agronomy

MDPI

Article

Mapping of Quantitative Trait Loci Underlying Nodule Traits in Soybean (*Glycine max* (L.) Merr.) and Identification of Genes Whose Expression Is Affected by the *Sinorhizobium fredii* HH103 Effector Proteins NopL and NopT

Hejia Ni †, Yang Peng †, Jinhui Wang †, Jing Wang, Yantong Yuan, Tingting Fu, Zikun Zhu, Jialin Zhang, Xipeng Pan, Zhuoling Cui, Chunyan Liu, Qingshan Chen * and Dawei Xin *

Key Laboratory of Soybean Biology of the Chinese Ministry of Education, Key Laboratory of Soybean Biology and Breeding, Genetics of Chinese Agriculture Ministry, College of Agriculture, Northeast Agricultural University, Harbin 150036, China; nhjwinner@163.com (H.N.); b210301014@neau.edu.cn (Y.P.); jinhuiwang113@126.com (J.W.); wangjing20211029@163.com (J.W.); ytyuan2022@163.com (Y.Y.); futingting1106@163.com (T.F.); zzk13009707019@163.com (Z.Z.); 100518star@163.com (J.Z.); panxipeng1997@163.com (X.P.); cuizhuoling@163.com (Z.C.); cyliucn@126.com (C.L.)
* Correspondence: qshchen@126.com (Q.C.); dwxin@neau.edu.cn (D.X.)
† These authors contributed equally to this work.

Abstract: Symbiotic nitrogen fixation provides most of the nitrogen required for soybean growth. Rhizobial nodulation outer proteins (Nops) have been reported to influence host specificity during symbiosis establishment. However, the host proteins that interact with Nops remain unknown. In this study, we generated *Sinorhizobium fredii* HH103 mutants (HH103Ω*NopL*, HH103Ω*NopT*, and HH103Ω*NopL*Ω*NopT*) and analysed the nodule number (NN) and nodule dry weight (NDW) of 12 soybean germplasms after inoculation with wild-type *S. fredii* HH103 or the mutant strains. The analysis of chromosome segment substitution lines revealed quantitative trait loci (QTLs) associated with NopL and NopT interactions. A total of 22 QTLs for the 2 nodule traits were detected and mapped to 12 different chromosomes in the soybean genome. Eight and fifteen QTLs were found to be associated with NN and NDW, respectively. Furthermore, 17 candidate genes were selected for further analyses. Considering the results of reverse-transcription quantitative PCR, we propose that the protein products of these 17 candidate genes interact with NopL and NopT.

Keywords: symbiosis; type III secretion system effector; soybean; quantitative trait locus; NopL; NopT

Citation: Ni, H.; Peng, Y.; Wang, J.; Wang, J.; Yuan, Y.; Fu, T.; Zhu, Z.; Zhang, J.; Pan, X.; Cui, Z.; et al. Mapping of Quantitative Trait Loci Underlying Nodule Traits in Soybean (*Glycine max* (L.) Merr.) and Identification of Genes Whose Expression Is Affected by the *Sinorhizobium fredii* HH103 Effector Proteins NopL and NopT. *Agronomy* **2022**, *12*, 946. https://doi.org/10.3390/agronomy12040946

Academic Editor: Michael Timko

Received: 19 March 2022
Accepted: 13 April 2022
Published: 15 April 2022

1. Introduction

Soybean (*Glycine max* (L.) Merr.) is an economically relevant crop that is widely grown worldwide and is considered a major protein source for humans and animals [1]. Rhizobia can enter soybean roots by infecting root hairs and then establishing a symbiotic relationship with soybean plants. Symbiosis enables soybean to fix atmospheric nitrogen through a process known as symbiotic nitrogen fixation (SNF) [2]. Nitrogen is an indispensable element for soybean growth and can be a limiting factor for crop production. In recent years, large amounts of nitrogen fertilisers have been used to increase crop production; these, however, can also cause serious negative impacts on the environment [3]. The legume–rhizobia symbiosis, characterised by highly efficient nitrogen fixation, produces 70% of the total SNF (Food and Agriculture Organization) and thus plays an important role in plant cultivation with reduced fertiliser application [4]. The recognition and acceptance of rhizobia by legumes is a complicated process. The main molecular determinant of this symbiotic interaction is the Nod factor. Rhizobial infection requires the activation of a genetic program in the plant root and the exchange of molecules between the symbiotic partners [5]. In addition, *Sinorhizobium fredii* nodulation in roots is influenced by nodulation

outer proteins (Nops), which are secreted through a type III secretion system (T3SS) [6]. Intriguingly, research suggests that the T3SS activates host nodulation signalling by bypassing Nod factor recognition [7]. The T3SS secretes effector proteins (T3Es) that are involved in nodulation efficiency and host range determination, and in some cases directly activate host symbiotic signalling in a Nod-factor-independent manner [8]. For example, the *Bradyrhizobium elkanii* T3SS triggers salicylic acid (SA)-mediated effector-triggered immunity (ETI) in soybean cultivars harbouring an *Rj4* allele, which consequently blocks symbiotic interactions [9].

S. fredii HH103, isolated from Chinese soil in the Hubei Province, is a fast-growing rhizobial species similar to the model strain *S. fredii* NGR234 [10,11]. In recent years, some Nops have been identified in the genome of *S. fredii* HH103. NopAA (GunA), showing specificity toward xyloglucan, may participate in the recognition of bacteria by plant roots as well as in the nodulation process [12]. NopP secreted by *S. fredii* HH103 inhibits soybean nodulation, and NopP secreted by the *Rhizobium* sp. strain NGR234 can be phosphorylated by plant kinases [13,14]. Conversely, NopC can be considered a rhizobium-specific effector that, when secreted by *S. fredii* HH103, promotes symbiosis with *G. max* [15]. Moreover, NopB, secreted by *Rhizobium* sp. NGR234 inhibits the interaction with *Pachyrhizus tuberosus* and promotes nodulation in *Tephrosia vogelii* [16]. In addition, NopM and NopD were first identified in *S. fredii* HH103; *S. fredii* HH103 NopM is homologous to the protein encoded by the gene *y4fr* of *Rhizobium* sp. NGR234, which is a NEL-domain E3 ubiquitin ligase [17,18]. The various functions of the Nops secreted by *S. fredii* HH103 are the result of complex interactions between each effector and host plants.

NopL, identified in *S. fredii* HH103 by genomic sequencing, is believed to be secreted within the host cell [19]. NopL can modulate host mitogen-activated protein kinase (MAPK) signalling and impair the function of MAPK substrates [20]. After inoculation of soybean with the *S. fredii* HH103Ω*NopL* mutant, a quantitative trait locus (QTL) for nodule dry weight (NDW) was found to be located on Gm02 [21]. NopT is a C58 cysteine protease that shares homology with YopT of *Yersinia* sp. and AvrPphB of *Pseudomonas syringae pv. phaseolicola* [22,23]. NopT_GS0123 has been shown to inhibit the host plant immune system and to exert a minimal effect on nodulation in *Robinia pseudoacacia* [23]. Moreover, NopT secreted by *S. fredii* HH103 directly affected the expression levels of *Glyma.02G135100* and *Glyma.02G100800* [24]. However, no proteins that directly interact with either NopL or NopT have yet been reported.

QTL mapping is a genome-wide inference of the relationship between the genotypes of different genomic positions and the phenotypes of a set of quantitative traits in terms of the number, genomic positions, effects, and interaction of QTLs [25]. The first QTL analysis for symbiotic nitrogen fixation activity traits was reported in 2012 [26]. Recent studies in soybean have identified several genes related to nodulation, such as *Glyma.04g060600*, *Glyma.18g159800*, and *Glyma.13g252600*, which might interact with *rhcJ*, belonging to the T3SS cluster [27]. Moreover, the expression of *Glyma.19g068600* and *Glyma.19g069200*, identified by QTL mapping, was found to be associated with the presence of NopD during the establishment of symbiosis between rhizobia and soybean plants [10]. Further identification of genes that interact with T3Es would contribute to an improved understanding of the signals exchanged between rhizobia and host plants during symbiosis establishment.

In this study, we identified QTLs encoding proteins that interact with NopL and NopT in a chromosome segment substitution lines (CSSLs) derived from a cross between soybean germplasms suinong14 and ZYD00006. Additionally, soybean genes whose expression is affected by NopL and NopT were identified.

2. Materials and Methods

2.1. Strains, Plasmids, and Plant Material

The used bacterial strains, including *Escherichia coli* and *Rhizobium* spp. strains and plasmids are listed in Table 1. *E. coli* DH5α cells were grown at 37 °C in Luria–Bertani (LB) medium [28]. *S. fredii* HH103 and its derived mutants were grown in tryptone yeast (TY)

medium [29] at 28 °C. Antibiotics, including rifampin, kanamycin, and spectinomycin, were used at a working concentration of 50 μg/mL. The germplasms used, derived from different ecoregions, were Jingshanpu, Heinong44, Heinong35, Suinong15, CN201, Hongfeng11, Nenfeng15, He00-23, Suinong14, Dongnong594, Dongnong50, and Charleston. Moreover, an experimental population comprising 213 chromosome segment substitution lines (CSSLs) was used in this study [30]. The CSSL population was constructed by crossing "suinong14" (provided by the Suihua Branch of the Heilongjiang Academy of Agricultural Sciences) and "ZYD00006" (provided by the Chinese Academy of Agricultural Sciences) and subsequent backcrossing with suinong14.

Table 1. Parental and population statistics for nodule traits in the soybean 'Suinong14' × 'ZYD00006' population.

		CSSLs (n = 213)			Parents (Average)	
	Traits	**Average**	**Standard Deviation**	**Coefficient of Variation**	**Suinong14**	**ZYD00006**
HH103	Nodule number	48.57	38.56	0.79	21.20 ± 19.88	14.67 ± 6.51 *
	Nodule dry weight (g)	0.04	0.03	0.69	0.03 ± 0.03	0.0013 ± 0.0038
HH103Ω*NopL*	Nodule number	13.65 **	8.03	0.58	17.00 ± 6.67	10.00 ± 1.00
	Nodule dry weight (g)	0.02 **	0.03	1.63	0.04 ± 0.02	0.0077 ± 0.0011
HH103Ω*NopT*	Nodule number	14.01 **	10.77	0.77	15.00 ± 3.61	8.33 ± 1.53
	Nodule dry weight (g)	0.02 **	0.02	1.28	0.02 ± 0.02	0.0074 ± 0.0038
HH103Ω*NopL*Ω*NopT*	Nodule number	16.77 **	11.56	0.69	12.20 ± 4.71	9.00 ± 6.08
	Nodule dry weight (g)	0.02 **	0.01	0.70	0.03 ± 0.02	0.0103 ± 0.0075

* indicates $p \leq 0.05$, ** indicates $p \leq 0.01$.

2.2. Generation of the *S. fredii* HH103Ω*NopL*Ω*NopT* Mutant

To generate the *S. fredii* HH103Ω*NopL*Ω*NopT* mutant, the HH103Ω*NopL* mutant was first constructed. A 1.1 kb fragment containing *nopL* was cloned into pGWC to generate pGWC-*NopL*1100. A *Spe*I restriction site close to the ATG codon of *nopL* was generated by PCR-based site-directed mutagenesis. The kanamycin Ω interposon marker (kanamycin resistance) was amplified from pEASY-T1 (TransGen Biotech, Beijing, China) using specific primers harbouring *Spe*I restriction sites (Supplemental Table S1), generating pGWC-*NopL*1100Ω. The construct was then cloned into the suicide vector pJQ200SK [31]. The resulting plasmid (pJQ-*NopL*1100Ω) was mobilised from *E. coli* DH5α into *S. fredii* HH103 by triparental mating using the pRK2013 helper plasmid [32]. Gene replacement was performed through selection for kanamycin resistance and growth on 5% (w/v) sucrose. The obtained mutant, HH103Ω*NopL*, was confirmed by PCR analysis (Supplemental Figure S1). At the same time, a 2.0 kb fragment containing *nopT* was cloned into pGWC to generate pGWC-*NopT*2000. An *Eco*RI restriction site of *nopT* was generated by PCR-based site-directed mutagenesis. The spectinomycin Ω interposon marker (spectinomycin resistance) was amplified from pSOY1 using specific primers harbouring *Eco*RI restriction sites (Supplemental Table S1), generating pGWC-*NopT*2000Ω. The construct was then cloned into the suicide vector pJQ200SK. HH103Ω*NopT* was constructed by triparental mating using the pRK2013 helper plasmid, pJQ-NopT2000Ω, and *S. fredii* HH103. The resulting plasmid (pJQ-*NopT*2000Ω) was mobilised from *E. coli* DH5α into *S. fredii* HH103Ω*NopL* by triparental mating using the pRK2013 helper plasmid. Gene replacement was performed through selection for kanamycin and spectinomycin resistance and growth on 5% (w/v) sucrose. The obtained mutant, HH103Ω*NopL*Ω*NopT*, was confirmed by PCR analysis (Supplemental Figure S2).

2.3. Nodulation Tests

Five seeds for each used soybean variety, including the germplasms and the CSSLs, were surface sterilised using chlorine gas, commercial Clorox bleach, containing 5.25%

bleach, and 3% (*v*/*v*) hydrogen peroxide and germinated in sterile plastic jars. In the latter, the upper layer was vermiculite, and the lower layer was a nitrogen-free nutrient solution, with cotton slivers connecting the upper and lower layers. All plants were cultivated under greenhouse conditions with 16 h of light at 26 °C and 8 h of dark at 25 °C [12,33]. Then, the plants were inoculated with 2 mL containing 1×10^9 bacteria (strain HH103 or its mutant derivatives) at the VC stage [20]. Non-inoculated plants were treated with 10 mm MgSO4 and served as a control. Three biological replicates were performed for nodulation tests for each variety of soybean. Four weeks after inoculation, the plants were harvested, the average NN and NDW were determined, and the NDW was determined again after drying at 65 °C for 48 h [34,35]. Student's *t*-tests were used to assess statistically significant differences in terms of nodulation phenotypes.

2.4. QTL Analysis

The experimental wild soybean CSSL population used in this study comprised 213 lines [36]. Genomic resequencing of these CSSLs and their parents has been completed (unpublished data); these genetic backgrounds provide support for identifying candidate genes in each QTL of interest. To map QTLs controlling nodule-related traits in the Suinong14 × ZYD00006 CSSL population, we performed composite interval mapping using WinQTL Cartographer [20,37]. The control marker number and window size were set to 5 and 10 cm, respectively. Additionally, a walking speed of 0.5 cm and the forward regression method were selected. Additive effects were assessed using the modified carbapenem inactivation method (mCIM) [38], and the proportion of variance was explained by each QTL. The logarithm of the odds (LOD) scores greater than 2.0 (WinQTL Cartographer default threshold) between plants independently inoculated with the wild-type and mutant strains used in this study indicated the existence of conditional QTLs for the two nodule traits. For the signals of additive effects, '+' indicates increasing allelic effects from ZYD00006, and '−' indicates decreasing allelic effects from Suinong14. Linkage was reported as significant if the two values for a marker were greater than the critical value at $p = 0.05$ [39].

2.5. Annotation of Genes in QTL Regions

The "Williams 82.a2.v1" genome, the first published soybean genome, can provide valuable information for QTL mapping of important traits in soybean [40]. A high-density genetic map of the recombinant inbred line (RIL) population in the early stage was constructed in our laboratory [40]. We used a BLAST search on SoyBase (http://soybase.org/gbrowse/cgi-bin/gbrowse/gmax1.01/, accessed on 26 December 2021) to search for soybean genes and their annotations. The predicted coding sequences in QTL regions of soybean were retrieved from the Phytozome website (http://www.phytozome.net/soybean, accessed on 26 December 2021) and were annotated by querying them against the *G. max* 'Wm82' proteome using BLASTX [41]. Predicted functional partners of the genes in the QTL regions were analysed using STRING (https://string-db.org/, accessed on 30 December 2021) and Cytoscape 3.9.1.

2.6. RNA-Seq KEGG Enrichment Analyses

To identify candidate genes interacting with NopL or NopT, reverse-transcription quantitative PCR (RT-qPCR) was performed to measure the relative expression levels of these genes in Suinong14. Root samples were collected at 4, 8, 12, and 24 h after independent inoculations with *S. fredii* HH103ΩNopLΩNopT or the relative parental strains. All RNA extractions were performed in three biological replicates, and each cDNA sample was analysed three times. Total RNA was isolated from the roots using the Trizol reagent (Invitrogen, USA) [20]. All the sets were sequenced with the Illumina HiSeq 2000 platform. The enrichment test was performed using Fisher's exact test and the Benjamini–Yekutieli false discovery rate p-value normalisation. Background terms were set to all GO terms in

G. max. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment was analysed by KOBAS 3.0 [42].

2.7. RT-qPCR Analysis of Candidate Genes

Each RNA sample was converted into cDNA using the HiScript®II Q RT SuperMix (Vazyme Biotech Co., Nanjing, China) under the following conditions: denaturation at 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Specific primers were designed to target the 3′ UTR of the Williams 82 gene sequences retrieved from the Phytozome website (http://www.phyzome.org/, accessed on 26 December 2021) [27]. GmActin (accession number, V00450.1) was used as the reference gene to normalise transcript abundance values among the different samples. Ct values were calculated using Roche LightCycler 480 II software. The primer sequences used for RT-qPCR of target genes are listed in Supplemental Table S1.

3. Results

3.1. Effect of NopL and NopT on Various Soybean Germplasms

In this study, we used 13 soybean germplasms derived from different ecoregions to elucidate the role of NopL and NopT in the symbiotic relationship between rhizobia and soybean. In most soybean germplasms, the nodule number (NN) was significantly different depending on the strain that was used for soybean inoculation. NopL had a positive effect on the NN of all germplasms except DN50, He00-23, Suinong14, Heinong44, Dongnong594, and ZYD00006. Similarly, NopT promoted the NN of all germplasms except the landraces Charleston, DN50, Suinong15, Suinong14, and ZYD00006. Moreover, inoculation with HH103Ω*NopL*Ω*NopT* had a positive effect on the NN of the landraces Charleston, Nenfeng15, Suinong14, Heinong44, and ZYD00006 (Figure 1). However, CN201, He00-23, Jingshanpu, and Heinong35 plants inoculated with HH103Ω*NopT* showed approximately 50% more nodules than those inoculated with HH103. When inoculated with HH103Ω*NopL* and HH103Ω*NopL*Ω*NopT*, half of the tested landraces exhibited higher NDW than that of plants inoculated with HH103, but the other half showed the opposite phenotype. Finally, NopT exerted a positive effect on the NDW of all germplasms, except for the landraces DN594, Jingshanpu, Suinong14, and ZYD00006 (Figure 2). Therefore, NopL and NopT may exert both positive and negative impacts on the symbiotic relationship between *S. fredii* HH103 and soybean; such differences depend on the genetic background of each germplasm.

3.2. Phenotypic Analysis

By comparing the outcome of inoculation with *S. fredii* HH103 or its mutants (Table 1), we observed significant differences between the parents of the CSSL population in terms of nodulation traits. The NN and NDW of the CSSL population varied, suggesting that both the *nopL* mutant and *nopT* mutant decreased NN and NDW in most CSSLs. Inoculation with the mutant HH103Ω*NopL*Ω*NopT* exerted a direct negative effect on nodule traits in the CSSL population, compared with *S. fredii* HH103 inoculation, resulting in a decrease from 48.5735 to 16.7689 in the average NN, and from 0.04393 to 0.020704 in the average NDW. These results indicate differences in nodulation ability within the CSSL population.

3.3. QTL Mapping of Nodulation-Related Traits

QTLs underlying the nodule traits NN and NDW were identified in the soybean CSSL population. In total, 22 QTLs were detected, located on 12 different chromosomes: Gm01 (one), Gm02 (two), Gm03 (four), Gm04 (one), Gm05 (two), Gm09 (one), Gm10 (three), Gm11 (one), Gm14 (three), Gm15 (two), Gm18 (one), and Gm19 (two). The LOD values of these QTLs ranged from 2.5426 to 70.6567, and the individual phenotypic variance (PVE) ranged from 1.0433% to 31.5547% (Table 2).

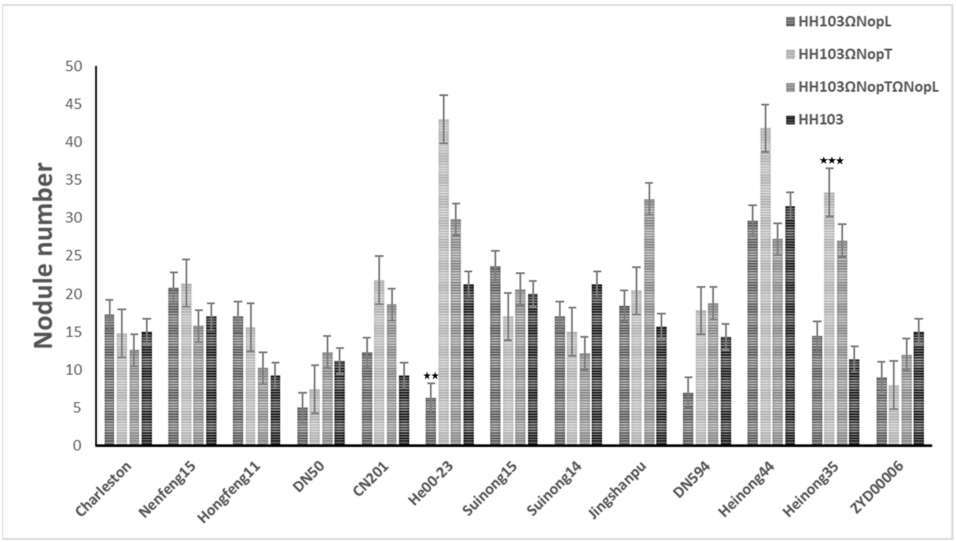

Figure 1. Nodule number phenotype of soybean germplasm after inoculation with *S. fredii* HH103 and *S. fredii* HH103Ω*NopL*, HH103Ω*NopT* and HH103Ω*NopL*Ω*NopT*. Nodulation tests were performed three times; *t*-test was performed comparing the NopP mutant to the wild-type strain; when significant ($0.01 \leq p \leq 0.05$), an asterisk is shown, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$. Ecoregions origin of germplasms: Charleston (America), Nenfeng15 (Heilongjiang), Hongfeng11 (Heilongjiang), Dongnong50 (Heilongjiang), CN201 (Sichuan), He00-23, Suinong15 (Heilongjiang), Suinong14 (Heilongjiang), Jingshanpu (Heilongjiang), Dongnong594 (Heilongjiang), Heinong44 (Heilongjiang), Heinong35 (Heilongjiang), ZYD00006.

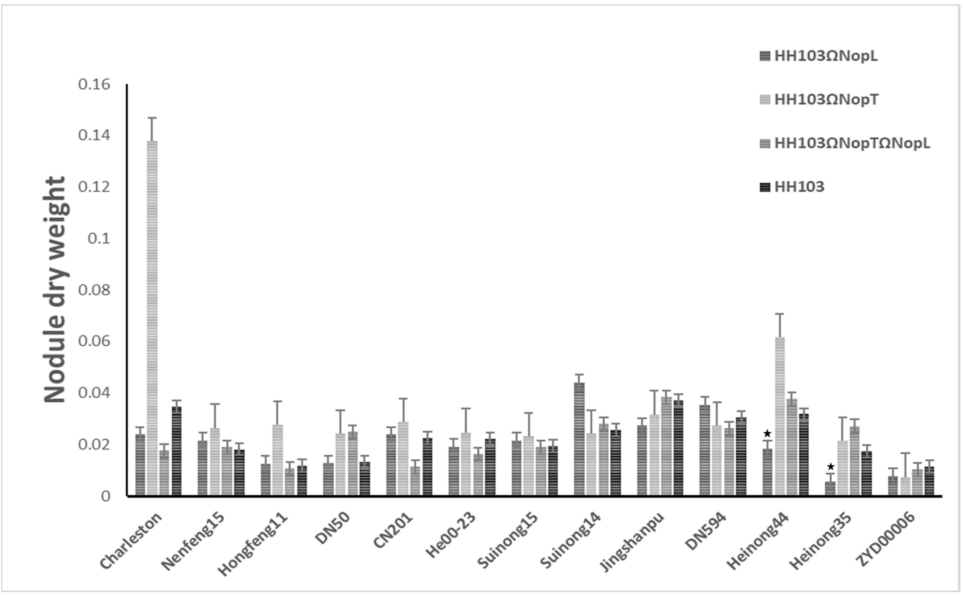

Figure 2. Nodule dry weight phenotype of soybean germplasm after inoculation with *S. fredii* HH103 and *S. fredii* HH103Ω*NopL*, HH103Ω*NopT* and HH103Ω*NopL*Ω*NopT*. Nodulation tests were performed three times; *t*-test was performed comparing the NopP mutant to the wild-type strain; when significant ($0.01 \leq p \leq 0.05$), an asterisk * is shown. Ecoregions origin of germplasms: Charleston (America), Nenfeng15 (Heilongjiang), Hongfeng11 (Heilongjiang), Dongnong50 (Heilongjiang), CN201 (Sichuan), He00-23, Suinong15 (Heilongjiang), Suinong14 (Heilongjiang), Jingshanpu (Heilongjiang), Dongnong594 (Heilongjiang), Heinong44 (Heilongjiang), Heinong35 (Heilongjiang), ZYD00006.

Table 2. Distribution of conditional quantitative trait locus (QTL) for nodule number (NN) and nodule dry weight (NDW) among linkage groups and chromosomes.

Strain	Trait	Gm	Start Position	End Position	PVE (%)	LOD	Additive Effect
HH103	NN	5	39,670,584	39,726,296	7.80	37.9	0.053
		5	3,976,808	39,891,168	5.19	28.4	−0.044
	NDW	2	7,376,054	7,504,904	8.52	12.3	−0.040
		2	8,200,157	8,248,549	15.60	20.4	0.051
		3	1933	234,034	4.32	6.8	0.015
HH103Ω*NopL*	NN	15	4,637,363	4,716,807	7.40	3.4	7.851
	NDW	15	4,119,708	4,302,572	1.75	2.5	0.011
		19	39,031,608	39,097,776	7.64	10.4	−0.028
		19	39,194,300	39,293,776	17.05	20.6	0.044
HH103Ω*NopT*	NN	3	6,743,461	6,777,452	17.00	8.6	5.881
	NDW	1	55,304,920	55,503,928	1.46	7.7	0.048
		3	2,244,023	2,274,724	1.18	5.8	0.010
		10	39,447,660	39,504,396	11.34	40.2	0.067
		10	39,660,168	39,754,196	12.85	43.2	0.058
		10	40,496,720	40,586,292	31.55	70.7	−0.128
		11	10,234,170	10,395,019	1.04	5.7	0.009
HH103Ω*NopL*Ω*NopT*	NN	3	1933	234,034	8.44	4.2	4.720
		4	8,807,067	8,829,123	5.55	2.8	5.273
		18	57,614,440	57,682,792	4.98	2.6	4.591
	NDW	9	41,985,024	42,131,960	3.55	14.7	29.446
		14	1,346,935	1,422,699	1.47	6.7	−23.711
		14	4,751,415	4,955,219	22.95	56.3	104.814
		14	4,956,367	5,109,562	17.36	47.7	−104.991

Gm: chromosome; R2 (%): the contribution rate of the QTL; LOD: log of odds.

After inoculation with *S. fredii* HH103, five QTLs were identified, distributed on three chromosomes: Gm05 (two), Gm02 (two), and Gm03 (one). After inoculation with HH103Ω*NopL*, four QTLs were detected, distributed on two chromosomes: Gm15 (two) and Gm19 (two). After inoculation with HH103Ω*NopT*, seven QTLs were found, distributed on four chromosomes: Gm01 (one), Gm03 (two), Gm10 (three), and Gm11 (one). Finally, after inoculation with HH103Ω*NopL*Ω*NopT*, seven QTLs were identified, distributed on five chromosomes: Gm03 (one), Gm04 (one), Gm09 (one), Gm14 (three), and Gm18 (one). Intriguingly, we found that QTLs on Gm03 controlled both tested traits after inoculation with HH103 and HH103Ω*NopL*Ω*NopT* and that these traits were associated with the same region, starting at base 1933 and ending at base 234,034.

3.4. Analysis of Candidate Genes

Gm03 was annotated to detect candidate genes after inoculation with *S. fredii* HH103Ω*NopL*Ω*NopT*. Additionally, we aimed to assess whether the QTLs that emerged in our study after inoculation with HH103Ω*NopL* and HH103Ω*NopT* were associated with nodulation. Predicted soybean protein-coding DNA sequences from the QTL regions and adjacent regions were retrieved from the Phytozome database (www.phytozome.net/soybean, accessed on 26 December 2021) and then annotated by using them as queries in BLASTX searches against the *G. max* Wm82 proteome. In total, 61 genes were annotated

(Supplemental Table S2). Finally, 17 genes associated with nodulation-related traits were selected (Table 3).

Table 3. Annotation of candidate genes.

Rhizobium	Chrom.	Gene	Predicted Function
HH103	05	*Glyma05g36870*	Zinc finger, RING-type
		Glyma05g36810	Phosphatidylinositol 3-/4-kinase, catalytic domain
		Glyma05g36700	Development/cell death domain
		Glyma05g36665	Domain of unknown function DUF2439
HH103Ω*NopL*	15	*Glyma15g06540*	Mg^{2+} transporter protein, CorA-like/Zinc transport protein ZntB
		Glyma15g06560	NET domain
		Glyma15g06590	PB1 domain
		Glyma15g06630	CTLH/CRA C-terminal to LisH motif domain
HH103Ω*NopT*	03	*Glyma03g06640*	Concanavalin A-like lectin/glucanase domain
HH103Ω*NopL*Ω*NopT*	03	*Glyma03g24141*	Nodulin-like
		Glyma03g00280	GOLD domain
		Glyma03g00370	K Homology domain
		Glyma03g00380	Acid phosphatase (Class B)
		Glyma03g00410	Thioredoxin
		Glyma03g00420	TAFII28-like protein
		Glyma03g00470	RNA recognition motif domain
		Glyma03g00460	WRKY domain

3.5. Differentially Expressed Genes (DEGs) Are Related to the Effectors

To better understand the mechanistic insights into the phenotype, RNA-seq analysis was performed. Root samples were collected after independent inoculations with *S. fredii* HH103Ω*NopL*Ω*NopT* or the relative parental strains. Pearson correlation and principal component analysis (PCA) among all RNA-seq libraries are plotted in Figure 3, A. Venn diagram showing the number of common differentially expressed genes in four different samples. Venn diagram analysis of these DEGs showed 751 common genes among different treatments for the roots (Figure 3b). To further receive an overview of the biological functions of proteins encoded by DEGs, we performed a Gene Ontology analysis in samples with *S. fredii* HH103 vs. samples with *S. fredii* HH103Ω*NopT*, samples with *S. fredii* HH103Ω*NopL* and samples with *S. fredii* HH103 Ω*NopL*Ω*NopT*. GO analysis demonstrated significant enrichments for plant–pathogen interaction, plant hormone signal transduction, MAPK signalling pathway, etc. (Figure 4).

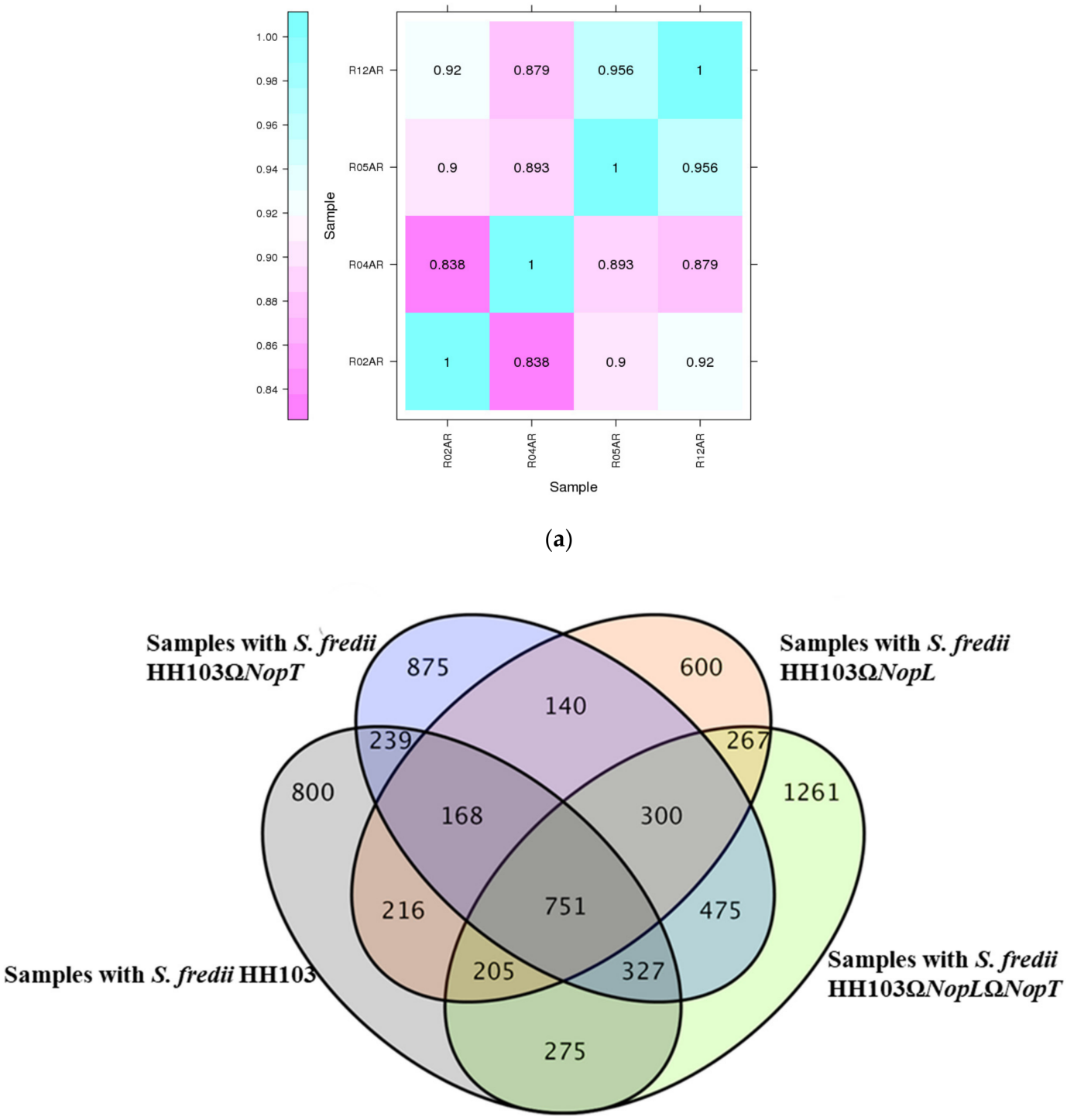

Figure 3. Transcriptomic analysis of roots of soybean inoculated with *S. fredii* HH103 and mutants: (**a**) Pearson correlation coefficients of all RNA-seq libraries; (**b**) Venn diagram showing the differentially expressed genes (DEGs) in the samples inodulated with different strains.

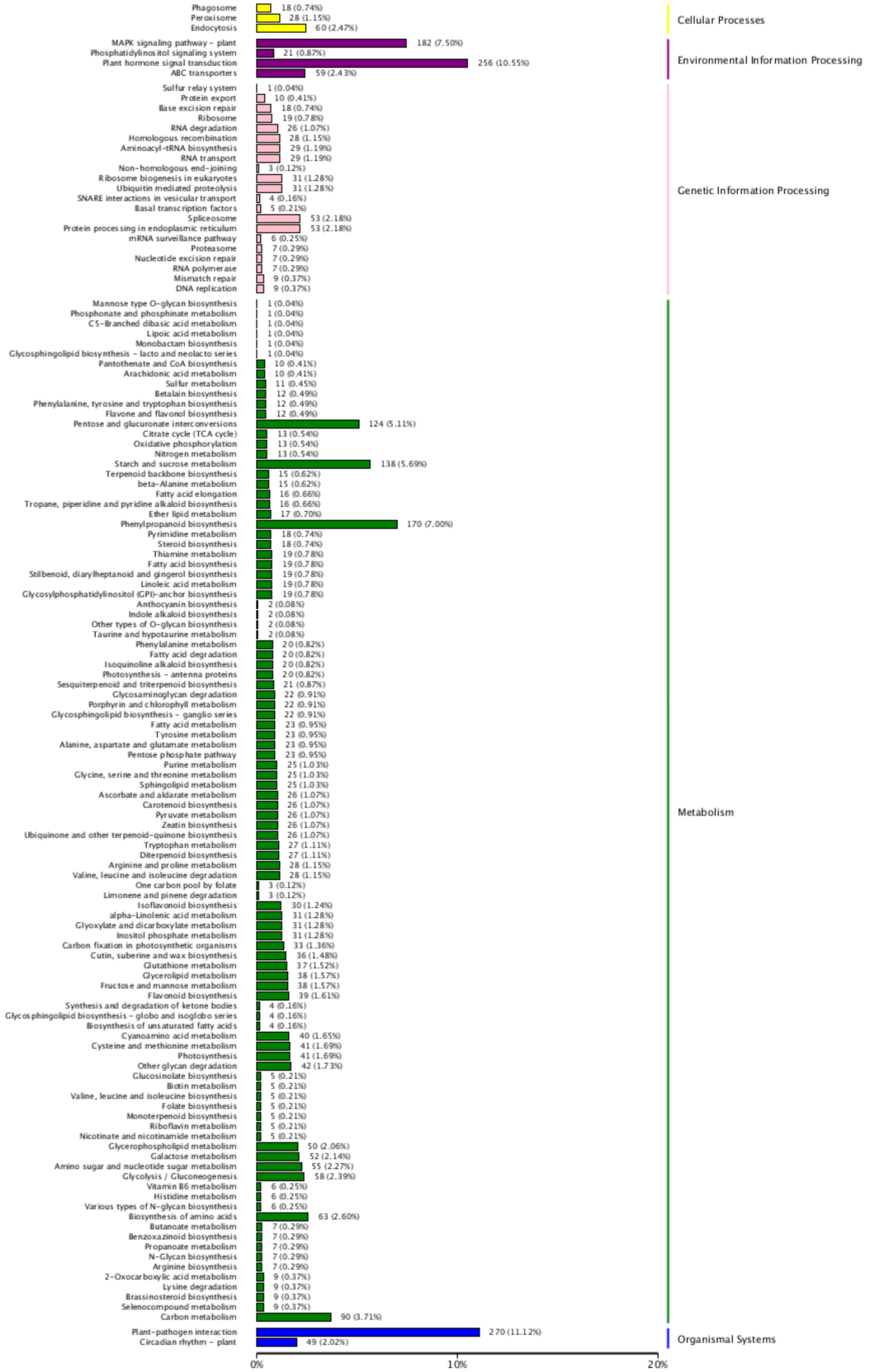

Figure 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) classifications of differentially expressed genes of the samples inodulated with different strains.

3.6. Validation of Candidate Gene Expression by RT-qPCR

In this study, a total of 22 QTLs for the 2 nodule traits of interest were detected and mapped to 12 different chromosomes in the soybean genome. Eight and fifteen QTLs were identified by analysing NN and NDW, respectively. Seventeen selected genes were validated with RT-qPCR (Figure 5). All candidate genes showed similar expression patterns between 12 and 24 h after inoculation with *S. fredii* HH103Ω*NopL* and *S. fredii* HH103Ω*NopT*. In particular, *Glyma03g00280*, *Glyma03g00370*, *Glyma03g00410*, *Glyma03g00470*, *Glyma03g24141*, *Glyma05g36665*, *Glyma05g36700*, *Glyma05g36810*, *Glyma15g06590*, and *Glyma15g06630* were upregulated after inoculation with *S. fredii* HH103Ω*NopL*, compared with their expression level after inoculation with *S. fredii* HH103, HH103Ω*NopT*, and HH103Ω*NopL*Ω*NopT*; however, the overall expression level was higher than that triggered by the *nopT* mutant. The other candidate genes were upregulated after inoculation with *S. fredii* HH103Ω*NopT*, compared with their expression level after inoculation with *S. fredii* HH103, HH103Ω*Nop*L, and HH103Ω*NopL*Ω*NopT*, showing higher expression levels than those triggered by the *nopL* mutant. The relative expression levels of the 17 candidate genes increased significantly by more than fivefold between soybean roots inoculated with HH103 and those inoculated with the mutants at 24 h post-inoculation. These results suggest that the 17 candidate genes interact with NopL and NopT.

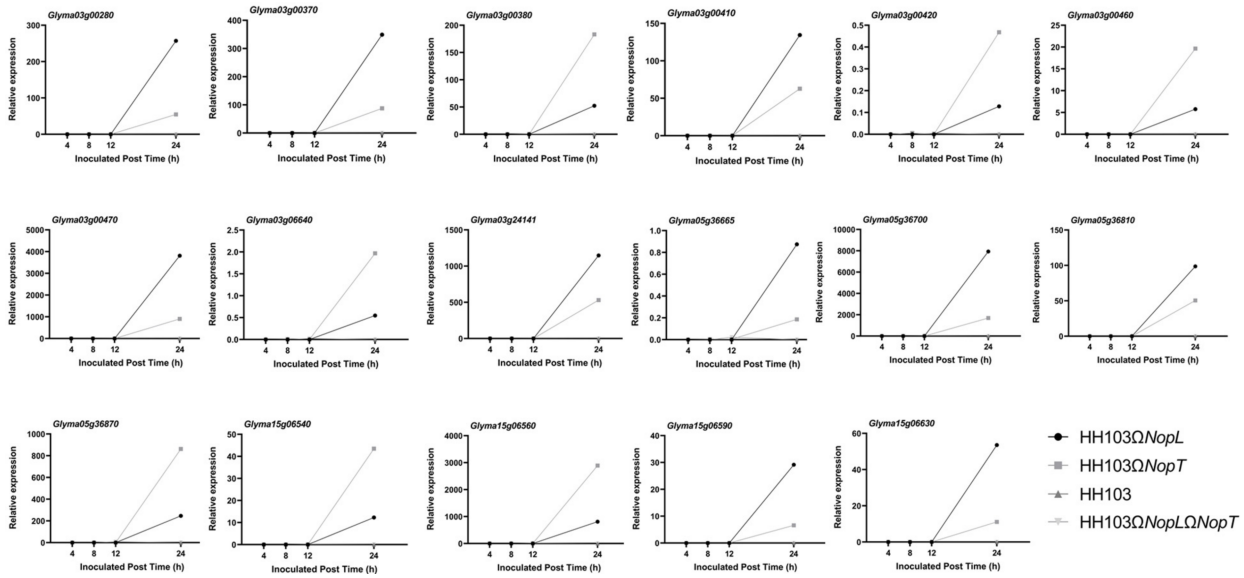

Figure 5. Relative expression levels of 17 candidate genes in 'Suinong14' soybean roots independently inoculated with *S. fredii* HH103, *S. fredii* HH103Ω*NopL*, HH103Ω*NopT,* and HH103Ω*NopL*Ω*NopT*. Non-inoculated plants were included as the control. The expression levels of target genes relative to the control samples at the indicated time points were calculated according to the $2^{-\Delta\Delta CT}$ method. Bars represent the mean ± standard error of three replicates.

4. Discussion

QTL clusters underlying different traits were observed on different chromosomes [43], and the detected associations between QTLs and nodule traits were in agreement with previous studies. For instance, it has been previously reported that QTLs on Gm03, Gm04, and Gm05 are associated with NN and that QTLs on Gm03, Gm10, and Gm14 are associated with NDW [24,27,41,44]. We found that the expression of 17 candidate genes was upregulated at 24 h post-inoculation with HH103Ω*NopL* or HH103Ω*NopT*. The expression of 10 of these genes—namely, *Glyma03g00280*, *Glyma03g00370*, *Glyma03g00410*, *Glyma03g00470*, *Glyma03g24141*, *Glyma05g36665*, *Glyma05g36700*, *Glyma05g36810*, *Glyma15g06590*, and *Glyma15g06630*, was more pronounced in the absence of *nopL*. These genes were confirmed to be upregulated at least twofold in Suinong14 at 24 h post-inoculation with HH103Ω*NopL* compared with their expression level after inoculation with HH103Ω*NopT*. This expression

pattern suggests that these 10 genes might be associated with the NopL-triggered signalling pathway that is activated after inoculation with rhizobia.

The other seven candidate genes showed higher expression levels when *nopT* was absent, with an upregulation of at least 3.4-fold at 24 h post-inoculation with HH103Ω*nopT*, compared with their expression level after inoculation with HH103Ω*NopL*. Interestingly, when the two effectors were simultaneously deleted, the expression of the candidate genes returned to levels similar to those of the wild type. This phenomenon may depend on the different signalling pathways mediated by NopL and NopT.

The candidate genes were introduced into the STRING database to identify putative interaction partners of the corresponding proteins. A protein interaction network was constructed and visualised using the STRING 11.0 database and Cytoscape 3.9.1 (Figure 6). The colour of the protein represents its importance in the interaction network. The redder the colour, the more important it is in the interaction network. Lines and arrows between proteins represent their interactions. GLYMA05G36810.1 shows interaction with GLYMA02G44560.1, GLYMA05G24700.1, GLYMA07G04200.1, GLYMA07G36460.1 and GLYMA15G06560.2. GLYMA03G00420.3 is an important protein in the protein interaction network showing interaction with 10 proteins.

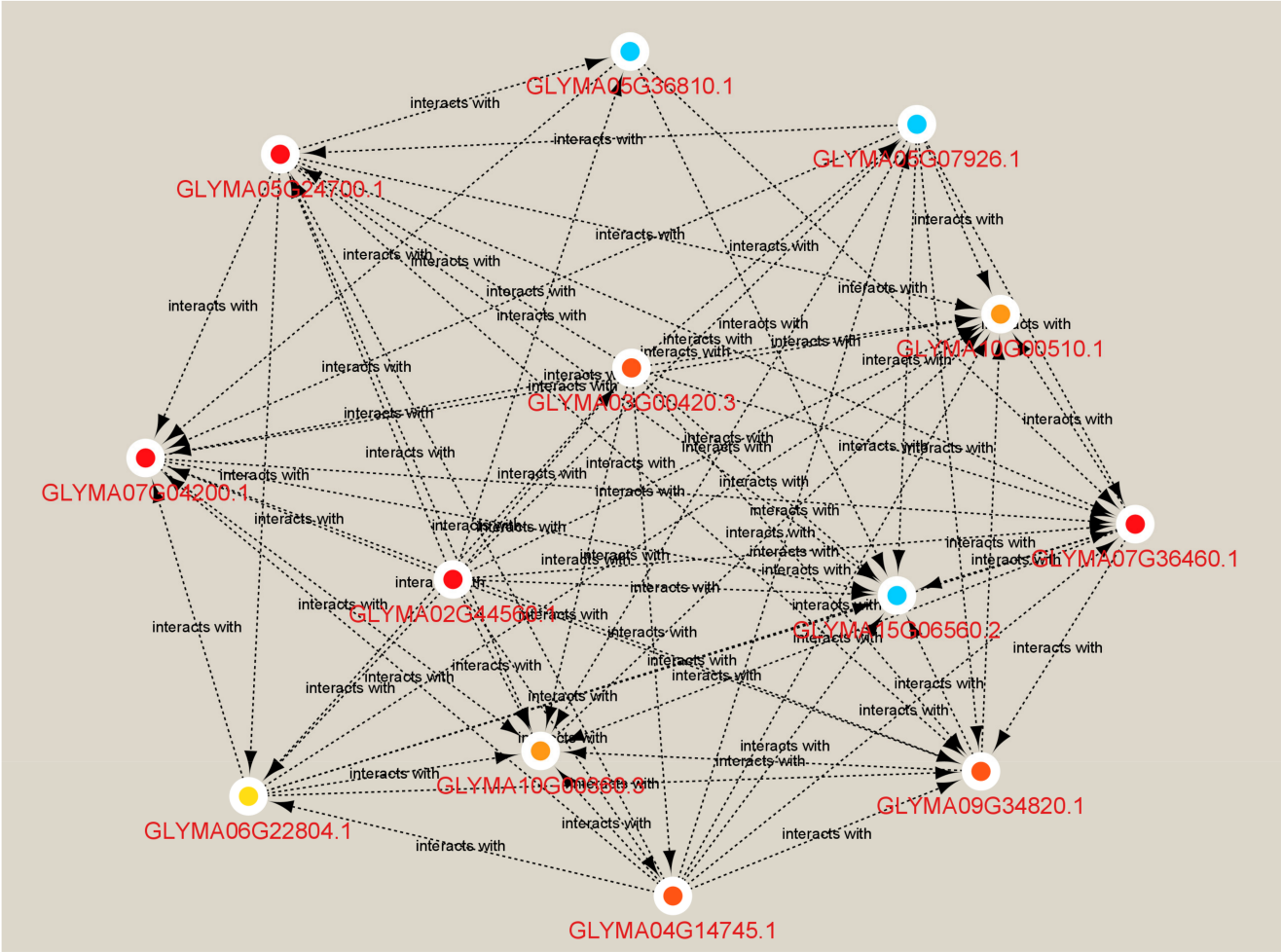

Figure 6. PPI network of candidate genes in soybean on the basis of STRING analysis.

Gene annotations for these candidate genes show that *Glyma15g06540*, *Glyma15g06560*, *Glyma15g06630*, *Glyma03g00370*, *Glyma03g00380*, and *Glyma03g00410* may be associated with genes in the root-specific co-expression subnetwork. In addition, *Glyma03g00420* may be associated with genes in the nodule-specific co-expression subnetwork, and *Glyma03g24141* may be associated with early nodulins. The protein encoded by *Glyma05g36810*

contains a phosphatidylinositol 3-/4-kinase catalytic domain, which is also present in a wide range of protein kinases that are involved in a variety of cellular functions, such as the control of cell growth, the regulation of cell cycle progression and DNA damage checkpoints, and recombination [45]. NopL was found to be multiply phosphorylated in yeast or *Nicotiana tabacum* cells expressing *nopL*. In addition, NopL mimics the MAPK substrate and impairs MAPK signalling in host cells [46]. Our RT-qPCR results confirmed that *Glyma05g36810* participates in the NopL-triggered signalling pathway in host cells. The Glyma03G00470.1 protein has been identified as eukaryotic translation initiation factor 3 subunit B. This protein is a component of the eukaryotic translation initiation factor 3 (eIF-3) complex, which is involved in protein synthesis and, together with other initiation factors, stimulates the binding of mRNA and methionyl-tRNAi to the 40 s ribosome. Additionally, the protein encoded by *Glyma03g00460* includes a WRKY domain and thus belongs to the WRKY family of transcription factors, one of the largest families of transcriptional regulators in plants, whose members play fundamental roles in both the repression and de-repression of crucial plant processes [47]. The protein encoded by *Glyma03g00280* carries a GOLD domain, through which it might simultaneously bind to membranes and other proteins, thereby playing a role in the assembly of membrane-associated complexes or that of cargo into membranous vesicles in the Golgi [48]. *Glyma05g36700* encodes a development and cell death (DCD) domain-containing asparagine-rich protein (NRP) that plays specific roles under different stress and developmental conditions; therefore, this gene is considered to be essential for plant adaptation and growth [49]. Thus, *Glyma05g36700* may be a key factor in both the legume–rhizobia symbiosis and developmental signalling. *Glyma05g36665* encodes a protein containing a domain of an unknown function, DUF2439, and belonging to a protein family whose members have been implicated in telomere maintenance in Saccharomyces cerevisiae and in meiotic chromosome segregation in *Schizosaccharomyces pombe*. Therefore, we speculate that this gene is involved in cell division, plant growth, and development. Moreover, the protein encoded by *Glyma05g36870* belongs to the zinc-finger superfamily, whose members perform a wide variety of tasks within cells by providing stable structural scaffolds and driving critical binding interactions, especially among proteins, DNA, and RNA [50]. *Glyma15g06590* encodes a protein containing the PB1 domain, which is a protein interaction module that is highly conserved in animals, fungi, amoebas, and plants, and participates in diverse biological processes [51]. For example, the PB1 domain mediates protein oligomerisation in Marchantia polymorpha, thereby affecting the function of the transcription factor *MpARF1* [52]. However, few studies have examined the function of the PB1 domain in soybean. Finally, the protein product of *Glyma03g24141* belongs to the nodulin-like protein family, which is widely distributed in Arabidopsis, rice, maize, poplar, and soybean [53]. In soybean, the genes encoding a nodulin-26-like protein and nodulin-16-like protein were found to be located on chromosome 5.

5. Conclusions

We identified 17 genes encoding proteins that potentially interact with NopL and NopT, two effectors that play critical roles in determining host specificity and interactions of rhizobia. Further analysis of these genes may be of great significance for determining the symbiotic mechanism of soybean and rhizobia, and for improving the nitrogen fixation ability of soybean. This study provides valuable candidate genes for detecting T3E-interacting proteins in soybean hosts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12040946/s1, Figure S1: PCR analysis of HH103Ω*NopL*; Figure S2: PCR analysis of HH103Ω*NopL*Ω*NopT*; Table S1: Primers for this research; Table S2: Gene annotation of candidate genes.

Author Contributions: Conceptualisation, D.X. and Q.C.; methodology, H.N., Y.P., and J.W. (Jinhui Wang); software, Y.Y. and J.W. (Jing Wang); validation, T.F. and Z.Z.; formal analysis, H.N.; investigation, J.Z., X.P., and Z.C.; resources, J.W. (Jinhui Wang); data curation, C.L.; writing—original draft preparation,

H.N., Y.P., and J.W. (Jinhui Wang); writing—review and editing, D.X.; visualisation, H.N. and Y.P.; supervision, Q.C.; project administration, C.L.; funding acquisition, D.X. and Q.C., H.N., Y.P. and J.W. (Jinhui Wang) contributed equally to this work. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Heilongjiang Provincial Natural Science Foundation of China (YQ2019C008).

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Westhoek, A.; Field, E.; Rehling, F.; Mulley, G.; Webb, I.; Poole, P.S.; Turnbull, L.A. Policing the legume-Rhizobium symbiosis: A critical test of partner choice. *Sci. Rep.* **2017**, *7*, 1419. [CrossRef] [PubMed]
2. Roy, S.; Liu, W.; Nandety, R.S.; Crook, A.; Mysore, K.S.; Pislariu, C.I.; Frugoli, J.; Dickstein, R.; Udvardi, M.K. Celebrating 20 Years of Genetic Discoveries in Legume Nodulation and Symbiotic Nitrogen Fixation. *Plant Cell.* **2020**, *32*, 15–41. [CrossRef] [PubMed]
3. Rong, L.; Chen, H.; Yang, Z.; Yuan, S.; Zhou, X. Research status of soybean symbiosis nitrogen fixation. *Oil Crop Sci.* **2020**, *5*, 6–10. [CrossRef]
4. Via, V.D.; Zanetti, M.E.; Blanco, F. How legumes recognize rhizobia. *Plant Signal. Behav.* **2016**, *11*, e1120396. [CrossRef]
5. Schechter, L.M.; Guenther, J.; Olcay, E.A.; Jang, S.; Krishnan, H.B. Translocation of NopP by *Sinorhizobium fredii* USDA257 into *Vigna unguiculata* Root Nodules. *Appl. Environ. Microbiol.* **2010**, *76*, 3758–3761. [CrossRef]
6. Okazaki, S.; Kaneko, T.; Sato, S.; Saeki, K. Hijacking of leguminous nodulation signaling by the rhizobial type III secretion system. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 17131–17136. [CrossRef]
7. Jiménez-Guerrero, I.; Acosta-Jurado, S.; Medina, C.; Ollero, F.J.; Alias-Villegas, C.; Vinardell, J.M.; Pérez-Montaño, F.; López-Baena, F.J. The *Sinorhizobium fredii* HH103 type III secretion system effector NopC blocks nodulation with Lotus japonicus Gifu. *J. Exp. Bot.* **2020**, *71*, 6043–6056. [CrossRef]
8. Yasuda, M.; Miwa, H.; Masuda, S.; Takebayashi, Y.; Sakakibara, H.; Okazaki, S. Effector-Triggered Immunity Determines Host Genotype-Specific Incompatibility in Legume-Rhizobium Symbiosis. *Plant Cell. Physiol.* **2016**, *57*, 1791–1800. [CrossRef]
9. Wang, J.; Wang, J.; Ma, C.; Zhou, Z.; Yang, D.; Zheng, J.; Wang, Q.; Li, H.; Zhou, H.; Sun, Z.; et al. QTL Mapping and Data Mining to Identify Genes Associated with the *Sinorhizobium fredii* HH103 T3SS Effector NopD in Soybean. *Front. Plant Sci.* **2020**, *11*, 453. [CrossRef]
10. Margaret, I.; Crespo-Rivas, J.C.; Acosta-Jurado, S.; Buendía-Clavería, A.M.; Cubo, M.T.; Gil-Serrano, A.; Moreno, J.; Murdoch, P.S.; Rodríguez-Carvajal, M.A.; Rodríguez-Navarro, D.N.; et al. *Sinorhizobium fredii* HH103 *rkp-3* genes are required for K-antigen polysaccharide biosynthesis, affect lipopolysaccharide structure and are essential for infection of legumes forming determinate nodules. *MPMI* **2012**, *25*, 825–838. [CrossRef]
11. Dorival, J.; Philys, S.; Giuntini, E.; Brailly, R.; de Ruyck, J.; Czjzek, M.; Biondi, E.; Bompard, C. Structural and enzymatic characterisation of the Type III effector NopAA (=GunA) from *Sinorhizobium fredii* USDA257 reveals a Xyloglucan hydrolase activity. *Sci. Rep.* **2020**, *10*, 9932. [CrossRef] [PubMed]
12. Skorpil, P.; Saad, M.M.; Boukli, N.M.; Kobayashi, H.; Ares-Orpel, F.; Broughton, W.J.; Deakin, W.J. NopP, a phosphorylated effector of *Rhizobium* sp. strain NGR234, is a major determinant of nodulation of the tropical legumes *Flemingia congesta* and *Tephrosia vogelii*. *Mol. Microbiol.* **2005**, *57*, 1304–1317. [CrossRef] [PubMed]
13. López-Baena, F.; Monreal, J.; Pérez-Montaño, F.; Guasch-Vidal, B.; Bellogín, R.A.; Vinardell, J.M.; Ollero, F.J. The Absence of Nops Secretion in *Sinorhizobium fredii* HH103 Increases GmPR1 Expression in Williams Soybean. *Mol. Plant-Microbe Interact.* **2009**, *22*, 1445–1454. [CrossRef] [PubMed]
14. Jiménez-Guerrero, I.; Pérez-Montaño, F.; Medina, C.; Ollero, F.J.; López-Baena, F.J. NopC Is a Rhizobium-Specific Type 3 Secretion System Effector Secreted by *Sinorhizobium (Ensifer) fredii* HH103. *PLoS ONE* **2015**, *10*, e0142866.
15. Saad, D.M.; Kobayashi, H.; Marie, C.; Brown, I.R.; Mansfield, J.W.; Broughton, W.J.; Deakin, W.J. NopB, a Type III Secreted Protein of *Rhizobium* sp. Strain NGR234, Is Associated with Pilus-Like Surface Appendages. *J. Bacteriol.* **2005**, *187*, 1173–1181. [CrossRef] [PubMed]
16. Rodrigues, J.A.; López-Baena, F.J.; Ollero, F.J.; Vinardell, J.M.; Mdel, R.E.; Bellogín, R.A.; Ruiz-Sainz, J.E.; Thomas, J.R.; Sumpton, D.; Ault, J.; et al. NopM and NopD Are Rhizobial Nodulation Outer Proteins: Identification Using LC-MALDI and LC-ESI with a Monolithic Capillary Column. *J. Proteome Res.* **2007**, *6*, 1029–1037. [CrossRef] [PubMed]
17. Xin, D.W.; Liao, S.; Xie, Z.P.; Hann, D.R.; Steinle, L.; Boller, T.; Staehelin, C. Functional analysis of NopM, a novel E3 ubiquitin ligase (NEL) domain effector of *Rhizobium* sp. strain NGR234. *PLoS Pathog.* **2012**, *8*, e1002707. [CrossRef]
18. Jiménez-Guerrero, I.; Pérez-Montaño, F.; Medina, C.; Ollero, F.J.; López-Baena, F.J. The *Sinorhizobium (Ensifer) fredii* HH103 Nodulation Outer Protein NopI Is a Determinant for Efficient Nodulation of Soybean and Cowpea Plants. *Appl. Environ. Microbiol.* **2017**, *83*, e02770-16. [CrossRef]
19. Alexander, V.B.; William, J.D.; Nawal, M.B.; McAlvin, C.B.; Stacey, G.; Malnoe, P.; Broughton, W.J.; Staehelin, C. NopL, an Effector Protein of *Rhizobium* sp. NGR234, Thwarts Activation of Plant Defense Reactions. *Plant Physiol.* **2004**, *134*, 871–879.

20. Zhang, Y.; Liu, X.; Chen, L.; Fu, Y.; Li, C.; Qi, Z.; Zou, J.; Zhu, R.; Li, S.; Wei, W.; et al. Mining for genes encoding proteins associated with NopL of *Sinorhizobium fredii* HH103 using quantitative trait loci in soybean (*Glycine max* Merr.) recombinant inbred lines. *Plant Soil* **2018**, *431*, 245–255. [CrossRef]
21. Dai, W.-J.; Zeng, Y.; Xie, Z.-P.; Staehelin, C. Symbiosis-Promoting and Deleterious Effects of NopT, a Novel Type 3 Effector of *Rhizobium* sp. Strain NGR234. *J. Bacteriol.* **2008**, *190*, 5101–5110. [CrossRef] [PubMed]
22. Miwa, H.; Okazaki, S. How effectors promote beneficial interactions. *Curr. Opin. Plant Biol.* **2017**, *38*, 148–154. [CrossRef] [PubMed]
23. Luo, Y.; Liu, D.; Jiao, S.; Liu, S.; Wang, X.; Shen, X.; Wei, G. Identification of Robinia pseudoacacia target proteins responsive to *Mesorhizobium amphore* CCNWGS0123 effector protein NopT. *J. Exp. Bot.* **2020**, *71*, 7347–7363. [CrossRef]
24. Liu, Y.; Sun, M.; Chen, Q.; Xin, D.; Sun, X. 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. *J. Plant Interact.* **2021**, *16*, 126–135. [CrossRef]
25. Zeng, Z.B. QTL Mapping. In *Brenner's Encyclopedia of Genetics*, 2nd ed.; Maloy, S., Hughes, K., Eds.; Academic Press: San Diego, MA, USA, 2001; pp. 8–12.
26. Tominaga, A.; Gondo, T.; Akashi, R.; Zheng, S.; Arima, S.; Suzuki, A. Quantitative trait locus analysis of symbiotic nitrogen fixation activity in the model legume *Lotus japonicus*. *J. Plant Res.* **2012**, *125*, 395–406. [CrossRef] [PubMed]
27. Zhu, J.; Wang, J.; Li, Q.; Wang, J.; Liu, Y.; Li, J.; Chen, L.; Shi, Y.; Li, S.; Zhang, Y.; et al. QTL analysis of nodule traits and the identification of loci interacting with the type III secretion system in soybean. *Mol. Genet. Genom.* **2019**, *294*, 1049–1058. [CrossRef]
28. Green, R.; Rogers, E.J. Transformation of chemically competent *E. coli*. *Methods Enzym.* **2013**, *529*, 329–336.
29. Methods: Optimization of culture medium for production of recombinant dengue protein in *Escherichia coli*. *Ind. Biotechnol.* **2009**, *5*, 179–183. [CrossRef]
30. Xin, D.; Qi, Z.; Jiang, H.; Hu, Z.; Zhu, R.; Hu, J.; Han, H.; Hu, G.; Liu, C.; Chen, Q. QTL Location and Epistatic Effect Analysis of 100-Seed Weight Using Wild Soybean (*Glycine soja* Sieb. & Zucc.) Chromosome Segment Substitution Lines. *PLoS ONE* **2016**, *11*, e0149380.
31. Quandt, J.; Hynes, M.F. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. *Gene* **1993**, *127*, 15–21. [CrossRef]
32. Figurski, D.H.; Helinski, D.R. Replication of an origin-containing derivative of plasmid *RK2* dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 1648–1652. [CrossRef] [PubMed]
33. Pueppke, S.G.; Broughton, W.J. *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host ranges. *Mol. Plant-Microbe Interact.* **1999**, *12*, 293–318. [CrossRef]
34. Hwang, S.; Ray, J.D.; Cregan, P.B.; King, C.A.; Davies, M.K.; Purcell, L.C. Genetics and mapping of quantitative traits for nodule number, weight, and size in soybean (*Glycine max* L. [Merr.]). *Euphytica* **2014**, *195*, 419–434. [CrossRef]
35. Yang, J.; Hu, C.; Hu, H.; Yu, R.; Xia, Z.; Ye, X.; Zhu, J. QTLNetwork: Mapping and visualizing genetic architecture of complex traits in experimental populations. *Bioinformatics* **2008**, *24*, 721–723. [CrossRef] [PubMed]
36. Shi, Y.; Zhang, Z.; Wen, Y.; Yu, G.; Zou, J.; Huang, S.; Wang, J.; Zhu, J.; Wang, J.; Chen, L.; et al. RNA Sequencing-Associated Study Identifies GmDRR1 as Positively Regulating the Establishment of Symbiosis in Soybean. *Mol. Plant-Microbe Interact.* **2020**, *33*, 798–807. [CrossRef]
37. Yu, M.; Chen, G.-y.; Zhang, L.-q.; Liu, Y.-x.; Liu, D.-c.; Wang, J.-r.; Pu, Z.-e.; Zhang, L.; Lan, X.-j.; Wei, Y.-m.; et al. QTL Mapping for Important Agronomic Traits in Synthetic Hexaploid Wheat Derived from *Aegiliops tauschii* ssp. *tauschii*. *J. Integr. Agr.* **2014**, *13*, 1835–1844. [CrossRef]
38. Jiang, C.; Zeng, Z.B. Multiple trait analysis of genetic mapping for quantitative trait loci. *Genetics* **1995**, *140*, 1111–1127. [CrossRef]
39. Williams, B.; Kantartzi, S.; Meksem, K.; Grier, R.L., IV; Barakat, A.; Lightfoot, D.A.; Kassem, M.A. Genetic Analysis of Root and Shoot Traits in the 'Essex' By 'Forrest' Recombinant Inbred Line (RIL) Population of Soybean [*Glycine max* (L.) Merr.]. *J. Plant Biol.* **2012**, *1*, 1–9.
40. Qi, Z.; Huang, L.; Zhu, R.; Xin, D.; Liu, C.; Han, X.; Jiang, H.; Hong, W.; Hu, G.; Zheng, H.; et al. A high-density genetic map for soybean based on specific length amplified fragment sequencing. *PLoS ONE* **2014**, *9*, e104871. [CrossRef]
41. Wang, J.; Wang, J.; Liu, C.; Ma, C.; Li, C.; Zhang, Y.; Qi, Z.; Zhu, R.; Shi, Y.; Zou, J.; et al. Identification of Soybean Genes Whose Expression is Affected by the *Ensifer fredii* HH103 Effector Protein NopP. *Int. J. Mol. Sci.* **2018**, *19*, 3438. [CrossRef]
42. Xie, C.; Mao, X.; Huang, J.; Ding, Y.; Wu, J.; Dong, S.; Kong, L.; Gao, G.; Li, C.-Y.; Wei, L. KOBAS 2.0: A web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res.* **2011**, *39*, W316–W322. [CrossRef] [PubMed]
43. Lv, H.; Wang, Q.; Liu, X.; Han, F.; Fang, Z.; Yang, L.; Zhuang, M.; Liu, Y.; Li, Z.; Zhang, Y. Whole-Genome Mapping Reveals Novel QTL Clusters Associated with Main Agronomic Traits of Cabbage (*Brassica oleracea* var. *capitata* L.). *Front. Plant Sci.* **2016**, *7*, 989. [CrossRef] [PubMed]
44. Wang, J.; Wang, J.; Tian, B.; Li, Q.; Zhu, J.; Liu, X.; Ma, C.; Li, C.; Qi, Z.; Zhu, R.; et al. QTL mapping and gene mining to identify genes on soybean (*Glycine max*) associated with NopB of *Sinorhizobium fredii HH103*. *Plant Breed.* **2019**, *138*, 677–685. [CrossRef]
45. Crespo, J.L.; Hall, M.N. Elucidating TOR Signaling and Rapamycin Action: Lessons from *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **2002**, *66*, 579–591. [CrossRef]
46. Zhang, L.; Chen, X.-J.; Lu, H.-B.; Xie, Z.-P.; Staehelin, C. Functional Analysis of the Type 3 Effector Nodulation Outer Protein L (NopL) from *Rhizobium* sp. NGR234: Symbiotic Effects, Phosphorylation, and Interference with Mitogen-Activated Protein Kinase Signaling. *J. Biol. Chem.* **2011**, *286*, 32178–32187. [CrossRef]

47. Rushton, P.J.; Somssich, I.E.; Ringler, P.; Shen, Q.J. WRKY transcription factors. *Trends Plant Sci.* **2010**, *15*, 247–258. [CrossRef]
48. Anantharaman, V.; Aravind, L. The GOLD domain, a novel protein module involved in Golgi function and secretion. *Genome Biol.* **2002**, *3*, research0023.
49. de Camargos, L.F.; Fraga, O.T.; Oliveira, C.C.; da Silva, J.C.F.; Fontes, E.P.B.; Reis, P.A.B. Development and cell death domain-containing asparagine-rich protein (DCD/NRP): An essential protein in plant development and stress responses. *Theor. Exp. Plant Phys.* **2019**, *31*, 59–70. [CrossRef]
50. Isalan, M. DNA Recognition/Processing | Zinc Fingers: Structure and Design. In *Encyclopedia of Biological Chemistry III*, 3rd ed.; Jez, J., Ed.; Elsevier: Oxford, UK, 2021; pp. 506–516.
51. Sumimoto, H.; Kamakura, S.; Ito, T. Structure and function of the PB1 domain, a protein interaction module conserved in animals, fungi, amoebas, and plants. *Sci. Stke Signal Transduct. Knowl. Environ.* **2007**, *2007*, re6. [CrossRef]
52. Kato, H.; Mutte, S.K.; Suzuki, H.; Crespo, I.; Das, S.; Radoeva, T.; Fontana, M.; Yoshitake, Y.; Hainiwa, E.; van den Berg, W.; et al. Design principles of a minimal auxin response system. *Nat. Plants* **2020**, *6*, 473–482. [CrossRef]
53. Denancé, N.; Szurek, B.; Noël, L.D. Emerging functions of nodulin-like proteins in non-nodulating plant species. *Plant Cell Physiol.* **2014**, *55*, 469–474. [CrossRef] [PubMed]