Transcriptomic and photosynthetic responses to grafting of the Nod1 gene in nodulated and non-nodulated soybeans

Qingyuan He,1,2 Shihua Xiang,3 Wubin Wang,2 Yingjie Shu,1 Zhengpeng Li,1 Songhua Wang,1 Lei Chen,1 Xiaoyan Yang,1 and Tuanjie Zhao4*

1College of Life and Health Science, Anhui Science and Technology University, Fengyang 233100, China
2Soybean Research Institute/National Center for Soybean Improvement, Ministry of Agriculture/Key Laboratory of Biology and Genetic Improvement of Soybean/Ministry of Agriculture/National Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, China
3Zigong Institute of Agricultural Sciences, Zigong 643000, China
4*Corresponding author: Email: tjzhao@njau.edu.cn

Abstract
Legume plants form symbiotic relationships with rhizobia to convert N₂ into ammonia, and the nodulation status can affect plant development including photosynthesis. However, the relationship between nitrogen fixation and photosynthesis during carbon and nitrogen metabolism remains unclear. This study was undertaken to unravel regulation of nodulation and photosynthesis using a spontaneous nonnodulated soybean mutant by grafting. The results of inheritance and gene mapping showed that the nonnodulated mutant was controlled by a recessive gene overlapped with the reported nodule locus, and might be a new nodule allele with 1 bp deletion in the fourth exon in comparison to the sequence of normal nodulation plants. According to grafting results, soybean nodulation is obviously determined by the roots, not the seedlings. Moreover, nitrogen content along with related metabolic enzyme activity, and photosynthetic capacity were enhanced by nonnodulated scions grafted with nodulated roots. Contrary results were obtained for nodulated scions grafted with nonnodulated roots. A total of 853 differentially expressed genes (DEGs) in the leaves and 1874 in the roots were identified by transcriptome analyses. Twenty DEGs interacting at translation level were selected, and the results of transcriptome analyses were verified by q-PCR. These findings indicated that the nodulation-related Nod1 allelic gene increases the nitrogen content of nonnodulated plants, which affects the enzymes involved in nitrogen metabolism, leading to changes in hormone levels and further regulation of photosynthesis and carbon metabolism.

Keywords: nodulation gene; soybean; grafting; photosynthesis; transcriptional expression

Introduction
Leaf nitrogen content is strongly related to photosynthetic capacity and other photosynthetic traits, including carboxylation capacity and electron transport rate (Kouki 2004). More than 90% of crop biomass is derived from photosynthetic products. Higher rates of photosynthesis in plants may be triggered by greater nitrogen allocation to ribulose biphosphate carboxylase oxygenase (Rubisco) (Aerts and Chapin 2000). However, nitrogen is an element that limits plant growth in many ecosystems (Hermans et al. 2006). Plants often preferentially allocate their biomass to their root system at the expense of shoot growth when nitrogen is limited (Makino 2011). However, the photosynthesis of legumes can be promoted by symbiotic nitrogen fixation.

Leguminous plants can make their nitrogenous nutrients by forming symbioses with rhizobia. Nitrogen-fixing root nodules are formed between the legumes and rhizobia. Rhizobium cells enter a legume host where they gain carbohydrates in exchange for nitrogen (Gresshoff 2003; Oldroyd et al. 2011). The nodules provide an ideal microenvironment for the reduction of molecular nitrogen to ammonia by rhizobia and nutrient exchange between the symbionts. The formation of this symbiosis and the resulting nitrogen fixation are the result of chemical communication between the plant and the rhizobia (Jin et al. 2016; Ling et al. 2016). Nodulation is the basis of nitrogen fixation. Furthermore, complex networks of nodules exist between plants and rhizobia involving different cell layers and molecular signaling functions (Madsen et al. 2010).

Soybean is an important leguminous crop, providing 69 and 30% of plant protein and vegetable oil, respectively (Lam et al. 2010). Their nodulation traits are controlled by genetic loci, namely Rj(s) or rj(s), and inoculation with compatible Bradyrhizobium or Ensifer species in soybean (Hayashi et al. 2012). The Rj/rj genes are classified into three categories: (i) recessive alleles (rj1, rj5, and rj6) leading to nonnodulation phenotypes (Williams and Lynch 1954; Pracht et al. 1993), (ii) recessive alleles (rj7/nts1, nitrate-tolerant symbiosis) resulting in supernodulation phenotypes (Akao and Kouchi 1992; Harper and Nickell 1995), and (iii) dominant alleles of Rj2, Rj3, Rj4, and Rfg1, which
inhibit nodulation in particular strains of *Bradyrhizobium japonicum* USDA122, Ensifer elkanii USDA33, B. elkanii USDA61, and Ensifer fredii USDA257, respectively (Hayashi et al. 2012). The Rj/rj1 gene, having a sequence of 3.4 kb, is related to the lipo-oligo chitin LysM-type receptor kinase gene, and is located on soybean chromosome 2 (Indrasumunar et al. 2011). Gene Rj1 expression controls the nodule number in soybeans, and overexpression of GmNFR1x can alleviate nodulation deficiency in acidic soil.

The homologous genes of GmNFR1x can also affect nodulation, however, the effects of these genes are not entirely consistent with the phenotype of nodulation (Zhang et al. 2007). Nodulation change may be related to alternative splicing of these genes (Williams and Lynch 1954). The regulation and expression mechanisms of these genes need to be further elucidated. Nodulation changes lead to nitrogen nutritional differences. It is not known whether these changes affect photosynthesis, carbon assimilation, and plant development, which warrants further study (Lawn and Brun 1974). The reciprocal grafting technique has been used to study the roles of shoots and roots in the uptake and transport of nutrients and nodulation in soybeans (Fujita et al. 1991; Perigio et al. 1993). A previous grafting study showed that differences in nodulation and N2 fixation are mainly controlled by root genotypes (Malik 1983) and that the supernodulating phenotype is controlled by the shoots (Perigio et al. 1993). Besides, the function of isoflavonoids in the process of nodulation has also been studied by grafting experiments (Cho and Harper 1991). The objectives of the instant research are to dissect regulation of photosynthesis and nitrogen metabolism during nitrogen fixation by grafting and transcriptome analysis using Nod1 mutant.

**Materials and methods**

**Plant materials and grafting experiment**

NN1138-2 (normal nodulation) and T3791 (natural nonnodulation mutant) were used as the female and male parents, respectively. Their F2 generation (184 plants) was used as the mapping population. In the summer of 2015, the F2 plants were grown in pots (filled with field soil) to V4 stage (Fehr et al. 1971) and then transplanted into the field. Nodulation was investigated individually before transplanting. After self-pollination, F3 families were obtained and used for determining the genotype of F2 individuals.

Seeds of NN1138-2 and T3791 were surface-sterilized with 70% ethanol for 1 minute and 0.1% HgCl2 for 6 minutes, and then washed five times with sterile water. These seeds were planted in pots filled in sterile soil under greenhouse condition (natural light). Grafting was conducted at V1 stage (Fehr et al. 1971) with four treatments: NN1138-2 roots + NN1138-2 scion (NN), NN1138-2 roots + T3791 scion (NT), T3791 roots + NN1138-2 scion (TN), and T3791 roots + T3791 scion (TT).

*B. japonicum* strain USDA110 was grown at 28°C in a darkroom in a liquid yeast extract mannitol broth medium (YMB; pH 6.8) with moderate shaking (120 rpm). After 6 days, cells of USDA110 were amassed by centrifugation (4000 rpm, for 10 minutes), washed three times with sterile water, and diluted in water to an optical density of OD600 = 0.8. Each pot of grafted plants was inoculated with 50 ml of the bacterial suspension. The plants were watered and cultured with sterile water in a greenhouse with natural light until flowering time.

**DNA isolation and linkage mapping**

Genomic DNA was extracted from young fresh leaves using the improved CTAB method (Saghai-Maroof et al. 1984). According to the simple sequence repeat (SSR) information from Soybase (https://soybase.org), 98 pairs of polymorphic primers were selected for genotypic screening in the parents and the F2 population. These SSRs were distributed across the whole genetic map. The PCR system, program, and production detection were the same as the description of He et al. (2015). Linkage map and location of nodulation gene were constructed and mapped using the inclusive composite interval mapping (ICIM) method (RSTESP-LRT-ADD model) in IciMapping V4.1 software (Wang et al. 2016).

**BSA analysis by genome sequencing**

The nodulation and nonnodulation bulks were constructed with 30 homozygous F3 plants. DNA samples were treated using sonication method to generate 350-bp fragments. These DNA fragments were end-repaired, A-tailed, ligated with full-length adapter, and amplified by PCR. The PCR products were purified using the AMPure XP system and the size distribution of the libraries was analyzed with an Agilent 2100 Bioanalyzer. Quantitative real-time PCR (qPCR) was used for accurate quantification. The libraries were sequenced on the Illumina HiSeq platform (Illumina, USA) at Genepioneer Biotechnologies (Nanjing, China). Raw sequence reads were filtered and the retained clean reads were aligned to the reference genome of Williams82 (Wm82.a2.v1). SNPs and InDels were detected using GATK software (McKenna et al. 2010).

**Candidate gene analysis**

To identify the sequence differences in the candidate genes, total DNA was isolated from leaves of NN1138-2 and T3791 using the Plant DNA Kit (TianGen Biotech, Beijing, China), and the genomic segments of the Glyma.02g270800 gene were sequenced. The primers used were designed by Primer Premier 5.0 (Supplementary Table S1). The target gene was amplified in sections, and then the products were sent to PersonalBi (Shanghai, China) for sequencing, splicing, and assembly. The sequences of NN1138-2 and T3791 were aligned. The function of the mutated protein was predicted.

**Chlorophyll content estimation, photosynthesis, and nitrogen metabolism**

Chlorophyll content was determined by spectrophotometry. Net photosynthetic rate (PN), transpiration rate (Tr), intercellular CO2 concentration (Ci), and stomatal conductance (Gs) were measured using the portable photosynthesis system LI-6400XT in the first flowering period between 10.00 and 11.00 a.m. Monoamine oxidase (MAO) and nitrate reductase (NR) activities were assayed according to aldehyde phenyl hydradrene colorimetry (Leagene Biotech) and an in vitro method, respectively. Dried samples were triturated to powder and their N content was determined by the Kjeldahl method. Nitrogen nitrogen (NO3-N) and ammoniacal nitrogen (NH4-N) were determined by the salicylic acid and indophenol blue spectrophotometry methods, respectively.

**RNA-sequence and transcriptome analysis of grafting**

A total of 12 samples from the four grafting treatments were collected and RNA-sequence analysis was conducted on the leaves, primitive roots of the rootstock, and the new scion roots. Total RNA was isolated from tissues using Trizol reagent (Invitrogen Life Technologies, Ambion®, UK). Transcriptomic libraries were constructed using NEBNext RNA super-speediness library preparation kits, including mRNA isolation and fragmentation, first-strand cDNA synthesis, second-strand cDNA synthesis, cDNA purification,
end-repair, and dA-tail addition, adaptor ligation, segment size selection (300–400 bp), library enrichment, and purification. The quality assessment and quantification of the libraries was performed. Then, sequencing was carried out on Illumina HiSeq platform (Beijing Ori-gene Science and technology, LTD.).

The sequencing results were aligned against the Williams 82 genome sequence (https://phytozome.jgi.doe.gov/pz/portal.html) using tophat-2.0.10. The percentages of saturation and coverage were analyzed using RSeQC (Wang et al. 2012). Novel genes were forecasted using Cufflinks and annotated by comparison with the Swiss-prot database. The abundance of transcripts, or gene expression, was calculated using FPKM (as follows). Correlations between treatments were measured in terms of the level of gene expression. Differences in gene expression among different samples were identified using the criterion of Trapnell et al. (2013). Alternative splicing of genes was analyzed by the rMATS method (Shen et al. 2014), gene ontology (GO)/KEGG enrichment analysis of differentially expressed genes was conducted based on a hypergeometric test, taking $P < 0.05$ as the threshold of significance (Young et al. 2010).

$$\text{FPKM} = \frac{\text{Unique}_{\text{map}} \cdot \text{ped} \cdot \text{fragment's number of transcript} \times 10^6}{\text{TotalUnique}_{\text{map}} \cdot \text{ped} \cdot \text{fragment's number} \times \text{base number of a transcript}}$$

Q-PCR was performed to validate the RNA-Seq results of 20 differentially expressed genes (DEGs) with interaction at translation level of RNA-Seq analysis differed across the 12 samples. Primers for q-PCR were designed using Primer Premier 5.0 (http://frodo.wi.nit.edu/primers) (Supplementary Table S1). Expression levels of these genes were normalized according to the tubulin gene (NCBI accession No. AY907703). Gene expression levels were quantified using the relative quantification method (ΔΔCT).

**Results**

**Genetic study for the nodulation trait and determination of the allele associated with nodulation gene**

**Genetic study for the nodulation trait**

Table 1 shows the results of segregation of nodulation/nonnodulation in the offspring and their parents. The NN 1138-2 and T3791 parents exhibited nodulation and nonnodulation traits, respectively. All F1 plants of NN 1138-2 × T3791 exhibited nodulation, indicating that nodulation is controlled by the dominant gene/allele. Segregation of nodulation to nonnodulation in the F2 population fitted a 3:1 ratio. The F3 population segregated at a ratio of 1 nonnodulation: 2 segregation: 1 nodulation (thus, 1:2:1). Therefore, the nonnodulation/nodulation trait was controlled by one mendelian factor.

**Conventional QTL analysis using the F2 and F3 populations**

A genetic map was constructed using 98 SSR markers from the 20 linkage groups in the 184 F2 individuals. This map spanned 1480.31 cM. The proportion of nodulated/nonnodulated F2 plants was considered as the phenotype of the F2 individuals. The putative allele controlling nodulation (Nod1), located between Satt459 and Satt271 on Chr.02, was identified by the ICIM method in IciMapping software (Figure 1A).

**Detection of QTL using BSA-seq analysis**

Four libraries (one for nodulation, one for nonnodulation, and two for parents) were constructed and subjected to whole-genome resequencing using Illumina HiSeq 2500. A total of 173,266,284, 111,850,845, 214,170,082, and 208,865,083 clean reads were obtained from the NN1138-2, T3791, nodulation, and nonnodulation pools, respectively. All F1 plants of NN 1138-2 × T3791 exhibited nodulation, indicating that nodulation is controlled by the dominant gene/allele. Segregation of nodulation to nonnodulation in the F2 population fitted a 3:1 ratio. The F3 population segregated at a ratio of 1 nonnodulation: 2 segregation: 1 nodulation (thus, 1:2:1).

Table 1 Segregation of nodulation in the NN 1138-2 × T3791 crossing

| Generation | Total plants | Nodulation | Nonnodulation | Segregation | $\chi^2$ | Expected ratio | $P$ |
|------------|--------------|------------|---------------|-------------|---------|----------------|-----|
| NN1138-2 (P1) | 100          | 100        | 0             | 0           | —       | —              | —   |
| T3791 (P2) | 100          | 0          | 100           | 0           | —       | —              | —   |
| F1 (P1 × P2) | 90           | 90         | 0             | 0           | —       | —              | —   |
| F2        | 1272         | 971        | 301           | 0           | 1.212   | 3:1            | 0.691|
| F3        | 847          | 222        | 205           | 420         | 0.740   | 1:1:2          | 0.271|

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(A) deletion at the position of 1918 bp in the fourth exon between the two parents, which leads to frame-shifts with premature termination of only 307 amino acids in T3791, while the normal protein has 611 amino acids in parent NN1138-2 (Figure 1D).

Effects on photosynthesis and nitrogen metabolism by mutual grafting

After grafting, the nodulation characteristic was unchanged. The primitive roots (roots of rootstocks) of NN1138-2 still showed...
nodulation, while the primitive roots of T3791 showed no nodulation. The new roots emerging from scions of the NN1138-2 roots showed nodulation, while T3791 showed no nodulation. There were significant differences in leaf color among the four treatments. The leaf color of the NT treatment was the greenest, followed by the two self-grafting treatments, while the leaf color of the TN treatment was more yellow (Figure 2A).

The chlorophyll content, net photosynthetic rate ($P_N$), transpiration rate ($Tr$), stomatal conductance ($Gs$) of NT treatment were significantly increased, while intercellular CO$_2$ concentration ($Ci$) exhibited significantly decreased. On the contrary, the chlorophyll content, and $P_N$, $Tr$, and $Gs$ were considerably reduced in TN treatment, while $Ci$ was increased. There was no significant difference between the two self-grafting treatments (Table 3). These results showed that nodulation can promote photosynthetic capacity and that nonnodulation led to a decrease in photosynthetic capacity.

The results showed that the distribution and metabolism of nitrogen were different in different tissues. There were significant differences between the roots and the aboveground parts in all five traits (the content of the total nitrogen, NH$_4^+$-N, and NO$_3^-$-N, the activity of MAO and NR). There were also significant differences between the primitive roots and the new roots in terms of NH$_4^+$-N, NO$_3^-$-N, and NR. Compared with TN treatment, the NT treatment significantly augmented the total nitrogen, NH$_4^+$-N, and NO$_3^-$-N contents in the roots and aboveground parts. Similar results were obtained for MAO and NR activity. The results showed that there were no significant differences in the MAO activity in new roots or the NR activity in leaves (Table 4). These results presented that the nodulated root of NN1138-2 increased the nitrogen content by nodulation and nitrogen fixation, while T3791 had its own regulation mechanism to ensure nitrogen balance and promote the formation of chlorophyll to improve its photosynthesis capacity under conditions of low nitrogen supply due to its lack of nodulation, such as by reducing NR activity.

**Analysis of transcriptome expression for grafting**

RNA-Seq data from the Illumina HiSeq platform produced 39.77–54.91 million clean reads with $\geq$60 bp and quality values $\geq$ Q30 and 5.55–7.64 G clean bases for 12 samples. There were $25.60–47.83$ million reads that passed the filtering criteria and mapped uniquely to the reference genome of Williams82 (Supplementary Table S5). The gene expression was basically saturated and the distribution of reads was relatively uniform in the genome.

Raw digital gene expression counts were normalized using a variation of the fragments/Kb/million (FPKM) method. Analysis of gene expression in the different sample groups showed that the number of expressed genes ranged from 31,937 to 37,004 (Supplementary Table S6). Analysis of the relationship between the four samples showed less difference between the two self-grafting samples, followed by those of NT and TN. The results indicated that the roots significantly affected the gene expression of the leaves. The aboveground parts of the plant can also affect the gene expressions of the roots. Therefore, the new root gene expression level of grafted scions of T3791 was less affected by the primitive root system, while that of NN1138-2 was more affected by the primitive root system (Figure 2B).

Comparative analysis of the different treatment libraries revealed significant expression changes in 0 to 1608 genes. Among them, significant DEGs in the leaves ranged from 70 to 270, and those in the roots ranged from 0 to 241 (Supplementary Figure 2).
Monoamine oxidase activity (NO2- average of different sample types. Values with different letters indicate significant difference among treatments (P<0.05).

Table 4 Nitrogen distribution and metabolism in the different tissues of the grafting treatments

| Trait                        | Sample type | Treatments | Average |
|------------------------------|-------------|------------|---------|
| Total nitrogen concentration (%) | Aboveground part | NN 2.75 ± 0.67 | 2.27 ± 0.21 | 2.72 ± 0.21 |
|                             | Primitive roots | NT 2.68 ± 0.67 | 2.68 ± 0.21 | 2.68 ± 0.21 |
| NH4+-N concentration (mg g⁻¹) | Aboveground part | NN 20.43 ± 0.67 | 19.33 ± 0.67 | 22.37 ± 0.67 |
|                             | Primitive roots | NT 20.83 ± 0.67 | 20.50 ± 0.67 | 21.76 ± 0.67 |
| NO3- N concentration (mg g⁻¹) | Aboveground part | NN 32.27 ± 0.67 | 34.55 ± 0.67 | 37.87 ± 0.67 |
|                             | Primitive roots | NT 34.66 ± 0.67 | 35.54 ± 0.67 | 39.72 ± 0.67 |
| Monoamine oxidase activity (NO2- µg g⁻¹ h⁻¹) | Leaves | NN 3.12 ± 0.67 | 2.71 ± 0.67 | 2.63 ± 0.67 |
|                             | Primitive roots | NT 4.62 ± 0.67 | 4.27 ± 0.67 | 4.37 ± 0.67 |
| Nitrate reductase activity (µg g⁻¹ h⁻¹) | Leaves | NN 3.76 ± 0.67 | 3.48 ± 0.67 | 3.63 ± 0.67 |
|                             | Primitive roots | NT 4.82 ± 0.67 | 4.58 ± 0.67 | 4.75 ± 0.67 |

Data are the mean value of three replicates ± standard derivation (SD). The first four columns are the compare among four treatments. The last column compares average of different sample types. Values with different letters indicate significant difference among treatments (P<0.05).
A total of 56 significant terms were identified among the leaf samples, ranging from 0 to 14, including metabolism (51), environmental information processing (4), and genetic information processing (4). In roots, 139 significant terms were identified, ranging from 0 to 10, including cellular processes (7), environmental information processing (22), genetic information processing (11), metabolism (91), and organismal systems (8) (Supplementary Table S9). The differential grafting treatments could affect the expression of genes related to receipt of environmental information, affect nitrogen metabolism through the nodulation response, and lead to changes in carbon metabolism.

**Network analysis of DEGs in grafting treatments**

The crucial factors affecting the metabolism of nodulation and nitrogen fixation were investigated by transcriptome analysis of the grafted plants. These included amino acid metabolism (16 out of 56 in leaves and 10 out of 139 in roots), flavonoid biosynthesis (1 in leaves and 9 in roots), carbohydrate metabolism (11 in leaves and 11 in roots), plant hormone signal transduction...
(4 in leaves and 5 in roots), and nitrogen metabolism (2 in roots), etc. (Supplementary Table S9). The coordination of these metabolic pathways leads to differences in nitrogen metabolism and photosynthesis.

There are often interactions between the different RNAs and proteins occurring in organisms. Based on the differential genes, we analyzed and revealed the interactions between differentially expressed transcripts and proteins from different perspectives. The correlation between the genes was screened using threshold correlation coefficient values of >0.99 or <-0.99 and a significance value of P > 0.05. A total of 9637 pairs of transcripts and 975 pairs of proteins were interacted with each other based on the analysis of gene expression in all 12 samples (Supplementary Tables S11 and S12). The Chr.20 had the lowest interacting transcripts, having only 25, with the highest on Chr.08 having 104 interacting transcripts. The Chr.12 had the lowest interacting proteins having only 2, with the highest on Chr.10 having 14 interacting proteins (Supplementary Tables S11 and S12). Gene transcripts and protein up- or down-regulation were compared based on comparisons between all groups. Network diagrams of the co-expressed transcripts and proteins involved in different gene interactions were plotted using Cytoscape (Supplementary Figure S3, A and B).

A total of 434 pairs of transcripts and 18 pairs of proteins interacted with each other according to the analysis of gene expression level in the eight root samples (Supplementary Tables S13 and S14). Network diagrams of the co-expressed transcripts and proteins involved in the different root gene interactions are presented in Figure 4, A and B. A total of 276 pairs of transcripts and ten pairs of proteins interacted with each other according to the analysis of the gene expression level in the four-leaf samples (Supplementary Tables S15 and S16). Network diagrams of the co-expressed transcripts and proteins involved in the different leaf gene interactions are listed in Figure 4, C and D.

Ten proteins were interacting with DEGs in the roots: Glyma.02g227200 (fatty acid desaturase), Glyma.05g046700 (arginosuccinate synthase family), Glyma.06g063200 (casein lytic peptidyl-prolyl cis-trans isomerase family protein), Glyma.07g007700 (ATPase E1-E2 type family protein/haloacid dehalogenase-like hydrolase family protein), Glyma.10g172200 (UDP-glycosyltransferase superfamily protein), Glyma.10g227300 (multidrug resistance-associated protein 14), Glyma.15g243100 (NAD(P)-binding Rossmann-fold superfamily protein), Glyma.14g156400 (alcohol dehydrogenase), and Glyma.12g036900 (NAD(P)-binding Rossmann-fold superfamily protein).

Discussion
Nodulation gene mapping

In this study, the segregation of nodulation genes conformed to the Mendelian law of a single dominance gene. The nodulation gene was located on the same position in Chr.02 in both the F2 and F3 populations based on the results of two methods: SSR markers and high-throughput whole-genome re-sequencing. The SNP-index, and Indel-index also obtained the same results via the BSA-Seq method. Hence, the mapping results are quite reliable. There are 682 candidate genes in this region, including the Nod1 gene (Indrasumunar et al. 2011). Based on previous studies and our sequencing results, there is 1-bp deletion in nonnodulated parent T3791 compared to nodulated parent NN1138-2 (Supplementary Figure S2) and terminated protein translation. Hence, the variation in nodulation could be caused by this variation in sequence.

Effects of the Nod1 gene on nodulation, photosynthesis, and nitrogen metabolism in grafted treatments

Previous studies have shown that the grafting of soybean scions and roots can affect plant nitrogen metabolism, hypernodulation regulation (Hamaguchi et al. 1992), and cadmium accumulation (Megumi et al. 2007). Control of the supernodulating phenotype resides in the shoots, while the nonnodulating phenotype is regulated by roots (Perigio et al. 1993). The factors causing the changes in the scions and roots could also affect the isoflavonoid content to regulate nodulation (Cho and Harper, 1991). The study also showed that nodulation is entirely controlled by roots and not affected by the scions, and that the nodulation is only controlled by the root genotype. The current results suggested that the Nod1 gene is solely expressed in roots. Nodulation can be controlled by certain genes using short- and long-distance signals to achieve equilibrium between cell proliferation and differentiation. The nodule primordials are regulated by shoot-root signaling known as autoregulation of nodulation (AON) (Suzuki and Nishiida 2019). GmNRK expression in the leaf has a major role in long-distance communication between nodules and lateral root primordials (Searle et al. 2003). The results indicated that the Nod1 gene is only expressed in roots, so it is considered that the Nod1 gene is a short-distance signal gene.

The differences in nodulation in underground root systems can also affect photosynthesis and nitrogen content. The grafted non-nodule seedlings with nodule roots had the highest photosynthetic capacity and nitrogen content. Conversely, grafted nodule seedlings with nonnodulated roots had the lowest photosynthetic capacity and nitrogen content. Similar results were also obtained for NH4-N, NO3-N, MAO, and NR. These results suggested that the nodulation gene Nod1 affects nitrogen metabolism and subsequently photosynthesis. The nitrogen fixation ability of nodules significantly increased the content of nitrogen, and enhanced the

Validation of RNA-Seq data by qRT-PCR

To validate the results of the expression patterns among the grafting treatments by RNA-Seq, we used q-PCR to analyze the expression levels of 20 DEGs with the interaction between at translation level. Although the log2-fold values of RNA-Seq showed slight differences to those of the q-PCR analyses, the expression levels detected by the two methods were basically the same (Supplementary Figure S4). The results showed that the interaction of DEGs was verified using q-PCR.
MAO and NR activity in the process of nitrogen metabolism. The high content of nitrogen may promote the increase of chlorophyll content and improve photosynthesis capacity.

**Effect of Nod1 gene expression in grafted plants**

RNA-Seq technology provides a powerful way to determine gene functions, regulatory networks, and expression profiles. The technology has been widely used to study the global expression profiling and regulation of various traits in soybean, such as nodulation (He et al. 2018), bacterial leaf pustulation (Kim et al. 2011), glabrousness (Hunt et al. 2011), and lipid biosynthesis (Chen et al. 2012). Nodulation occurs by the interaction of a series of genes and can affect the expression of other genes. The LysR-family transcriptional regulatory protein triggers the horizontal gene transfer (HGT) process in response to plant flavonoids (Ling et al. 2016). This study identified a total of 853 DEGs among leaves and 1874 among roots, 285 differential GO terms among leaves and 856 among roots, and 57 differential pathway terms among leaves and 207 among roots in the grafting treatments.

Nodulation genes affect a series of genes by altering nitrogen metabolism, such as those related to photosynthesis, plant hormone signal transduction, and so on. The Nod1 gene (Glyma.02g270800) was not expressed in leaves, on the contrary, it was highly expressed in the new roots of NN1138-2. This indicates that Nod1 gene may be possible for nodulation; hence, further study will be needed to elucidate mechanism underlying this gene and the phenotype. A regulatory network was formed based on the ten DEGs in leaves, including the regulation of photosynthesis, energy metabolism, and so on. Another regulatory network was developed based on the ten DEGs in roots, including the regulation of energy metabolism, ammonium transportation, etc., due to differences in nodulation. These networks give a clue about possible interaction in regulating nodulation in soybean. Therefore, functional validation of few genes (especially hub-genes and highly interconnected genes in the network) are recommended for future study.

**Signaling regulation of the Nod1 gene**

Previous studies have identified several host legume genes involved in Nod factor (NF) perception and subsequent symbiotic signal transduction, bacterial infection, nodule organogenesis, and the regulation of nitrogen fixation (Radutoiu et al. 2003; Kouchi et al. 2010; Suzaki and Nishida 2019). The Nod1 gene is a LysM-type receptor kinase gene with putative Nod factor receptor components in soybean (Indrasumanar et al. 2011). Nodulation and nitrogen fixation consume energy from plant photosynthesis and form a feedback autoregulation system to balance nitrogen fixation and photosynthesis (Heckmann et al. 2006; Reid et al. 2011). This study showed that the Nod1 gene affects nodulation, thereby affecting nitrogen metabolism and ammonium transportation, leading to changes in photosynthesis in the different treatments. The nodulation/nonnodulation root grafting treatments caused differences in nitrogen levels, resulting in changes in NR activity, thus affecting the expression of hormone-related genes. Co-enzymatic gene expression in the energy metabolism pathway was altered through network regulation, and changing the rate of photosynthesis in leaves. The results provided here offer deeper understanding of the regulation of nodulation in soybean, which will aid in our future breeding efforts to breed for prolific nodulation genotype.
Conclusions
The results in this study suggest that the Nod1 gene located Chr:02:43030619-48324669 bp region. There was one base pair (A) deletion at the position of 1918 bp in the fourth exon which leads to frame-shifts with premature termination. The gene increased nitrogen content and photosynthetic capacity when nonnodulated scions were grafted onto nodulated roots; in contrast, nitrogen content and photosynthetic capacity decreased when nonnodulated scions were grafted onto nonnodulated roots. 853 DEGs were identified among leaves and 1874 among roots, and there were 285 and 856 differential GO terms among leaves and roots, respectively. Also, through KEGG pathway enrichment analysis, 57 and 207 differential pathways were detected in roots and leaves, respectively. As a long-distance regulatory nodulation gene, Nod1 increases nitrogen content after nodulation, which affects enzymes related to nitrogen metabolism, leading to changes in hormone levels and further regulation of photosynthesis and carbon metabolism.

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Q.H. had completed manuscript writing and grafting experiment. S.X. had completed location analysis of Nod1 gene using BSA resequencing. W.W. analyzed transcriptome data. Y.S. measured photosynthesis. Z.L. identified phenotype of nodule. S.W. and X.Y. identified SSR. T.Z. designed experimental design and revised manuscript.

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Data availability
Supplementary material and data are available at figshare: https://doi.org/10.25387/g3.14742693.

Conflicts of interest
None declared.

Literature cited
Aerts R, Chapin FSII. 2000. The mineral nutrition of wild plants revisited: a re-evaluation of processes and patterns. Adv Ecol Res. 30:1–67.
Akao S, Kouchi H. 1992. A supernodulating mutant isolated from soybean cultivar Enrei. Soil Sci Plant Nutr. 38:183–118.
Chen H, Wang FW, Dong YY, Wang N, Sun YP, et al. 2012. Sequence mining and transcript profiling to explore differentially expressed genes associated with lipid biosynthesis during soybean seed development. BMC Plant Biol. 12:122.
Cho MJ, Harper JE. 1991. Root isoflavonoid response to grafting between wild-type and nodulation-mutant soybean plants. Plant Physiol. 96:1277–1282.
Fehr WR, Caviness CE, Burmood DT, Pennington JS. 1971. Stage of development descriptions for soybeans, Glycine max (L.) Merrill. Crop Sci. 11:929–931.
Fujita K, Masuda T, Ogata S. 1991. Analysis of factors controlling dinitrogen fixation in wild and cultivated soybean (Glycine max) plants by reciprocal grafting. Soil Sci Plant Nutr. 37:233–240.
Gresshoff PM. 2003. Post-genomic insights into plant nodulation symbioses. Genome Biol. 4:201.
Hamaguchi H, Kokubun M, Akao S. 1992. Shoot control of nodulation is modified by the root in the supernodulating soybean mutant En6500 and its wild-type parent cultivar Enrei. Soil Sci Plant Nutr. 38:771–774.
Harper JE, Nickell CD. 1995. Genetic analysis of nonnodulating soybean mutants in a hypernodulated background. Soybean Genet. NewsL. 22:185–190.
Hayashi M, Saeki Y, Haga M, Harada K, Kouchi H, et al. 2012. Bj(rj) genes involved in nitrogen-fixing root nodule formation in soybean. Breed Sci. 61:544–553.
He QY, Yang HY, Xiang SH, Tian D, Wang WB, et al. 2015. Fine mapping of the genetic locus L1 conferring black bolls using a chromosome segment substitution line population of soybean. Plant Breed. 134:437–445.
He QY, Yang HY, Li ZP, Wang SH, Zhu CW, et al. 2018. Transcriptomic characterization of soybean (Glycine max) roots in response to rhizobium infection by RNA sequence. Pakistan J Bot. 50:389–398.
Heckmann AB, Lombardo F, Miwa H, Perry JA, Bunnewell S, et al. 2006. Lotus japonicus nodulation requires two GRAS domain regulators, one of which is functionally conserved in a non-legume. Plant Physiol. 140:1739–1750.
Hermans C, Hammond JP, White PJ, Verbruggen N. 2006. How do plants respond to nutrient shortage by biomass allocation? Trends Plant Sci. 11:610–617.
Hunt M, Kaur N, Stromvik M, Vodkin L. 2011. Transcript profiling reveals expression differences in wild-type and glabrous soybean lines. BMC Plant Biol. 11:145.
Indrasumunar A, Searle I, Lin MH, Kereszt A, Men A, et al. 2011. Nodulation factor receptor kinase 1x controls nodule organ number in soybean (Glycine max L. Merr). Plant J. 65:39–50.
Jin Y, Liu H, Luo DX, Yu N, Dong WT, et al. 2016. DELLA proteins are common components of symbiotic rhizobial and mycorrhizal signalling pathways. Nat Commun. 7:12433.
Kim KH, Kang YJ, Kim DH, Yoon MY, Moon JK, et al. 2011. RNA-Seq analysis of a soybean near-isogenic line carrying bacterial leaf pustule-resistant and -susceptible alleles. DNA Res. 18:483–415.
Kouchi H, Imaiizumi-Anraku H, Hayashi M, Hakoyama T, Nakagawa T, et al. 2010. How many peas in pod? Legume genes responsible for mutualistic symbioses underground. Plant Cell Physiol. 51:1381–1397.
Kouki H. 2004. Interspecific different in the photosynthesis–nitrogen relationship: patterns, physiological causes, and ecological importance. J Plant Res. 117:481–494.
Lam HM, Xu X, Liu X, Chen WB, Yang GH, et al. 2010. Resequencing of 31 wild and cultivated soybean genomes identifies patterns of genetic diversity and selection. Nat Genet. 42:1053–1059.
Lawn RJ, Bunwa VA. 1974. Symbiotic nitrogen fixation in soybeans. I. Effect of photosynthetic source-sink manipulations. Crop Sci. 14:11–16.
Ling J, Wang H, Wu P, Li T, Tang Y, et al. 2016. Plant nodulation inducers enhance horizontal gene transfer of Azorhizobium.
caulinodans symbiosis island. Proc Natl Acad Sci USA. 113: 13875–13880.

Madsen LH, Tirichine L, Jurkiewicz A, Sullivan JT, Heckmann AB, et al. 2010. The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus. Nat Commun. 1:10.

Makino A. 2011. Photosynthesis, grain yield, and nitrogen utilization in rice and wheat. Plant Physiol. 155:125–129.

Malik NSA. 1983. Grafting experiments on the nature of the decline in N₂ fixation during fruit development in soybean. Physiol Plant. 57:561–564.

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, et al. 2010. The Genome analysis toolkit: a MapReduce framework for analyzing next generation DNA sequencing data. Genome Res. 20: 1297–1303.

Megumi S, Noriharu A, Tomohito A. 2007. Role of roots in differences in seed cadmium concentration among soybean cultivars-proof by grafting experiment. Plant Soil. 295:1–11.

Oldroyd GE, Murray JD, Poole PS, Downie JA. 2011. The rules of engagement in the legume-rhizobial symbiosis. Annu Rev Genet. 45: 119–144.

Perigio B, Francisco JR, Shoichiro A. 1993. Autoregulation and nitrate inhibition of nodule formation in soybean cv. Enrei and its nodulation mutants. J Exp Bot. 44:547–553.

Pracht JE, Nickell CD, Harper JE. 1993. Genes controlling nodulation in soybean: Rj5 and Rj6. Crop Sci. 33:711–713.

Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, et al. 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. Nature. 425:585–592.

Reid DE, Ferguson BJ, Hayashi S, Lin YH, Gresshoff PM. 2011. Molecular mechanisms controlling legume autoregulation of nodulation. Ann Bot. 108:789–795.

Saghai-Maroo M, Soliman KM, Jorgensen RA, Allard RW. 1984. Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA. 81:8014–8018.

Searle IR, Men AE, Laniya TS, Buzas DM, Iturbe-Ormaetxe I, et al. 2003. Long-distance signaling in nodulation directed by a CLAVATA1-like receptor kinase. Science. 299:109–112.

Shen SH, Park JW, Lu ZX, Lin L, Henry MD, et al. 2014. rMATS: robust and flexible detection of differential alternative splicing from replicate RNA-Seq data. Proc Natl Acad Sci USA. 111:E5593–E5601.

Suzaki T, Nishida H. 2019. Autoregulation of legume nodulation by sophisticated transcriptional regulatory networks. Mol Plant. 12: 1179–1181.

Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, et al. 2013. Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat Biotechnol. 31:46–53.

Wang JK, Li HH, Zhang LY, Meng L. 2016. User’ Manual of QTL IciMapping. p. 1–256.

Wang L, Wang S, Li W. 2012. RSeQC: quality control of RNA-seq experiments. Bioinformatics. 28:2184–2185.

Williams LF, Lynch DL. 1954. Inheritance of a non-nodulating character in the soybean. Agronj. 46:28–29.

Young MD, Wakefield MJ, Smyth GK, Oshlack A. 2010. Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol. 11:R14.

Zhang XC, Wu XL, Findley S, Wan JR, Libault M, et al. 2007. Molecular evolution of lysine motif-type receptor-like kinases in plants. Plant Physiol. 144:623–636.

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