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Effects of *GmERF5*-Responsive Effector on Soybean Symbiotic Nodulation

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Abstract: The type III secretion system (T3SS) of *Rhizobium* plays a crucial role during the establishment of the soybean (*Glycine max* (L.) Merr.)-*Rhizobium* symbiosis system. Additionally, host-specific nodulation may also depend on the Nops (nodulation outer proteins) secreted by the T3SS of *Rhizobium*. However, there is limited understanding of the response of soybean genes to Nops. In this study, a *NopC* mutant, HH103Ω*NopC*, was constructed from *Sinorhizobium fredii* HH103 and then utilized to assess the impact of the NopC on nodulation. An RNA-seq analysis revealed that the *GmERF5* (*Glycine max* Ethylene Responsive Factor 5) gene was induced by NopC, which was confirmed by qRT-PCR. Furthermore, an overexpression of the *GmERF5* hair-root system was constructed to investigate the effect of *GmERF5* on nodulation and its interaction with NopC. Differences in nodule number and nodule dry weight in the overexpression of the *GmERF5* hair-root system supported the conclusion that *GmERF5* can regulate soybean nodule phenotype via NopC. These results elucidate the genetic mechanisms that regulate nodule phenotype in soybean.

Keywords: nodule; soybean–*Rhizobium* interaction; NopC; *GmERF5*

1. Introduction

Soybeans (*Glycine max*) are a significant food and feed crop prized for their high-quality protein and oil content, which are valuable for both human and animal consumption. To enhance soybean yields, the excessive use of nitrogen fertilizers is commonly employed [1,2]. However, the overapplication of chemical nitrogen fertilizers can lead to soil acidification and deterioration of the soil environment, ultimately having detrimental effects on crop growth and yield [1,2]. Fortunately, soybeans possess the unique ability to acquire nitrogen through the establishment of a symbiotic relationship with rhizobia.

Rhizobia secrete *Nop*s (nodulation outer proteins) (type III effectors secreted via type III secretion system) during the early stages of symbiosis (infection thread formation) as well as in fully developed nodules to regulate soybean–rhizobium interaction [3–8]. Rhizobial Nops share characteristics with those of pathogenic bacteria. Once secreted into the host cell, most rhizobial Nops may manipulate cellular processes to inhibit defense responses against invading bacteria. There is growing evidence that inhibiting plant defense responses can have both positive and negative effects on the establishment and maintenance of symbiotic relationships [9].

Type III effectors have been found to play a crucial role in the symbiotic interactions between rhizobia and legumes. During the establishment of the legume–*rhizobium* symbiotic

system, the type III secretion system (T3SS) of rhizobia can deliver multiple type III effectors directly to eukaryotic host cells, influencing the plant's immune recognition process and its nodule-bearing capacity. These effectors can function either as extracellular device components of the T3SS or as secreted type III effectors [10]. In rhizobia, secreted type III effectors typically target components of the host immune system to enhance bacterial survival and virulence [11,12]. Similar to pathogenic bacteria, the rhizobial genes required for the synthesis of T3SS are concentrated in the genome. The organization of these genes and the classification of rhizobial T3SS have been recently reviewed [13,14]. Host-specific nodulation also depends on Nops secreted by the *Rhizobium* T3SS (type III secretion system). To date, fewer than ten Nops of *Rhizobium* have been identified as playing pivotal roles in the establishment of symbiosis, including NopM, NopP, NopT, NopL, NopAA, and NopC. NopM and NopT have been proven to possess specific enzymatic activities [15]. NopM belongs to a novel Ubiquitin ligase family, supporting the ubiquitination of targets in the host to underpin nodule formation. NopT is a member of the YopT protein family and has the ability to cleave itself as well as NFR5 [16]. The effector NopC, a conserved type III effector, has been reported to play an active role in nodule formation in some legumes, and mutations in NopC do not affect the secretion of other effectors [17]. However, the host gene response to NopC has not been well characterized.

The ERF protein family (ERF, Ethylene Responsive Factor) is a plant-specific family of transcription factors characterized by a highly conserved DNA-binding domain known as the ERF structural domain, which is a unique function of this protein family [15]. It is hypothesized that the expression of many ERF genes is regulated by environmental stresses in various patterns, reflecting their role in stress tolerance. Several *ERF* genes are regulated by disease-associated stimuli and components of stress signaling pathways, such as the phytohormones jasmonic acid (JA), ethylene (ET), and salicylic acid (SA), as well as by pathogen infections [18]. The key roles of SA, JA, and ET as mediators of pathogen defense response signals are well documented [16]. Generally, resistance to biotrophic and semi-biotrophic pathogens (e.g., *Pseudomonas syringae* and *Aspergillus parasiticus*) is mediated through SA signaling, whereas resistance to necrotrophic pathogens (e.g., *Aspergillus grey mold*) is mediated through JA/ET signaling [19,20]. Extensive crosstalk exists between these two signaling pathways, with most studies reporting a mutually antagonistic interaction [21,22]. Recent findings suggest that the ERF protein family plays a significant regulatory role in soybean symbiotic nodulation, though its interaction with Rhizobial type III effectors has not been reported [23].

To identify soybean genes responsive to NopC, RNA-seq analysis was conducted to discern differentially expressed genes (DEGs) following inoculation with single mutants of NopC. *GmERF5* was identified as a candidate gene responsive to NopC. To further investigate the interaction between NopC and GmERF5, an overexpression line of *GmERF5* was constructed.

2. Materials and Methods

2.1. Materials

The test soybean seeds used were of the cultivar Suinong 14 (SN14), and the *Nicotiana tabacum* seeds were preserved in our laboratory. The bacterial strains included *Escherichia coli* DH5α and *E. coli* BL21 (DE3), *Agrobacterium rhizogenes* K599, *A. tumefaciens* EHA105, and *S. fredii* strain HH103. The vectors included pET28b, FU28, FU76, FU79, pSOY1, pSOY2, pJQ200SK, pRK2013, and pCas9. They were all maintained in the laboratory of Northeast Agricultural University.

2.2. Type III Effector NopC Gene Cloning and Mutation

The NopC single mutant HH103Ω*NopC* was constructed via triparental mating [24,25]. The mutant sequence, including the *EcoR* I cleavage site, was designed immediately after the start codon ATG. Primers with sequences matching the end of the upstream region and the beginning of the downstream region were designed as homology arms. The upstream

and downstream sequences were then amplified using the *NopC* fragments as templates (the *NopC* gene of the type III secretion system starts at about 900 bp upstream of codon ATG as the upper arm and 600 bp downstream of ATG as the lower arm) (Table 1). These sequences were joined via PCR to create a large linear *NopC* mutant fragment. A pair of homologous recombination ligation primers (sequences provided below) was designed to connect to the pJQ200SK vector, generating pJQ200SK-*NopC*-Mut. For the triparental mating process, *E. coli* carrying pJQ200SK-*NopC*-Mut, the Helper strain, and *S. fredii* HH103 (referred to as HH103) were mixed in a 1:1:3 ratio [26]. The *NopC* coding region and the mutant homologous arm fragment CDS sequences were amplified using genomic DNA as a template and high-fidelity enzyme (KOD Plus Neo, Toyobo, Japan). PCR primer design was performed using Primer 5.0, with sequences as follows:

Table 1. Amplification primers for *NopC* homology arm fragments.

Primer	Sequences (5′→3′)	Target Segment Length
NopC-F	gggtttgttgaagtggatg	1461
NopC-R	cgccgccttcttggttg	
NopC-SY-R	tcagggtcatcagaattctcatcatcacatcagctactcctgcct	1488
NopC-XY-F	tgatgatgagaattctgatgaccctgagtcggagtgattggaagtggag	

2.3. Soybean Nodulation Experiment

In this experiment, cultivar Suinong 14 (SN14), HH103, and the mutant HH103Ω*NopC* were utilized. SN14 seeds were subjected to surface sterilization using a chlorine method for 12–14 h. Subsequently, the sterilized soybean seeds were placed in Petri dishes, partially submerged in sterile water, sealed, and kept in darkness at room temperature for germination. Once the seedlings reached a length of 2 cm, robust and uniform seedlings were transplanted into autoclaved pots. Growing conditions included a 12 h light/12 h dark photoperiod, with temperature maintained at 28 °C during light periods and 25 °C during dark periods. The plants were watered with B&D nutrient solution [27]. Upon reaching the stage where unifoliate leaves were fully uncovered, each plant received 1 mL of rhizobial bacterial solution with an OD_{600} value of 0.4, inoculated directly under the soil layer of soybean roots using a syringe, ensuring approximately 10^9 bacteria were inoculated per plant (with 15 biological replicates). The number of rhizomes was investigated 25 days post-inoculation.

B&D media: $MgSO_4$ 0.5 mol·L^{-1}, $Na_2 MoO_4$ 0.2 mmol·L^{-1}, $MnSO_4$ 2 mmol·L^{-1}, H_3BO_3 4 mmol·L^{-1}, $CaCl_2$ 2 mol·L^{-1}, $CoSO_4$ 0.2 mmol·L^{-1}, K_2SO_4 0.5 mol·L^{-1}, $CuSO_4$ 4 mmol·L^{-1}, KH_2PO_4 1 mol·L^{-1}, $ZnSO_4$ 1 mmol·L^{-1}, and $C_6H_5FeO_7$ 20 mmol·L^{-1}.

2.4. mRNA-Seq (mRNA Sequencing) Analysis

For RNA-seq analysis, the soybean cultivar SN14 was chosen. Roots inoculated with MgSO4 (as controls), HH103, and the mutant HH103Ω*NopC* were collected at 0.5 h and 6 h post-inoculation. Root segments, approximately 1 cm in length near the hypocotyl, were excised from each of the three biological replicates, weighing about 0.1 g each. RNA-seq data analysis was conducted using DEseq2-edgeR, with a false discovery rate (FDR) threshold of less than 0.01 and fold change (FC) threshold greater than 1.5. Heatmaps were generated using TBtools-II v2.003.

2.5. RNA Extraction and qRT-PCR Analysis

Root samples were harvested at 0.5 h and 6 h post-rhizobial inoculation for RNA extraction using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using HiScript II Reverse Transcriptase, and qRT-PCR was performed on a Roche LightCycler 480 System with TB Green (Takara Biomedical Technology, Shiga, Japan). Each sample underwent

three biological and technical replicates. Ct values were utilized to determine relative gene expression employing the $\log_2$ $(-\Delta\Delta Ct)$ method.

2.6. Transcriptional Activity Analysis Assay

The CDS sequences of *GmERF5* transcriptional regulators were retrieved from the soybean Ws82 genome database on Phytozome (https://phytozome-next.jgi.doe.gov/, accessed on 9 May 2023). Subsequently, Primer5.0 was employed to design homology arm amplification primers for PCR amplification (Table 2). The *GmERF5* transcription factors were then constructed onto the GFP-tagged starter vector Fu28 using the In-Fusion method, resulting in the Fu28-*GmERF5*: GEP. The sequences of the homology arm primers are as follows:

Table 2. *GmERF5* homologous primer sequence.

Primer	Homologous Primer Sequence (5′→3′)	Target Segment Length
GmERF5-TY-F	GATCTTCTAGAGATATCACTAGATGGCAAACGCTCTTGAAGT	828 bp
GmERF5-TY-R	GCAATGCATATTAATGTCGACACAACCATAAGCTGTGGAA	

After successfully constructing the Fu28 entry vector, it was integrated into the expression vector pSOY1 by LR reaction. Following successful sequencing to confirm the construct, the extracted plasmid was introduced into *A. rhizogenes* K599 using either heat shock or electroporation methods. The expression vector plasmid was transferred into *A. rhizogenes* K599 for downstream applications.

2.7. Nodules Identification Method

Transgenic soybean plants with positive roots were selected, and they were transplanted into sterilized special plastic boxes filled with sterilized vermiculite. After incubation for 1–2 days, the plants were inoculated with 1 mL of *Rhizobium* solution (OD_{600} = 0.4) directly under the soil layer of soybean roots using a syringe. The growth conditions included a photoperiod of 12 h light/12 h dark, with temperatures maintained at 28 °C during the light period and 25 °C during the dark period. The plants were watered with B&D nutrient solution. The nodule phenotype was analyzed 4 weeks after inoculation with both HH103 and its derived *NopC* mutant.

2.8. Phenotypic Statistics and Data Analysis

SPSS22.0 (Statistical Product and Service Solutions) software was utilized for the statistical analysis of both nodule number and nodules dry weight. Given the small sample size ($n < 30$), an ANOVA was employed for analysis, including the qRT-PCR results. Significance levels were set at $p < 0.01$ for extreme significance and $p < 0.05$ for significance.

3. Results

3.1. Construction of the NopC Mutant

The primers *NopC-F* and *NopC-R* were specifically designed for PCR amplification, using the wild-type S. fredii HH103 genome as the template. The resulting amplified fragment size was approximately 1.5 kb, consistent with the predicted theoretical size of the fragment encoded by the corresponding gene (Figure 1).

A large fragment of *NopC* was utilized as a template to amplify an approximately 900 bp fragment upstream of ATG (Figure 2A) using primers *NopC-F* and *NopC-SY-R*. Simultaneously, a 600 bp fragment downstream insertion was amplified using primers *NopC-R* and *NopC-XY-F* (Figure 2A). A mutant sequence was inserted into the fragment, and the predicted enzyme cleavage site on this sequence was identified using SnapGene. The mutant sequence 'tgatgatgagaattctgatgaccctga' was incorporated. Subsequently, the upstream and downstream fragments were linked to construct the *NopC* mutant fragment using primers *NopC-F* and *NopC-R*. Sequencing confirmed that the constructed fragment

was approximately 1500 bp, consistent with predictions, and this was further validated through PCR analysis (Figure 2B).
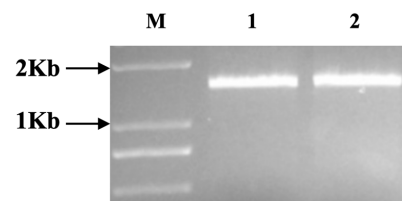
Figure 1. Amplification of *NopC* big fragment. Note: M: Trans 2K Plus DNA marker; 1, 2: *NopC* (1, 461 bp).
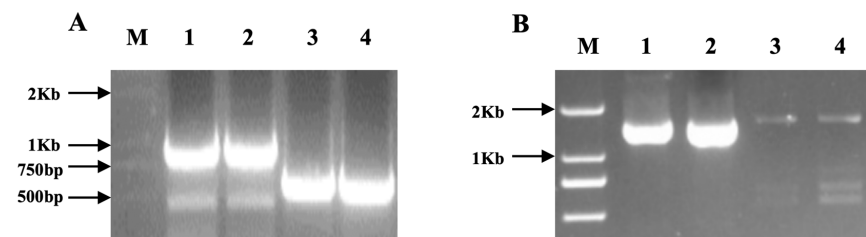
Figure 2. *NopC* fragment insertion at mutation site. Note: M: Trans 2K Plus DNA marker; (**A**): lanes 1 and 2 for *NopC* upstream fragments; lanes 3 and 4 for *NopC* downstream fragments; (**B**): amplification of *NopC* mutant fragments.

3.2. Characterization of the Effect on Nodulation of NopC

The phenotypic investigation of nodules is depicted in Figure 3. Regarding the nodule count, the HH103Ω*NopC* mutant induced three nodules, whereas wild-type HH03 induced four nodules, indicating a difference of one, showcasing a distinction between the two. The dry weight of the nodules induced by HH103Ω*NopC* was 0.07 g, while that induced by wild-type HH103 was 0.03 g, with a disparity of 0.4 g, indicating a notable contrast. The number of nodules with a diameter of ≥2 mm was consistent, with both HH103Ω*NopC* and wild-type HH103 inducing two nodules, demonstrating no difference. However, concerning nodules with a diameter of <2 mm, HH103Ω*NopC* induced eight, whereas wild-type HH103 induced twenty-two, illustrating a highly significant difference. These findings underscore the pivotal role of NopC in regulating both the nodule count and the dry weight of nodules.
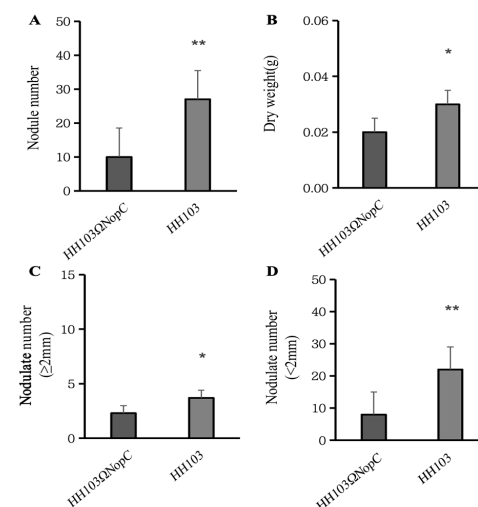
Figure 3. Identification of nodules of different strains inoculated with SN14. RNA-seq analysis of *NopC* induced different expression genes. Note: (**A**) number of nodules; (**B**) dry weight of nodules; (**C**) number of large nodules (diameter ≥ 2 mm); (**D**) number of small nodules (diameter < 2 mm) (where "******" represents $p < 0.01$ and "*****" represents $p < 0.05$).

3.3. Analysis of GmERF5 Response Effector Regulation

Through RNA-seq analysis, we assessed the differential genes in SN14 soybean roots inoculated with wild-type HH103 and *NopC* mutant, leading to the identification of a total of 4711 differential genes. Among these, 2623 genes were specifically induced by HH103, 1453 by *NopC*, and 635 were common differential genes (Figure 4a). Notably, the analysis of DEGs unveiled the involvement of five *GmERF* family genes in the symbiotic process, shared between HH103 and *NopC*.
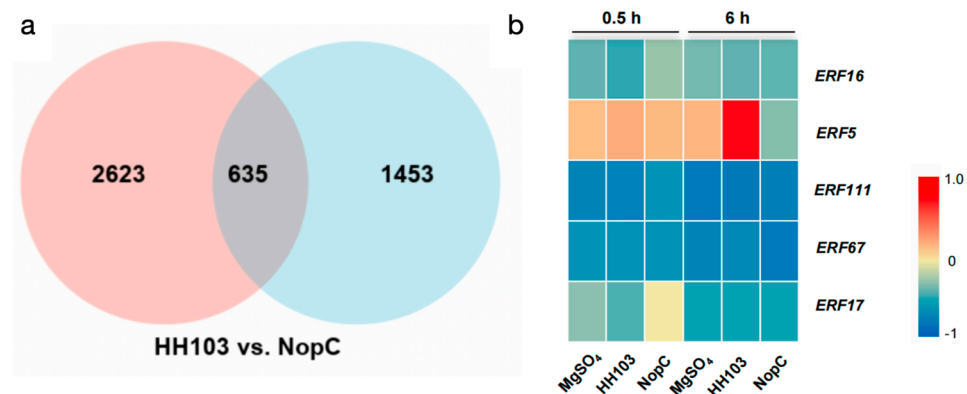

Figure 4. Venn diagram and heatmap of ERF family expression. (**a**) Identified DEGs induced by HH103 and *NopC* mutant; (**b**) five GmERF genes expression pattern.

To delve deeper into the role of *GmERF* family genes in the symbiotic process, we scrutinized the expression changes in these genes. Remarkably, *GmERF5* exhibited peak expression at 6 h post-inoculation with HH103, whereas, following inoculation with the *NopC* mutant, the expression of *GmERF5* at the same time point was significantly reduced compared to wild-type HH103 inoculation (Figure 4b). These observations strongly indicate that *GmERF5* plays a crucial role as a key gene in response to *NopC*.

The expression changes in *GmERF5* in response to the effector NopC were further investigated using qRT-PCR. Interestingly, at 0.5 h post-inoculation, there was no significant difference in the expression level of *GmERF5* between wild-type HH103 and *NopC* mutants. However, at 6 h and 12 h post-inoculation, the expression of *GmERF5* was notably higher in plants inoculated with wild-type HH103 compared to those inoculated with the *NopC* mutant (Figure 5). These findings align with the RNA-seq analysis, providing additional evidence that *GmERF5* is the primary gene within the ERF family responding to NopC.
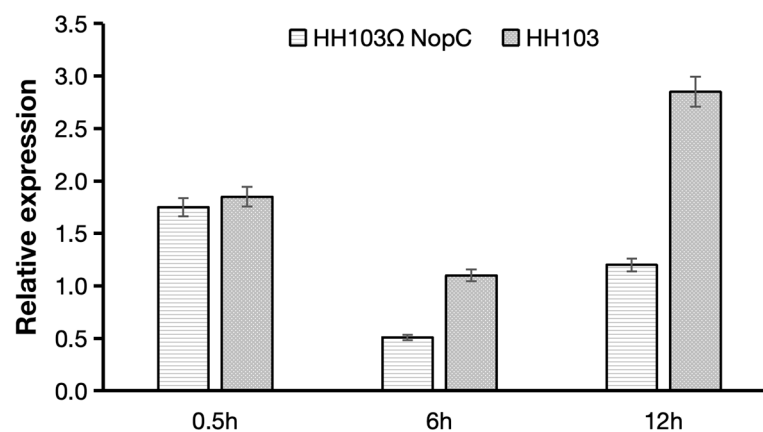

Figure 5. Real-time quantification results of *GmERF5* gene.

3.4. Functional Analysis of Plant Candidate Genes

GmERF5 Gene Functional Studies

To investigate the impact of *GmERF5* gene on SN14 nodulation, transgenic soybean root hairs with *GmERF5* gene overexpression were developed. Initially, *GmERF5* was inserted into the entry vector Fu28 (Figure 6A). Subsequently, *GmERF5* was integrated into the overexpression vector pSOY1 by LR reaction. PCR analysis of the positive bacterial colony confirmed that the inserted fragment matched the expected size and was sequenced accurately, resulting in the formation of pSOY1-*GmERF5*:GFP (Figure 6B). The engineered plasmid was transferred into A. rhizogenes K599 through heat shock, and PCR amplification validated the presence of the desired fragment of approximately 821 bp, in accordance with the anticipated size (Figure 6C).
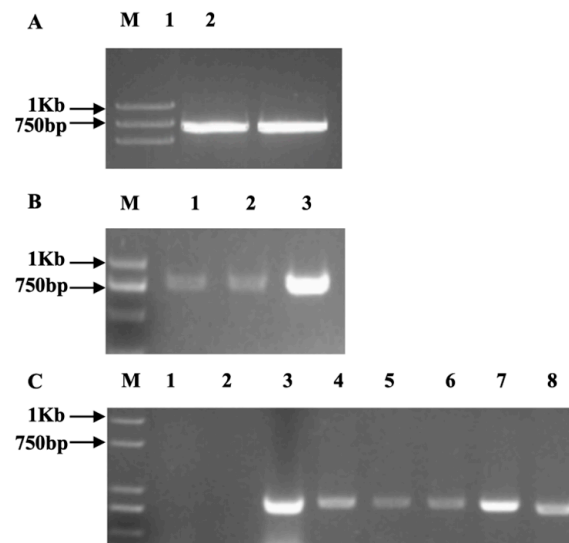

Figure 6. Construction of *GmERF5* overexpression vector. Note: M: Trans 2K Plus DNA marker; (**A**) PCR identification of Fu28-*GmERF5* vector construct; (**B**) PCR identification of pSOY1-*GmERF5* vector construct; (**C**) PCR identification of *A. rhizogenes* K599 transformant.

Through hypocotyl excision and Agrobacterium co-cultivation, plant roots were induced by root-generating Agrobacterium, and the transformed roots were confirmed as positive through GFP labeling detection, as illustrated in Figure 7, and validated via real-time quantitative analysis. The presence of the GFP tag facilitated the identification of transgenic soybean hairy roots under UV fluorescent light, where positive roots emitted a green fluorescence while negative roots appeared red. Subsequently, the identified positive plants were transplanted into sterilized vermiculite and separately inoculated with the HH103Ω*NopC* mutant and wild-type HH103.

Following the overexpression of the *GmERF5* gene, the verification of hairy root transformation was conducted through qRT-PCR analysis, revealing that the expression levels in *GmERF5*-overexpressing plants were approximately four times higher compared to transformed plants (Figure 8).

Upon overexpressing the *GmERF5* gene, its expression level was significantly elevated compared to its baseline expression in SN14. In transgenic hairy roots, the expression of the *GmERF5* gene was observed to be lower at 12 h post-inoculation, but it was up-regulated at 24 h both in wild-type HH103 and mutant HH103Ω*NopC*. The expression levels of the *GmERF5* gene in plants inoculated with HH103 were higher than those in plants inoculated with HH103Ω*NopC*. The highest expression level of the *GmERF5* gene was recorded in plants inoculated with HH103 at 24 h post-inoculation with *Rhizobium*, while the lowest expression level was detected in plants inoculated with HH103Ω*NopC* (Figure 9). These findings further substantiate that the *NopC* effector can promote the expression of the *GmERF5* gene.
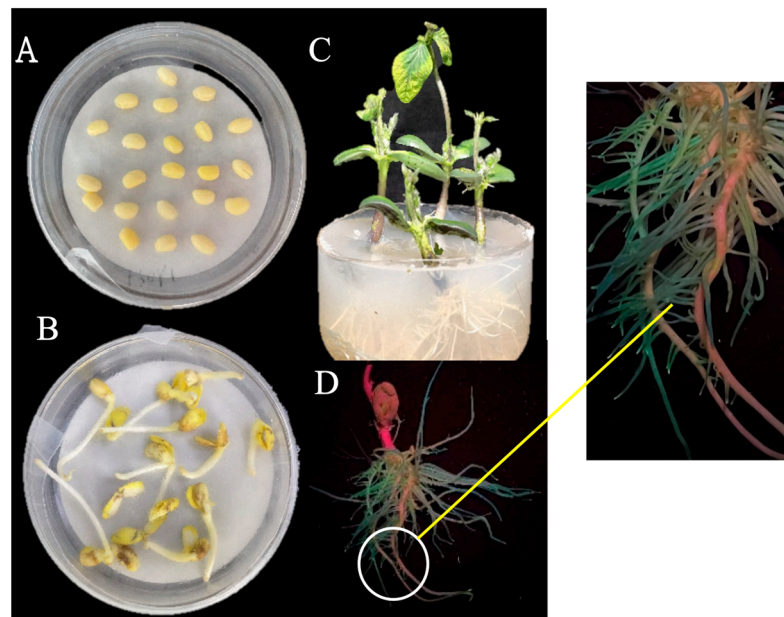
Figure 7. *GmERF5* gene rooting transformation method and identification results. Note: (**A**–**C**) soybean rooting transformation process; (**D**) UV lamp irradiation fluorescence GFP labeling detection.
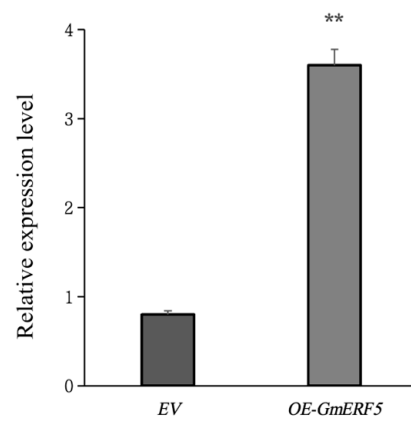

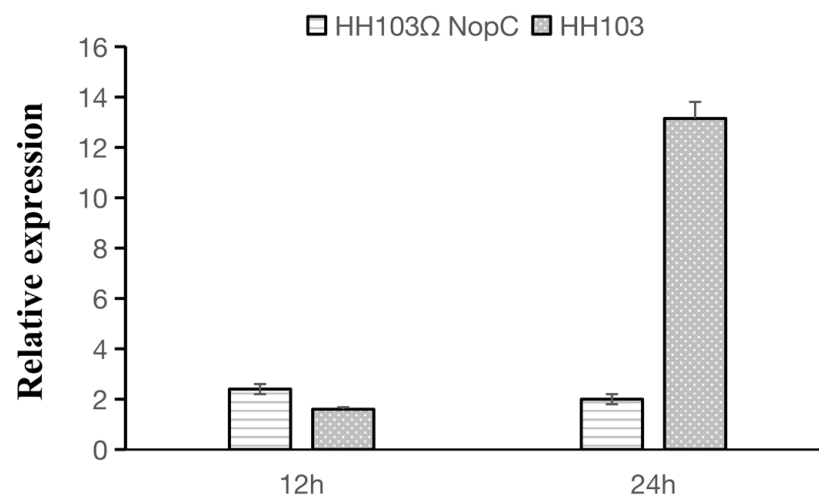**Figure 8.** qRT-PCR analysis of OE-*GmERF5* plants (where "******" represents $p < 0.01$).

Figure 9. The qRT-PCR result of *GmERF5* gene overexpression plants.

The phenotypic assessment of nodules is presented in Figure 10. Regarding the nodule count, mutant HH103Ω*NopC* displayed 9 nodules, while wild-type HH03 had 26 nodules, indicating a notable difference of 17 nodules (Figure 10A). The dry weight of the nodules induced by HH103Ω*NopC* was 0.02 g, whereas those induced by wild-type HH103 weighed 0.03 g, demonstrating a disparity (Figure 10B). In terms of nodules with a diameter ≥2 mm, mutant HH103Ω*NopC* had two nodules, whereas wild-type HH103 had three, indicating a significant contrast (Figure 10C). The nodule count with a diameter <2 mm was 7 in plants inoculated with HH103Ω*NopC* and 22 in those inoculated with HH103, displaying a highly significant distinction.
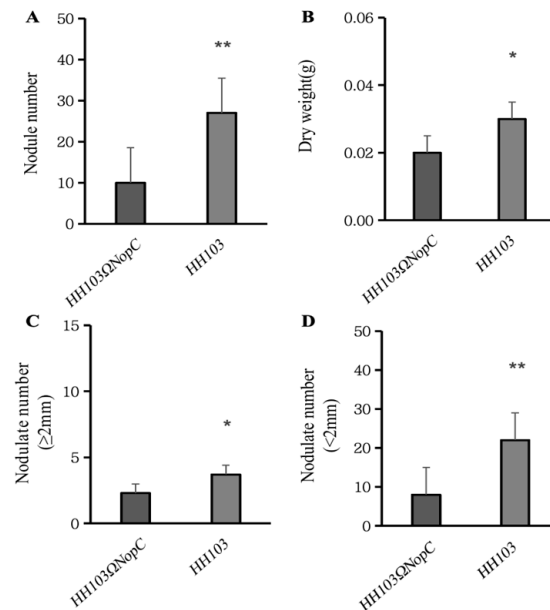

Figure 10. Phenotypic identification of overexpression *GmERF5*. Note: (**A**) number of nodules; (**B**) dry weight of nodules; (**C**) number of large nodules (diameter ≥ 2 mm); (**D**) number of small nodules (diameter < 2 mm) (where "****" represents $p < 0.01$ and "***" represents $p < 0.05$).

In the context of the average nodule count, mutant HH103Ω*NopC* exhibited a decrease in the number of nodules compared to wild-type HH103, as shown in Table 3. This reduction could be associated with the overexpression of *GmERF5* and the potential inter-regulatory effect between *GmERF5* and *NopC*, leading to divergent impacts of various mutants on nodule formation.

Table 3. Phenotypic identification of transgenic hairy root nodules.

Strains	Nodulate Number (nos.)	Dry Weight (g)	Number of Large Nodules (≥2 mm)	Number of Small Nodules (<2 mm)
HH103Ω *NopC*	9.69 ± 1.759	0.02 ± 0.003	2.31 ± 0.624	7.38 ± 0.1470
HH103	26.20 ± 4.957	0.03 ± 0.005	3.87 ± 1.046	22.07 ± 4.794

4. Discussion

Rhizobium, a nitrogen-fixing bacterium, typically infects legumes by entering through root hairs, forming lines of infestation. Nitrogen-fixing bacteria convert atmospheric nitrogen to ammonia. Host-specific nodulation may depend on the secretion of Nops (nodulation outer proteins) by the T3SS (type III secretion system) of rhizobia. The T3SS of Gram-negative bacteria is a complex multiprotein secretion apparatus that actively exports proteins through needle-like lumens. In pathogenic or commensal bacteria, T3SS has the ability to deliver effector proteins (type III effectors) directly into eukaryotic host

cells. The rhizobial T3 effector plays a role in symbiotic interactions between rhizobia and legumes [28]. By constructing T3SS knockout mutants (genes encoding T3SS device proteins, NopA, NopB, or the regulatory gene ttsI) and testing them for nodulation on legume host and non-host plants, specific legumes were identified that would nodulate in a better or worse way with this T3SS knockout. It was also observed that various mutant strains showed altered competitiveness in co-inoculation experiments with parental strains [29,30]. The effector NopC has no homologs in pathogenic bacteria and does not possess any structural domains or conserved features; therefore, NopC can be considered a rhizobial-specific T3SS-secreted protein that may provide clues to its role in symbiosis. NopC may act as a chaperone to facilitate the assembly of the secretion apparatus or the secretion of effectors into the interior of the host cell. Chaperones are usually retained as small, acidic cytoplasmic proteins within bacterial cells, some of which are encoded by genes within operons that carry genes encoding components of the secretory apparatus. Additionally, effector-bound chaperones exhibit a continuous set of conserved structural folds (α-β-β-β-β-α-β-β-α), and their absence leads to the undersecretion of their corresponding effectors [31]. However, NopC was found to be translocated into the interior of soybean cells, the secretion of other Nop was not abolished upon NopC inactivation, and the analysis of the secondary structure of NopC revealed a low content of α-helices at its carboxyl terminus, with no conserved structure detected in effector-bound chaperones. Further studies are needed to determine the specific function of this *rhizobium*-specific effector protein in plant cells.

The ERF protein family is an important factor in regulating nodule formation [32]. In our study, we found that the overexpression of ERF5 significantly increased the number of nodules. Additionally, the expression of *GmERF5* increased after inoculation with HH103 but not with NopC, updating our understanding of symbiosis in legumes. In the *Lotus japonicus, ERFs* are involved in infection thread formation, and NopC is secreted into the host cell during the establishment of symbiosis. Our findings demonstrate that NopC may also play a key role in the regulation of nodule formation by interacting with signaling pathways, including the *GmERF5* gene. We also found that the expression of *GmERF5* significantly decreased when NopC was mutated, which was consistent with nodule phenotype analysis showing fewer nodules upon inoculation with the NopC mutant. This suggested a clue that NopC affected nodule formation through the *GmERF5*-regulated signaling pathway. However, we did not find evidence that NopC directly interacts with *GmERF5*, indicating that further experiments are needed to elucidate the connection between NopC and *GmERF5*.

5. Conclusions

In this study, we identified the positive effect of NopC on nodulation. Furthermore, RNA-seq analysis revealed that five members of the *GmERF* gene family responded to NopC, with *GmERF5* showing a significant and specific response. The interaction between NopC and *GmERF5* was confirmed through qRT-PCR. The overexpression of *GmERF5* in hair roots supported the notion that *GmERF5* regulated nodulation via interaction with NopC. Our findings suggested a novel pathway in which the soybean *GmERF5*-regulated signaling pathway interacted with the rhizobial type III effector NopC. This offered insights into the molecular mechanisms underlying the symbiosis between soybean and *rhizobium*, which is regulated by both host genes and rhizobial type III effectors.

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Data Availability Statement: The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

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