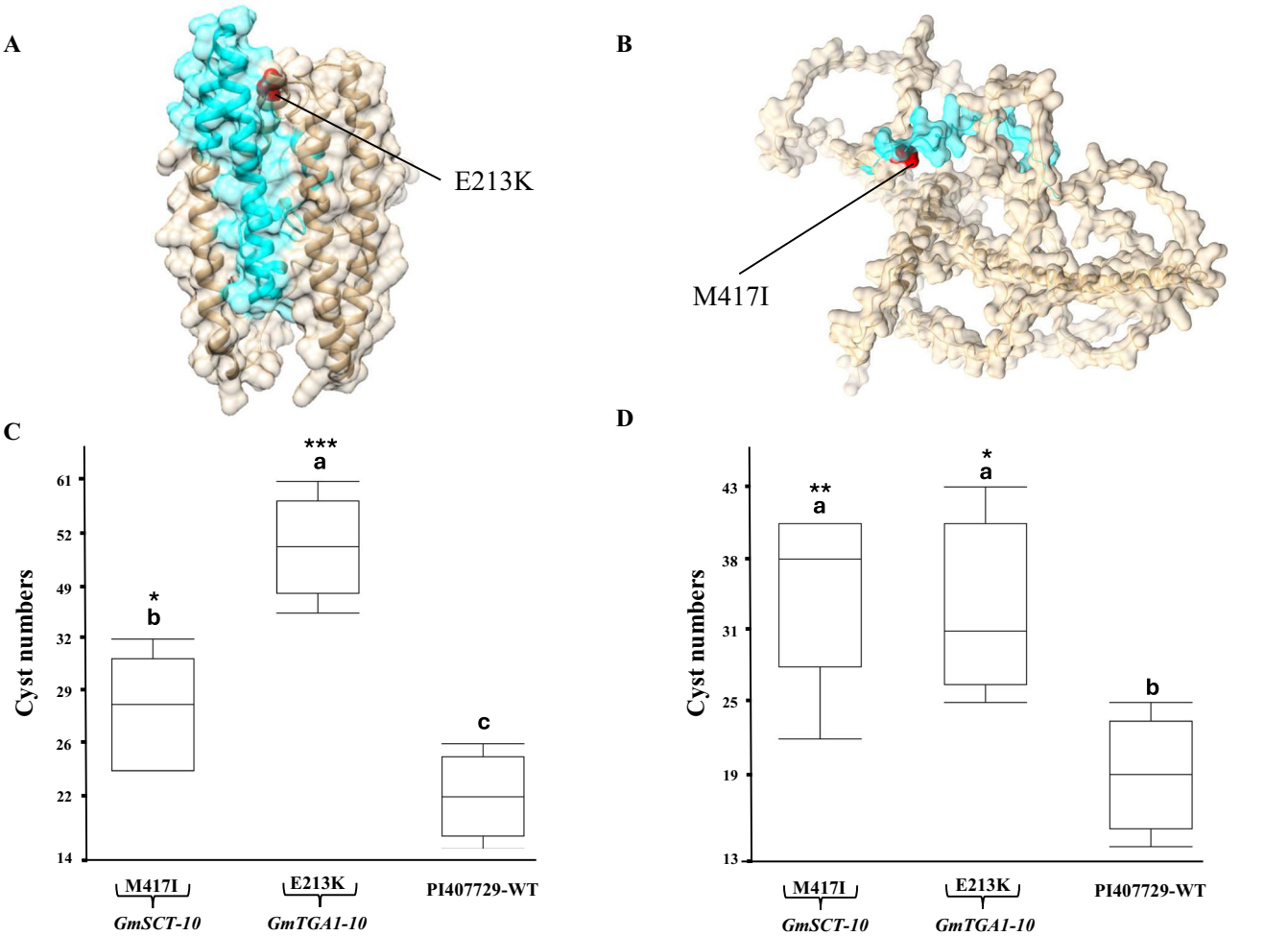


Fig. 7 | Protein homology modeling of the GmTGA1-10 (A) and GmSCT-10 (B) proteins and SCN screening of the isolated *Gmtga1-10* and *Gmsct-10* TILLING mutants. In cyan, DOG1 conserved domain (**A**) at the GmTGA1-10 protein (conserved residues 47-124; NCBI domain architecture ID 10624400) which controls seed dormancy and (**B**) Shugoshin C-terminus at the GmSCT-10 protein (conserved residues 395-419; NCBI domain architecture ID 10540851) which protects Rec8 at centromeres during meiosis. SCN HG type 0 (race 3) (**C**) and HG type 2.5.7 (race 5) (**D**) screening of the isolated EMS TILLING *Gmtga1-10* and *Gmsct-10* mutants. Connecting letters indicate significant differences between the tested lines as determined by ANOVA (***P <0.001, **P < 0.005, *P <0.05) (n = 5, 6) (Supplementary Data 9).

compared to the wild-type (Fig. 7C). The *Shugoshin C-terminus* $GmSCT_{M417I}$ mutant showed also increased CN to 26 in average (max of 32 CN) when compared to the wild-type (Fig. 7C).

Under HG type 2.5.7 SCN, PI 407729 wild-type showed a moderate resistant reaction to SCN with 18 CN on average. The *transcription factor TGA1-related GmTGA1-10E213K* mutant showed increased CN to 33 in average (max of 43 CN) (P <0.05) when compared to the wild-type (Fig. 7D). The Shugoshin C-terminus GmShugoshin-C-terM417I mutant showed increased CN to 35 in average (max of 41 CN) (P <0.005), when compared to the wild-type (Fig. 7D). Therefore, the TILLING mutants validated the role of the *Shugoshin C-terminus* and *transcription factor TGA1-related* genes in SCN resistance (Fig. 7D).

Discussion

Novel genes for broad-spectrum SCN resistance

The present study discovered two novel genes in PI 567516C that are required to confer resistance to SCN. Unraveling the genes responsible for SCN resistance has historically presented a significant challenge to the soybean community. Two major SCN resistance types are known, the PI 88788-type that requires the resistant *rhg1-b* allele and the Peking-type requires both the *Rhg4-a* and *rhg1-a* resistant alleles. PI 88788 type resistance requires 8 copies of the *rhg1-b* allele, while the Peking-type resistance requires both the *Rhg4-a* (2 copies) and *rhg1-a* (3 copies) resistant alleles and this allele combination is absent in PI 567516C[28]. Unlike the Peking and PI 88788-type of resistance, PI 567516C is a unique source that shows broad-spectrum resistance to SCN at least against five HG types of SCN (2.5.7 (race 1), 1.2.5.7 (race 2), 0 (race 3), 2.5.7 (race 5), and 1.3.5.6.7 (race 14))[15]. Additionally, although PI 567516C carries the *rhg1- a* like Peking-type, it contains the *Rhg4-b* susceptible allele (unlike the *Rhg4-a* resistant allele). Moreover, the *Rhg4* promoter haplotype at PI 567516C is also different than the resistant haplotype promoter in Peking and PI88788 type of resistance (Supplementary Data 7). Thus, our previous studies of haplotype clustering and copy number variation[28] (Supplementary Data 7) and fine-mapping analysis[24] demonstrated that PI 567516C shows different haplotype and copy number variations at the *rhg1* and *Rhg4* loci as compared to the resistant haplotypes contained in PI 88788 and Peking types of resistance. Mapping the *qSCN10* locus on chr. 10 in PI 567516C facilitated the discovery of a new SCN resistance type (independent of resistant *rhg1* and *Rhg4-a* loci), leading to the identification of novel SCN-resistant genes in soybeans[24]. In this study, the *qSCN10* region was narrowed down to 142 kb (Fig. 1), in which 20 genes were identified and further investigated in detail based on the haplotype (Figs. 2, 3), gene expression analyses (Fig. 4), coupled with the presence of non-synonymous SNPs. The resequencing of diverse genetic populations represents a potent strategy for uncovering traits and has been applied across a range of organisms, encompassing humans[43] and a number of other species[44,45]. The WGRS dataset enables the detection of functional variations and offers an extensive inventory of genome-wide polymorphisms in closely related accessions. Among all the genes, two genes namely *GmSCT-10* and *GmTGA1-10* were identified as potential candidates harboring non-synonymous SNPs underlying the *qSCN10* locus regulating SCN resistance. The functional validation of candidate genes was investigated in two ways; overexpression of candidate genes and mutagenesis through the $TbyS^{+}$ approach. Overexpression of *GmSCT-10* and *GmTGA1-10* into susceptible W82 roots demonstrated reduced cyst count (more than 80%). PI 567516C presents a resistant reaction to PA5 (FI=2), but a moderate reaction to PA1 (FI = 27), PA2 (FI = 18), PA3 (FI=15), and PA14 (FI=15)[28]. Overexpression of both genes in the SCN- susceptible variety Williams 82 via composite hairy root transformation significantly reduced the cyst numbers from 96.6 on average contained in WI82::EV (control) to 15.03 (84.6%) and 18.69 (81.2%), when compared to 28 cyst numbers on average contained in PI 567516C. The observed effect of both *GmSCT-10* and *GmTGA1-10* genes was even stronger than the previously identified *GmPR08-Bet VI* gene, whose overexpression in transgenic Williams 82 roots decreased SCN cysts by 65%[21]. Therefore, the obtained data revealed the effectiveness of those genes in conferring resistance out of the PI 567516C genetic background.

Additionally, *GmSCT-10* and *GmTGA1-10* mutants from PI 407729 showed increased CN compared to the wild-type (Fig. 7C-D) under HG type 0 and HG type 2.5.7 indicating the crucial involvement of *Shugoshin C-terminus* and *transcription factor TGA1-related* genes in SCN resistance. The obtained data show that both genes at the *qSCN10* locus not only are effective in conferring resistance in PI 567516C, but also in the closely related sources of SCN such as PI 407729, as shown via mutational analysis. Although overexpression analysis showed that individual tested genes decrease cyst numbers by more than 80%, and since the overexpression analysis and mutagenized lines have shown the effect of these genes individually, it is not possible at this time to rule out the effect of both genes to provide resistance in PI567516C.

This is not the first time that several genes within the same locus have been shown to play a role in SCN resistance. Previous studies have also demonstrated the involvement of several genes within the *rhg1-b* locus in conferring resistance[46,47]. The three genes; an amino acid transporter, alpha-SNAP, and wound-inducible protein were reported to play a role in SCN resistance in PI 88788 source of resistance, although their mode of action and link among their functional role remains unclear.

Shugoshin/microtubule mediated novel regulation of SCN resistance

Shugoshin-1 (Sgo1) is a crucial protein involved in the maintenance of centromeric cohesion and pairing during mitosis, preventing premature separation of homologous chromosomes before recombination is complete[48,49]. In addition to regulating various aspects of chromosome segregation and stability during cell division. *Shugoshin* is involved in the regulation of kinetochore-microtubule attachments and cohesin, which are crucial for chromosome movement during mitosis and maintaining chromosomal stability during cell division respectively.

Microtubules are involved in organizing and guiding the movement of the stylet within the plant cells. Upon nematode invasion, soybean plants activate defense responses to restrict the nematode's movement and development. In this case, microtubules may play a role in the establishment and regulation of these defense responses. Microtubules are an essential component of the plant's cytoskeleton and have important functions in cell division, and cell shape maintenance and actively involved in the establishment and regulation of cellular and defense responses to various stresses, including nematodes[50]. Microtubules are vital components in the complex interaction between nematodes and plants[50–52].

SCN induces the formation of feeding sites called syncytia within the plant roots. The syncytia are multinucleated cells formed by the fusion of neighboring plant cells, and they serve as feeding sites for the nematode. Microtubules are involved in the reorganization of the plant cytoskeleton during syncytium formation and involved in regulating gene expression in response to nematode (SCN) infection and therefore, participate in the control of gene transcription, translation, and the transport of RNA molecules, influencing the plant's ability to mount an effective defense response against the nematode[52]. It has been reported that a "giant cell mini cell plate" may form a physical barrier to separate the two daughter nuclei that are required for the multiple rounds of mitosis in developing giant cells, which result in a functional feeding site[53]. MAP65-3 plays a key role in microtubule arrays organization during mitosis and cytokinesis during plant cell division. A defect in giant cell mini cell plate formation in the absence of MAP65-3 would lead to the accumulation of mitosis defects (cell wall stubs and connected nuclei) during repeated mitoses[53]. These defects may prevent the development of functional feeding cells, resulting in the death of the nematode.

Altogether, these studies confirm critical role of microtubule in nematode resistance including soybean cyst nematode. SCN resistance involves a sophisticated interplay between the nematode and the plant's defense responses. Understanding the roles of microtubules in SCN

resistance can provide valuable insights into the molecular mechanisms of defense and may contribute to the development of more effective strategies to combat this damaging nematode pest.

Transcription factor-mediated novel regulation of SCN resistance

Transcription factors (TFs) regulate the expression of a specific set of genes by binding to promoter regions. They play essential roles in various cellular processes, including plant's response to biotic and abiotic stresses[54–56]. While limited information is available regarding the role of TGA (TGACG-binding), TFs in SCN resistance, and/or their specific involvement in microtubule dynamics related to SCN resistance; it is worth mentioning that the field of plant- nematode interactions are complex and multifaceted. Transcription factors can be part of the signaling pathways that are activated upon nematode infection and may modulate the expression of genes involved in the plant's defense response[57–59].

A *transcription factor TGA1-related* was previously reported to regulate effector-triggered immunity and hormonal signaling networks[25]. Nematode effectors are crucial components of the interaction between plant-parasitic nematodes and their host plants (SCN-soybeans). Effectors are molecules produced by nematodes and secreted into the plant to manipulate plant cellular processes, suppress the plant's defense mechanisms, and facilitate the nematode's successful infection and establishment[60–62]. In soybeans, HgSLP-1, an esophageal-gland protein that is secreted by the nematode during plant parasitism, was co-purified with the SCN resistance protein Rhg1 α-SNAP, suggesting that these two proteins physically interact[7]. Several pathogen effectors and their host targets involving transcriptional regulation during plant immunity were identified in several plant species, including *Arabidopsis thaliana*, chili pepper (*Capsicum annuum*), rice (*Oryza sativa*), and black cottonwood (*Populus trichocarpa*)[63–67].

Mutations in TFs, including WRKYs, TGAs, NACs, CBP60s/SARD1, ERFs, bZIPs, bHLHs, MYBs, CAMTAs, and TCPs, altered plant disease resistance against different pathogens[68–73]. The *GmTGA1-10* (Glyma.10G194800) has 30 paralogs including genes for TGA transcriptional factor, Basic leucine zipper (*bZIP*) transcription factor, and camp-response element binding protein. Out of the 30 *GmTGA1-10* paralog genes, the *GmTGA2-13* (Glyma.13G193700) member was previously shown to respond to SCN resistance[21]. *GmTAG2- 13* transcripts were induced in both compatible and incompatible reactions under SCN infections, whereas *GmTAG2-13* transcripts were significantly induced in the resistant line only after treatment with salicylic acid[21]. Overexpression of the *At-TGA2* in transgenic soybean roots also showed a reduction of SCN cysts by less than 50%[74]. However, no other study reported the direct involvement of TGA in resistance to SCN. Thus, to the best of our knowledge, this is the first report showing the involvement of the *GmTGA1-10* and *GmSCT-10* in conferring resistance to SCN in PI 567516C type of resistance. Unlike the other type of SCN resistance requiring either the *rhg1-b* locus (case of PI 88788) or the *rhg1-a* and *Rhg4-a* loci (case of Peking-type) *Rhg4*, the PI 567516C type resistance requires *GmTGA1-10* and *GmSCT-10* for its resistance to SCN.

Furthermore, the presence of NAC domain *cis-element* in *GmSCT-10* and *GmTGA1-10* promoter in the SCN susceptible soybean suggested that this *cis-element* may play a role in SCN susceptibility. Previous studies have shown the implication of the NAC domain-containing proteins in root-knot nematode stressed soybean roots[75], and also was found within a QTL region for resistance to the three SCN HG Types, 0, 2.5.7, and 1.2.3.5.6.7, in the common bean (*Phaseolus vulgaris*)[76]. In soybeans, NAC domain-containing proteins were shown to be induced under HG Type 2.5.7 SCN infection in roots[77].

Although the present study revealed the roles of *GmSCT-10* and *GmTGA1-10* at the *qSCN10* (O) locus in resistance to SCN, their involvement and mode of action in cell division, microtubule dynamics, and effector-triggered immunity still need to be further elucidated.

Material and methods

Genetic population and progeny test of additional recombinants

A novel QTL (*qSCN10*) was mapped for SCN resistance in a cross of Magellan and PI 567516C and subsequently confirmed in the near-isogenic background to confer resistance to multiple SCN HG types[15]. Our previous study developed a BC_4F_2 backcross population to introgress the *qSCN10* region from the exotic resource to the elite parent, Magellan, using marker-assisted backcrossing (MABC) method[24]. The initial fine-mapping effort was able to narrow the *qSCN10* region to 379-kb interval between the markers SNP2 (Gm10:42,430,713) and SNP-O8 (Gm10:42,809,800)[24]. In the current study, 18 additional recombinant progenies were identified and advanced to homogenize the recombinant chromosomal segments. Further, these recombinants were planted in a greenhouse of the SCN phenotyping facility at the University of Missouri.

SCN bioassays

For SCN bioassays, several soybean accessions, progenies of the Magellan × PI 567516C population, and SCN indicator lines were evaluated for resistance to SCN HG Type 1.3.5.6.7 (race 14), while transgenic hairy roots developed in this study were screened with HG type 0 (race 3) as it is known historically as the most abundant race in the United States field. Homogenous nematode population of HG Type 1.3.5.6.7 and HG Type 0 have been maintained for more than 30 generations. In each experiment, all 18 recombinants were tested with parental lines and indicator lines (Peking, PI 88788, PI 90763, PI 437654, PI 209332, PI 89772, PI 54831 along with Pickett) as well as susceptible lines 'Hutcheson' and 'Lee 74' for HG type test[78]. SCN bioassays were performed in a greenhouse at the University of Missouri following a well-established method[24,79,80]. Briefly, soybean seeds were germinated in paper pouches for 3–4 days and then transplanted into PVC tubes (100 cm^3) (one plant per tube). The tubes were filled with steam-pasteurized sandy soil and packed into plastic containers prior to transplanting. Each container held 25 tubes and was suspended over water baths maintained at 27 °C. Five plants of each indicator line were arranged in a randomized complete block design (RCBD). Two days after transplanting, each plant was inoculated with approximately 2000 SCN eggs. Thirty days post-inoculation, nematode cysts were washed from the roots of each plant and counted using a fluorescence-based imaging system[80]. The female index (FI %) was estimated to evaluate the response of each plant to each HG type of SCN using the following formula: FI (%) = (average number of female cyst nematodes on a given individual/ average number of female nematodes on the susceptible check). Five replications were included for each tested line and organized in an RCBD per experiment. Two independent experiments were performed, and the final values are the averages of the female index of two independent experiments.

Development of new markers and Kompetitive allele-specific PCR (KASP) assay

New SNP markers for KASP assay (LGC Genomics, UK) were developed to dissect the chromosomal crossover points of the newly identified recombinants. Briefly, whole genome resequencing (WGRS) data for PI 567516C[29] and Magellan[81] were used to obtain the genome sequence information of the *qSCN10* locus. The sequence data were further rechecked by investigating read alignments in the Integrative Genomics Viewer tool[82]. We selected a set of SNPs and designed two allele-specific forward primers (along with tail sequences) and one common reverse primer for KASP assays (Table S1). For the genotyping assay, genomic DNA was extracted from young trifoliate leaves of the parental lines, Magellan and PI 567516C, and individual recombinant progenies using a standard CTAB (cetyl trimethyl ammonium bromide) method[83] with some minor modifications. KASP reactions were performed as follows; we added 5 µl of 2X premade KASP master mix, 5 µl of 10–25 ng/µl gDNA, and 0.14 µl of primers mix for 10 µl reaction volume. These reactions were kept in the PCR cycling conditions at 95°C 15 min, followed by ten touch-down cycles at 94°C of 20 s, at 65–57 °C of 20 s (drop 0.8 °C per cycle) and then 23 cycles at 94°C of 20 s and at 57°C of 1 min.

All the reactions were performed in Roche Light Cycler 480 Instrument II (Roche Applied Sciences, Indianapolis, IN, USA) using the fluorescent end-point genotyping method.

Analysis of Putative Cis-Elements at the *GmTGA1-10*, *GmSCT-10*, and *GmLRR-PK-10* Promoters

Putative *cis-elements* in the upstream region (-2Kb upstream) of *GmTGA1-10*, *GmSCT-10*, and *GmLRR-PK-10* promoters were searched using the programs PLACE, Plant PAN 2.0, and MatInspector[84–86]. Additional filtering was carried out based on motif score and redundant repeated motifs. Next, significant motifs were searched manually using PLACE for the putative role in effector-triggered immunity, hormonal signaling networks, and biotic stress[84].

Expression analysis

Soybean root tissue from SCN-infected and non-infected Magellan and PI 567516C that were used for RNAseq expressions analysis were compiled as shown earlier[35]. Briefly, gene expression profiles of PI 567516C and Magellan in response to the SCN inoculation at different time points (0, 2, 3, 5, 10 DPI) were performed. Three independent experiments were conducted using a randomized complete block design (RCBD). Ten seedlings per line were included for each replication. Total RNAs were extracted using RNeasy QIAGEN KIT and RNA-seq library preparation and sequencing were performed at Novogen INC using the Illumina Hiseq 2500 platform. Briefly, messenger RNA has been purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the 1st strand cDNA was synthesized using random hexamer primers followed by the second strand synthesis. The library was prepared using end repair, A- tailing, adapter ligation, size selection, amplification, and purification steps. These libraries were checked and quantified and then pooled for RNA sequencing on the Illumina platform. Raw RNA reads were processed through fastp software and clean reads were obtained by following Q30 content analysis. After quality check, clean reads were mapped to the Glycine max v2 reference genome using Hisat2 v2.0.5[87]. To identify differentially regulated genes between two samples, the following criteria were selected: 2-fold, with a t-test *p value* <0.05, and the absolute signal intensity difference between the two samples >10. DESeq2 has been used to study differential expression analysis between samples. Functional classification of differentially regulated genes was analyzed using MapMan. The SRA data was submitted to NCBI with bioproject accession number: PRJNA 1163880.

Haplotype clustering analysis

The WGRS data of 106 diverse soybean lines sequenced at approximately 17X genome coverage[29] have been utilized to identify allelic variants in the *qSCN10* QTL. Haplotype analysis of *qSCN10* QTL was conducted using a previously described pipeline[27]. Briefly, SNPs from *qSCN10* QTL were extracted from the whole genome resequencing data using SNPviz (V2.0) tool (https://soykb.org/SNPViz2/). The imported data file converted into map (.map) and genotype (.geno) file to upload into Flapjack software to visualize the graphical genotypes and identify associated haplotypes as detailed manual[30]. This SNPs underlying *qSCN10* were further analyzed by calculating the similarity matrix and principal components to generate dendrogram. The dendrogram and genotype matrix (light blue and black color) was then converted to image files, followed by assigning haplotype number (H1 – H6) based on the major clades in dendrogram. Furthermore, gene IDs were manually inserted in the exact position on the genotype matrix (Fig. 2). Next, the major genes with large effect SNP and higher expression upon SCN infection (Fig. 3) such as *GmTGA1-10*, *GmSCT-10*, and *GmLRR-PK-10* were clustered as mentioned above followed by overlaying SCN phenotypic data in excel. Transcript sequence-based annotation (Wm82.a2.v1) was used to classify synonymous and non-synonymous SNPs by translating into amino acid sequences. The haplotype analysis and SCN cyst count phenotypic data were overlayed with SNP matrix (haplotypes) corresponding to individual lines and specific clusters.

Cloning and transgenic soybean composite hairy root transformation

The functional characterization of the selected two candidate genes, including the *transcription factor TGA1-related* (Glyma.10G194800) and *Shugoshin C-terminus* (Glyma.10G196400) genes, has been validated using the transgenic composite hairy root system[21,88]. cDNA sequence from both genes were amplified from soybean PI 567516C root cDNA by RT–PCR, in addition to the *GmSHMT08* (*Rhg4-a*) that used as a positive control, were cloned into the *pG2RNAi2* vector under the control of the soybean *ubiquitin* (*GmUbi*) promoter. Cloning was carried out between AscI and AvrII sites in the *pG2RNAi2* vector to generate *pG2RNAi2::GmTGA1-10* and *pG2RNAi2::GmSCT-10*. Williams 82 composite hairy roots transformed with *pG2RNAi2::empty vector* were used as a negative control. The *pG2RNAi2* vector carries a GFP-selectable marker for planta selection. Transgenic Williams 82 composite hairy roots transformed with *pG2RNAi2::GmTGA1-10* and *pG2RNAi2::GmSCT-10* were produced by injecting *Agrobacterium* (K599) suspensions three times into the hypocotyl directly below soybean cotyledons using a 3-mL syringe (BD#309578). After injection, composite hairy roots from at least 50 independent soybean transgenic plants per construct were grown and propagated in vermiculite. Plants were covered with plastic domes and sprayed consistently with water to maintain humidity in a growth chamber for 1–2 weeks. Plants were fertilized once a week with NPK 20-20-20. Plants with GFP-positive composite hairy roots at ~2–3 inches long were transferred to sandy soil before SCN screening. Growth conditions and SCN screenings with HG type 0 was performed, as mentioned earlier. SCN HG type 0 is known historically as the most abundant race in the United States field. After 30 days, cysts were counted under a stereomicroscope. The experiment was independently conducted three times with a minimum of 15 to 20 independent composite hairy root lines per construct per experiment. The results were plotted and analyzed for statistical significance by using analysis of variance (ANOVA) using the JMP Pro V12 software[88].

Development of an EMS-mutagenized Forrest population

The soybean lines PI 407729 and PI 567516C were used to develop two EMS mutagenized populations. The wild-type seeds from the two soybean genotypes were mutagenized with 0.6% EMS[89], and planted to harvest families of M2 generation. It included 1,536 from PI 407729 and 1,440 from PI 567516C, which were subsequently advanced to the M3 generation at the Horticulture Research Center at Southern Illinois University, Carbondale, IL. In total, 2,976 M3 EMS mutants (from PI 407729 and PI 567516C) were used to pool EMS mutants within the two candidate genes (*GmTGA1-10* and *GmSCT-10*) using TILLING-by-Sequencing^{+}.

TILLING-by-Sequencing^{+}

A two-dimensional pooling strategy was implemented to reduce the number of pools. Samples were vertically pooled into pools of 24 samples and horizontally into pools of 48 samples, as shown earlier[37,39,42]. Then, specific probes were designed to target regions of interest at the *transcription factor TGA1-related* gene (Glyma.10G194800) and *shugoshin C-terminus* gene (Glyma.10G196400). After library preparation and probe design, the developed TILLING-by- Sequencing^{+} workflow (based on capture-seq enrichment and target recovery technology) uses magnetic beads for targeting desired genes with higher efficiency and specificity before proceeding with next-generation sequencing of the pooled DNA. The result was saved as a FASTQ file, then raw reads were subject to quality control using FastQC[90], trimming, and filtering of low-quality reads using Trimmomatic[91]. The clean reads were aligned to the Williams 82 reference genome (Wm82.a4) using BWA[92]. SAM tools were used to filter and sort the bam files to serve as input for variant calling using Freebayes[93] and CRISP[94]. The VCF files resulting from variant calling were filtered by VCFtools and visualized in IGV for demultiplexing[95]. Using the VCF files, the SNPs corresponding to the induced mutations

within the mutant population were found. After identifying the desired PI 407729 *GmTGA1-10* and *GmSCT-10* TILLING mutants, their seeds were phenotyped for their HG type 0 (race 3) and HG type 2.5.7 (race 5) SCN reaction, since PI 407729 wild-types show resistance to moderate resistance to both SCN races.

Homology modeling of Transcription Factor TGA1-related and Shugoshin C-Terminus protein and mutational analysis

Homology modeling of putative Transcription Factor TGA1-related and Shugoshin C- Terminus protein structures was conducted with Deepview[96] and Swiss Model Workspace software[97] using the protein sequence from "Williams 82" and an available crystal structure as a template; PDB accession *I1NHU8.1.A* for Transcription Factor TGA1-related and *I1LCL1.1.A* for Shugoshin C-Terminus. All residues were modeled against the two templates with a sequence identity of 100%. Mutation mapping and visualizations were performed using the UCSF Chimera package[98].

Statistics and reproducibility

The data presented in Fig. 6 were performed with the analysis of variance by a T-student test means comparison, using JMP Pro V18 software. The data shown represents the averages from all three biological repeats. Connecting letters indicate significant differences between the tested lines as determined by ANOVA (P <0.0001). The experiments were repeated three times, and similar results were obtained. The data presented in table S2 were generated using the mean of 10 replications and statistical analysis was performed by using Tukey's HSD test which demonstrated the significant difference between the tested lines (P <0.0001).

Conclusion

The exotic soybean line, PI 567516C, harbors previously unknown SCN resistance genes that provide broad-spectrum resistance to SCN, distinct from the known *Rhg4* and *rhg1* genes. In this study, we further conducted fine-mapping the *qSCN10* QTL region, which was detected in PI 567516C. Through rigorous genotyping, expression analysis, and QTL-based haplotyping, we successfully selected the two most promising candidate genes within a 62.4-kb interval. Among these candidates, *GmTGA1-10* and *GmSCT-10* exhibited distinctive allelic variations in both coding and promoter regions, providing the basis for further gene functional characterization. Overexpression of *GmTGA1-10* and *GmSCT-10* genes in susceptible genotype (Wm82) significantly reduced the FI by (84.6%) and (81.2%), respectively. These genes responsible for SCN resistance in PI 567516C hold significant potential in countering shifts in SCN populations and virulence. These genes can be introduced to high-yielding varieties by using newly developed molecular markers for MAS to enhance the durability of soybean resistance to SCN. Moreover, the obtained results provide a basis for novel molecular mechanisms involving cell division, microtubule dynamics, and effector-triggered immunity that warrants in-depth investigation. The current study's findings provided valuable genetic resources for the sustainable management of SCN.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data supporting the findings of this study are available within the paper. Supplementary data associated with this study can be found in the online version. The SRA data was submitted to NCBI with bioproject accession number: PRJNA1163880. All other data provided in this manuscript are available from the corresponding author. Numerical source data for the graphs presented in Figs. 6, 7 can be found in Supplementary Data 1 & 2, respectively. Source data of the ANOVA analysis for the graph presented in Fig. 6 can be found in Fig. S3.

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Author contributions

H.T.N. and K.M. oversaw the project and conceived the research idea; N.L. and S.S.C. designed the project experiments and wrote the manuscript; N.L. conducted composite hairy root transformation analysis and gene over-expression, Tilling-by-seq^{+}, EMS mutants development and identification, gene cloning, protein homology modeling, *cis-element* promoter analysis, expression analysis, and data interpretation; SSC conducted fine mapping, progeny testing, SCN phenotyping, KASP marker development, cloning, expression analysis, SCN testing of transgenic plants and data interpretation; V.D. and G.P. performed haplotype analysis and edited the manuscript; H.Y. and T.V. re-confirmed the earlier discovery of novel SCN resistance source from PI 567516C by developing the mapping population, recombinant progeny testing, and data analysis, D.K. and A.E.B. performed *cis-*

element analysis. All authors discussed the results and contributed to the final manuscript

Competing interests
The authors declare no competing interests.

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