RNA Sequencing-Associated Study Identifies *GmDRR1* as Positively Regulating the Establishment of Symbiosis in Soybean

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In soybean (*Glycine max*)-rhizobium interactions, the type III secretion system (T3SS) of rhizobium plays a key role in regulating host specificity. However, the lack of information on the role of T3SS in signaling networks limits our understanding of symbiosis. Here, we conducted an RNA sequencing analysis of three soybean chromosome segment substituted lines, one female parent and two derived lines with different chromosome-substituted segments of wild soybean and opposite nodulation patterns. By analyzing chromosome-linked differentially expressed genes in the substituted segments and quantitative trait loci (QTL)-assisted selection in the substituted-segment region, genes that may respond to type III effectors to mediate plant immunity-related signaling were identified. To narrow down the number of candidate genes, QTL assistant was used to identify the candidate region consistent with the substituted segments. Furthermore, one candidate gene, *GmDRR1*, was

identified in the substituted segment. To investigate the role of *GmDRR1* in symbiosis establishment, *GmDRR1*-overexpression and RNA interference soybean lines were constructed. The nodule number increased in the former compared with wild-type soybean. Additionally, the T3SS-regulated effectors appeared to interact with the *GmDRR1* signaling pathway. This finding will allow the detection of T3SS-regulated effectors involved in legume-rhizobium interactions.

Keywords: dirigent protein, *GmDRR1*, RNA-seq, segment-substituted lines, soybean, wild soybean

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Soybean [*Glycine max* (L.) Merrill] is the most important legume crop worldwide, supplying more than half of the world's edible oil and protein (He et al. 2016; Lam et al. 2010). *Glycine soja* Sieb. & Zucc. is the wild progenitor species of soybean, which was domesticated in China 5,000 years ago (Carter et al. 2004; Hymowitz 1970). Like other legumes, soybean is capable of symbiotic nitrogen fixation, which has significant advantages for agricultural sustainability in both developing and developed countries (Foyer et al. 2018). This symbiosis results in the formation of root nodules, and the nodule organogenesis process requires complex signal exchanges between the symbiotic partners (Chiasson et al. 2014; Miwa and Okazaki 2017). Understanding the signal exchange mechanism is necessary for applying biological nitrogen fixation in agriculture.

The type III secretion system (T3SS) is important for nodulation and host-range determination (Staehelin and Krishnan 2015). The T3SS is used by many phytopathogenic bacteria to deliver a collection of type III effector proteins (T3Es) that dampen host defenses (Blüher et al. 2017; Jones et al. 2018). Functional analyses of T3Es (nodulation outer proteins [Nops]) have been performed for diverse mutualistic species of rhizobia, including *Sinorhizobium fredii* (*Ensifer fredii*), *Bradyrhizobium japonicum*, and *Mesorhizobium loti* (Jones and Dangel 2006). After flavonoid induction in the host,

the expression of T3SS-related genes, including *rhc* and *nop* genes, is activated in the rhizobium (Staehelin and Krishnan 2015). A mutation of *RhcN* (strain HH103 Ω *RhcN*) abolishes the secretions of all Nops (Wang et al. 2018a). The Nop proteins act as positive and negative effectors of symbiosis, depending on the host genetic background (Xin et al. 2012). More than 11 secreted Nop proteins, including NopA, NopB, NopC, NopD, NopJ, NopL, NopM, NopI, NopP, NopT, and NopX, have been identified in *Bradyrhizobium* spp. and *S. fredii*. Elucidating the exact symbiotic and biochemical roles of these effectors is important for understanding the establishment of legume-rhizobium symbiosis (Wang et al. 2018a).

Here, we performed fine gene mapping and transcriptomic analyses, using chromosome segment substitution lines (CSSLs) carrying wild-soybean genome fragments (Xin et al. 2016). We obtained 180 RNA sequencing (RNA-seq) datasets and used them to identify the locations of genes in the substituted segments from wild soybean. A dirigent (DIR) protein, GmDDR1, was predicted to interact with the T3SS. We determined that the effect of GmDDR1 on nodule phenotype may depend on the T3SS. Our results show that changes in gene expression during soybean domestication target the formation of symbiosis-related traits. These results provide new insights into soybean domestication and reveal candidate genes for improving soybean symbioses.

RESULTS

Nodulation phenotype of CSSLs induced by *S. fredii* HH103 and its T3SS mutants.

In this study, we used an introgressed population of cultivated variety *G. max* SN14 with wild-soybean ZYD00006. We previously reported a soybean CSSL population in which marker-defined regions of SN14 were replaced with homologous intervals of the wild species *G. soja* ZYD00006 (Li et al. 2018; Xin et al. 2016).

Two CSSL lines, F1680 and F1011, showed opposite nodulation phenotypes after inoculation with *S. fredii* HH103. The

nodule number (NN) was higher in F1680 but lower in F1011 compared with that in SN14 (Fig. 1A and B). These two lines were derived from the CSSLs developed by crossing SN14 and ZYD00006, with the former being the recurrent parent. Phenotypic differences in nodulation were dependent on the F1680 and F1011 genetic backgrounds. After 10 years of backcrossing, only a few chromosome segments of ZYD00006 were substituted in SN14. Genome resequencing of F1680, F1011, and SN14 revealed four substituted fragments of ZYD00006 in F1011 on chromosomes 08 (1), 11 (1) and 16 (2), and eight substituted fragments of ZYD00006 in F1680 on chromosomes 02 (1), 05 (2), 07 (1), 11 (1), 13 (1), 17 (1), and 19 (1) (Fig. 1C; Supplementary Dataset S1).

To determine whether the phenotypic difference in nodulation was related to the T3SS, we constructed two null mutants of *S. fredii* HH103, HH103 Ω *RhcN* and HH103 Ω *NopAA*. Compared with SN14, F1680 and F1011 showed increased NNs after inoculation with HH103 Ω *NopAA* and HH103 Ω *RhcN* (Fig. 1A and B). However, F1680 had a higher NN than SN14 after being inoculated with HH103, HH103 Ω *NopAA*, and HH103 Ω *RhcN*. In F1011, the NN decreased after being inoculated with HH103 compared with being inoculated with HH103 Ω *NopAA* or HH103 Ω *RhcN* (Fig. 1B). These fragments may harbor genes that affect the NN, and there may be some unknown interaction between segments and T3SS-regulated T3Es.

Soybean transcriptome signatures influenced by the T3SS of *S. fredii* HH103.

We profiled the soybean root transcriptome under 45 different conditions (180 samples in total) (Fig. 2A; Supplementary Dataset S2). Three HH103 strains were used for these analyses. SN14 and the two CSSL lines F1101 and F1680 were used for root transcriptome analyses at 0.5, 3, 6, 12, and 24 h after inoculation independently with HH103, HH103 Ω *NopAA*, and HH103 Ω *RhcN*. At least 42, 302, and 202 reads from each line, respectively, were mapped to the Williams 82 reference

Fig. 1. The nodule phenotype and genotype of F1011 and F1680. **A**, Nodule and root phenotypes of SN14, F1011, and F1680. **B**, Comparisons of nodule numbers among SN14, F1011, and F1680 after inoculation with *Sinorhizobium fredii* HH103, HH103 Ω *RhcN*, and HH103 Ω *NopAA*. Error bars indicate standard deviations ($n = 5$). One asterisk (*) indicates $P < 0.05$ and two (**) $P < 0.001$, denoting significant differences (Student's t test). **C**, Schematic structures of substituted segments in F1011 and F1680.

genome (Fig. 2B). The mapping rate ranged from 88.87 to 94.34%. The significantly up- and downregulated genes after inoculation with HH103, HH103 Ω NopAA, and HH103 Ω RhcN were identified (Fig. 2B, C, and D).

A *k*-means clustering analysis was performed to investigate the overall gene expression patterns in SN14 and the two specific CSSL lines after inoculation with HH103 and its derived T3SS mutants. This analysis quantified the transcript levels of genes over time under different conditions. The 8,439 detected differentially expressed genes (DEGs) in soybean were clustered into 16 gene clusters containing 91 (C13) to 912 (C15) genes (Fig. 2D). In general, genes in the same cluster showed similar expression

patterns, while different clusters had distinct expression patterns. On the basis of the gene expression levels in each cluster, significant differences in gene expression under certain conditions were detected between SN14 and the two CSSL lines.

Four clusters (C4, C8, C12, and C16) showed clear differences after induction by HH103 and its derived mutants, with significant differences detected between SN14 and both F1101 and F1680 after inoculation with HH103 (Fig. 2C and D; Supplementary Dataset S3).

The genes in C12 were enriched in the gene ontology (GO) terms response to stimulus and metal ion transport (Fig. 2E). The genes in C4, C8, and C16 were enriched in

Fig. 2. Soybean transcriptome profiles under various conditions. **A**, Soybean genotypes, rhizobial strains, and conditions used in this study. **B**, General differentially expressed genes in soybean after inoculation independently with *Sinorhizobium fredii* HH103, HH103 Ω RhcN, and HH103 Ω NopAA. **C**, Gene expression patterns of SN14, F1011, and F1680 after inoculation independently with *S. fredii* HH103, HH103 Ω RhcN, and HH103 Ω NopAA. **D**, Gene clusters identified in 180 samples. Sixteen gene clusters (C1 to C16) were identified using *k*-means clustering. In each cluster, the y axis represents $\log_2$ fragments per kilobase of exon per million fragments mapped derived from RNA-seq data for each biological sample, and the x axis represents biological samples (H1 to H4, L1 to L4, and S1 to S4 for F1680, F1011, and SN14, respectively). Red, green, and blue curves represent average gene expressions in each tissue of the three soybean lines, respectively. Statistical differences are indicated (one asterisk indicates [*] $P < 0.05$ and two [*] $P < 0.01$, Student's *t* test). **E**, Heatmap of the $-\log_{10} P$ of the biological process category enrichment among the 16 clusters as determined by AgriGO.

the GO terms establishment of localization, localization, response to stimulus, transport, cellular developmental process, cation transport, ion transport, positive regulation of biological process, response to carbohydrate stimulus, response to oxidative stress, and response to temperature stimulus (Fig. 2E).

Genes in the response to stimulus-encoded DIR-like protein, glutamate dehydrogenase 2, and CLAVATA3/ESR-RELATED 45 were identified in C12, these genes might be involved in plant-bacteria interaction. In total, 105 genes were involved in cellular biosynthetic processes and 108 genes were involved in membrane-bounded organelle. Additionally, genes in C12 encoding basic helix-loop-helices (bHLHs) and WRKY transcription factors (TFs) were identified. These TFs affect nodule development and activities in soybean and *Medicago truncatula* (Chiasson et al. 2014; Liu et al. 2011).

Characterization of DEGs in substituted fragments.

To determine whether the genes in the substituted fragments affected gene expression, we identified the DEGs located in the substituted fragments at each timepoint during infection in the soybean lines. The DEGs were identified using the following criteria: false discovery rate (FDR) ≤ 0.01 and $\log_2 \geq 1$ (fragments per kilobase of exon per million fragments mapped [FPKM]_{inoculated}/[FPKM]_{mock}). The number of DEGs

varied from 11 to 1,135 on chromosomes 02, 05, 07, 11, 13, 16, 17, and 19. Among these DEGs, 2,186 and 1,472 were up- and down-regulated, respectively, in response to rhizobial infection in SN14 and F1680. In total, 3,112 genes were commonly up- and down-regulated between SN14 and F1680. The number of DEGs located in the substituted regions was 67 in SN14, 53 in F1680, and 103 in both lines (Fig. 3A; Table 1; Supplementary Dataset S4-1).

On chromosome 2, genes coding DUF707 domain protein and MADS-BOX PROTEIN SVP in the AGL17 subfamily were identified, which promote root development and nodulation in soybean (Heard and Dunn 1995). Genes encoding proteins involved in cell development and signal transduction were found on chromosome 5 (Pietrzykowska et al. 2014).

On chromosome 11, genes encoding CYCLIN, CMPG1, a DIR-like protein, XK1, and TRX were identified, which are all related to signal transduction and cell development (Heise et al. 2002). These proteins are mainly involved in cell development and responses to pathogens (Upson et al. 2018).

In SN14 and F1011, 1,406 genes and 2,491 genes were up- and down-regulated by rhizobial infection, respectively (Supplementary Dataset S4-2). In total, 3,892 genes were commonly up- and down-regulated between SN14 and F1011. Fifteen genes were specifically expressed in SN14, 20 genes were specifically expressed in F1011, and 24 genes were commonly

Fig. 3. Differentially expressed genes (DEGs) among SN14, F1011, and F1680. **A**, Venn diagram of DEGs located in substituted segments among SN14, F1011, and F1680. **B**, Quantitative reverse transcription-PCR analysis of 14 symbiosis-related DEGs in SN14 at 0.5, 3, 6, 12, and 24 h after inoculation independently with *Sinorhizobium fredii* HH103, HH103 $\Omega RhcN$, and HH103 $\Omega NopAA$.

expressed in both. These genes were located on chromosomes 08, 11, and 16 (Fig. 3A).

To validate the RNA-seq results, we performed quantitative reverse transcription-PCR (qRT-PCR) experiments using 14 genes involved in nodule formation, ammonium transport, signal transduction, and oxidative stress responses. The results are shown in Figure 3B.

Identification of candidate genes underlying symbiosis and interactions with T3SS.

To further investigate the correlations between the detected DEGs and soybean nodule traits, we performed a linkage analysis of the DEGs, using quantitative trait loci (QTL) and selection sweeps. We focused on the chromosomes containing substituted fragments from the wild-soybean genome. By analyzing the QTL information in Soybase and the QTL identified in our laboratory, we found 89 QTL underlying nodule traits on chromosomes 02, 05, 07, 11, 13, 17, and 19 (Supplementary Fig. S1). We modeled chromosome 11 as a representative. In the substituted region Block6281, the genes *Glyma.11g150700* and *Glyma.11g150400* were among the DEGs. Block6281 contained 27 annotated genes, including three duplicated genes (*Glyma.11g150300*, *Glyma.11g150400*, and *Glyma.11g150700*) predicted to encode DIR-like disease resistance-responsive (DRR) protein family members (Fig. 4A). These genes formed a gene cluster and were designated as

GmDRR1 (*Glyma.11g150400*), *GmDRR2* (*Glyma.11g150300*) and *GmDRR3* (*Glyma.11g150700*) (Fig. 4A).

The three *GmDRR* proteins belonged to the DIR-like DRR protein family and had domain structures characteristic of DRR proteins, including a DIR domain. Because DIR proteins (from the Latin *dirigere*, to align, guide) have been implicated in plant responses to biotic stress (Chen et al. 2018; Hosmani et al. 2013), these three genes were selected for further investigation as candidate genes underlying symbiosis establishment. Only *Glyma.11g150400* and *Glyma.11g150700* were among the DEGs identified from the RNA-seq data.

Sequence comparisons of the *GmDRR* gene clusters in SN14, F1011, and F1068 revealed several nucleotide polymorphisms in F1011 (R28), including a premature termination caused by a single nucleotide deletion in *GmDRR1* (Fig. 4C, Supplementary Dataset S1). No single nucleotide polymorphisms were found in the coding regions of *GmDRR2* and *GmDRR3*. However, polymorphisms were found in the promoter regions of all three genes (Supplementary Dataset S1). Thus, the nucleotide polymorphisms in *GmDRR1-GmDRR3* might be responsible for the different nodule phenotypes in the CSSLs and their parents.

To explore the roles of the *GmDRR* genes in symbiosis establishment, we detected the transcript levels of *GmDRR1-GmDRR3* in roots of SN14 after inoculation independently with HH103, HH103Ω*RhcN*, and HH103Ω*NopAA*. The qRT-PCR

Table 1. The genes shared between SN14 and F1011/F1680

Gene	Gene ID	Annotation
SN14/F1680		
Chr02	Glyma.02G039900	Protein of unknown function (DUF707)
	Glyma.02G041500	K-box region and MADS-box transcription factor family protein
Chr05	Glyma.05G128000	Chlorophyll A/B binding protein 1
	Glyma.05G095400	Ferritin 2
	Glyma.05G096800	Granulin repeat cysteine protease family protein
	Glyma.05G127800	Starch synthase 4
	Glyma.05G082200	Disease resistance protein (coiled coil-nucleotide binding site-leucine-rich repeat [NBS-LRR] class) family
Chr13	Glyma.05G092800	Cooperatively regulated by ethylene and jasmonate 1
	Glyma.13G003200	DCD (Development and Cell Death) domain protein
	Glyma.13G005800	Leucine-rich repeat (LRR) family protein
	Glyma.13G028100	Disease resistance protein (toll interleukin 1 receptor-NBS-LRR class), putative
	Glyma.13G004400	Zinc transporter 5 precursor
	Glyma.13G007200	Protein kinase superfamily protein
	Glyma.13G008000	
	Glyma.13G004400	Zinc transporter 5 precursor
	Glyma.13G007200	Protein kinase superfamily protein
	Glyma.13G008000	
	Glyma.13G003700	Heat-shock protein 70 (Hsp 70) family protein
	Glyma.13G005100	VQ motif-containing protein
	Glyma.13G029900	UDP-glucosyl transferase 85A3
	Glyma.13G030100	High mobility group B2
	Glyma.13G030200	Polyketide cyclase/dehydrase and lipid transport superfamily protein
SN14/F1011		
Chr08	Glyma.08G212900	Fatty acid desaturase 5
	Glyma.08G213100	Phosphatidic acid phosphatase-related / PAP2-related
	Glyma.08G213400	Molecular chaperone Hsp40/DnaJ family protein
Chr11	Glyma.08G216200	NAD(P)-binding Rossmann-fold superfamily protein
	Glyma.11G108500	Plant protein of unknown function (DUF827)
	Glyma.11G109100	PLATZ transcription factor family protein
	Glyma.11G109400	Prohibitin 2
	Glyma.11G111800	Subtilase family protein
	Glyma.11G112600	Xylulose kinase-1
	Glyma.11G117100	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein
	Glyma.11G118300	cyclase associated protein 1
	Glyma.11G118600	Coatomer gamma-2 subunit, putative / gamma-2 coat protein, putative / gamma-2 COP, putative
	Glyma.19G243500	Duplicated homeodomain-like superfamily protein
Chr19	Glyma.19G243600	NIMA-related kinase 2
	Glyma.19G243700	IQ-domain 19
	Glyma.19G244100	HXXXD-type acyl-transferase family protein

analyses confirmed that the three *GmDRRs* were strongly induced in soybean roots by the presence of the rhizobium and its derived mutants (Fig. 5A), and *GmDRR1* was induced at the early stage of nodule establishment. The T3SS mutation promoted *GmDRR* expression, suggesting that *GmDRR* might interact with the effectors secreted by the T3SS during nodule formation.

To further explore the role of *GmDRR1*, we generated a *GmDRR1*-overexpression line (*GmDRR1*-OX) and a *GmDRR1* RNA interference line (*GmDRR1*-RNAi) (Supplementary Fig.

S2). Compared with wild-type soybean, transgenic *GmDRR1*-OX soybean showed significantly higher NNs after inoculation with HH103 Ω RhcN and HH103 Ω NopAA (Fig. 5B). However, there were no significant differences in NNs among the *GmDRR1*-RNAi lines inoculated independently with HH103, HH103 Ω RhcN, and HH103 Ω NopAA. The *GmDRR1*-OX line had a similar nodular phenotype as F1011, the NN was increased after inoculation with HH103 Ω RhcN and HH103 Ω NopAA compared with after inoculation with HH103. These results suggested that *GmDRR1* is a gene in the substituted segment that

Fig. 4. Quantitative trait loci (QTL) locations within substituted segments of chromosome 11. **A**, Confidence region of a multi-QTL related to the nodule phenotype on chromosome 11 and the overlap between substituted regions in F1011 and F1680. **B**, Epistasis analysis of QTL among chromosome 07, 11, and 12 in the recombinant inbred line population. Results are from two-year field tests of nodulation in high generation recombinant inbred lines. **C**, Haplotype analyses of *GmDRR1* in F1011 and F1680.

underlies the establishment of symbiosis, which could be T3SS- and T3E-dependent.

To determine the effects of *GmDRR1* on the nodule phenotype, nodules of *GmDRR1*-OX lines were embedded in paraffin and sections were examined under a microscope. The nodule structures of *GmDRR1*-OX lines were the same after inoculation independently with HH103, HH103 Ω NopAA, and HH103 Ω RhcN (Fig. 5C).

DISCUSSION

Studies of the symbiosis between legumes and rhizobia in the last few decades have revealed two major forms of plant innate immunity, microbe-associated molecular pattern-triggered (MTI) and effector-triggered (ETI) (Cao et al. 2017; Gourion et al. 2015). Both MTI and ETI are highly effective in restricting symbiosis establishment. However, how MTI and ETI regulate rhizobial growth is unknown, and it is difficult to study their mechanisms in detail. In this study, we conducted an RNA-seq analysis of roots inoculated with T3SS-related mutants and wild-type rhizobia in soybean lines having similar genetic backgrounds. We were able to link the activation of various immune-signaling pathways to specific changes in gene expression in the T3SS null mutant.

Using a combination of RNA-seq and QTL analyses, we identified specific genes in the substituted chromosome segments that were expressed in response to rhizobial infection. We found that immune-responsive genes expressed at the early stage of infection were involved in cell development and metabolism. One of the immune-responsive genes expressed early during infection encodes a DIR protein. The DIR proteins are involved in the stereo-specific coupling of *E*-coniferyl alcohol to produce the lignin (+)-pinorensinol (Khan et al.

2018). In general, DIR and DIR-like proteins are involved in disease resistance responses (Marquez et al. 2018; Sedivy et al. 2017). We found that transgenic plants overexpressing *GmDRR1* showed increased NNs after inoculation with the T3SS null mutant but not after inoculation with the T3E *NopAA* null mutant. This result supported the idea that unidentified T3Es may regulate DIR expression to promote symbiosis establishment.

Lignins and phenolics contribute to plant resistance to fungal and insect attacks (Sedivy et al. 2017; Sun et al. 2018; Tobimatsu and Schuetz 2019). In cotton, lignification can block the spread of *Verticillium dahliae* (Shi et al. 2012). Overexpression of a DIR protein in rice conferred broad-spectrum resistance against several pathogens (bacteria, fungi, and oomycetes) (Van Holle and Van Damme 2018). In *Medicago truncatula*, the expression of a gene encoding a DIR protein in the plasma membrane was modified by arbuscular mycorrhizal symbiosis (Aloui et al. 2018). We believe that DIR proteins have unusual regulation mechanisms during rhizobial infection and nodule formation. The DIR proteins are involved in the final stage of cell-wall lignin synthesis, which could allow them to direct important changes in cell-wall structures during symbiosis establishment. Changes in the expression of *GmDRR1* may result in changes in lignin content and distribution in the nodule to regulate nodule formation.

In previous studies, DIR-like proteins were down-regulated by *GmNSP1* and *GmNSP* during nodule organogenesis (Liu et al. 2011), and a *GmbHLH* TF important for nodule growth down-regulates DIR-like proteins during symbiosis establishment (Chiasson et al. 2014). The mutant *GmbHLHm1* forms significantly fewer and smaller nodules than the wild type. In *Medicago truncatula*, *MtbHLH1* is expressed in nodule primordia, nodule vascular tissues, and in uninfected cells of the nitrogen-fixing

Fig. 5. Nodule phenotypes in soybean Tianlong1 (TL1) and TL1 overexpressing *GmDRR1*. **A**, Quantitative reverse transcription-PCR analysis of *GmDRR* transcript levels in SN14 at 0.5, 3, 6, 12, and 24 h after inoculation independently with *Sinorhizobium fredii* HH103, HH103 Ω RhcN, and HH103 Ω NopAA. **B**, Nodule number in *GmDRR1*-overexpression lines after inoculation independently with *S. fredii* HH103, HH103 Ω RhcN, and HH103 Ω NopAA. **C**, Images of paraffin sections showing structures of nodules in soybean TL1 and TL1 lines overexpressing *GmDRR1*.

zone (Diédhiou and Diouf 2018). However, the roles of DIR-like proteins in soybean-rhizobium symbiosis is unknown.

GmDRR1 belongs to the DIR-b cluster, whose members have no known biochemical or physiological functions in the formation of pinosresinol. This cluster includes *DIR4* from *Gossypium hirsutum*, which catalyzes the atroposelective coupling of two achiral hemigossypol molecules into (+)-gossypol (Corbin et al. 2018). Additionally, DIR expression is regulated by bHLH TFs in soybean and cotton (Song and Peng 2019). In soybean, the gene *Symbiotic Ammonium Transporter 1* (*SAT1*) encodes a bHLH TF that is important for soybean nodule growth and is linked to the activity of a unique class of ammonium channels and to signaling cascades affecting the nodule circadian clock (Chiasson et al. 2014). This TF can suppress the expression of DIR genes during soybean-rhizobium interactions. This suggests that *GmDRR2* might be regulated by *GmSAT1*. According to our qRT-PCR results, the expression of *GmSAT1* was affected by the T3SS (Fig. 4). However, more work is needed to identify which T3E specifically interacts with the *GmDRR* signaling pathway.

We detected a QTL on chromosome 07 that had an epistatic effect on chromosome 11. This locus coincided with the region containing *GmDRR1* (Fig. 4B). The genes *Glyma.07g077400* and *Glyma.07g077500* on chromosome 07 were among the DEGs detected in F1011. A STRING analysis showed that *Glyma.07g077400* encodes a protein related to defense responses and cell-wall structure.

Cultivated soybean was domesticated from its annual wild relative *G. soja* in China (Carter et al. 2004). The genetic variations that occurred during soybean domestication have been studied (Wang et al. 2018b) but are still poorly understood. Here, we used CSSLs with a wild-soybean genetic background and compared their transcriptomes after induction with a rhizobium and its derived T3SS-related mutants to shed new light on soybean domestication and the genetic improvement of symbiosis. We found 47 genes located in the substituted segments of chromosome 16 that were expressed in SN14 and F1068 but not in F1011. This suggested that the TFs controlling the expression of these genes were inhibited or destroyed in F1011. Because there are only three substituted segments of wild soybean in F1011, the lower NN in F1011 compared with that in SN14 might be related to these unexpressed genes. Some of these genes were involved in nitrogen compound metabolism and cellular metabolism.

MATERIALS AND METHODS

Genetic population.

A wild-soybean CSSL population comprising 213 lines was constructed in this study. In 2017, 213 CSSLs were obtained, based on 165 elementary CSSLs, by marker selection. A fine mapping population was created by selecting homozygous recombinant BC₃F₂, BC₃F₃, BC₃F₄, and BC₃F_n lines (Xin et al. 2016). The CSSL population was constructed by crossing with the cultivar Suinong14 (recurrent parent, provided by the Suihua Branch of Heilongjiang Academy of Agricultural Sciences) and ZYD00006 (donor parent, provided by the Chinese Academy of Agricultural Sciences) as the F₁. These lines were further developed through three backcrosses of selected F₁ lines to Suinong14 and then self-pollination to obtain the F₂ generation (Xin et al. 2016).

Construction and confirmation of the *S. fredii* HH103 *RhcN* (HH103Ω*RhcN*) and *NopAA* (HH103Ω*NopAA*) mutants.

For mutant construction, 2.1- and 1.4-kb fragments of *RhcN* and *NopAA*, respectively, were amplified. These fragments

were cloned independently into pGWC digested with *SpeI* and *KpnI* to generate pGWC-*RhcN* and pGWC-*NopAA*, respectively. Through PCR-mediated site-directed mutagenesis, *SpeI* and *KpnI* restriction sites were generated close to the start codons (ATG) of *RhcN* and *NopAA*. The kanamycin gene was amplified from pEASY-T1 (Transgen Biotech), with specific primers, to generate pGWC-*RhcN*-km and pGWC-*NopAA*-km, respectively. The amplified fragments and suicide vector were digested and were ligated independently and were then transformed into *Escherichia coli* DH5α and sequenced. The resulting constructs were named pJQ200SK-*RhcN*-km and pJQ200SK-*NopAA*-km, respectively (Quandt and Hynes 1993). *E. coli* harboring pJQ200SK-*RhcN*-km, *E. coli* with the helper plasmid pRK2013 (Figurski et al. 1979), and HH103 were triparentally hybridized and were grown on tryptone yeast solid medium with 5% (wt/vol) sucrose and rifampin and kanamycin antibiotics at 28°C. The selected mutants were named HH103Ω*RhcN* and HH103Ω*NopAA*, respectively. The candidate mutants HH103Ω*RhcN* and HH103Ω*NopAA* were confirmed by Southern blot analysis using DIG-High Prime DNA labeling and detection starter kit II (Roche). The probe designed for Southern blotting was 500 bp and included part of the target fragment and a kanamycin-resistance fragment and was labeled with DIG-High Prime (Roche). All the plasmid constructs were verified by sequencing. The relevant PCR primers are listed in Supplementary Table S1.

Plant growth conditions and nodulation assays.

The soybean cultivar used in the study was SN14 and the lines were F1680 and F1011. The seeds were surface-sterilized in chlorine gas for 12 h, were kept under sterile airflow for 30 min on an aseptic bench, and were then germinated in sterile plastic plates with sterile water. The soybean plants were transferred to two Magenta jars linked with a cotton wick. One end of the wick was covered with vermiculite and small stones in the upper vessel, and the other end was immersed in nitrogen-free nutrient solution in the lower vessel; all parts of this system were sterile (Skorpil et al. 2005). The *S. fredii* HH103 strain was cultured for 2 or 3 days, and the mutants HH103Ω*RhcN* and HH103Ω*NopAA* were cultured for 3 or 4 days, until the optical density at 600 nm value reached 0.6. The number of cells was adjusted to 10⁹ ml⁻¹ in 10 mmol liter⁻¹ MgCl₂ solution, and 2 ml rhizobium liquid was inoculated onto soybean roots with a syringe. Plants were grown in a plant growth cabinet at 25°C under a 16-h light and 8-h dark photoperiod. The nodules were counted at 21 days after inoculation.

The CSSL populations and their parents were grown in a field at Northeast Agricultural University, Harbin (45.75°N, 126.53°E). Each line was planted in a 5-m-long row with row spacing of 0.65 m and 5 cm between seeds. General field management conditions are described elsewhere (Zhang et al. 2018). At the soybean V10 to V13 stage, nodule phenotype traits were evaluated in the CSSLs and their parental lines. The nodule traits included small NN (diameter ≤ 2 mm), big NN (diameter > 2 mm), total NN, and total nodule dry weight. Five biological repeats were used for every trait. The average value was used for QTL identification. Nodule dry weight was determined after oven-drying at 65°C for 48 h.

RNA isolation and sequencing.

Different root samples (Supplementary Dataset S2) were collected at 0.5, 3, 6, 12, and 24 h after inoculation for RNA-seq. Three biological replicates were analyzed. Soybean root total RNA was extracted using TRIzol reagent (Invitrogen). First-strand cDNA was prepared with 1 µg soybean total RNA, using a PrimeScript RT reagent kit (TaKaRa Biomedical Technology).

Libraries were constructed using the Illumina TruSeq kit (RS-200-0012; Illumina Inc.). Agilent 2100 and NanoDrop instruments were used to determine the integrity and purity of the total RNA, respectively. mRNA sequencing was performed on the Illumina HiSeq 4000 PE 150 platform to determine the DNA strand forward or reverse orientation to more accurately count the number of transcripts and determine gene structure.

Analysis of transcriptome data and whole-genome resequencing.

We analyzed the RNA-seq datasets using a modified version of a previously published protocol (Liu et al. 2012). The quality of the raw transcriptome data was controlled using the FastQC toolkit (Babraham Bioinformatics). The raw RNA-seq datasets were aligned to the *Glycine max* Wm82.a2.v1 genome (Joint Genome Institute [JGI] Phytozome portal) and were annotated using HISAT2 (Kim et al. 2015). Read numbers for each gene were counted using HTseq-count (Anders et al. 2015), based on a GTF-formatted file generated by Cufflinks and Cuffcompare (Trapnell et al. 2012). The FPKM (Mortazavi et al. 2008) value for each gene was calculated and normalized, using DESeq2 (Love et al. 2014) in the R package, with global normalization parameters.

The DEGs for each condition compared with the corresponding control were identified using DESeq2 with default parameters (Love et al. 2014). *P* values were adjusted using the FDR. Genes with an absolute value of $\log_2$ ratio (condition/control) ≥ 1 and an adjusted *P* value ≤ 0.01 were declared differentially expressed. Annotation information from the soybean genome was obtained for the DEGs. The DEG expression patterns were classified by *k*-means clustering (Huang et al. 2012). The number of 16 cluster was decided by the silhouette coefficient. The GO enrichment analysis was performed using AgriGO, with a *P*-value < 0.01 . The Kyoto Encyclopedia of Genes and Genomes enrichment analysis was performed using the clusterProfiler R package.

RNA isolation and qRT-PCR analyses of candidate genes.

To determine whether the candidate genes were involved in nodulation, total RNA was extracted using total RNA extraction reagent (Transgene Biotech Co.). Reverse transcription was performed with the HiScript II First-strand cDNA synthesis kit (Vazyme Biotech). The qRT-PCR analyses were conducted using a Roche LightCycler 480 quantitative PCR instrument. Primers were designed based on locus information in Phytozome, and *GmUKN1* (*Glyma.12g020500*) was used as a reference gene for relative quantification of target gene transcript levels (Hu et al. 2009). The qRT-PCR program was as follows: predenaturation at 95°C for 30 s, and 40 cycles of release and annealing at 58°C for 10 s. Three biological replicates and three technical replicates were used to improve the accuracy of the results.

Construction of GmDRR1 transgenic soybean plants.

To generate transgenic *GmDRR1*-OX soybean plants, the full-length coding sequence was amplified using gene-specific primers (5'-ATGGATGGAGACG-3' and 5'-CTAAGGATGAGAAA CAGTGACA-3'). The amplified fragment was digested with *Xba*I and *Asc*I and was then inserted into the transformation vector pFGC5941, containing the cauliflower mosaic virus 35S promoter, and an additional green fluorescent protein (GFP) tag was added. The RNAi vector pB7GWING2-RNAi was constructed by transferring the RNAi sequence from pGWC-RNAi by the LR reaction. The RNAi sequence was amplified using primers (forward: ACTTTCATGATGGACAATGT and reverse: GGCAATGGCA TAGCCCTT) and was then ligated into the pGWC vector.

Each plasmid was transformed independently into the soybean cultivar Tianlong 1 using *Agrobacterium*-mediated transformation (Paz et al. 2006). Transformants were identified

for resistance to Basta herbicide. Transgenic soybeans over-expressing the *GmDRR1* transgene were identified using qRT-PCR and Western blotting with a GFP antibody (Supplementary Fig. S2). The RNAi soybean lines were identified using qRT-PCR. Paraffin embedding and sectioning of nodules were performed as described previously, using a microwave processor (Zhao 2009).

Morphological characteristics and structures of nodules at different developmental stages.

Roots were embedded in paraffin and were cut into sections to observe morphological characteristics and structures (Zhao 2009). Soybean root samples collected at different times (0.5, 3, 6, 12, and 24 h) after inoculation with rhizobium were fixed in 90 ml of 50% ethanol, 5 ml of oxymethylene, and 5 ml of glacial acetic acid for at least 24 h. The specimens were then transferred from the fixing solution to an ethanol gradient (1.5 h each in 85, 90, 100, and 100% ethanol) for dehydration. Then, the samples were immersed in a series of different ratios of xylene and ethanol (1/3 xylene and 2/3 ethanol, 1/2 xylene and 1/2 ethanol, 2/3 xylene and 1/3 ethanol, and pure xylene twice) for 1 h each. Wax was added to the pure xylene until it reached saturation, and the sample was kept at 37°C for 12 h. Half the volume of xylene and paraffin was then poured out and melted wax was added. This process was repeated three times; the last time all xylene and paraffin was poured out and was replaced with melted wax. The sample was then removed to a make a small cube, and a paraffin slicer was used to cut the sample into slices. The sections were observed under a microscope.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

AgriGO toolkit and database: <http://bioinfo.cau.edu.cn/agriGO>
JGI Phytozome portal: <https://phytozome.jgi.doe.gov>

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