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GmENOD40 Expression Decreased in *NORK* Knock-down Transgenic Soybean Roots

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ABSTRACT

Root nodules are highly organized root organs where the nitrogen fixation take place in, its formation are the results of complicated interactions between legumes and rhizobia. The nodule formation and nitrogen fixation are energy-demanding processes. During nodule formation and nitrogen fixation, a large amount of sucrose, as the major end product of photosynthesis is required to be transported into nodules. In legume root nodules, hydrolysis of sucrose by Sucrose synthase (SuSy) is a necessary prerequisite for normal nodule development and a key step in nitrogen fixation. Deficient SuSy activity in nodules renders them incapable of effective nitrogen fixation. ENOD40 play a role in the regulation of sucrose utilization in nodules through the soyabean ENOD40 peptides directly binding to SuSy.In both legumes and non-legumes, ENOD40 expression is important in nodule organogenesis and development. However, during symbiotic development, whether Nod factor signaling associate with in the regulation of sucrose utilization in nodules is unknown. NORK the immediately downstream component of these Nod factor receptors, is central to the Nod factor signalling cascade. NORK functions not only in the early signaling pathway operative in root hairs, but also in later stages of nodule formation. In this study, we found that the GmENOD40expression level decreased in GmNORKRNAisoybean transgenic root by rhizobial inoculation. Thus provide important detail information toward understanding the functions of NORK and GmENOD in symbiotic signaling and nodule development.

Key words: ENOD40, NORK, Soybean, nodule, rhizobial

INTRODUCTION

Soybean (*Glycine max*) as an important food and animal feed is the major crop legume in the world. Soybean has the capability to establish endosymbiotic relationships with nitrogen-fixing bacteria, called rhizobia[1].

The establishment of legume-rhizobium symbiosisis a multistage process including signal perception, signal transduction, and subsequent downstream developmental events that eventually give rise to a new organ, the nodule, in which the intracellular bacterial symbiontsthrive[2]. In nodules, the bacteria are enclosed in a membrane of plant origin giving rise to vesicular-like compartments, termed symbiosomes to draw attention to their quasi-organellar like status[3]. Within the symbiosomes, the *rhizobia* differentiate into nitrogen-fixing formcalled bacteroids[4]. Then bacteroids convert atmospheric nitrogen (N_2) into ammonia (NH_3) for plant usein exchange of photosynthate from the plant host[5].

The formation of symbiotic nodules requires two parallel signaling pathways, one that promotes nodule organogenesis while the other allows bacterial infection[6]. These processes are coordinated in both a spatial and temporal manner to ensure successful symbiotic development[7].

Both processes require plant recognition of the Nod factor (NF). Nod factors (NF) are signaling molecules secreted by

rhizobia and are perceived by plant hosts through two plasma membrane-located receptor-like kinases (RLK) NFR1 and NFR5[9,10], and activated a leucine rich repeat (LRR) serine/threonine kinase then triggers downstream signaling cascades.

The leucine rich repeat (LRR) serine/threonine kinase is one of important genes involves nodule initiation. These receptor-like kinase were given different names depending on plant species, SYMbiosis Receptor-like Kinase (SymRK) in *L. japonicus* or Nodulation Receptor Kinase (NORK) in *Medicago sativa*, SYM19 in *Pisumsativum*, and DMI2 (Does not Make Infections 2) in *Medicagotruncatula*, and NORKa/b in *Glycine max*[11-14]. Recently, SYMRK/NORK was shown to be a co-receptor interacting with NFR5 and fine-tuning the symbiotic signaling cascade[15].

The Nodulation Receptor Kinase (NORK) gene is essential for Nod factor perception/transduction in Medicago sativa since it is involved in the common signaling pathway of symbiosis[11]. Many researchers have suggested that NORK is involved in a protein complex with the Nod factor receptor or/and transduces the signaling of Nod factor receptors to subsequent events[16,17]. However, there is little mechanistic information as to how NORK actually does work. NORK is a 919 amino acid leucine rich repeat (LRR) receptor-like kinase, containing a 325 amino acid serine/threonine kinase domain[18].

The nodule formation and nitrogen fixation are energy-demanding processes. During nodule formation and nitrogen fixation, a large amount of sucrose, as the major end product of photosynthesis is required to be transported into nodules [19]. Sucrose synthase (SuSy) is one of the most abundant proteins in mature legume nodules[20, 21] and plays a major role in the degradation of sucrose in root nodules.In legume root nodules, hydrolysis of sucrose by Sucrose synthase (SuSy) is a necessary prerequisite for normal nodule development and a key step in nitrogen fixation[22].

The early nodulin gene ENOD40 encoded two short Peptides, Peptide A (12 aa residues) and peptide B (24 aa residues)[19,23]. ENOD40 play a role in the regulation of sucrose utilization in nodules through the soyabeanENOD40 peptides directly binding to SuSy[19].ENOD40 is also a regulatory RNA[24].The secondary structure of ENOD40 mRNA has been shown to be a key element in the signaling process underlying nodule organogenesis [25].In both legumes and non-legumes, ENOD40expression is important in nodule organogenesis and development[26].However, the molecular mechanisms of its activity are unclear[24]. During nodule development, cross-talk between ENOD40and phytohormonesignallingexists [27].Recent study show that ENOD40 is regulated by *miR172c-NNC1,* a regulatory module of nodulation [28].

However, During symbiotic development, whether Nod factor signaling associate with in the regulation of sucrose utilization in nodules is unknown.

In this study, we report that the *GmENOD*expression level decreased in *GmNORKRNAi*soybean transgenic root by rhizobial inoculation. Thus provide important detail information toward understanding the functions of NORK and GmENOD in symbiotic signaling and nodule development.

EXPERIMENTAL SECTION

Alignment and phylogenetic analysis.
NCBI BLAST searches using GmNORK1a and GmNORK1b detected a number of highly similar peptide sequences. The alignments of the SymRK (NORK) genes are in terms of neighbor joining (NJ) tree in newick format.

Plasmid construction and transformation.
A 188-bp DNA fragment from the 42 bp downstream of the *NORK1b* stop codon to the 229-bp was amplified from William 82 using *NORK1b*-RNAi F/R primer pairs (Table 1.), then cloned into the binary vector pDONR222 to generate gateway entry plasmid using BP reaction. Then the 188-bp DNA fragment was cloned into pCAM-GWi(GWY RNAi) using LR reaction to generate *ICNO1a*-RNAi plasmid.Empty vector and CGT5200 (GUS RNAi) was used as the RNAi control vector, as described previously [29, 30]. This vector contains an RNAi construct directed against GUS.

Table 1 Primers used in this work

Primers for making NORK1b RNAi constructs	
NORK1b-RNAi F	GGGGACAAGTTTGTACAAAAAAGCAGGCTactgatttagtcatgatacatttcaaat
NORK1b-RNAi R	GGGGACCACTTTGTACAAGAAAGCTGGGTAatcaattttgtttacgcaaattttacca
Primers for quantitative real-time RT-PCR	
qNORK1b F	AAATTGTGAGCGGCAGGGAA
qNORK1b R	GCATGGACCATACCCATTCA
qGmENOD40 F1	TGGACAACACCCTCTAAACCA
qGmENOD40 R1	GTGAGGGAGTGTGAGGAGTGA
GmCons4 F	GATCAGCAATTATGCACAACG
GmCons4 R	CCGCCACCATTCAGATTATGT
GmCons6 F	AGATAGGGAAATGGTGCAGGT
GmCons6 R	CTAATGGCAATTGCAGCTCTC
Primers for Protein expression constructure	
*NORKbKD*F	aaaGAATTCTGCCGCTATAGACAAAAGTTGATT
*NORKbKD*R	aaaGTCGACCTATCTTGGCTGTGGATGGGATAA

A. rhizogenes-mediated hairy root transformation.

The constructs were electroporated into A. rhizogenes strain K599 using a GenePulser apparatus with pulse controller (Bio-Rad Laboratories, Hercules, California) with settings at 25 μF, 200 Ω, 1.8 kV. After electroporation, 100 μL LB medium was added to the competent cells, which were allowed to recover at 30^{o}C with shaking at 180 rpm for at least 2 h, then plated onto LB agar supplemented with appropriate antibiotics. Plates were incubated at 28°C for 2-3 days. A. rhizogenes-mediated hairy root transformations were performed as described in our laboratory protocol.

Seedlings preparation: Seeds of Glycine max (L.) Merrill cultivar Williams 82 were surface sterilized by 10% bleach, rinsed several times with autoclaved distilled water (diH2O), once with 0.8% HCl for 10 min, and then several times with diH2O. Sterilized seeds were then sewn onto 1% agar round plates (20 cm diameter) and incubated in chambers at 27^{o}C and 80% humidity for 3 days in darkness and 3 days at 22 ^{o}C with a light regime of 16h light and 8h darkness. Transformation: A. rhizogenes K599 carrying the respective constructs was inoculated into liquid LB medium with appropriate antibiotics and incubated overnight at 30 ^{o}C with shaking at 200 rpm. Approximately 500μl of the overnight bacterial culture were plated onto LB plates with antibiotics and bacteria were grown overnight in a 30°C chamber. The 6-day old seedlings were cut at the base of the hypocotyl and the shoot was dipped into the bacterial confluent lawn that had developed overnight on the plates. The seedlings were placed onto square Petri dishes with Fahräeusmedium [31]supplemented with 1mM $CaCl_2$ and 1mM KNO_3. Plantlets were incubated in the growth chamber at 22^{o}C for 2 days in darkness. Plantlets were transferred into plastic growth pouches (Mega International, Minneapolis, MN) containing 50 ml solid Fahraeus medium supplemented with $CaCl_2$ and $KNO_3$and grown under a 16-h photoperiod for 5-7 days at 22°C. After 5-7 days, adventitious roots were removed and the composite plants transferred into growth pouches with liquid Fahraeus medium supplemented with $CaCl_2$ and KNO_3. Approximately 3 weeks after transformation, formation of transgenic roots was checked by GFP florescence and non-transgenic roots were removed. If the roots were long enough (at least ca. 3 cm), the seedlings were planted into a vermiculite and perlite mixture (3:1), grown in greenhouse conditions and inoculated with the soybean rhizobialsymbiont, *Bradyrhizobiumjaponicum.*

Nodulation assay

For soybean inoculation with rhizobia, B. japonicum wild-type strain USDA110[32], or a derivative strain constitutively expressing β-glucuronidase (GUS)[33] were grown at 30^{o}C for 3 days prior to inoculation in liquid HM medium[34]supplemented with appropriate antibiotics (50 μg/ml tetracycline, 100 μg/ml spectinomycin) Cells were grown to an OD600 between 0.5 and 1.0. Cells were then pelleted by centrifugation at 4,000 rpm at 20^{o}C for 10min and resuspended in sterilized MilliQ water to a final OD600 of 0.05. Composite plants growing in a vermiculite and perlite mixture were inoculated 2 days later with 2 mL of the respective B. japonicum strain. Plants were left in the laboratory for 2 days for acclimatization and then transferred into the greenhouse.

The nodulation phenotypes of soybean roots inoculated with B japonicum were analyzed 4 weeks post-inoculation (wpi) using a Leica M205 FA stereo microscope with a high resolution Leica DFC295 color camera. Only the nodules formed on transgenic roots, as determined by expression of the GFP marker, were analyzed.

ß-glucuronidase assay.

For histochemical ß-glucuronidase staining, transgenic roots of composite plants were cut and were placed into 15-ml Falcon tubes containing 10 ml of GUS staining solution as previously described [35]. Vacuum was applied three times for 3 min, and then the roots were incubated at 37°C in the dark for 2days overnight. Infected roots and nodules were checked for blue staining under a stereomicroscope. Evaluation and documentation of nodules and primordia were performed using stereomicroscope (Olympus SZX12; Olympus, Tokyo, Japan) fitted with an Olympus DP10 camera.

Quantitative Real-Time RT-PCR analysis.

Transgenic root were collected at 28 d after inoculation (DAI), All samples were immediately frozed in liquid nitrogen and stored at -80℃untile use. Total RNA from transgenic root tissues used in this study were isolated by TRizol (Ambion, Austin, TX, USA), followed by a DNase treatment (Turbo DNase, Ambion), and 1 micrograms root total RNA were used for complementary DNA synthesis using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions.

qPCR experiments were conducted with gene-specific primers (Table 1.) in the reaction system of SYbGreen mix (Bio-Rad) on the 7500 System (Applied Biosystems) according to the manufacturer's instructions. The thermal profile of the qRT-PCR reactions was 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The geometric mean of cons4 and cons6, encoding an ATP-binding cassette transporter and an F-box protein [29], were used as reference genes to normalize the expression levels.

Recombinant protein purification and kinase assay.

The plasmid pGE*NORK* was transformed into BL21 (DE3). Protein production was induced by adding 0.3 mM isopropyl b-D-thiogalactopyranoside (IPTG) when the culture reached an OD600 of 0.6. The protein was extracted after inducing the cultures for 24 hours at 16^{o}C. Recombinant protein purification was done according to the following procedure. Bacterial cells from 200 ml LB medium were pelleted by centrifugation at 4 ^{o}C at 8,000 rpm for 10 min and resuspended in 10 ml 1×PBS solution supplemented with 1×EDTA-free protease inhibitor (Roche, Indianaplois, IN), 0.5% Triton X-100, 1 mg/ml lysozyme and put on ice for 30 min. The cells were lysed by sonication before centrifuging at 13,000 rpm for 10 minutes at 4^{o}C. Supernatants were used for protein affinity purification using Glutathione Sepharose 4B (GE Healthcare, Milwaukee, WI). The column was washed with at least 20 bed volumes of 1×PBS solution. The eluted proteins, including GST and GSTNORKKD, were dialyzed with buffer (50 mMTris (PH 7.5), 50 mMKCl, 2 mM DTT, and 10% glycerol).

The in vitro kinase assays used 1mg purified protein in a buffer containing 50 mMTris (PH 7.5), 50 mMKCl, 2 mM DTT, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 10% glycerol, 10 mM ATP and 5mMCi[γ-32P]-ATP. The assay mix was incubated at 28^{o}C for 30 minutes and the reaction was stopped by adding 1×SDS loading buffer. The samples were separated on 10% SDS-PAGE gel and the gel imaged by autoradiography using phosphor screens and a phosphorimager [36].

RESULTS AND DISCUSSION

NORK structure

GmNORK1a and GmNORK1b genomicDNA both have 15 Exons and 14 introns,both have signal peptides (SP) at N-terminal, leucine-rich -repeatdomain (LRR), trans-membrane domain (TM) andSerine-Threonine /tyrosine kinase domain (PK) (Fig. 1).*GmNORK1a* and *GmlNORK1b*both contain 919a.a and share 94.9% identity (Fig.2).

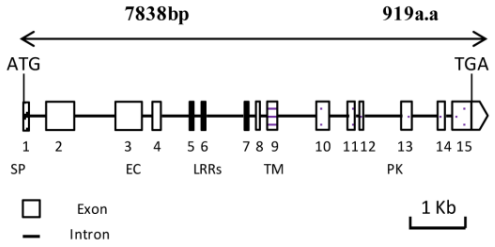

Fig. 1.*GmNORK1a and GmNORK1b* genestructure.Genomic structure of Gm*NORK1a* and *GmNORK1b* with the indicated predicted protein domains. Exons are indicated as boxes, introns as black lines.SP, predicted signal peptide; EC, extracellular domain; LRR, Leucine-rich repeat motifs; TM, transmembrane domain; PK, protein kinase domain

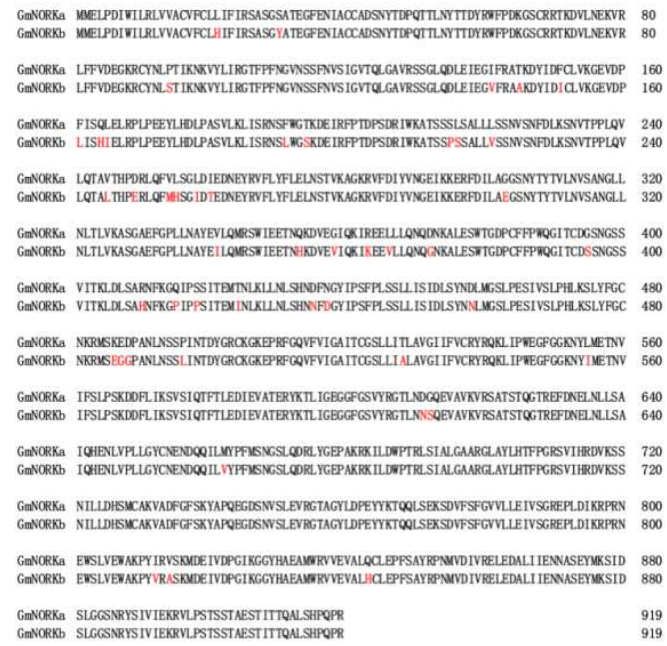

Fig. 2.Protein sequence of GmNORK1a and GmNORK1b. GmNORK1a and GmNORK1b show 94.9% similarity to each other,unconserved amino acids are indicated with red letters

Molecular phylogenetic analysis of *GmNORK1a and GmNORK1b*

To investigate the relationship of GmNORK1aand GmNORK1b proteins with other homologous proteins in soybean and other plant species, a phylogenetic tree based on amino acid sequences was generated(Fig. 3). GmNORK1aand GmNORK1b share 94.9% identity, and they are the closest homologs. They likely derive from a common ancestor and have close homologs in*Phaseolus vulgaris* (*ADQ74920.1*) and *Sesbaniarostrata*（*AAV88623.1*）, both are legumes that support a rhizobium symbiosis. It shows that the *SymRK* (*NORK*) orthologsare divided into three groups, Rhizobium*(Fabaceae*, legume), actinorhizal (non-legume) nodule-forming plants and non-nodule-forming plants.*G.max NORK*and *P. vulgaris SymRK* are highly similar. Within the legume orthologs, *A. hypogaea*is the most distant ancestor since the *SymRK* of *A. hypogaea* shows the lowest similarity to*GmNORK*（Fig. 3）.

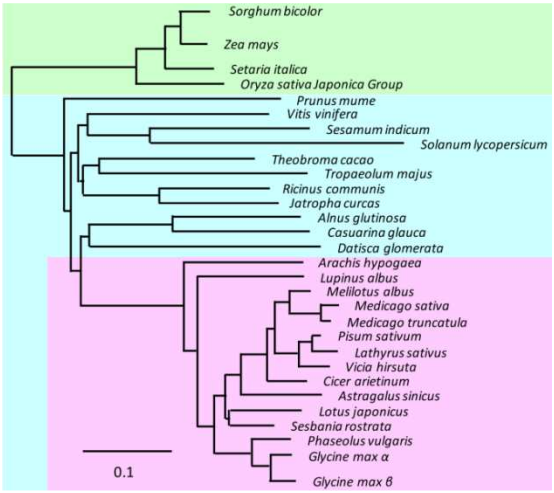

Fig. 3.Molecular phylogenetic analysis.Monocot and dicot NORKs group into distinct sub-clades: light green and light blue color sub-clades, respectively. In each dicot sub-clade, legume NORKs also sort into specific clusters (pink)

The expression of *GmNORK1a,GmNORK1b*and *GmENOD40*in soybean roots was significantly reduced in *GmNORKRNAi* lines.

Due to the lack of homozygous knockout mutants for *GmNORK1a* and *GmNORK1b* in soybeans, RNA silencing was applied to investigate their functions in nodulation. Soybean roots were transformed with *GmNORKRNAi,*an empty vector and a vector expressing an RNAi construct specifically targeted to β-glucuronidase (GUS) (*GUS* RNAi) as controls. *GmNORK1a*and *GmNORK1b*expression levels in *GmNORK*RNAiare approximately 37% and 24%of that

in the control transgenic roots, respectively (Fig. 4A , 4B).

In order to study whether *NORK* involves the *GmENOD40* associated nodulation, we analyzed the *GmENOD40* expression in *GmNORKRNAi* lines. *GmENOD40*expression levels in *GmNORKRNAi* are approximately 36% of that in the control transgenic roots (Fig. 4C).

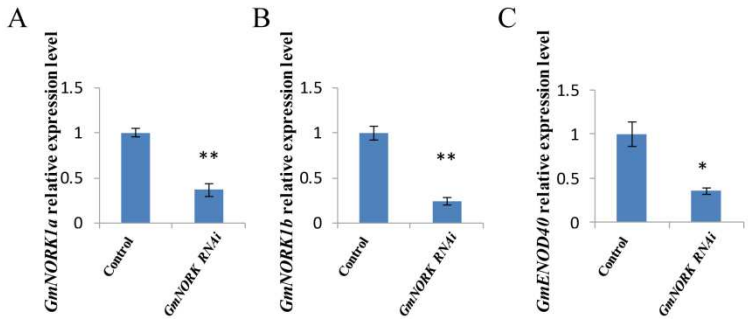

Fig. 4.The expression of *GmNORK1a, GmNORK1b,* and *GmACP* in soybean roots was significantly reduced in *GmNORKRNAi* lines

(A) Relative expression levels of *GmNORK1a* in roots of soybean transformed with negative control vector,*GmNORKRNAi*plasmids. (B) *GmNORK1b* relative expression levels in roots of soybean transformed with negative control vector, *GmNORKRNAi* plasmids.(C) *GmENOD40*relative expression levels in roots of soybean transformed with negative control vector, *GmNORKRNAi*plasmids. ** denotes significantly differences at $P < 0.01$. Bars indicate the standard error of the mean of 3 plants. Control indicates the geometric mean of *GUS RNAi* and *Empty vectorRNAi*

RNA silencing *GmNORK RNAi* resulted in reduced nodule number.

Transgenic roots could be identified by the green fluorescent protein (GFP) marker expressed by the binary vector (Fig. 5A). Nodule morphology was examined by using GUS staining. The nodules from the RNAi roots showed no obvious structural changes relative to the controls (Fig.5B, 5C). The number of nodules formed on the *GmNORKRNAi* were approximately 6.4% that of the controls (with $P < 0.01$)(Fig.5D). The results demonstrated that *GmNORK1a* and *GmNORK1b* are involved in nodule formation in soybean plants when inoculated with B. japonicum.

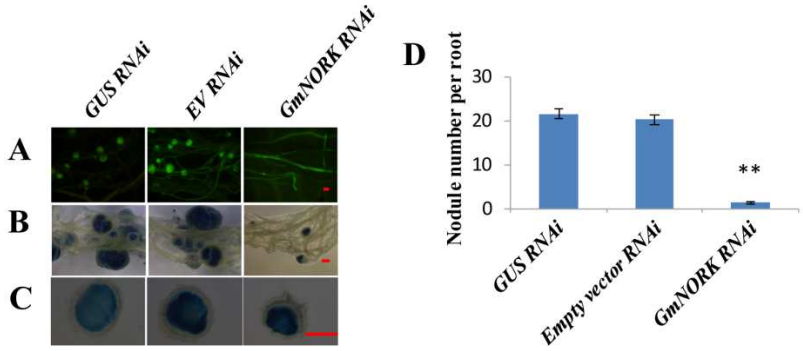

Fig. 5.RNA silencing of *GmNORK*resulted in a reduced nodule number

(A) Representative transgenic root and nodules expressing *GUS RNAi*, *EVRNAi*, and *GmNORKRNAi* constructs. Scale bars, 1 mm. (B) Stained micrographs of nodule derived from RNAi transgenic roots at 21 d after inoculate with *B. japonicum.* Scale bars, 1 mm. (C) Stained micrographs of nodule sections derived from RNAi transgenic roots at 21 d after inoculation with *B. japonicum.* The nodule morphology was similar among the nodules formed on roots expressing the *GUS RNAi*, *Empty vector (EV),*and *GmNORKRNAi* constructs. Scale bars, 1 mm. *EV RNAi* indicates *Empty vector RNAi*. (D) Nodulation was measured as nodule number per transgenic root. The nodule numbers per root in *GmNORKRNAi* transgenic lines decreased significantly compared to those in the *GUSRNAi* and empty vector transgenic lines. ** denotes significant differences at $P < 0.01$. Bars indicate the standard error of the mean of 30 plants.

Recombinant protein purification and kinase assay.

In order to confirm NORK phosphorylating activation, we performed in vitro phosphorylation assays. NORK kinase domain (538 a.a. to 919a.a.) was expressed by fusing with the glutathione sulfotransferasedomain. As shown in Figure 6, GmNORK was shown to exhibit autophosphorylation in vitro, consistent with previous reports [18, 37].

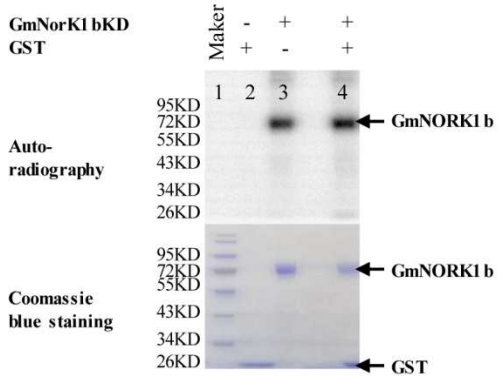

Fig. 6.Recombinant protein purification and Kinase assay

Upper panel is an autoradiography image. Lower panel is a Coomassie blue staining image. Lane 1 is the maker. Lane 2 is the GST input. Lane 3 is the GmNORK1b kinase domain input. Lane 4 is the combined GmNORK1b kinase domain and GST inputs.

The nodule formation and nitrogen fixation are energy consuming processes. A large amount of sucrose, as the major end product of photosynthesis is required to be transported into nodules [19]. Sucrose synthase (SuSy) is one of the most abundant proteins in mature legume nodules[20，22] and plays a major role in the degradation of sucrose in root nodules.Sucrose synthase (SuSy) catalyzes the cleavage of sucrose into UDP-glucose and fructose. The hydrolysis of sucrose by SuSy provides substrates for rapidly growing tissues and sink organs（e.g. legume root nodules）.

In legume root nodules, hydrolysis of sucrose by SuSyplays an important role in nodule nitrogen fixation. Deficient SuSy activity in nodules decreases the effective nitrogen fixation[22].The regulation of SuSy activity and stability the protein against proteolysis, would lead to an increase in sink strength in nodules.ENOD regulates SuSy activitythrough peptide A activating sucrose cleavage activity [19]. ENOD40is one of the earliest nodulin genes specifically induced by nodulation factor-secreting rhizobia and appears to play an important role in root nodule organogenesis[26].

In both legumes and non-legumes, ENOD40 expression is important in nodule organogenesis and development [27,26].However, the molecular mechanisms of its activity are unclear [24]. The activity of the ENOD40 genes is associated with new organ formation with high expression observed during the development of the nodule, an organ specific for symbiotic nitrogen fixation [26]. ENOD40 is also a regulatory RNA [38].The secondary structure of ENOD40 mRNA has been shown to be a key element in the signaling process underlying nodule organogenesis[25].Alone ENOD40 expression is not sufficient for nodule primordium formation [39]. ENOD40 interaction with other plant factors is probably required for the initiation of nodule development.During nodule development, cross-talk between ENOD40and phytohormonesignalling exists[27]. Recent studies show that ENOD40 expression is regulated by *miR172c-NNC1,* a regulatory module of nodulation [28].

The formation of symbiotic nodules requires two parallel signaling pathways : bacterial infection and nodule development[6]. These processes are coordinated in both a spatial and temporal manner to ensure successful symbiotic development [7]. Both processes require plant recognition of the Nod factor (NF). NORK (also called Mt DMI2 or Lj SYMRK), immediately downstream component of these Nod factor receptors, is central in the Nod factor signalling cascade[11, 12].Several interacting proteins of NFRs and SYMRK/NORK have been identified, including SINA4 arbuscularmycorrhizationarbuscularmycorrhizationarbuscularmycorrhization (SEVEN IN ABSENTIA 4), HMGR1 (3-hydroxy-3-methylglutaryl-CoA reductase 1), PUB1 (Plant U-box protein 1), SYMREM1 (Symbiotic Remorin 1). The means by which the signal is transferred from the plasma membrane to the nuclear envelop is still unclear. Three components of nuclear pore (NUP85, NUP133, and NENA) are required for the generation of symbiotic calcium oscillations. Nucleus-localized calcium- and calmodulin (CaM)-dependent serine/threonine protein kinase (CCaMK) and CYCLOPS are involved in decoding the calcium oscillations and activate the transcription factor (TF) for symbiosis-associated gene expression [15]. However, wheather Nod factor signaling affect the hydrolysis of sucroseor not is still unkown.

CONCLUSION

Our studies show *GmENOD* expression level is decreased in *GmNORKRNAi* transgenic soybean roots. These data

suggest *NORK* can affect hydrolysis of sucrose by decreasing the *GmENOD*expression level. The significant decrease in nodule number of RNAi roots suggests a role for *NORK* and *ENOD* at an earlier stage than rhizobial release from infection threads. Possibly the *NORK* and *GmENOD*are active at the stage of root hair infection, when rhizobia, entrapped in a root hair curl. The detail mechanism of *NORK* affect *GmENOD*expression level need further elucidate.

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Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

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