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Mapping quantitative trait loci related to nodule number in soybean (*Glycine max* (L.) Merr.) in response to the *Sinorhizobium* (Ensifer) *fredii* HH103 NopT type III effector

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ABSTRACT

Type III effectors play a vital role in the establishment of symbiosis. In this study, positive and negative effects of NopT on nodulation were detected in 30 soybean germplasms. The quantitative trait loci (QTL) related to nodule number were identified and their responses to NopT in recombinant inbred lines and chromosome segment substitution lines were evaluated. Ten QTL were identified on chromosomes 02, 05, 06, 10, and 13. One overlapping region on chromosome 02 was responsive to the wild-type rhizobium and the NopT mutant. The expression patterns of the genes in this overlapping region were determined and single nucleotide polymorphisms and haplotypes were analyzed to identify the candidate genes. Four candidate genes were identified as significantly responsive to NopT. *Glyma.02G135100* and *Glyma.02G100800* expression levels were directly affected by NopT. These results form the basis of future efforts to identify the plant host genes affected by type III effectors.

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Quantitative trait loci; rhizobia; symbiosis; recombinant inbred line; NopT

Introduction

Soybean (*Glycine max* (L.) Merr.), which is one of the most important oil and protein crops worldwide, has evolved the ability to host N_2-fixing bacteria (i.e. rhizobia) in specialized organs called root nodules (Kibido et al. 2020; Liu et al. 2020). Previous studies revealed that up to 90% of the seed nitrogen (N) content may be derived from N_2 fixation (Mastrodomenico and Purcell 2012). In agricultural environments, approximately 80% of the biologically fixed N is the result of symbiotic N fixation (Li et al. 2020; Maseko et al. 2020). In addition to influencing soybean quality, soybean–rhizobium interactions are crucial for the sustainable intensification of global farming systems (Liu et al. 2020).

Type III effectors (T3Es) are inserted into host cells via the Type III secretion system (T3SS) (Alfano and Collmer 2004). Rhizobial T3Es, known as nodulation outer proteins (Nops), are vital for establishing regulatory symbiosis (Bent and Mackey 2007; Miwa and Okazaki 2017). Various Nops positively and negatively affect the nodulation of leguminous plant hosts (Staehelin and Krishnan 2015), including NopT, NopM, NopL, and NopP from *Rhizobium* sp. NGR234 (Skorpil et al. 2005; Dai et al. 2008; Zhang et al. 2011; Xin et al. 2012). The *Sinorhizobium fredii* HH103 NopL mutant positively influences the nodulation of several soybean cultivars, including Huangpingshanzibai, Wanhuangdadou, Heidou, Suinong14, ZYD00006, Dongnong594, and Charleston (Zhang et al. 2018). In contrast, an inoculation with *S. fredii* HH103Ω*NopL* was observed to negatively affect the nodulation of the Williams 82, Baimaodou, and Qingdou varieties (Jiménez-Guerrero et al. 2017; Zhang et al. 2018). Additionally, NopM, an E3 ubiquitin ligase produced by *Rhizobium* sp. NGR234, enhances the nodulation of *Lablab purpureus* (Xin et al. 2012). Another study identified NopP of *Bradyrhizobium diazoefficiens* USDA 122 as the determinant of the symbiotic incompatibility with the *Rj2*-genotype soybean cultivar. In *Rj2* soybean (Hardee), NopP is required for nodule formation, but in *rj2* soybean (Lee), NopP is unnecessary. In a recent investigation, GmNNL1 was identified as the host protein that interacts directly with NopP based on a genome-wide association study of nodulation (Zhang et al. 2021). Additional studies are needed to identify the genes responsive to T3Es. Moreover, the symbiotic and biochemical functions of these effectors have not been elucidated. Identifying the affected genes in the host would further our understanding of the molecular mechanisms involved in soybean–rhizobium interactions.

NopT, which belongs to the YopT-AvrPphB effector family, has autoproteolytic properties and elicits a rapid hypersensitive reaction when transiently expressed in tobacco (Dai et al. 2008; Palace et al. 2018). YopT can promote infections by pathogenic *Yersinia* species, which can modulate RhoG activity and localization. Nodulation experiments with an NGR234 NopT mutant indicated that NopT affects nodulation either positively (*Phaseolus vulgaris* cv. Yudou No. 1 and *Tephrosia vogelii*) or negatively (*Crotalaria juncea*) (Dai et al. 2008). Although NopT is an important

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symbiotic effector, its target(s) in soybean and role(s) are still unclear.

Until recently, symbiotic N fixation-related traits have rarely been studied as quantitative traits (Diaz et al. 2017). Most of the quantitative trait locus (QTL) mapping studies conducted for symbiosis-related traits in soybean have involved low-density genetic maps constructed using simple sequence repeat markers (Hwang et al. 2014). However, advances in soybean genomic sequencing have resulted in the application of single nucleotide polymorphisms (SNPs) in QTL mapping studies (Qi et al. 2014; Lee et al. 2015; Zhang et al. 2019). The QTL and candidate genes underlying the symbiosis-related traits were identified in recombinant inbred lines (RILs) based on a linkage map with SNP markers. The responses of QTL to NopL, NopP, and NopD have been studied (Wang et al. 2018; Zhang et al. 2018; Wang et al. 2020). Although previous research has identified several QTL associated with symbiosis-related traits in soybean, more work is needed to identify the genes involved in QTL responses to different T3Es.

In this study, an investigation of the effects of NopT on soybean revealed that NopT significantly regulates soybean nodule formation. The QTL responsible for nodule number (NN) were identified following the inoculation of RIL and chromosome segment substitution line (CSSL) populations with the wild-type rhizobium and a NopT mutant. One conserved QTL was identified and localized on a chromosome, and two genes within the QTL were responsive to NopT during the establishment of symbiosis. These findings provide the basis for identifying the genes mediating soybean responses to rhizobial T3Es.

Materials and methods

Generation of the S. fredii *HH103 NopT mutant and complementation*

The *NopT* gene sequence was amplified from the *S. fredii* HH103 genome. A 1.5 kb fragment (i.e. from approximately 600 bp upstream to 800 bp downstream of the *NopT* gene start codon) was cloned into the pEASY-Blunt vector. An *Eco*RI restriction site was introduced 10 bp downstream from the *NopT* gene for the insertion of a spectinomycin resistance gene (Fig. S1A). The resulting sequence was amplified by PCR using a primer with a *Spe*I restriction site and then cloned into the pJQ200SK suicide vector (Quandt and Hynes 1993). The resulting pJQ200SK-*NopT*2400 recombinant plasmid was inserted into *S. fredii* HH103 via tri-parental mating involving the pRK2013 helper plasmid (38). Marker exchange was confirmed on solid TY medium containing 5% (w/v) sucrose. The integration of the Ω interposon at the correct position in *S. fredii* HH103 was verified by Southern blotting. The *NopT* complementation strain was constructed by inserting pFAJ1702-*NopT* into *S. fredii* HH103Ω*NopT* to generate *S. fredii* HH103Ω*NopT*::pFAJ1702-*NopT* via tri-parental mating. The full-length *NopT* gene sequence was amplified from the genome of *S. fredii* HH103 and incorporated into the pEASY-Blunt vector after digestions with *Kpn*I and *Xba*I. The *Kpn*I–*Xba*I fragment was excised from pGWC-*NopT* and cloned into the pFAJ1702 vector.

Mapping of QTL related to nodule traits

The QTL responses to NopT were determined using the RIL population derived from a cross between Charleston and Dongnong594 (Xin et al. 2008). Because the RIL population was an advanced generation population, it was suitable for identifying stable QTL underlying target traits. A total of 150 lines in this population were used to construct a high-density genetic map via specific locus amplified fragment sequencing (20). The genetic map included 5308 markers on 20 linkage groups and was 2655.68 cM long. The NN data for each RIL were used for the QTL mapping in WinQTL Cartographer (version 2.5). The average values for five plant nodule traits were used for the QTL mapping, which was completed using the recommended parameter settings of WinQTL Cartographer. A logarithm of odds (LOD) score ≥ 2.4 was set as the threshold for identifying potential QTL. The CSSL population was derived from a cross between Suinong14 and ZYD00006, which produced 200 lines covering 95% of the wild-type soybean genetic background (Xin et al. 2016). Each line included one or more genomic segments derived from wild-type soybean, implying the phenotypic differences were due to the substituted genomic segments.

The QTL were localized based on the nodule trait phenotype. The average values for five plant nodule traits were used for the QTL mapping of the CSSL population with the IciMapping program (Meng et al. 2015). The QTL localization results for the RIL and CSSL populations were combined to precisely identify the target QTL region.

Soybean plants were inoculated with the wild-type rhizobium (*S. fredii* HH103) or the NopT mutant (*S. fredii* HH103Ω*NopT*), after which the QTL localization results were compared to identify consistent and novel QTL. The only difference between the wild-type rhizobium and the NopT mutant was an insertion mutation to the *NopT* coding sequence in the mutant. Accordingly, the differences and consistency among the identified QTL were likely related to NopT. Plants were grown in sterile vermiculite in a greenhouse at 25°C with a 12-h light/12-h dark cycle. The B&D medium with trace nitrogen was used as the nutrient medium (Shi et al. 2020). At the Vc stage (i.e. cotyledon with sufficiently unrolled unifoliolate leaves in the vegetative growth stage), the plants were inoculated with rhizobia (>10^5 bacterial cells). Nodule phenotypes were analyzed 4 weeks later.

Sequence and haplotype analyses of candidate genes

Seqman 1.0 was used for sequence assembly (Swindell and Plasterer 1997). On the basis of the sequence alignment results, DnaSP (version 5) was used to calculate the nucleotide diversity and Tajima's D statistics. Haploview 4.2 was used for estimating the haplotype frequencies in the CSSL population (Valliyodan et al. 2016). The coding sequence and the 3000 bp upstream region were used for the haplotype analysis of each candidate gene. The Williams 82 genome served as the reference sequence (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax).

RNA extraction and qRT-PCR analysis

Charleston cultivar root samples collected at 0.5, 6, and 12 h after inoculations with *S. fredii* HH103 or *S. fredii*

HH103Ω*NopT* were ground to a fine powder in liquid nitrogen using a pestle and mortar. Total RNA was extracted from the ground material using the TRIzol Reagent. After removing residual DNA from the RNA samples using DNase (Applied Biosystems/Ambion, Austin, TX, USA), cDNA was synthesized from 1 µg RNA using a commercial cDNA synthesis kit (Toyobo Biotech Co. Ltd., Shanghai, China). The qRT-PCR analysis was completed using the iQ SYBR Green Supermix and the LightCycler® 480 II system (Roche, West Sussex, United Kingdom). Each reaction mixture consisted of 1 µL (250 ng) cDNA, 1 µL (100 µM) each primer, and 5 µL iQ SYBR Green Supermix in a final volume of 10 µL. Each reaction was performed in duplicate in 96-well optical grade PCR plates sealed with optical sealing tape (Bio-Rad Laboratories, Hercules, CA). The *GmUKN1* gene (*Glyma.12g020500*) was used as the reference control for calculating relative gene expression levels (Hu et al. 2009). The qRT-PCR program was as follows: 95°C for 30 s; 40 cycles of release and 58°C for 10 s. Three biological replicates and three technical replicates were used to improve the accuracy of the results.

Results

Inactivation of NopT negatively and positively affects nodule formation

The NopT mutant was constructed by inserting a spectinomycin resistance gene downstream of the *NopT* start codon (Fig. S1A). A positive mutant rhizobial clone was identified by Southern blotting. Significant band shifts were detected in the digested genomic DNA of wild-type *S. fredii* HH103 and the candidate mutant *S. fredii* HH103Ω*NopT* (Fig. S1B). The consistent results for the *Xho*I and *Sac*I digestions provide evidence that the spectinomycin resistance gene was inserted at the correct site. Whether the NopT mutant affects soybean nodule formation was subsequently investigated.

To explore the effects of NopT during the establishment of symbiosis, nodulation assays were conducted using *S. fredii* HH103 and the mutant *S. fredii* HH103Ω*NopT* with an in-frame insertion in the *NopT* gene. Thirty soybean germplasms from different ecoregions were used to elucidate the role of NopT in the symbiosis established between rhizobia and soybean plants. The NN differed significantly among the germplasms inoculated with *S. fredii* HH103 or *S. fredii* HH103Ω*NopT* (Figure 1(A)). Interestingly, NopT negatively affected the NN of the Dongnong44, Heinong35, Beifeng11, GongyeA03-5570, Dawason, and Dongnong50 cultivars, but positively affected the NN of the Heihe35, Heinong44, He00-23, Hongfeng11, ZihuaNo.2, Nattosan, Hefeng37, Heihe14, Suinong15, Jingshanpu, Sui02-339, Aika166, Gongye04-L15, Charleston, Suinong14, and ZYD00006 varieties (Figure 1(A)). In Heihe18, Dongnong42, and Heinong75, there were no differences in the nodule traits following inoculations with *S. fredii* HH103 or *S. fredii* HH103Ω*NopT*. Furthermore, *S. fredii* HH103Ω*NopT*::pFAJ1702-*NopT* (i.e. complementation of the mutant NopT strain) was used to

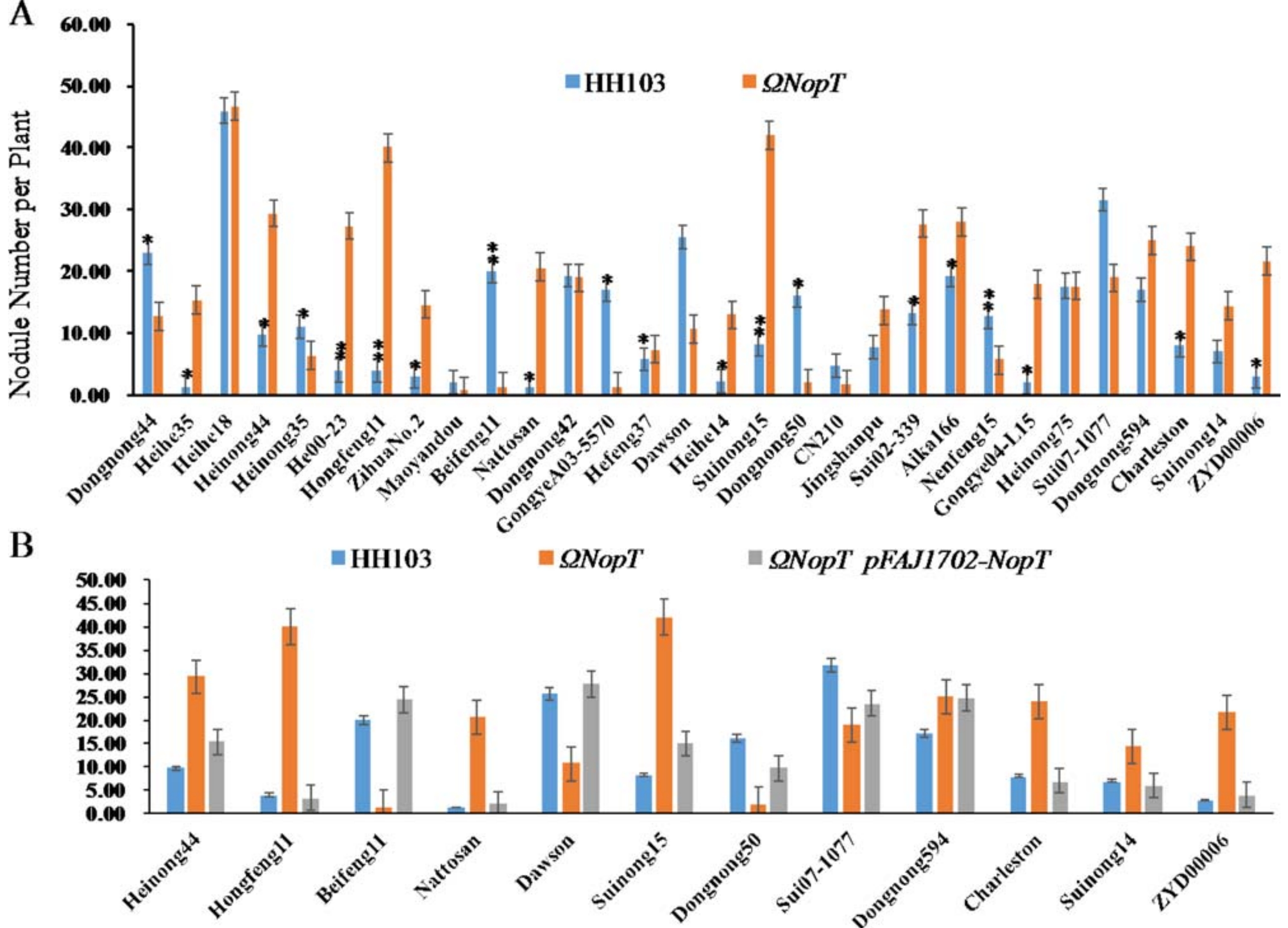

Figure 1. Results of the soybean germplasm nodulation tests, which were completed using five plants per variety. Data were analyzed by Student's *t*-test (**p* < .05).

confirm the effect of NopT on nodulation (Figure 1(A)). Nodulation assays involving 10 of 30 germplasms and inoculations with *S. fredii* HH103, *S. fredii* HH103Ω*NopT*, and *S. fredii* HH103Ω*NopT*::pFAJ1702-*NopT* revealed that *S. fredii* HH103Ω*NopT*::pFAJ1702-*NopT* and the wild-type *S. fredii* HH103 similarly affected nodulation (Figure 1(B)). These findings imply that NopT is crucial for soybean nodule formation and its symbiosis-related function is influenced by the genetic background of soybean.

QTL underlying symbiosis-related traits induced by S. fredii HH103 and S. fredii HH103ΩNopT

The negative and positive effects of NopT suggest that it affects soybean genes to regulate nodule formation. To test this hypothesis, two genetic populations were used to identify the responses of QTL to NopT. In the RIL population, the NN ranged from 1 to 31 following the inoculation with wild-type *S. fredii* HH103. The NN of the parents was 9.6 (Dongnong594) and 3.4 (Charleston) (Table 1). However, the NN was higher for the plants inoculated with the NopT mutant. More specifically, the inoculation with *S. fredii* HH103Ω-*NopT* resulted in an NN of 17 and 24 for the Dongnong594 and Charleston plants and an NN of 3–78 for the plants in the RIL population. These results imply that NopT negatively affected the nodulation of the RIL population and the parents. This enabled the identification of the QTL responsive to NopT in soybean.

In the CSSL population, the NN was 1–39 following the inoculation with *S. fredii* HH103. Additionally, the difference in the NN between the parents Suinong14 and ZYD00006 was minimal. However, a significant difference was detected between the Suinong14 and ZYD00006 cultivars inoculated with the NopT mutant (Table 2). Interestingly, the NN of Suinong14 plants inoculated with the NopT mutant was approximately 2-fold higher than that of the Suinong14 plants inoculated with the wild-type rhizobium. In contrast, the NN of ZYD00006 plants inoculated with the NopT mutant was not significantly different from the NN of the plants inoculated with the wild-type rhizobium. Regarding the CSSL population, the NN increased after the inoculation with the NopT mutant. These results suggest that the substituted segments of the wild-type soybean genome influenced the NN and may include some genes responsive to NopT.

To identify the candidate genes responsive to NopT, we performed a QTL analysis of the RIL population to reveal the QTL underlying the NN. Two and three QTL related to the NN were identified in the RILs inoculated with *S. fredii* HH103 and *S. fredii* HH103Ω*NopT*, respectively. The two QTL identified after the inoculation with *S. fredii* HH103 were detected on chromosome 02 and the three QTL identified following the inoculation with *S. fredii* HH103Ω*NopT* were detected on chromosomes 02 and 10. Regarding the QTL on chromosome 02, qNN2-1 was located in a region from 14,397,227 to 14,497,539 and had a LOD score of 3.7 and an R^2 value of 13.01%; qNN2-2 was located in a region from 9,287,538 to 9,387,851 and had a LOD score of 3.3 and an R^2 value of 1.83%; and qNN2-3 was located in a region from 9,297,538 to 9,587,851 and had a LOD score of 2.4 and an R^2 value of 5.97%. The two QTL on chromosome 10 were qNN10-1 and qNN10-2, which respectively had LOD scores of 2.9 and 2.8 and R^2 values of 6.53% and 4.50%. An overlapping region between qNN2-2 and qNN2-3 was identified (Table 3 and Figure 2).

The CSSL population inoculated with *S. fredii* HH103 and *S. fredii* HH103Ω*NopT* was also analyzed to identify the QTL underlying the NN. The five identified QTL were distributed on chromosomes 02, 05, 06, and 13. Three QTL related to NN (qCNN2-1, qCNN6-1, and qCNN13-1) were identified following the inoculation with *S. fredii* HH103 (Table 4). Of these QTL, qCNN2-1 was detected on chromosome 02, with a LOD score of 5.9 and an R^2 value of 13.88%. In contrast, qCNN6-1 and qCNN13-1 were detected on chromosomes 06 and 13, respectively. The LOD scores for qCNN6-1 and qCNN13-1 were 2.8 and 3.6, respectively, and their R^2 values were 6.36% and 7.98%, respectively. Two QTL were identified after the inoculation with *S. fredii* HH103Ω*NopT*, including qCNN2-2, which was detected in the same region as qCNN2-1, with a LOD

Table 1. Nodule phenotypes of the RIL population and the parents.

				RIL population	
Trait	Rhizobium	Dongnong594	Charleston	Mean	Range
Nodule number	HH103	9.6 ± 1.32	3. 4 ± 0.98	7.20 ± 2.37	1.0–31.0
	HH103Ω*NopT*	17 ± 2.72	23.1 ± 1.23	15.69 ± 8.65	3.0–78.0

Table 2. Localization of the QTL in the RIL population after inoculations with HH103 and HH103Ω*NopT*.

Rhizobium	Trait	QTL	Chrom.	Confident interval	LOD	R^2	Additive effect
HH103	NN	qNN2-1	2	14,397,227–14,497,539	3.7	13.01	3.9929
		qNN2-2	2	9,287,538–9,387,851	3.3	1.83	3.6321
HH103Ω*NopT*	NN	qNN2-3	2	9,297,538–9,587,851	2.4	5.97	3.9775
		qNN10-1	10	11,601,134–11,701,442	2.9	6.53	−2.2522
		qNN10-2	10	11,118,474–11,318,746	2.8	4.50	−1.9955

Table 3. Nodule phenotypes of the CSSL population and the parents.

				CSSL population	
Trait	Rhizobium	Suinong14	ZYD00006	Mean	Range
Nodule number	HH103	5.25 ± 1.32	3.84 ± 0.98	6.183 ± 1.48	1.0–39.0
	HH103Ω*NopT*	38.8 ± 5.72	4 ± 1.23	10.03 ± 7.69	1.0–69.0

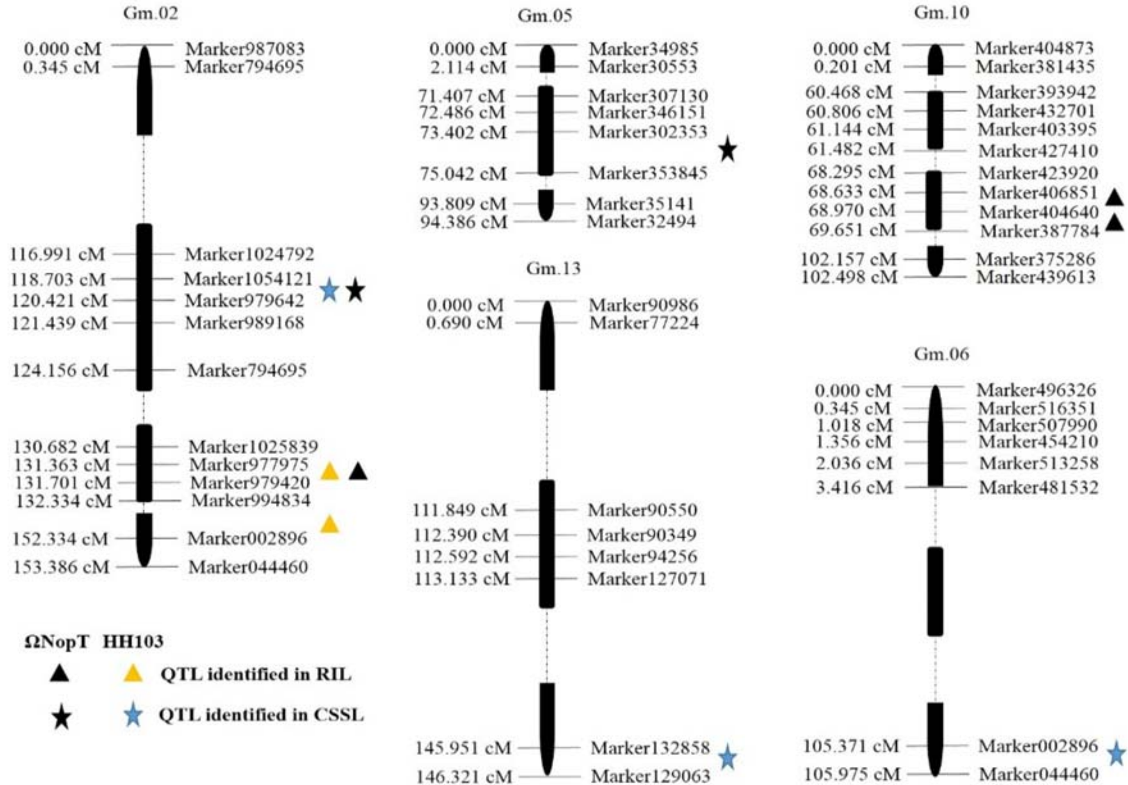

Figure 2. Distribution of quantitative trait loci on linkage groups. QTL for NopT mutant are shown in black triangle and pentagram, while yellow triangle and blue pentagram represent the wild type rhizobium induced QTL, respectively.

score of 3.2 and an R^2 value of 9.66%. The other QTL, qCNN5-1, was detected on chromosome 05, with a LOD score of 3.5 and an R^2 value of 10.58%.

On the basis of the QTL analysis, 10 QTLs were identified in the two examined genetic populations. Chromosome 02 included five QTL (Figure 2), whereas chromosome 10 contained two QTL and chromosomes 05, 06, and 13 had one QTL each. None of the QTL regions overlapped between the two genetic populations. However, in each population, one QTL region on chromosome 02 identified in plants inoculated with *S. fredii* HH103 was also detected in plants inoculated with *S. fredii* HH103Ω*NopT* (Figure 2). These results suggest that the QTL on chromosome 02 might be important for nodulation. Moreover, candidate genes responsive to NopT might be included in the chromosome 02 QTL region.

Candidate genes responsive to NopT

To identify the candidate genes affected by NopT in the QTL on chromosome 02, the NN of the CSSLs inoculated with *S. fredii* HH103 was analyzed. Ten CSSLs differed regarding their NN, with F169, F073, and F1011 forming fewer nodules than F1680, F121, F140, F100, F014, F175, and F914. To clarify the genomic diversity among these CSSLs, the differences in the substituted chromosomal segments were determined (Figure 3(A)). The substituted chromosome 02 segments were similar in F169, F073, and F1011 and were derived from Suinong14. The substituted chromosome 02 segments in the other seven lines were consistent and were from ZYD00006 (Figure 3(A)). These results indicate that the overlapping chromosome 02 region in F169, F073, and F1011 might contain NopT-responsive candidate genes underlying the NN. Thus, we analyzed the genes located in the overlapping region. Single nucleotide polymorphisms in the candidate genes in this region were detected in the CSSL population. The SNPs and the haplotypes of *Glyma.02G135100*, *Glyma.02G100800*, *Glyma.02G109100*, and *Glyma.02G113800* were identified (Figure 4). The SNPs and haplotypes among specific CSSLs with different nodule phenotypes indicated these four genes are potentially responsive to NopT. Four haplotypes were identified for the *Glyma.02G135100* coding sequence and four SNPs were not associated with haplotype blocks. Eleven haplotypes were identified for the *Glyma.02G100800* coding sequence. Regarding *Glyma.02G109100* and *Glyma.02G113800*, only a single haplotype block was detected. These findings support the possibility that these four genes influence the NN of soybean.

We also used previously generated RNA-seq data (25) to evaluate whether the genes distributed in the candidate region

Table 4. Localization of the QTL in the CSSL population after inoculations with HH103 and HH103Ω*NopT*.

Rhizobium	Trait	QTL	Chrom.	Confident interval	LOD	R^2	Additive effect
HH103	NN	qCNN2-1	2	6,554,934–6,764,681	5.9	13.88	5.3602
		qCNN6-1	6	3,540,629–3,606,857	2.8	6.36	4.4129
		qCNN13-1	13	30,441,889–30,464,844	3.6	7.98	3.3546
HH103Ω*NopT*	NN	qCNN2-2	2	6,554,934–6,764,681	3.2	9.66	5.7054
		qCNN5-1	5	36,931,375–37,003,875	3.5	10.58	5.2107

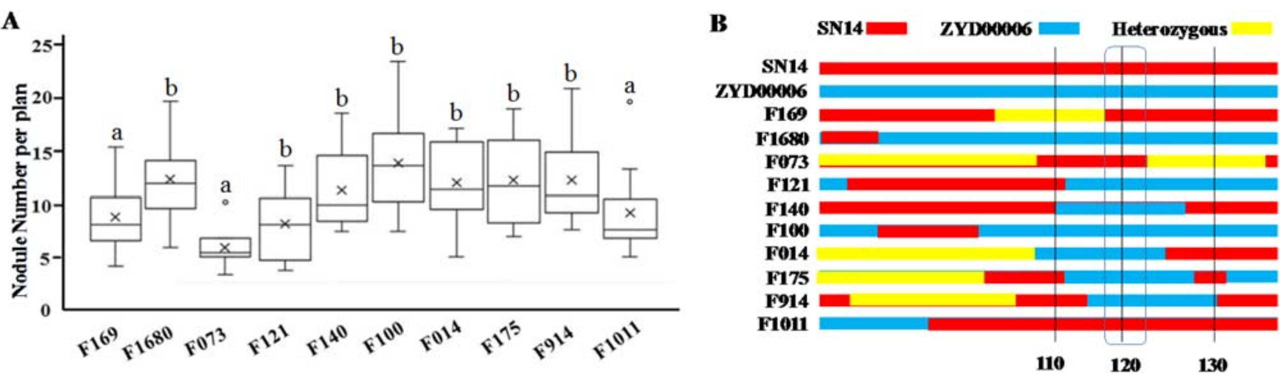

Figure 3. Phenotypic and genotypic analyses of specific CSSLs. (A) *Sinorhizobium fredii* HH103 nodulation test results. (B) Genotypic analysis results. A specific region of chromosome 02 is presented. A two-way ANOVA with a Tukey-Kramer multiple comparison post-test was used for statistical analyses in (A) ($p < .05$).

on chromosome 02 are responsive to T3Es. We analyzed all 110 genes located in the QTL region on chromosome 02. A comparison of the *Glyma.02G135100*, *Glyma.02G100800*, *Glyma.02G109100*, and *Glyma.02G113800* expression patterns based on the published RNA-seq data revealed differences (Figure 5). Additionally, *Glyma.02G078400*, *Glyma.02G109700*, *Glyma.02G100800*, *Glyma.02G080200*, *Glyma.02G109100*, *Glyma.02G123500*, and *Glyma.02G132500* expression levels were upregulated in F1011 and F1680 inoculated with the wild-type rhizobium and the derived T3E

Figure 4. Single nucleotide polymorphism analysis of candidate genes in the identified region of the substituted segment in chromosome 02. Generated by Haploview (version 4.2), and each box represents the D′ value between pairs of SNPs (ranging from 0 to 1). Dark red, strong LD; light red, weak LD.

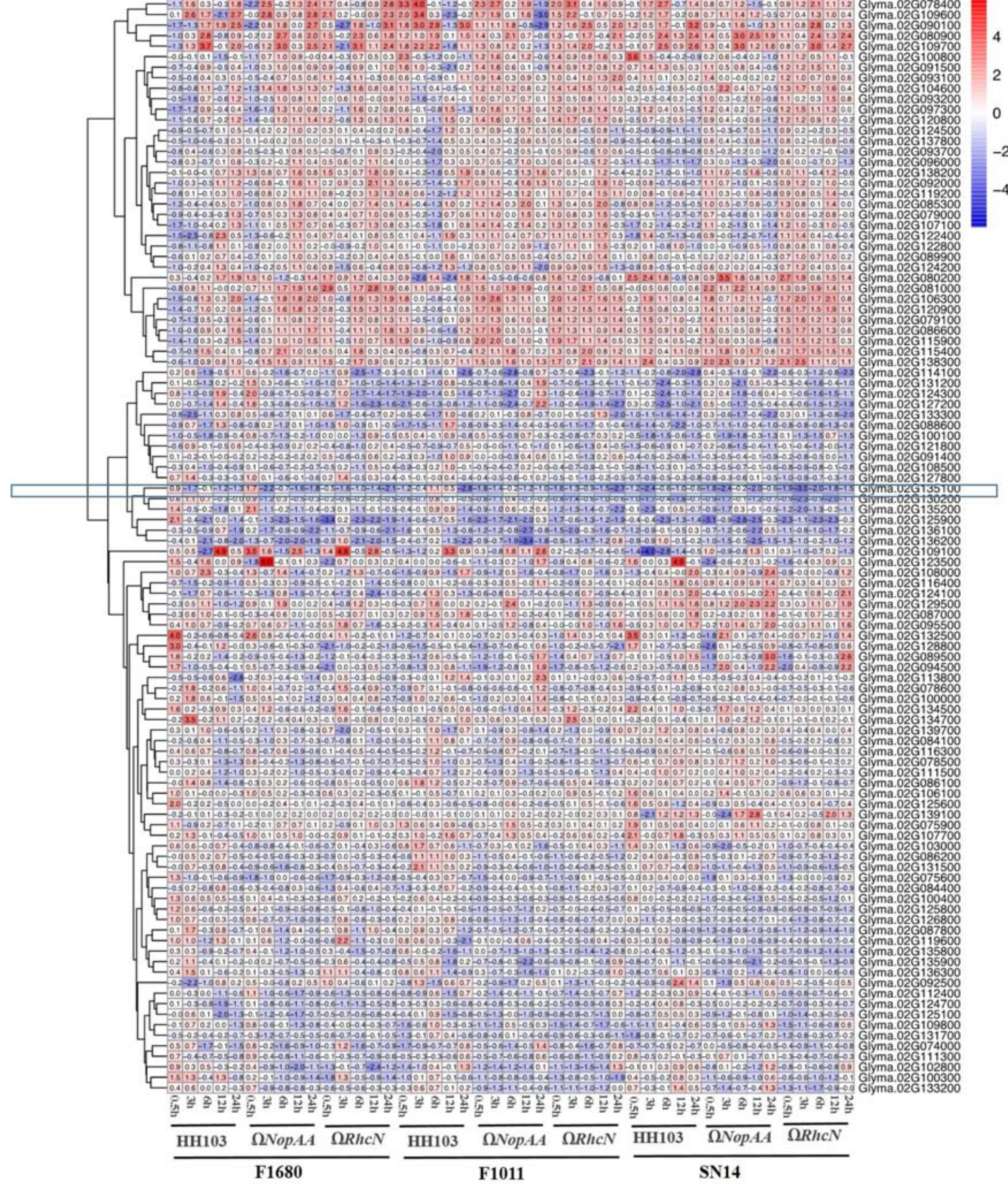

Figure 5. Expression patterns of genes within the QTL region of chromosome 02 in CSSLs. Data for the relative expression levels of genes were obtained by DGE data after taking log 10 (FPKM+1). Color from red to blue, indicated that the log 10 (FPKM+1) values were from large to small, red color indicates high expression level and blue color indicates low expression level.

mutant. In contrast, the *Glyma.02G109600*, *Glyma.02G125900*, and *Glyma.02G136200* expression levels were downregulated. Because the haplotype analysis revealed differences in the *Glyma.02G135100*, *Glyma.02G100800*, *Glyma.02G109100*, and *Glyma.02G113800* genomic sequences, we compared the expression levels of these four genes. The data indicated *Glyma.02G135100* was highly expressed in nodules, but not in the other tissues, and was exclusively expressed in response to rhizobia. The *Glyma.02G113800* gene was highly expressed in symbiotic leaves and following N treatments. The RNA-seq data confirmed that *Glyma.02G135100*, *Glyma.02G100800*, *Glyma.02G109100*, and *Glyma.02G113800* expression can be induced by wild-type rhizobia and T3E-related mutants.

A qRT-PCR assay proved that *Glyma.02G135100* and *Glyma.02G100800* expression was induced by NopT, whereas *Glyma.02G109100* and *Glyma.02G113800* expression was not. Moreover, *Glyma.02G135100* expression was upregulated between 0.5 and 12 h after the inoculation with *S. fredii* HH103, whereas *Glyma.02G135100* expression was downregulated at 6 h after the inoculation with *S. fredii* HH103Ω*NopT* (Figure 6). The *Glyma.02G135100* expression trends were similar after the inoculations with *S. fredii* HH103 and *S. fredii* HH103Ω*NopT*, but the overall expression level was downregulated by the NopT mutant. No significant differences were detected in the *Glyma.02G109100* and *Glyma.02G113800* expression levels after the inoculations with *S. fredii* HH103 and *S. fredii* HH103Ω*NopT* (Figure 6). These results suggest that *Glyma.02G135100* and *Glyma.02G100800* are candidate genes responsive to NopT in soybean during the establishment of symbiosis.

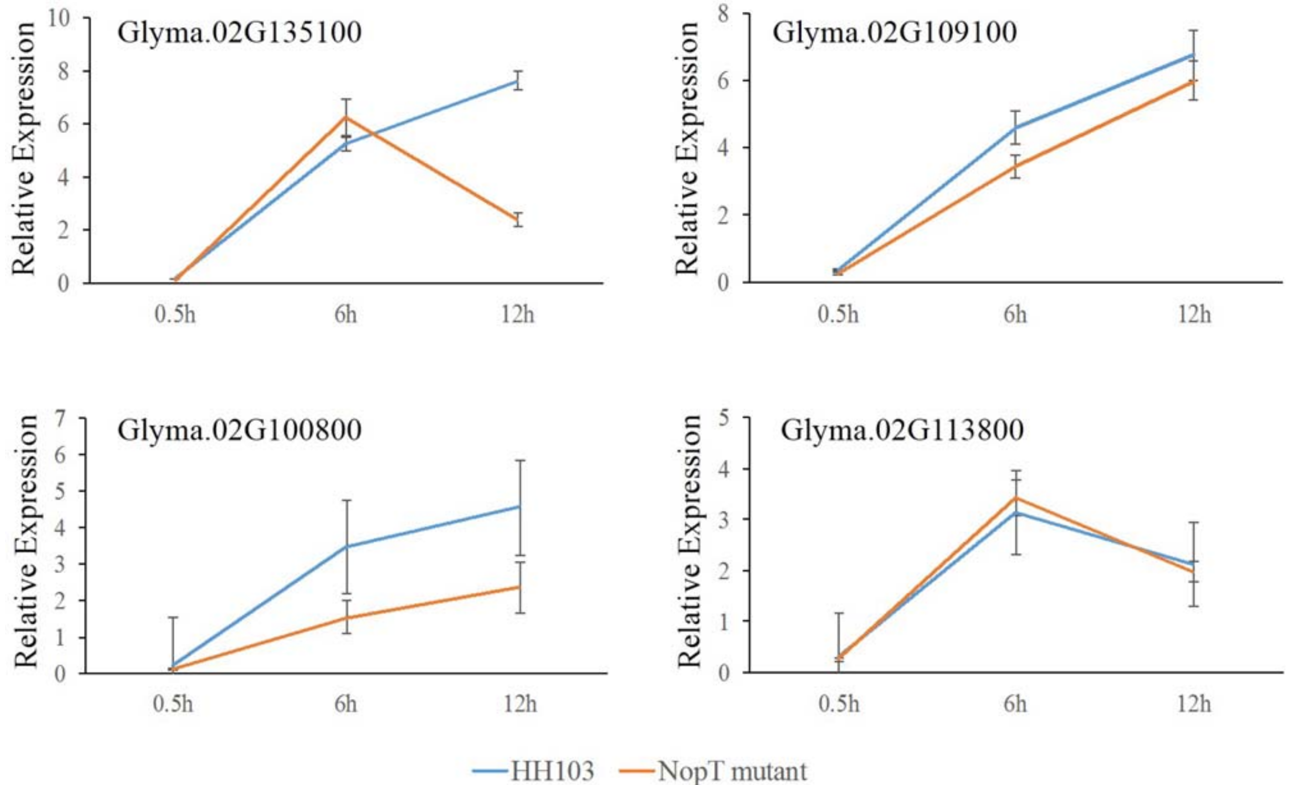

Figure 6. qRT-PCR analysis of candidate gene expression levels. *GmUNK1* (*Glyma.12G020500*) was used as the reference gene to calibrate the transcript abundance values among different samples. Statistical significance of the results was assessed using the pairwise Student's *t* test. *$p < .05$.

Discussion

All identified and analyzed QTL had an R^2 value between 1.83% and 13.88%. These QTL were detected after inoculations with different rhizobial strains. Two genetic populations were used to identify the conserved QTL. There were no overlapping QTL regions between the two populations; however, the QTL were detected in similar regions on the same chromosome. This suggests that the genetic background of the populations is the primary factor influencing the establishment of symbiosis. This is because the efficiency of N fixation is reportedly controlled by quantitative inheritance (Devine and Kuykendall 1996). The compatibility between cultivars and rhizobial strains must be determined to identify the QTL underlying nodule traits.

In this study, we determined that the same QTL regions can be identified on specific chromosomes via the simultaneous inoculation of soybean plants with wild-type rhizobium or the NopT mutant (Figure 2). This result may reflect the substantial conservation of the QTL regions. Because the NopT mutant varies from the wild-type rhizobium only because of an insertion mutation in *NopT*, the QTL identified following the inoculation with the NopT mutant may contain genes responsive to NopT. The QTL were also detected in the plants inoculated with the wild-type rhizobium. Together, these results suggest that the QTL are crucial determinants of the NN. In a recent genome-wide association study of soybean inoculated with the wild-type rhizobium, *GmNNL1* was identified as a novel gene, and the encoded protein can directly interact with the T3E NopP (Zhang et al. 2021). The detection of the same QTL region following the inoculation with the wild-type rhizobium and the derived T3E mutant in this study implies the identified QTL might comprise genes responsive to NopT.

The localization of qCNN6 in a region on chromosome 06 was consistent with the results of an earlier investigation of a KS4895 × Jackson population (Hwang et al. 2014). Additionally, QTL clusters were detected on chromosome 02 in a region similar to that identified in a previous study on the nodulation of a RIL population derived from Peking × Tamahomare (Ramongolalaina et al. 2018). In the current study, QTL related to NN were also detected on chromosomes 05, 10, and 13 in the RIL population. The QTL underlying the genetic interrelationships affecting the compatibility between *Bradyrhizobium* sp. and plants were identified in an earlier study (Ramongolalaina et al. 2018). This is consistent with the proposed regulatory effects of T3Es on the compatibility between soybean and rhizobia (Yasuda et al. 2016; Temprano-Vera et al. 2018). On the basis of these previous reports, we concluded that the QTL identified in the present study are stable QTL. Notably, not all of the identified QTL were localized to the same regions in populations with different genetic backgrounds. These results might be related to the specificity of legume–rhizobium symbioses critical for soybean–rhizobium interactions. The *Rj2* and *Rj4* soybean genotypes are reportedly important for restricting the nodulation induced by specific rhizobial strains (Tsukui et al. 2013). Several recent studies proved that Rj2 can indirectly interact with NopP, supporting the hypothesis that T3Es contribute to symbiosis specificity.

In this study, a NopT mutant was constructed and two genetic populations were used to clarify QTL responses to NopT. The QTL on chromosome 02 identified following the inoculations with the wild-type and NopT mutant rhizobia were consistently localized, implying the genes affected by NopT are located in the identified regions. The SNP and RNA-seq analyses revealed that *Glyma.02G135100* and *Glyma.02G100800* expression is induced by NopT. An earlier

study demonstrated that *Glyma.02G135100* encodes a peroxidase superfamily protein that regulates the removal of H_2O_2, the oxidation of toxic reductants, the biosynthesis and degradation of lignin, suberization, auxin catabolism, and responses to various stresses (e.g. wounding, pathogen attacks, and oxidative stress) (Bindschedler et al. 2006). In fresh bean (*Phaseolus vulgaris*), peroxidases can prevent oxidative bursts in response to a fungal elicitor and enhance the susceptibility to a broad range of fungal and bacterial pathogens (O'Brien et al. 2012). The expression of *Glyma.02G135100* increases in the absence of NopT, suggesting *Glyma.02G135100* may inhibit the accumulation of reactive oxygen species (ROS) induced by rhizobia. Moreover, NopT can cleave the N-terminal of the GTPases RhoA, Rac, and Cdc42 at the prenylated cysteine to produce geranylgeranyl cysteine methyl esters (Shao et al. 2003). Recent research has raised the interesting possibility that ROS and reactive nitrogen species can directly regulate Rho GTPases through redox-mediated post-translational modifications (Clayton and Ridley 2020; Holmes et al. 2020). Furthermore, NopT may modulate the ROS signaling pathway to regulate the establishment of symbiosis. *Glyma.02G100800* encodes aspartyl protease family protein 2. In Arabidopsis, an atypical aspartic protease (ASPR1), which regulates primary root growth and lateral root development, is sensitive to ROS (Soares et al. 2019). These findings imply that NopT is involved in the peroxidase and aspartyl protease signaling pathways. The data generated in the present study may be useful for detecting plant host genes regulated by rhizobial T3Es during the establishment of symbiosis.

Conclusion

We proved that NopT is a pivotal regulator of the soybean–rhizobium interaction. We identified the novel QTL underlying the NN as well as the candidate genes responsive to NopT. The data presented herein provide insights useful for elucidating the precise mechanism mediating symbiosis establishment. Additional experiments will need to be conducted to clarify how NopT regulates nodule formation and affects host signaling pathways.

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