Shoot-to-root translocated GmNN1/FT2a triggers nodulation and regulates soybean nitrogen nutrition

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Abstract

Symbiotic nitrogen fixation (SNF) provides sufficient nitrogen (N) to meet most legume nutrition demands. In return, host plants feed symbionts carbohydrates produced in shoots. However, the molecular dialogue between shoots and symbionts remains largely mysterious. Here, we report the map-based cloning and characterization of a natural variation in GmNN1, the ortholog of Arabidopsis thaliana FLOWERING LOCUS T (FT2a) that simultaneously triggers nodulation in soybean and modulates leaf N nutrition. A 43-bp insertion in the promoter region of GmNN1/FT2a significantly decreased its transcription level and yielded N deficiency phenotypes. Manipulating GmNN1/GmFT2a significantly enhanced soybean nodulation, plant growth, and N nutrition. The near-isogenic lines (NILs) carrying low mRNA abundance alleles of GmNN1/FT2a, along with stable transgenic soybeans with CRISPR/Cas9 knockouts of GmNN1/FT2a, had yellower leaves, lower N concentrations, and fewer nodules than wild-type control plants. Grafting together with split-root experiments demonstrated that only shoot GmNN1/FT2a was responsible for regulating nodulation and thereby N nutrition through shoot-to-root translocation, and this process depends on rhizobial infection. After translocating into roots, shoot-derived GmNN1/FT2a was found to interact with GmNFYA-C (nuclear factor-Y subunit A-C) to activate symbiotic signaling through the previously reported GmNFYA-C-ENOD40 module. In short, the description of the critical soybean nodulation regulatory pathway outlined herein sheds novel insights into the shoot-to-root signaling required for communications between host plants and root nodulating symbionts.

Introduction

With the inclusion of nearly 750 genera and 20,000 species, legumes constitute the third largest family of angiosperms [1]. Most leguminous plants are capable of establishing symbiotic associations with soil-dwelling rhizobia that lead to the formation of nodules where symbiotic nitrogen fixation (SNF) takes place [2]. This association enables leguminous plants to reduce atmospheric N₂ gas to biologically available ammonia [3]. In this mutual exchange, soybean
Plants provide rhizobia with carbohydrates derived from photosynthesis in return for nitrogen (N) provided by rhizobia through SNF [4]. It has been reported that the N provided through SNF could cover up to 70% of the total N needed for growth and development of soybean plants, as well as N rich residue for subsequent or intercropped planting [2,5,6].

The mutual symbiosis between legumes and rhizobia involves complicated biochemical dialogue through symbiotic signaling networks [7–11]. To date, several important regulators of symbiotic signaling have been identified, including nodule inception (NIN), nuclear factor-Y subunit A1 (NF-YA1), and nodulation signaling pathway 1 and 2 (NSP1/NSP2) [8,11–13]. Furthermore, SNF is an energy-intensive metabolic process requiring tight control by the host legume to balance the trade-offs between allocation of resources for growth or nodulation [14]. Long distance signaling through autoregulation of nodulation (AON) is one reported pathway employed by legume host shoots to control nodulation in the roots [15–17]. In addition, environmental conditions, such as high N supply, have been reported to significantly inhibit nodulation [10,11,18–20]. Recently, long distance light signaling has also been reported to be involved in the regulation of nodulation through the CaMK-STF-FT module [17,21]. However, specifics of the molecular dialogue between host shoots and symbionts and the fine tuning of resource division between nodulation and development remain largely mysterious.

In this study, we identified a quantitative trait locus (QTL) and the major gene GmNN1 in this locus that is responsible for shoot control of root nodulation. We further identified a naturally occurring variant with a 43-bp insertion in the promoter region of GmNN1 that leads to lower expression of GmNN1 and inhibits its translocation from shoots to roots. Further observation revealed that the GmNN1/FT2a-GmNFYA-C module regulated nodulation through association with the promoter of GmENOD40. Overall, this work elucidates a novel regulatory component in soybean nodulation and provides new insights into communications between host plants and symbionts through shoot-to-root translocated signaling components.

Results

Discovery of natural variation in nitrogen nutrition in soybeans

In the field, we found 2 genotypes segregating from the same sub-progeny that displayed distinct nitrogen (N) nutrition phenotypes, with the ND (N deficient) line exhibiting yellower leaves than the NS (N sufficient) line (Fig 1A), which was verified by 33.3% and 48.5% lower SPAD values and leaf N concentrations in ND than in NS plants (Fig 1B and 1C). To dissect the genetic basis for this natural variation in leaf N nutrition, we first re-sequenced the ND and NS genotypes. The results showed that only 4 regions on chromosome 16 were highly heterozygous (S1 Fig and S1 Table). To validate the role of qNN1 in controlling N nutrition, we generated a 132 member sub-mapping population that was derived from 5 individual F2 residual heterozygous plants. One QTL for N concentration was localized to a region between the Gm16_29370959 and Gm16_31348398 markers located in region 4 and was subsequently named as nitrogen nutrition locus 1 (qNN1) (Fig 1F). To determine which region is required for controlling N nutrition, we generated a 132 member sub-mapping population that was derived from 5 individual F3 residual heterozygous plants. One QTL for N concentration was localized to a region between the Gm16_29370959 and Gm16_31348398 markers located in region 4 and was subsequently named as nitrogen nutrition locus 1 (qNN1) (Fig 1F). To validate the role of qNN1 in controlling N nutrition, we developed the following near isogenic lines (NILs), NIL-Gmnn1, which carries the ND allele, and NIL-GmNN1, which carries the homologous segment from NS. Phenotyping analysis showed that NIL-Gmnn1 plants have yellower leaves and lower SPAD values than NIL-GmNN1 lines (Fig 1D and 1E).

Map-based cloning of GmNN1

Fine mapping narrowed down the location of qNN1 to an interval between the 2 markers Chr.16G_31103873 and Chr.16G_31122406 containing 2 putative genes, Glyma.16G150700 and Glyma.16G150800 (Fig 1F). Phenotype of the recombinant plants were also evaluated in
progeny lines. Consistently, #3B lines harboring the ND allele had lower SPAD values than #3A lines harboring the NS allele and progeny lines exhibited large variations in SPAD values (Fig 1G). We then sequenced these 2 genes in both ND and NS plants. In comparison with NS plants, the Glyma.16G150700 (also known as Flowering Locus T, FT2a) gene in ND contains one 43-bp deletion in the promoter region (Fig 1H), while Glyma.16G150800 encodes an exocyst complex protein not displaying any variation between NS and ND plants. Therefore, we considered Glyma.16G150700 as the causal gene underlying phenotypic variation associated with qNN1, and hereafter the gene was renamed GmNN1/FT2a.

To investigate whether the 43-bp variation in the promoter of GmNN1/FT2a is critical for gene expression, the isolated promoters of ND and NS were fused to separate GUS reporter genes and then transferred to both hairy roots of transgenic composite soybean plants and tobacco leaves (Figs 1I and S2A). Although GmNN1/FT2a promoters were able to drive GUS gene expression, transgenic hairy roots and tobacco leaves harboring proGmNN1/FT2a::GUS...
amplified from ND plants exhibited lower GUS activity than those harboring constructs from the NS genotype. The expression level of GUS and its protein activity mediated by GmNN1/FT2a from NS were 4.39-fold and 2.86-fold in hairy roots and 2.84-fold and 4.31-fold higher in transient transgenic tobacco compared with the impacts of GmNN1/FT2a from ND (Figs 1J, 1K, S2B, and S2C). Moreover, GmNN1/FT2a transcription was also evaluated in NILs, with GmNN1/FT2a transcript levels significantly higher in NIL-GmNN1 lines than in NIL-Gmnn1 plants (S2D Fig). These results suggest that variation in the promoter region of GmNN1/FT2a leads to differences in mRNA accumulation that might be responsible for the improved N nutrition in NS relative to ND soybean plants.

**Manipulating GmNN1/GmFT2a significantly impacts nodulation and N nutrition in soybean**

To further outline potential roles filled by GmNN1/FT2a in soybean N nutrition, we also generated stable transgenic soybeans in the Williams 82 (Ws82, wild type, WT) background carrying the NS allele, including 2 independent CRISPR/Cas9 GmNN1/FT2a knockout lines (Gmnn1/ft2a, S3 Fig), as well as 2 independent lines overexpressing GmNN1/FT2a (OX-GmNN1/FT2a, S4 Fig). The 2 knockout lines exhibited obviously delayed flowering (S4A and S4C Fig) and yellower leaves than WT plants, while OX-GmNN1/FT2a lines transcribing GmNN1/FT2a 12.1- to 16.6-fold higher than WT displayed opposite phenotypes (Figs 2A and S4B). The SPAD value decreased by 23.0% and 26.6% in the 2 Gmnn1/ft2a mutants and increased by 16.9% and 17.7% in the OX-GmNN1/FT2a lines (Fig 2B). Meanwhile, leaf N concentrations decreased by averages of 22.4% and 20.8% in knockout mutants and increased by 22.1% and 21.7% in OX-GmNN1/FT2a lines (Fig 2C).

Since SNF is a vitally important and irreplaceable N source for legumes, nodulation traits for WT and mutant lines were therefore investigated in hydroponics at 21 d after rhizobium inoculation (dai). Intriguingly, we found that manipulating GmNN1/GmFT2a significantly affected soybean nodulation, and as shown in Fig 2D, nodules were less numerous in mutants and more numerous in OX-GmNN1/FT2a lines in comparisons with each other and with WT plants. Nodule counts declined by 42.9% and 32.9%, and nodule weights decreased by 37.6% and 32.7% in knockout lines compared with WT plants. Conversely, overexpression of GmNN1/FT2a led to 43.0% and 52.9% increases in nodule numbers, accompanied by 54.9% and 52.6% increases in nodule biomass relative to nodulation on WT plants (Fig 2E and 2F). This strong genetic component was also confirmed in NILs, as NIL-Gmnn1 lines displayed fewer nodules and lower nodule dry weights than NIL-GmNN1 lines (Fig 2G–2I). In addition, the nodulation characteristics of ND and NS genotypes were further evaluated in hydroponics after rhizobium inoculation. Consistent with the observations in mutant lines and naturally occurring variants outlined above, ND plants with yellow leaf phenotypes harbored fewer nodules (S5A Fig). The total number of nodules was 36.4% lower in ND lines than in NS lines (S5B Fig), with concomitant nodule dry weight losses totaling 41.0% (S5C Fig). Taken together, these results suggest that GmNN1/FT2a simultaneously triggers soybean leaf N limitation responses and nodulation on roots.

**Manipulating GmNN1/GmFT2a regulates plant growth, nodulation, and N content**

To evaluate the effects of GmNN1/FT2a on plant growth and N nutrition at the whole plant level, 21 dai roots and shoots were separately harvested for further measurement. As shown in Fig 3A, knockout of GmNN1/FT2a significantly inhibited root growth, while OX of GmNN1/FT2a obviously promoted root elongation when compared with WT plants. The total root
length and root fresh weight decreased by 21.3% to 21.9% and 26.2% to 26.9% in GmNN1/FT2a knockout lines and increased by 19.1% to 20.9% and 11.3% to 17.1% in OX lines compared with WT, respectively (Fig 3B and 3C). Since root growth was modified by GmNN1/FT2a, nodule number per root length was further analyzed and found that manipulating GmNN1/FT2a increased or decreased nodule number per root length 23.1% to 44.1% or 16.4% to 21.6% in overexpressions or knockout mutants, respectively (Fig 3D), suggesting that GmNN1/FT2a is directly involved in nodulation beyond regulating root growth. Moreover, compared with WT lines, overexpression or knockout of GmNN1/FT2a led to 20.8% to 21.5% and 20.6% to 21.1% decrease or 23.4% to 38.2% and 23.1% to 28.6% increase in plant dry weight and N content, respectively (Fig 3E and 3F). Taken together, these results suggest that GmNN1/FT2a is indeed an important contributor to plant growth, nodulation, and N nutrition in soybean.

**GmNN1/FT2a impacts nodulation and N nutrition through shoot-to-root translocation**

Previous studies revealed that FT products are synthesized in leaves and then travel through the vasculature to the shoot apical meristem to trigger flowering [22]. In this study, we also
investigated the expression pattern of GmNN1/FT2a and found that transcripts of GmNN1/FT2a localized primarily in leaves rather than stems, roots, and nodules (S6 Fig). To further investigate whether shoot-derived GmNN1/FT2a is truly able to systemically enhance nodule formation in roots, we also conducted an experiment of reciprocal grafting between WT (Ws82) and Gmnn1/ft2a mutant soybeans. Knockout of GmNN1/FT2a in scions led to significant decreases in nodule number and nodule dry weight on either WT or Gmnn1/ft2a rootstocks (Fig 4A–4C). Moreover, scions knockout of GmNN1/FT2a significantly reduced root length, root fresh weight, nodule number per root length, plant dry weight as well as N content in the WT rootstocks to those equivalent to Gmnn1/ft2a rootstocks (Figs 4D–4F and S7). In contrast, grafting between WT scions and Gmnn1/ft2a rootstocks did not produce significant effects on nodulation traits, plant growth, and N content compared with self-grafted WT plants. Taken together, grafting results suggest that shoot GmNN1/FT2a systemically restores nodule formation and root growth capabilities to Gmnn1/ft2a roots.

Fig 3. Plant growth, nodule formation, and N content as affected by manipulating of GmNN1/FT2a. (A) Photographs showing soybean root growth performance. (B) Total root length (n = 10). (C) Root FW (n = 10). (D) Nodule number per RL (n = 10). (E) Plant dry weight (n = 10). (F) N content (n = 7). WT and transgenic soybean lines with knockout or overexpression of GmNN1/FT2a inoculated with rhizobia were grown in hydroponic for 21 dai. All data are given as mean ± SD. Different letters denote significant differences (P < 0.05) according to Duncan’s multiple range comparison tests. Data underlying the graphs in the figure can be found in S1 Data. dai, day after inoculation; FW, fresh weight; N, nitrogen; RL, root length; WT, wild type. 

https://doi.org/10.1371/journal.pbio.3001739.g003
Another grafting experiment was further carried out to determine whether GmNN1/FT2a itself functions as the long distance signal moving from shoot to root to mediate soybean nodulation. Transcripts of GmNN1/FT2a-GFP were detected only in shoots, but not in roots through quantitative reverse transcription PCR (qRT-PCR) analysis of GmNN1/FT2a-GFP/Ws82 grafts (Fig 4G). Further immunoprecipitation and immunoblot testing revealed that GmNN1/FT2a-GFP products in both shoots and roots of GmNN1/FT2a-GFP/Gmnn1/ft2a grafts (Fig 4H). These results suggest that the GmNN1/FT2a protein might be moved from shoots to roots. Additionally, to dissect the relationship between GmNN1/FT2a mobility and rhizobial infection, we established a split-root hydroponic setup, and 1 side of the split roots was inoculated with rhizobia (+R) while the other side without inoculation (−R) (Fig 4I). Then, 5 dai roots were separately harvested for immunoprecipitation and immunoblotting analysis. Surprisingly, the GmNN1/FT2a-GFP signal was only detected in roots with inoculated with rhizobium (Fig 4J), suggesting that shoot-to-root translocation of GmNN1/FT2a depends on rhizobial infection. Collectively, these results paint GmNN1/FT2a as a mobile...
shoot-derived signal directed to nodulating roots where it plays critical roles in nodule development.

**Shoot-derived GmNN1/FT2a modulates nodulation through symbiotic signaling pathway genes**

To investigate molecular mechanisms underlying the GmNN1/FT2a-mediated regulation of nodulation, we first checked for differential expression of symbiotic signal pathway genes between ungrafted Ws82 (WT) and Gmnn1/ft2a mutant plants at 3 dai. In these observations, knockout of GmNN1/FT2a inhibited most examined marker genes in symbiotic signaling pathway, including GmCHS1 (Chalcone synthase 1), GmNFR1, GmNSP1a, GmNINa, GmERN1 (require for nodulation), GmNYFA-C, and GmENOD40 (Fig 5A). Among them, transcripts of GmNYFA-C and GmENOD40 were 4.61 and 7.57 times lower in Gmnn1/ft2a mutants than in Ws82 plants. Then, GmNN1/FT2a impacts on the root expression of symbiotic marker genes were verified in reciprocally grafting treatments using Ws82 (WT) and Gmnn1/ft2a mutant roots and scions (Fig 5B). In general, the expression level of symbiotic genes was severely inhibited in root when grafting included Gmnn1/ft2a in either the scion or rootstock, though impacts tended to be even declined with rootstock expression. The most notable exception was that GmENOD40 expression was only inhibited with shoot expression of Gmnn1/ft2a (Fig 5B), suggesting that shoot-derived GmNN1/FT2a stimulates the expression of GmENOD40.

The results reported here verify previous work in which GmNFYA-C-GmENOD40 was reported as an important symbiotic signaling molecule [11]. Taken together with the present results showing that shoot-derived GmNN1/FT2a stimulates GmENOD40 expression (Fig 5), we then investigated the possibility of whether GmNN1/FT2a could interact with GmNFYA-C in triggering soybean nodulation. Interestingly, the results revealed a direct interaction between GmNN1/FT2a and GmNFYA-C via a yeast 2-hybrid assay (Fig 6A), which was further confirmed in a bimolecular fluorescence complementation (BiFC) assay (Figs 6B and S8) and co-immunoprecipitation (Co-IP) assays using tobacco leaves co-transformed with GmNN1/FT2a-GFP and MYC-NFYA-C (Fig 6C). To test whether the GmNN1/FT2a-GmNFYA-C module directs the expression of GmENOD40, we performed chromatin immunoprecipitation (Ch-IP)-qPCR assays using the transgenic soybean composite plants, consisting of a GmNN1/FT2a-GFP shoot with or without GmNFYA-C knockout transgenic hairy roots (Figs 6D, 6E and S9). After 5 dai, the positive hairy roots were separately harvested for ChIP-qPCR analysis, and the results revealed that GmNN1/FT2a-GmNFYA-C regulated the expression of GmENOD40 through associating with P1, P2, P4, and P5 regions in the promoter, which harbored the canonical CCAAT binding site for GmNFYA protein [23]. All of these results are consistent with the conclusion that GmNN1/FT2a directly interact with GmNFYA-C.

More conclusively, using hypocotyl graft chimeras, we observed that in comparisons with Ws82 self-grafted controls, nodule numbers and dry weights declined significantly with grafting between Gmnn1/ft2a and Ws82, or between Ws82 and nfy-a-c, and dropped even further with grafting between Gmnn1/ft2a scions and nfy-a-c rootstocks (Figs 6F, 6G and S10). These biochemical and genetic results conclusively revealed that GmNN1/FT2a interacts with GmNFYA-C to regulate nodulation through activating the GmENOD40 symbiotic signaling pathway (Fig 7).

**Discussion**

This work uncovered natural variation in the promoter region of GmNN1/FT2a and further identified the novel regulatory module GmNN1/FT2a-GmNYFA-C-GmENOD40 as a key
element in shoot regulation of root nodulation in soybean plants (Fig 7). To accomplish this, we first bred progeny populations from residual heterozygous lines exhibiting contrasting nitrogen (N) nutrition phenotypes under field conditions (Fig 1A and 1F). Then, we identified

![Graph showing relative expression levels of symbiotic marker genes in the roots of different plant materials.](https://doi.org/10.1371/journal.pbio.3001739.g005)

Fig 5. Relative expression levels of symbiotic marker genes in the roots of different plant materials. (A) The mRNA abundance of marker genes in WT (Ws82) and GmNN1/FT2a knockout mutant (Gmnn1/ft2a) plants. (B) Relative expression of marker genes in the roots of reciprocal grafting treatments conducted between Ws82 and Gmnn1/ft2a. All data were acquired by qRT-PCR of roots at 3 dai. Data are given as means ± SEM (n = 3 technical repeats). Three biological replications were performed with similar results. Data underlying the graphs in the figure can be found in S1 Data. dai, day after inoculation; qRT-PCR, quantitative reverse transcription PCR; WT, wild type.

https://doi.org/10.1371/journal.pbio.3001739.g005
a segregating locus and its candidate gene * Glyma.16G150700*, which is homologous to *FT* genes (Figs 1F, 1G and S1 and S1 Table) [24]. Further experimentation demonstrated that the observed N deficiency phenotype resulted from a decline in SNF due to a significant decrease in nodulation (Fig 2). This is consistent with previous reports in which nodulation has been tightly associated with N status in soybeans [2, 18, 25]. Further detailed analysis herein delimited differential expression effects of *Gmnn1/ft2a* versus *GmNN1/FT2a* to a 43-bp insertion in the promoter region of *Gmnn1/ft2a* that significantly inhibited expression (Figs 1H–1K and S2). In addition, overexpression of *GmNN1/FT2a* increased nodulation and simultaneously increased plant growth as well as N nutrition, while knockout of *GmNN1/FT2a* by CRISPR/Cas9 decreased nodulation, plant growth, and N nutrition (Figs 2 and 3). This suggests that N

![Diagram](https://doi.org/10.1371/journal.pbio.3001739.g006)
supplied through SNF might be manipulated through altering the expression of \( \text{GmNN1/FT2a} \). Therefore, the natural variation in \( \text{GmNN1/FT2a} \) discovered presently could be useful for breeding elite cultivars with high SNF capacities.

It has long been known that \( \text{GmNN1/FT2a} \) is mainly expressed in leaves and that it encodes a mobile polypeptide involved in the regulation of the transition to flowering [22,26]. In the present study, we demonstrate alternative functionality in which nodulation on roots appears to be primarily controlled by shoot-to-root translocated \( \text{GmNN1/FT2a} \), but not derived from root (Fig 4G and 4H). This is consistent with a previous report that FT regulates the formation of potato tubers [27], as well as a recent publication in which \( \text{GmNN1/FT2a} \) was found to be translocated from leaves to regulate nodulation in soybean roots [17]. Here, we also demonstrated that the translocation of \( \text{GmNN1/FT2a} \) depends on rhizobium infection, with \( \text{GmNN1/FT2a} \) specifically accumulating in the rhizobium infected half of split-root assays (Fig 4I and 4J). We presume that the translocation of \( \text{GmNN1/FT2a} \) is regulated by an unidentified signal originating from rhizobium infection sites. Other researchers have reported that above-ground systematic signals could be equally distributed among roots [28], so it remains possible that the \( \text{GmNN1/FT2a} \) translocated to uninfected roots might be degraded by unknown factors. However, the lack of information currently available on the stability of FT protein in planta indicates that these hypotheses require further investigation to sort out [29].

Previous reports have proposed working models with \( \text{GmNN1/FT2a} \) acting mainly through associations with transcription factors [24]. Here, we demonstrated that \( \text{GmNN1/FT2a} \) associates with \( \text{GmNFYA-C} \) (Fig 6A–6E), which has been identified along with homologous genes

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**Fig 7. A proposed working model of how the \( \text{GmNN1/FT2a-GmNYFA-C-GmENOD40} \) module regulates nodulation in soybean.** Shoot-derived \( \text{GmNN1/FT2a} \) translocates to roots, with its mobility being determined by rhizobial inoculation. In soybean roots, \( \text{GmNN1/FT2a} \) directly interacts with \( \text{GmNYFA-C} \) to promote nodulation, and thereby facilitate N nutrition, via activation of \( \text{GmENOD40} \) expression.

https://doi.org/10.1371/journal.pbio.3001739.g007
in other species as essential for nodule initiation and development in *Medicago truncatula*, *Lotus japonicus*, and *Glycine max* [11,30,31]. Moreover, GmNFYA-C and GmNN1/FT2a exhibited additive effects in the regulation of nodule formation, as the number of nodules declined in grafting assays using Gmnn1/ft2a and nfy-a-c mutants (Figs 6F, 6G and S10), though redundancy effects of other NYFAs or FTs might obscure some impacts [31,32].

Symbiotic signaling was significantly repressed in the Gmnn1/fa2a mutant, which was reversed for specific symbiotic signaling markers by shoot-derived GmNN1/FT2a (Fig 5). This is consistent with previous reports of grafting effects on nodule formation (Fig 4A–4C) and further validates the hypothesis that nodule formation is controlled largely by shoot-derived GmNN1/FT2a. In this study, upon translocation from shoots to roots, GmNN1/FT2a was found to directly interact with GmNFYA-C (Fig 6A–6E) and then to promote GmENOD40 (Fig 5), which is a direct target of GmNFYA-C [11]. The expression of GmNFYA-C was demonstrated previously to be specifically induced by rhizobial inoculation under low N conditions [11], the interaction between GmNN1/FT2a and GmNFYA-C proved that the regulation of GmNN1/FT2a in nodule formation and N nutrition through shoot-to-root translocation is dependent on rhizobial infection and also affected by N conditions. Finally, the expression levels of other symbiotic signaling genes were also influenced in the Gmnn1/ft2a mutant, but not through shoot dependent translocation, indicating that GmNN1/FT2a could also regulate nodule formation through other pathways [17].

Taken together, our findings identify a novel regulatory component in soybean nodule formation and provide new insights into communications between host plants and symbionts through shoot-to-root translocated signaling elements.

**Materials and methods**

**Plant materials and growth conditions**

Experiments in this study included plants grown in the field, pot cultures, and hydroponics. For the field experiments, ND and NS plants were identified at the Dishang experimental farm (E 114.48˚, N 38.03˚) of the Institute of Cereal and Oil Crops, Hebei Academy of Agricultural and Forestry Sciences, Shijiazhuang City, Hebei Province.

For the pot experiments, seeds of ND and NS lines, as well as seeds from a 132 member sub-mapping population were germinated in pots filled with soil inoculated with the rhizobium strain *Bradyrhizobium* sp. BXYD3 (OD$_{600}$ = 0.8). Seedlings were fertilized once per month with 530 μM low-N nutrient solution as described previously [33]. At the seed-filling stage, plants were harvested to analyze SPAD values, measure N concentrations, and conduct resequencing. Two independent experiments were performed and 15 replicates were investigated for SPAD value, and 7 plants were harvested for N concentration measurement.

For the hydroponic experiment, seeds of soybean used for phenotypic analysis were surface sterilized in 3% H$_2$O$_2$ for 1 min, rinsed with distilled water, and germinated in sand for 7 d. Uniform seedlings were inoculated with BXYD3 by immersing roots in a rhizobial suspension (OD$_{600}$ = 0.8) for 1.5 h, prior to transplanting into a low-N nutrient solution containing 530 μM N as described above. Plants were grown in growth chambers (day/night: 12 h/12 h, 26˚C/24˚C). Nutrient solution was refreshed weekly, and the pH was maintained at 5.8 to 6 with diluted H$_2$SO$_4$ or KOH.

For the grafting assay, plants were sown on sand culture for 4 d. Uniform seedlings were cut at the basal hypocotyls, and scions were grafted on rootstocks. Grafted plants were grown in hydroponics cultures containing 530 μM low-N nutrient solution for 3 d in darkness and then 5 d under light/dark conditions. Subsequently, grafted plants were inoculated with BXYD3 (OD$_{600}$ = 0.8) for 1.5 h as described above. For split-root grafts, part of the main root...
system was separated and after another 2 d of recovery, 1 side was inoculated with BXYD3 and the other side without inoculation was used as the control. At 5 dai, roots and shoots of grafts were harvested for qRT-PCR, immunoprecipitation (IP), and immunoblotting analysis. At 21 dai, nodules were harvested for phenotyping analysis. Sampled roots were scanned as digital images using a specialized color scanner. Total root length was quantified using a computer image program.

**Resequencing of ND and NS plants**

ND and NS were grown in pots and on day 15 of growth, entirely expanded young leaves were harvested. DNA was isolated using a DNeasy Plant Mini Kit. Resequencing libraries were quantified using Bioanalyzer and then sequenced (paired-end, 100-mer each) in the Illumina genome analyzer.

**QTL analysis**

Since 4 highly heterozygous sites were found in ND and NS, and to isolate the gene responsible for N nutrition, we separately searched F5 residual heterozygous plants derived from the parents of ND and NS. Then, QTL analysis was carried out in MapQTL 6.0 (https://mybiosoftware.com/) to further narrow down the position. An LOD threshold score of 2.5 was considered as the minimum necessary to declare that given QTLs were significant. Among them, we identified a QTL for N concentration in leaves named as qNN1 (N nutrition locus1) on chromosome 16, which was close to site 4 (S1 Fig) identified in the 132 member sub-mapping population.

**Positional cloning of qNN1**

A line derived from the mapping population displaying heterozygosity at the qNN1 locus was selected for fine mapping of qNN1. The genes of interest were narrowed down to Gm16_29370959 and Gm16_31348398 in sub-F3:4 progeny. Progeny testing of homozygous recombinants localized qNN1 to approximately 18-kb interval between Chr.16G_31103873 and Chr.16G_31122406, which contains 2 putative genes, Glyma.16G150700 and Glyma.16G150800. Primer sequences of the markers for mapping are listed in S2 Table.

**DNA extraction and PCR assays**

Genomic DNA was extracted from soybean leaves by a modified CTAB method [34]. PCR was performed in 25 μL volumes containing 50 ng genomic DNA, 0.5 μL of specific primers (S2 Table), 2.5 μL Ex Taq HS buffer, 2 μL dNTP, 0.125 μL Ex Taq HS DNA polymerase, and distilled, deionized water up to 25 μL. The procedure for PCR amplification were as follows: denaturation at 94˚C for 2 min and then 32 cycles of denaturation at 94˚C for 30 s, annealing at 50˚C to 57˚C for 30 s, extension at 72˚C for 30 s, and a final extension at 72˚C for 10 min. PCR products were digested using corresponding restriction endonucleases in a final volume of 20 μL according to kit protocols. Then, digested products were visualized after electrophoresis on a 3% polyacrylamide gel.

**RNA extraction and qRT-PCR analysis**

Total RNA was extracted from various tissues of different plant tissue as described in the text using TRIzol reagent and was reverse transcribed using the M-MLV Reverse Transcriptase kit based on protocols from the supplier. qRT-PCR was performed in 10 μL volumes containing 1 μL of 1:50 diluted cDNA, 0.3 μL of specific primers, 3.4 μL of distilled, deionized water, and
5 μL of Trans Start Top Green qPCR SuperMix using a LightCycler96 PCR system. The elongation factor EF-1α gene from soybean (TefS1; accession no. X56856) or from tobacco (NtEF1a; accession no. AF120093) [35] was used as a reference gene to evaluate relative transcript abundance. Relative transcript abundance was calculated as the ratio of the expression value of the target gene to that of TefS1 or NtEF1a using the 2^ΔΔCT (threshold cycle) method. Primers used for detecting target genes are listed in S3 Table.

**Vector construction and plant transformation**

For promoter activity assays, the 2-kb upstream sequence of GmNN1/FT2a was amplified from ND and NS genotypes using the primers proGmNN1/FT2a-F1/proGmNN1/FT2a-R1. The PCR products were cloned into EcoRI and BamHI sites of the pTF102 vector to create proGmNN1/FT2a::GUS constructs using the ClonExpress II One Step Cloning Kit.

For CRISPR/Cas9 gene editing, 2 single-guide RNAs were designed using Primer Design software. Two respective U6 promoters were used for the guide RNA oligonucleotide pair. U6 promoters driving single-guide RNA cassettes were cloned into the pGEL201 vector.

To construct the GmNN1/FT2a overexpression or GmNN1/FT2a-GFP plasmid, the ORF of GmNN1/FT2a was amplified by PCR using the primers GmNN1/FT2a-OE-F/GmNN1/FT2a-OE-R and GmNN1/FT2a-GFP-F/GmNN1/FT2a-GFP-R. Amplified fragments were cloned into Ncol and BamHI sites of the pFGC5941-p35S vector and AscI sites of the pFGC5941-p35S-GFP vector. All primers are listed in S4 Table.

All constructs for soybean transformation were introduced into Agrobacterium tumefaciens strain EHA105 and then transformed into Ws82. In tobacco leaves, recombinant plasmids were introduced into the A. tumefaciens strain GV3101 and then transiently transferred into tobacco leaves by infiltration. After 2 d, transgenic leaves were harvested for GUS staining and activity analysis.

**GUS staining and fluorometric GUS activity assay**

For GUS staining, tobacco leaves were incubated in GUS staining solution containing 50 mm inorganic phosphate-buffered saline (Na₂HPO₄-NaH₂PO₄ buffer (pH 7.2)), 0.1% (v/v) Triton X-100, 2 mm K₃Fe(CN)₆, 2 mm K₄[Fe(CN)₆]·3H₂O, 10 mm EDTA-2Na, and 2 mm 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid at 37˚C overnight. After staining, samples were incubated in 70% (v/v) and then in 100% ethanol until the chlorophyll was completely removed.

For fluorometric GUS assay, protein quantities in transgenic tobacco leaves were first determined based on published methods using bovine serum albumin as a standard [36]. Then, GUS enzyme activity was measured by the fluorescence of 4-methylumbelliferone produced by GUS cleavage of 4-methylumbelliferyl-β-D-glucuronide according to published procedures as described previously [37,38]. GUS activity was calculated as nanomoles of methylumbelliferone per minute per milligram of protein.

**Immunoprecipitation (IP) and immunoblotting**

Proteins were extracted in IP buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA (pH 8.5), 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF)) containing the complete protease inhibitor mixture. Extracted GmNN1/FT2a-GFP proteins were captured on anti-GFP magarose beads for 1 h at 4˚C, washed 3 times with wash buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA (pH 8.5), 0.1% Triton X-100), and transferred into a new tube and incubated at 95˚C for 10 min before elution with SDS sample buffer (0.5 M Tris·HCl (pH 6.8), 10% SDS, 20% glycerol, 2% β-mercaptoethanol, 1% (w/v) bromophenol blue).
For the detection of GmNN1/FT2a-GFP proteins, eluted samples were separated by SDS-PAGE in an 8% agarose gel and then transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in 5% skim milk for 1 h at room temperature prior to incubating with the primary anti-GFP antibodies overnight at 4°C. Horseradish peroxidase (HRP)-conjugated secondary antibodies were then added at 1:5,000 dilutions for 45 min.

**Protein–protein interaction assays**

For the yeast 2-hybrid assay, the GmNN1/FT2a ORF was amplified using the primers GmNN1/FT2a-F1/GmNN1/FT2a-R1 and subcloned into the prey plasmid pGADT7. The ORF of GmNFYA-C was amplified using the primers GmNFYA-C-F1/GmNFYA-C-R1 and subcloned into the bait plasmid pGBK7. Yeast 2-hybrid assays were performed using the *Saccharomyces cerevisiae* strain AH109. Interaction was determined by co-transforming the prey and bait vectors into yeast and growing the yeast colonies on SD-His-Trp-Leu in 30 mM 3AT plates. Yeast transformation and growth were performed as described in the Yeast Protocols Handbook.

For semi-in vivo pull-down assays, the ORF of *GmNFYA-C* was amplified with the primers MYC-GmNFYA-C-F1/MYC-GmNFYA-C-R1 and then inserted into the pENTR 1A Entry vector. Entry clones were subcloned into Gateway compatible pEarleyGate203 (35S-MYC-Gene) vectors using the LR reaction. Both the MYC-tagged GmNFYA-C (MYC-NFYA-C) and GmNN1/FT2a-GFP were introduced into *A. tumefaciens* strain GV3101 and then transiently co-expressed in tobacco leaves using the same IP method as described above to enrich the FT2a–GFP protein. Eluted samples were analyzed on immunoblots using anti-GFP (diluted 1:1,000) and anti-MYC antibodies (diluted 1:5,000).

For in vivo bimolecular fluorescence complementation (BiFC) assays, the ORFs of GmNN1/FT2a, GmNFYA-C, GmPHR9, and GmSPX3 were amplified using the primers as listed in S4 Table prior to separately inserting them into the vectors pCAMBIA1300-nYFP and pCAMBIA1300-cYFP. pCAMBIA1300-nYFP is the vector for N-terminal fusion to yellow fluorescent protein (nYFP), and pCAMBIA1300-cYFP is the vector for C-terminal fusion to YFP (cYFP). Among them, GmPHR9 was reported to be interacted with GmSPX3 in yeast assay [39], and here, leaf epidermal cells of *N. benthamiana* were co-transformed with GmPHR9-cYFP and GmNN1/FT2a-nYFP or GmNFYA-C-cYFP and GmSPX3-nYFP was used as control according to previous description [40]. All of the different plasmid combinations were introduced into *A. tumefaciens* strain GV3101 and then transiently transferred into tobacco leaves through infiltration. After 2 d, transgenic leaves were examined and imaged using a confocal laser scanning microscope.

**Chromatin immunoprecipitation (Ch-IP)-qPCR assay**

For ChIP-qPCR analysis, seeds of the stable transgenic plant harboring GmNN1/FT2a-GFP were surface sterilized in 3% H2O2 for 1 min, rinsed with distilled water, and germinated in sand for 5 d. Uniform seedlings with unfolded cotyledons were infected on the hypocotyl with *A. rhizogenes* strain K599 carrying the pGEL201 vector to generate *nfya-c* mutant hairy roots. When the emerged hairy roots were approximately 10-cm long, the main roots were removed and the hairy roots were collected for DNA extraction and sequencing. Selected transgenic soybean composite plants were inoculated with rhizobial strain BXYD3 and then grown in sand irrigated with low-N nutrient solution for 5 d. Four biological replicates of samples were collected, and each replicate was collected from 3 independent hairy roots. A 2-g aliquot of tissue was then fixed by formaldehyde cross-linking, and the ChIP-qPCR assay procedures were performed as described previously [41]. Target chromatin was immunoprecipitated using
anti-GFP magnetic beads. Enrichment of DNA fragments was determined by qRT-PCR analysis. The fold-enrichment was calculated by normalizing target fragment against internal reference gene GmELF1B (Glyma.02G276600). The relevant primer sequences are given in S5 Table.

**Measurement of N concentration**

For N determination, shoots were dried at 105°C for 30 min, followed by heating at 65°C until completely dry. Samples were ground into powder and digested by H₂SO₄ [42]. N concentrations were measured by the sodium hypochlorite salicylic acid colorimetric method using a continuous flow analyzer running samples at a wavelength of 660 nm and a temperature of 40°C. Colorimetric signals were recorded using FlowAccess as recommended by manufacturer.

**Data analysis**

For phenotypic evaluation, at least 4 individual plants were analyzed and 2 independent experiments were performed. For expression analyses using qRT-PCR, at least 3 individual plants were pooled per tissue sample, and at least 3 qRT-PCR reactions (technical replicates) for each sample were performed. The exact number of replicates (n) is stated in the figure legends.

Mean values for each measured parameter were compared using 2-sided Student t tests or Duncan’s multiple range comparison tests when appropriate.

**Supporting information**

S1 Fig. Distribution of variation between the ND and NS genotypes across soybean chromosomes. 1–4 represent highly heterozygous sites. Data underlying the graphs in the figure can be found in S2 Data. ND, N deficient; NS, N sufficient. (TIF)

S2 Fig. GmNN1/FT2a expression analysis. (A) GUS staining of tobacco leaves harboring different GmNN1/FT2a promoters constructed from ND and NS plant materials. (B) Relative expression of the GUS gene (n = 7). (C) Quantitative GUS activity analysis of the transgenic tobacco leaves by fluorimetric assay (n = 7). (D) Relative expression of GmNN1/FT2a in NILs (n = 3). Three independent lines of each NIL were generated, and 3 biological replicates for each line were harvested for qRT-PCR analysis. All data are given as mean ± SD. Asterisks denote significance of differences (threshold P = 0.05) according to Student t tests; **P < 0.001; ***P < 0.001. Data underlying the graphs in the figure can be found in S2 Data. ND, N deficient; NIL, near-isogenic line; NS, N sufficient; qRT-PCR, quantitative reverse transcription PCR. (TIF)

S3 Fig. Genotype of the Gmnn1/ft2a mutants edited by the CRISPR/Cas9 system. (A) The target site of CRISPR/Cas9 editing in the first exon led to a 1-bp insertion when compared with the WT plant sequence. (B) The target site of CRISPR/Cas9 editing in the first exon led to 1-bp deletion compared with the WT sequence. WT, wild type. (TIF)

S4 Fig. Phenotype and molecular identification of GmNN1/FT2a in stable transgenic soybean plants. (A) Flowering phenotype as affected by overexpression or knockout of GmNN1/FT2a. (B) Relative expression of GmNN1/FT2a in leaves (n = 4). (C) Flowering time in various plants (n = 8). All data are given as mean ± SD. Different letters denote significant differences.
$P < 0.05$) according to Duncan’s multiple range comparison tests. Data underlying the graphs in the figure can be found in S2 Data.

S5 Fig. Comparison of nodulation traits between ND and NS soybean materials. (A) Phenotype of nodules. Scale bars, 2 cm. (B) Nodule number per plant. (C) Nodule dry weight. Soybean plants inoculated with rhizobia were grown in hydroponics for 21 d prior to harvesting nodules for trait analysis. All data are given as mean from 2 independent experiments ± SD ($n = 15$). Asterisks denote significance of differences (threshold $P = 0.05$) according to Student $t$ tests; $^{***} P < 0.001$. Data underlying the graphs in the figure can be found in S2 Data. ND, N deficient; NS, N sufficient.

S6 Fig. Expression of GmNN1/FT2a in various tissues of soybean plants. Ws82 was inoculated with rhizobial BXD3 and transplanted into low-N nutrient solution for 14 d. Then, roots, nodules, stems, YL, and OL were separately harvested for qRT-PCR analysis. Data underlying the graphs in the figure can be found in S2 Data. N, nitrogen; OL, old leaf; qRT-PCR, quantitative reverse transcription PCR; YL, young leaf.

S7 Fig. Plant dry weight and N content assays for grafts between Gmnn1/ft2a mutant and Ws82 at 21 dai. (A) Plant dry weight ($n = 10$). (B) N content ($n = 7$). Gmnn1/ft2a mutant: stable transgenic soybean with knockout of GmNN1/FT2a; Ws82: WT control. Data are given as mean ± SD. Different letters denote significant differences ($P < 0.05$) according to Duncan’s multiple range comparison tests. Data underlying the graphs in the figure can be found in S2 Data. dai, day after inoculation; N, nitrogen; WT, wild type.

S8 Fig. GmNN1/FT2a interacted with GmNYFA-C in the BiFC assay. Leaf epidermal cells of N. benthamiana were co-transformed with GmPHR9-cYFP and GmNN1/FT2a-nYFP or GmNYFA-C-cYFP and GmSPX3-nYFP was used as control according to previous description [40]. BF, bright field; Merge, overlay of the YFP and bright field images.

S9 Fig. Genotype of the nfya-c mutant edited by the CRISPR/Cas9 system from soybean hairy roots. The target site of CRISPR/Cas9 editing in the first exon led to a 2-bp deletion when compared with the WT plant sequence. WT, wild type.

S10 Fig. Nodule dry weight on grafts. Nodules were harvested at 21 dai. Gmnn1/ft2a mutant: stable transgenic soybean with knockout of GmNN1/FT2a; nfya-c: stable transgenic soybean with knockout of GmNYFA-C; Ws82: WT control. Data are given as mean ± SD ($n = 6–8$). Different letters denote significant differences ($P < 0.05$) according to Duncan’s multiple range comparison tests. Data underlying the graphs in the figure can be found in S2 Data. dai, day after inoculation; WT, wild type.

S1 Table. Variation between the ND and NS across soybean chromosomes. 0|0 and 1|1 from both ND and NS represent reference and mutant single-nucleotide polymorphisms, respectively. ND, N deficient; NS, N sufficient.
S2 Table. Gene specific primers used for GmNN1/FT2a fine mapping.
(XLSX)

S3 Table. Gene-specific primers used for qRT-PCR analysis.
(XLSX)

S4 Table. Gene-specific primers used for vector constructs.
(XLSX)

S5 Table. Primer sequences used for ChIP-qPCR assay.
(XLSX)

S1 Raw Images. Raw images of the western blot shown in Figs 4H, 4J and 5C.
(PDF)

S1 Data. Data underlying Figs 1B, 1C, 1E, 1J, 1K, 2B, 2C, 2E, 2F, 2H, 2I, 3B, 3C, 3D, 3E, 3F, 4B, 4D, 4E, 4F, 4G, 5A, 5B, 6E and 6G.
(XLSX)

S2 Data. Data underlying S1, S2B–S2D, S4B, S4C, S5B, S5C, S6, S7A, S7B and S10 Figs.
(XLSX)

Acknowledgments
We would like to thank Prof. Yuefeng Guan and Dr. Shikui Song for help with generating stable transgenic soybean plants, and Xing Fu, Wenqin Ai, Xiao Zhang, and Wenting Lian for help with plant growth, sampling, and counting of nodules. We also thank Thomas Walk of Golden Fidelity LLC for critical reading.

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References

1. Doyle JJ, Luckow MA. The rest of the iceberg. Legume diversity and evolution in a phylogenetic context. Plant Physiol. 2003; 131(3):900–910. https://doi.org/10.1104/pp.102.018150 PMID: 12644643

2. Herridge DF, Peoples MB, Boddey RM. Global inputs of biological nitrogen fixation in agricultural systems. Plant Soil. 2008; 311(1):1–18.

3. Ferguson BJ, Indrasumunar A, Hayashi S, Lin MH, Lin YH, Reid DE, et al. Molecular analysis of legume nodule development and autoregulation. J Integr Plant Biol. 2010; 52(1):61–76. https://doi.org/10.1111/j.1744-7909.2010.00899.x PMID: 20074141
Laporte P, Lepage A, Fournier J, Catrice O, Moreau S, Jardinau MF, et al. The CCAAT box-binding
21. Taylor BN, Menge DNL. Light regulates tropical symbiotic nitrogen fixation more strongly than soil nitrogen. Nat Plants. 2018; 4(9):655–661. https://doi.org/10.1038/s41477-018-0231-9 PMID: 30127409
22. Corbesier L, Vincent C, Jang S, Formara F, Fan Q, Searle I, et al. FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science. 2007; 316(5827):1030–1033. https://doi.org/10.1126/science.1141752 PMID: 17446353
23. Laporte P, Lepage A, Fournier J, Catrice O, Moreau S, Jardinau MF, et al. The CCAAT box-binding transcription factor NF-YA1 controls rhizobial infection. J Exp Bot. 2014; 65(2):481–494. https://doi.org/10.1093/jxb/ert392 PMID: 24319255
24. Yue L, Li X, Fang C, Chen L, Yang H, Yang J, et al. FT5a interferes with the Dt1-AP1 feedback loop to control flowering time and shoot determinacy in soybean. J Integr Plant Biol. 2021; 63(6):1004–1020. https://doi.org/10.1111/jipb.13070 PMID: 33458938

25. Qin L, Zhao J, Tian J, Chen L, Sun Z, Guo Y, et al. The high-affinity phosphate transporter GmPT5 regulates phosphate transport to nodules and nodulation in soybean. Plant Physiol. 2012; 159(4):1634–1643. https://doi.org/10.1104/pp.11.160796 PMID: 20864544

26. Kong F, Liu B, Xia Z, Sato S, Kim BM, Watanabe S, et al. Two coordinately regulated homologs of FLOWERING LOCUS T are involved in the control of photoperiodic flowering in soybean. Plant Physiol. 2010; 154(3):1220–1231. https://doi.org/10.1104/pp.110.160796 PMID: 20864544

27. Navarro C, Abelenda JA, Cruz-Oró E, Cuéllar CA, Tamaki S, Silva J, et al. Control of flowering and storage organ formation in potato by FLOWERING LOCUS T. Nature. 2011; 478(7367):119–122. https://doi.org/10.1038/nature10431 PMID: 21947007

28. Wigge PA. FT, a mobile developmental signal in plants. Curr Biol. 2011; 21(9):R374–R378. https://doi.org/10.1016/j.cub.2011.03.038 PMID: 21549960

29. Lee N, Imaizumi T. Uncoupling FT protein transport from its function. Plant Cell Physiol. 2018; 59(8):1487–1489. https://doi.org/10.1093/pcc/pcy123 PMID: 30103002

30. Combier JP, Frugier F, de Billy F, Boualem A, El-Yahyaoui F, Moreau S, et al. MtHAP2-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in Medicago truncatula. Genes Dev. 2006; 20(22):3084–3088. https://doi.org/10.1101/gad.402806 PMID: 17114582

31. Soyan T, Kouchi H, Hirota A, Hayashi M. Nodule inception directly targets NF-Y subunit genes to regulate essential processes of root nodule development in Lotus japonicus. PLoS Genet. 2013; 9(3):e1003352. https://doi.org/10.1371/journal.pgen.1003352 PMID: 23555278

32. Li X, Fang C, Yang Y, Lv T, Su T, Chen L, et al. Overcoming the genetic compensation response of soybean florigens to improve adaptation and yield at low latitudes. Curr Biol. 2021; 31(17):3755–3767. https://doi.org/10.1016/j.cub.2021.06.037 PMID: 34270946

33. Li X, Zheng J, Yang Y, Liao H. INCREASING NODULE SIZE1 expression is required for normal rhizobial symbiosis and nodule development. Plant Physiol. 2018; 178(3):1233–1248. https://doi.org/10.1104/pp.18.01018 PMID: 30266750

34. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 1980; 8(19):4321–4325. https://doi.org/10.1093/nar/8.19.4321 PMID: 7433111

35. Schmidt GW, Delaney SK. Stable internal reference genes for normalization of real-time RT-PCR in tobacco (Nicotiana tabacum) during development and abiotic stress. Mol Genet Genomics. 2010; 283(3):233–241. https://doi.org/10.1007/s00438-010-0511-1 PMID: 20098998

36. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248–254. https://doi.org/10.1006/abio.1976.9999 PMID: 942051

37. Jefferson RA. Plant reporter genes: the GUS gene fusion system. Genet Eng. 1988; 10:247–263.

38. Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 1987; 6(13):3901–3907. https://doi.org/10.1002/j.1460-2075.1987.tb02730.x PMID: 3327686

39. Zhuang Q, Xue Y, Yao Z, Zhu S, Liang C, Liao H, et al. Phosphate starvation responsive GmSPX5 mediates nodule growth through interaction with GmNF-YC4 in soybean (Glycine max). Plant J. 2021; 108(5):1422–1438. https://doi.org/10.1111/pj.15520 PMID: 34587329

40. Kudla J, Bock R. Lighting the way to protein-protein interactions: recommendations on best practices for bimolecular fluorescence complementation analyses. Plant Cell. 2016; 28(5):1002–1008. https://doi.org/10.1105/tpc.16.00043 PMID: 27099259

41. Hou X, Zhou J, Liu C, Liu L, Shen L, Yu H. Nuclear factor Y mediated H3K27me3 demethylation of the SOC1 locus orchestrates flowering responses of Arabidopsis. Nat Commun. 2014; 5:4601. https://doi.org/10.1038/ncomms5601 PMID: 25105952

42. Liu S, Liao LL, Nie MM, Peng WT, Zhang MS, Lei JN, et al. A VIT-like transporter facilitates iron transport into nodule symbiosomes for nitrogen fixation in soybean. New Phytol. 2020; 226(5):1413–1428. https://doi.org/10.1111/nph.16506 PMID: 32119117