



# Regulation of nodule number by GmNORK is dependent on expression of *GmNIC* in soybean

Lijun Wang · Lingwei Deng · Xue Bai · Yongqing Jiao · Yangrong Cao · Yufeng Wu

Received: 29 October 2016 / Accepted: 13 March 2019 / Published online: 19 March 2019  
© Springer Nature B.V. 2019

**Abstract** Root nodules are highly organized root organs where nitrogen fixation takes place in, and its formation are the results of complicated interactions between legumes and rhizobia. Nodule number is tightly controlled by Legumes to ensure optimal growth without energy losses wasted by excessive number of nodules. One of major factors controlling of nodule number is nitrate availability in the soil. However, during symbiotic development, the details of Nod factor signaling associated with nitrate

regulation of nodulation are unknown. NORK, the immediately downstream component of these Nod factor receptors is central to the Nod factor signaling. NIC1, a CLE peptide-encoding gene in soybean (*Glycine max*), play an important role in nitrate regulation of nodulation. In this study, specified RNAi construct of *GmNORK* was generated and transformed into soybean roots by agrobacterium rhizogenes-mediated hairy root transformation. We found that the nodule number decreased substantially in *GmNORK* knock-down soybean transgenic roots. The expression levels of *GmNIC1* in the *GmNORK* RNAi soybean transgenic roots is substantially reduced after rhizobial inoculation compared with

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s10457-019-00382-8>) contains supplementary material, which is available to authorized users.

L. Wang (✉)  
The College of Life Science, Yangtze University,  
Jingzhou 434025, China  
e-mail: ljwang516@126.com

L. Deng  
Tillage Institute, Heilongjiang Academic of Agriculture  
Sciences, Harbin 150086, China

X. Bai  
Jingzhou No.1 People's Hospital and First Affiliated  
Hospital of Yangtze University, Jingzhou 434000, China

Y. Jiao (✉)  
Oil Crops Research Institute of Chinese Academic of  
Agriculture Sciences, Wuhan 430062, China  
e-mail: yqjiao@126.com

Y. Cao (✉)  
The College of Life Science, Huazhong Agricultural  
University, Wuhan 430070, China  
e-mail: yrcao@mail.hzau.edu.cn

Y. Wu (✉)  
The State Key Laboratory of Crop Genetics and  
Germplasm Enhancement, Bioinformatics Center, Jiangsu  
Collaborative Innovation Center for Modern Crop  
Production, Nanjing Agricultural University,  
Nanjing 210095, China  
e-mail: yfwu@njau.edu.cn

the control soybean transgenic roots. Our data suggest that nitrate regulation of nodulation was affected by Nod factor signaling during nodule development in soybean, providing valuable information toward understanding the functions of *GmNORK* and *GmNIC1* in symbiotic signaling and nodule development.

**Keywords** Soybean · *GmNORK* · *GmNIC* · Nodule · Rhizobia

## Introduction

Soybean (*Glycine max*) is the major crop legume in the world, which is mainly used as an important food and animal feed. Soybean has the capability to establish endosymbiotic relationships with nitrogen-fixing bacteria, called rhizobia (Gonzalez-Guerrero et al. 2014). The establishment of legume-rhizobium symbiosis is 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 symbionts thrive (Broughton et al. 2000). 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-organelle-like status (Emerich and Krishnan 2014). Within the symbiosomes, the rhizobia differentiate into nitrogen-fixing form called bacteroids (Udvardi and Poole 2013). Then bacteroids convert atmospheric nitrogen (N<sub>2</sub>) into ammonia (NH<sub>3</sub>) for plant use in exchange of photosynthate from the plant host (Gibson et al. 2008).

The formation of symbiotic nodules requires two parallel signaling pathways, one that promotes nodule organogenesis while the other allows bacterial infection (Madsen et al. 2010). These processes are coordinated in both a spatial and temporal manner to ensure successful symbiotic development (Oldroyd et al. 2011). Both processes require plant recognition of the Nod factors (NF). NF as signaling molecules secreted by rhizobia, are perceived by plant hosts through two plasma membrane-located receptor-like kinases (RLK) NFR1 and NFR5 (Arrighi et al. 2006; Indrasumunar et al. 2010; Limpens et al. 2005; Madsen et al. 2003), 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 *Pisum sativum*, and DMI2 (Does not Make Infections 2) in *Medicago truncatula*, and *GmNORKa/b* in *Glycine max* (Indrasumunar et al. 2015; Capoen et al. 2005; Endre et al. 2002; Mitra et al. 2004; Stracke et al. 2002). Recently, SYMRK/NORK was shown to be a co-receptor interacting with NFR5 to regulate the symbiotic signaling cascade (Antolin-Llovera et al. 2012). Almost all the knowledge regarding the function of SymRK/NORK was obtained from model legumes, such as Lotus and Medicago, but not from soybean.

The NORK/SymRK gene is characterized with a signal peptide, three LRR motifs, a transmembrane domain and a Ser/Thr kinase domain. Mutations in SymRK/NORK not only affect nodulation, but also impair arbuscular mycorrhization (Endre et al. 2002; Stracke et al. 2002). The NORK gene is essential for Nod factor perception/transduction in *Medicago sativa* since it is involved in the common signaling pathway of symbiosis (Endre et al. 2002). Many researchers have suggested that NORK/SymRK is involved in a protein complex with the Nod factor receptor or/and transduces the signaling of Nod factor receptors to subsequent events (Hogg et al. 2006; Riely et al. 2004). However, there is little mechanistic information as to how NORK/SymRK actually does work. NORK/SymRK is a protein containing 919 amino acid, leucine rich repeat (LRR) receptor-like kinase, containing a 325 amino acid serine/threonine kinase domain (Yoshida and Parniske 2005). Previous research had shown that NORK/SymRK is an active kinase (Kevei et al. 2007).

NIC1, a CLE peptide-encoding genes in soybean (*Glycine max*), play an important role in nitrate regulation of nodulation. NIC1 strongly responds to nitrate treatment but not to *Bradyrhizobium sp.* Inoculation. NIC1 functions locally to inhibit nodulation depending on root GmNARK activity. A SDI-like nitrate induced inhibitor (NII) of nodulation produced by the interaction of NIC1 with NARK in the root lead to local nitrate inhibition of nodulation (Reid et al.

2011). GmNARK is a common component during autoregulation of nodulation (AON) and nitrate regulation of nodulation. Both systemic and local regulations of nodulation respond to nitrate (Jeudy et al. 2010).

In this study, we reported that rhizobial inoculation decreases the expression level of *GmNIC1* in *GmNORK RNAi* soybean transgenic roots, suggesting that NORK directly regulates AON directed by GmNIC. Our results provide important information in understanding of the functions of *GmNORK* and *GmNIC1* in symbiotic signaling and nodule development.

## Materials and methods

### Alignment and phylogenetic analysis

NCBI BLAST searches using GmNORKa and GmNORKb 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 *GmNORKb* stop codon to the 229-bp was amplified from Williams 82 using *GmNORKb*-RNAi F/R primer pairs (Table S1), and 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 (Govindarajulu et al. 2009; Libault et al. 2010). This vector contains an RNAi construct directed against GUS.

### Agrobacterium rhizogenes-mediated hairy root transformation

The constructs were electroporated into *Agrobacterium rhizogenes* strain K599 using a GenePulser apparatus with pulse controller (Bio-Rad Laboratories, Hercules, California) with settings at 25  $\mu$ F, 200  $\Omega$ , 1.8 kV. After electroporation, 100  $\mu$ L LB medium was added to the competent cells, which were

allowed to recover at 30 °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. *Agrobacterium 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 ( $\text{dH}_2\text{O}$ ), once with 0.8% HCl for 10 min, and then several times with  $\text{dH}_2\text{O}$ . Sterilized seeds were then sown onto 1% agar round plates (20 cm diameter) and incubated in chambers at 27 °C and 80% humidity for 3 days in darkness and 3 days at 22 °C with a light regime of 16 h light and 8 h darkness.

**Transformation:** *Agrobacterium rhizogenes* K599 carrying the respective constructs was inoculated into liquid LB medium with appropriate antibiotics and incubated overnight at 30 °C with shaking at 200 rpm. Approximately 500  $\mu$ L 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 Fahraeus medium (Fahraeus 1957) supplemented with 1 mM  $\text{CaCl}_2$  and 1 mM  $\text{KNO}_3$ . Plantlets were incubated in the growth chamber at 22 °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  $\text{CaCl}_2$  and  $\text{KNO}_3$  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  $\text{CaCl}_2$  and  $\text{KNO}_3$ . Approximately 3 weeks after transformation, formation of transgenic roots was checked by GFP fluorescence 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 rhizobial symbiont, *Bradyrhizobium japonicum*.

## Nodulation assay

For soybean inoculation with rhizobia, *B. japonicum* wild-type strain USDA110 (Halverson and Stacey 1986), or a derivative strain constitutively expressing  $\beta$ -glucuronidase (GUS) (Loh et al. 2001) were grown at 30 °C for 3 days prior to inoculation in liquid HM medium (Cole and Elkan 1973) supplemented with appropriate antibiotics (50  $\mu$ g/ml tetracycline, 100  $\mu$ g/ml spectinomycin). Cells were grown to an OD600 between 0.5 and 1.0. Cells were then pelleted by centrifugation at 4000 rpm at 20 °C for 10 min 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 and Leica Application Suite Software. Only the nodules formed on transgenic roots, as determined by expression of the GFP marker, were analyzed.

## $\beta$ -glucuronidase assay

For histochemical  $\beta$ -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 (Toth et al. 2012). Vacuum was applied three times for 3 min, and then the roots were incubated at 37 °C in the dark for 2 days 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 °C until 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 S1.) 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 s, and 60 °C for 1 min. The geometric mean of *cons4* and *cons6*, encoding an ATP-binding cassette transporter and an F-box protein (Govindarajulu et al. 2009), were used as reference genes to normalize the expression levels. The relative expression levels of the gene between the two samples is calculated using  $2^{-\Delta\Delta Ct}$  method. The average of the expression levels between three different replicates was calculated.

## Recombinant protein purification and kinase assay

The plasmid pGENORK 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 h at 16 °C. Recombinant protein purification was done according to the following procedure. Bacterial cells from 200 ml LB medium were pelleted by centrifugation at 4 °C at 8000 rpm for 10 min and resuspended in 10 ml 1  $\times$  PBS solution supplemented with 1  $\times$  EDTA-free protease inhibitor (Roche, Indianapolis, 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 min at 4 °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  $\times$  PBS solution. The eluted proteins, including GST and GSTNORKKD, were dialyzed with buffer [50 mM Tris (PH 7.5), 50 mM KCl, 2 mM DTT, and 10% glycerol].

The in vitro kinase assays used 1 mg purified protein in a buffer containing 50 mM Tris (PH 7.5),

50 mM KCl, 2 mM DTT, 5 mM MnCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 10% glycerol, 10 mM ATP and 5mM[Ci[γ-<sup>32</sup>P]-ATP. The assay mix was incubated at 28 °C for 30 min 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 phosphor imager (Emerich and Krishnan 2014).

### Statistical analysis

Data for nodule numbers were statistically analyzed using a one-way analysis of variance (ANOVA). The expression levels of genes were analyzed using Student's *t* test.

## Results

### GmNORK structure

As a diploidized ancient tetraploid species, soybean has two copies of NORK genes in its genome, referred to as *GmNORKa* and *GmNORKb*. Through analysis of their genomic features, both *GmNORKa* and *GmNORKb* have 15 Exons and 14 introns. Both the predicted proteins have signal peptides (SP) at N-terminal, leucine-rich-repeat domain (LRR), trans-membrane domain (TM) and Serine-Threonine/tyrosine kinase domain (PK) (Fig. 1). Both *GmNORKa* and *GmNORKb* both contain 919 a.a and share 94.9% identity (Fig. 2).

### Molecular phylogenetic analysis of GmNORKa and GmNORKb

To investigate the relationship of *GmNORKa* and *GmNORKb* proteins with other homologous proteins in soybean and other plant species, a phylogenetic tree based on amino acid sequences was generated (Fig. 3). *GmNORKa* and *GmNORKb* 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 *Sesbania rostrata* (*AAV88623.1*), both are legumes that support a rhizobium symbiosis. It shows that the *SymRK* (*NORK*) orthologs are divided into three groups, Rhizobium (*Fabaceae*, legume), actinorhizal (non-legume) nodule-forming plants and non-nodule-

forming plants. *G.max NORK* and *P. vulgaris NORK* 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).

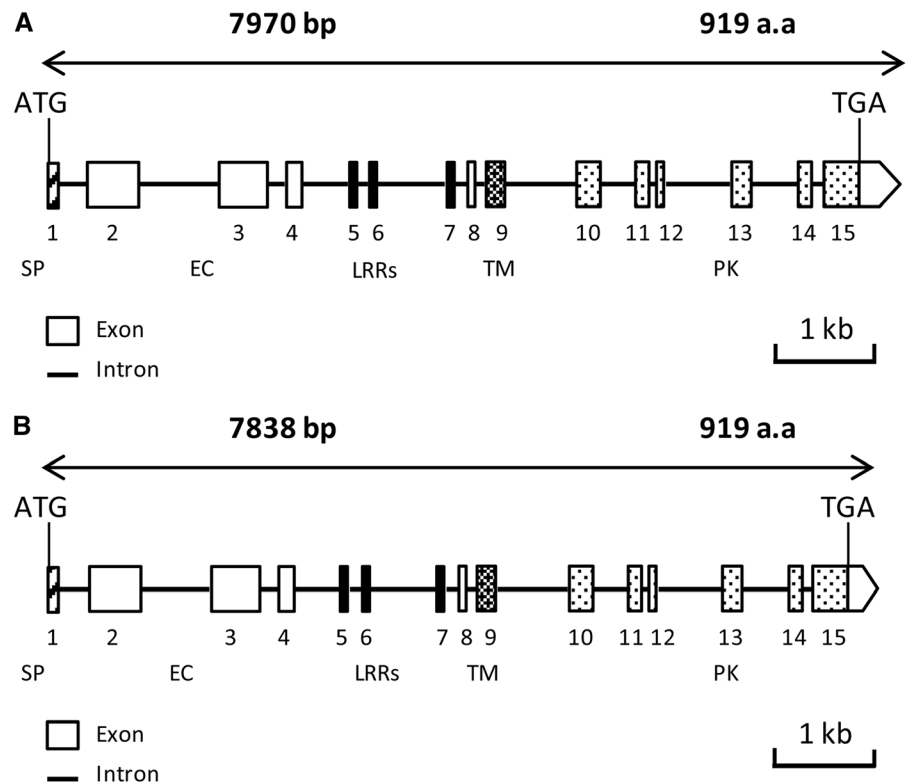
*RNA silencing GmNORK* resulted in reduced nodule number

To study the function of *GmNORK* involved in root nodule symbiosis in soybean, specified RNAi construct of *GmNORK* was generated and transformed into soybean roots by *agrobacterium rhizogenes*-mediated hairy root transformation. Transgenic roots were identified with the green fluorescent protein (GFP) marker expressed in the binary vector (Fig. 4a). Nodule morphology was examined by using GUS staining. The nodule morphology from the RNAi roots showed no obvious structural changes relative to the controls (Fig. 4b, c). The number of nodules formed on the *GmNORK RNAi* was approximately 6.4% that of the controls (with *P* < 0.01) (Fig. 4d). The results demonstrated that *GmNORKa* and *GmNORKb* are involved in nodule formation in soybean plants after inoculated with *B. japonicum*.

The expression of *GmNORKa*, *GmNORKb*, and *GmNIC1* in *GmNORK RNAi* lines

Due to the lack of homozygous knockout mutants for *GmNORKa* and *GmNORKb* in soybeans, RNA silencing was applied to investigate their functions in nodulation. Soybean roots were transformed with *GmNORK RNAi*, an empty vector and a vector expressing an RNAi construct specifically targeted to β-glucuronidase (GUS) (*GUS RNAi*) as controls. *GmNORKa* and *GmNORKb* expression levels in *GmNORK RNAi* are approximately 37% and 24% of that in the control transgenic roots, respectively (Fig. 5a, b). The results suggest that *GmNORK* plays a critical role in mediating root nodule symbiosis (RNS). *GmNIC1* play a critical role in AON system, which tightly control the soybean nodule numbers. In order to study whether *GmNORK* involves the *GmNIC1* associated nodulation, we analyzed the *GmNIC1* expression in *GmNORK RNAi* lines. *GmNIC1* expression levels in *GmNORK RNAi* are approximately 21% of that in the control transgenic roots (Fig. 5c).

**Fig. 1** *NORK1a* and *NORK1b* gene structure. Genomic structure of *NORK1a* and *NORK1b* with indicated predicted protein domains. Exons are indicated as boxes, introns as black line. SP, predicted signal peptide; EC, extracellular domain; LRR, Leucine-rich repeat motifs; TM, transmembrane domain; PK, protein kinase domain



### Recombinant protein purification and kinase assay

In order to confirm GmNORK phosphorylating activation, we performed in vitro phosphorylation assays. GmNORK kinase domain (538 a.a. to 919 a.a.) was expressed by fusing with the glutathione S-transferase domain. As shown in Fig. 6, GmNORK was shown to exhibit autophosphorylation in vitro, consistent with previous reports (Samaddar et al. 2013; Yoshida and Parniske 2005).

### Discussion

GmNIC1 functions locally to inhibit nodulation depend on root GmNARK activity and strongly responds to nitrate treatment but not to *Bradyrhizobium sp.* Inoculation (Reid et al. 2011). The interaction of NIC1 with NARK in the root probably produces a SDI-like nitrate induced inhibitor (NII) of nodulation leading to local nitrate inhibition of nodulation. Thus, GmNARK is a common component during AON and

nitrate regulation of nodulation (Reid et al. 2011). Both systemic and local regulations of nodulation respond to nitrate (Jeudy et al. 2010).

SYMRK/NORK, a component immediately downstream of these Nod factor receptors, is central to the Nod factor signaling cascade (Indrasumunar et al. 2010; Stracke et al. 2002). Several interacting NFRs and SYMRK/NORK proteins have been identified, including SINA4 (Seven In Absentia 4), HMGR1 (3-hydroxy-3-methylglutaryl-CoA reductase 1), PUB1 (Plant U-box protein 1) and SYMREM1 (Symbiotic Remorin 1). How the signal is transferred from the plasma membrane to the nuclear envelope remains unclear. Three nucleoporin (NUP85, NUP133, and NENA) and two cation channel proteins (CASTOR and POLLUX) are involved in symbiotic calcium oscillations generation. The nucleus-localized  $Ca^{2+}$ -calmodulin dependent protein kinase and CYCLOPS are used in decoding these calcium oscillations, and activate the transcription factor (TF) for symbiosis-associated gene expression (Mitra et al. 2004). However, the mechanism via which Nod factor signaling

GmNORKa	MMELPDIWILRLVAVCFLLIFIRSASGSATEGFENIACCADSNYTDPTTLNYTTDYRWFPDKGSCRRTKDLNEKVR	80
GmNORKb	MMELPDIWILRLVAVCFLLHIFIRSASGYATEGFENIACCADSNYTDPTTLNYTTDYRWFPDKGSCRRTKDLNEKVR	80
GmNORKa	LFFVDEGKRCYNLPTIKNKVYLIRGTFPFNGVNSSFVNSIGVTQLGAVRSSGLQDLEIEGIFRATKDYIDFCLVKGEVDP	160
GmNORKb	LFFVDEGKRCYNLSTIKNKVYLIRGTFPFNGVNSSFVNSIGVTQLGAVRSSGLQDLEIEGVFRAAKDYIDI CLVKGEVDP	160
GmNORKa	FISQLELRPLPEEYLHDLPASVCLKISRNSFWGTKDEIRFPTDPSDRIWKATSSSLSALLSSNVSNFDLKSNTVTPPLQV	240
GmNORKb	LISHIELRPLPEEYLHDLPASVCLKISRNSLWGSKDEIRFPTDPSDRIWKATSSPSSALLVSSNVSNFDLKSNTVTPPLQV	240
GmNORKa	LQTAVTHPDLRQFVLSGLDIEDNEYRVFLYFLELNSTVKAGKRVFDIYVNGEIKKERFDILAGGSNYTYTVLNVSANGLL	320
GmNORKb	LQTAALTHPERLQFMHSGIDTEDNEYRVFLYFLELNSTVKAGKRVFDIYVNGEIKKERFDILAEGSNYTYTVLNVSANGLL	320
GmNORKa	NLTLVKASGAIEFGPLLNAEYVLMRQSWIEETNQKQVEGIQKIREELLQNDKALESWTGDPCFPWQGITCDGSSNGSS	400
GmNORKb	NLTLVKASGAIEFGPLLNAEYILQMRQSWIEETNHKQVEVIQKIKEEVLLQNGKALESWTGDPCFPWQGITCDSSNGSS	400
GmNORKa	VITKLDLSARNFKGQIPSSITEMTNLKLNLSHDNDFNGYIPSFPLSSLLISIDLSYNDLMGSLPESIVSLPHLKSLYFGC	480
GmNORKb	VITKLDLSAHNFKGPIPPSSITEMINLKLNLSHNFDGYIPSFPLSSLLISIDLSYNNLMGSLPESIVSLPHLKSLYFGC	480
GmNORKa	NKRMSKEDPANLNSSPINTDYGRCKGKEPRFGQVFVIGAITCGSLLI TLAVGI IFVCRYRQKLIPWEGFGGKNYLMETNV	560
GmNORKb	NKRMSEGGPANLNSSLINTDYGRCKGKEPRFGQVFVIGAITCGSLLI ALAVGI IFVCRYRQKLIPWEGFGGKNYIMETNV	560
GmNORKa	IFSLPSKDDFLIKSVSIQTFTLEDIEVATERYKTLIGEGFGSVYRGTLNDGQEVAVKVRSAATSTQGTREDFNELNLLSA	640
GmNORKb	IFSLPSKDDFLIKSVSIQTFTLEDIEVATERYKTLIGEGFGSVYRGTLNNSQEVAVKVRSAATSTQGTREDFNELNLLSA	640
GmNORKa	IQHENVPLPLGYCNENDQQILMYPFMSNGSLQDRLYGEPAKRRI LDWPTRL SIALGAARGLAYLHTFPGRSVIHRDVKSS	720
GmNORKb	IQHENVPLPLGYCNENDQQILVYPFMSNGSLQDRLYGEPAKRRI LDWPTRL SIALGAARGLAYLHTFPGRSVIHRDVKSS	720
GmNORKa	NILLDHSMCAKVADFGFSKYAPQEGDSNVSEVRGTAGYLDPEYYKTQQLSEKSDVFSFGVVLEIVSGREPLDIKRPN	800
GmNORKb	NILLDHSMCAKVADFGFSKYAPQEGDSNVSEVRGTAGYLDPEYYKTQQLSEKSDVFSFGVVLEIVSGREPLDIKRPN	800
GmNORKa	EWSLVEWAKPYIRVSKMDEIVDPGIKGGYHAEAMWRVVEVALQCLEPFSAYRPNMVDIVRELEDALIENNASEYMKSID	880
GmNORKb	EWSLVEWAKPYVRAASKMDEIVDPGIKGGYHAEAMWRVVEVALHCLEPFSAYRPNMVDIVRELEDALIENNASEYMKSID	880
GmNORKa	SLGGSNRYSIVIEKRVLPSTSSTAESTITTQALSHPPQR	919
GmNORKb	SLGGSNRYSIVIEKRVLPSTSSTAESTITTQALSHPPQR	919

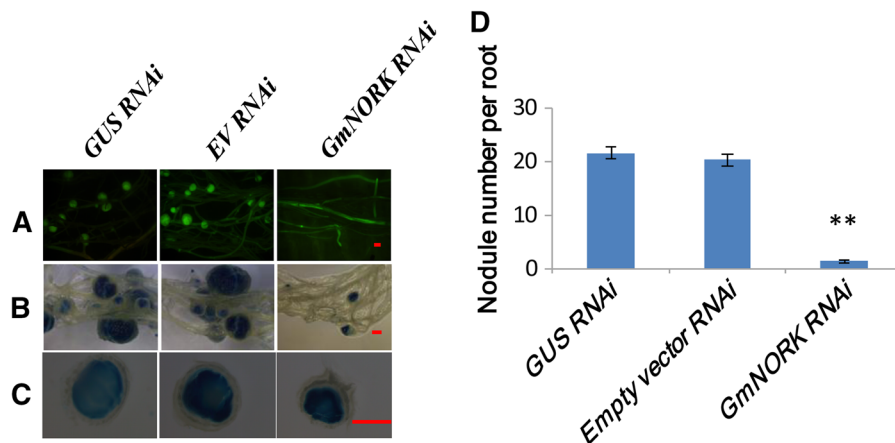
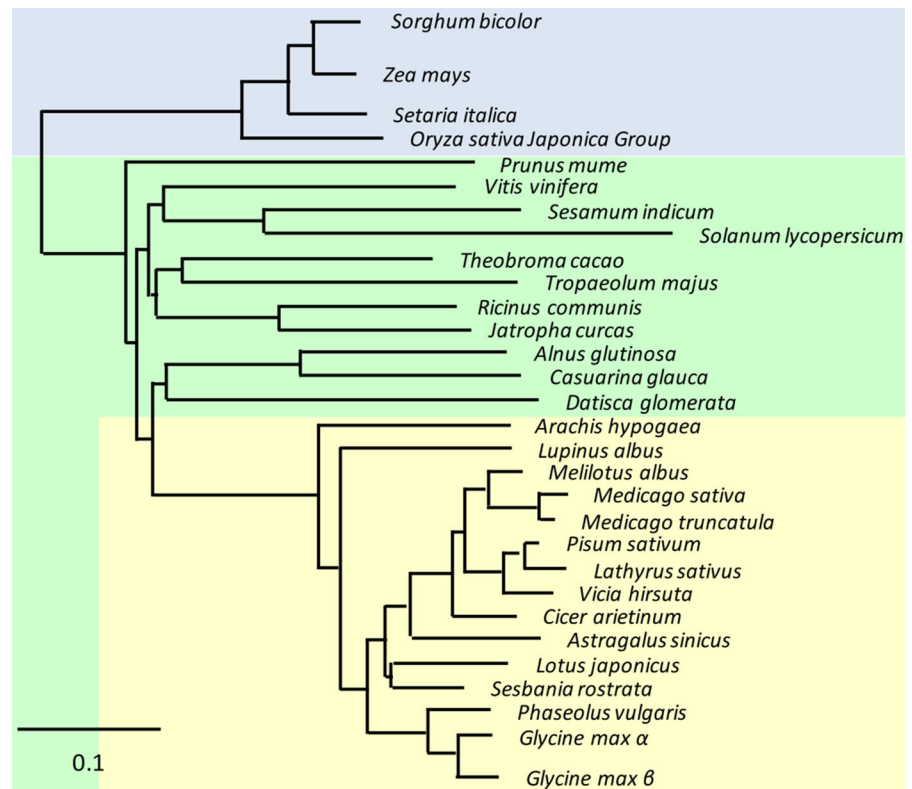
**Fig. 2** Protein sequence of NORK1a and NORK1b. NORK1a and NORK1b show 94.9% similarity each other, unconserved amino acids are shown with red letters. (Color figure online)

affects nodule development, nuclear size and the chromatin structure remains unknown.

Soybean nodule numbers are tightly controlled by AON. *GmNIC1* play a critical role in AON system (Reid et al. 2011). GmNORK plays a critical role in mediating root nodule symbiosis (RNS). *GmNORK* probably involves the *GmNIC1* associated nodulation. As seen in Fig. 4, RNA silencing *GmNORK* resulted in reduced nodule number, consistent with previous

reports (Indrasumunar et al. 2015). Our studies show that the *GmNIC1* expression level is decreased in *GmNORK RNAi* transgenic soybean roots. These data suggest that *GmNORK* affect nodule development and nitrate regulation of nodulation by decreasing the *GmNIC1* expression level. The details of the mechanism behind the *GmNORK* affect *GmNIC* expression level require further elucidation.

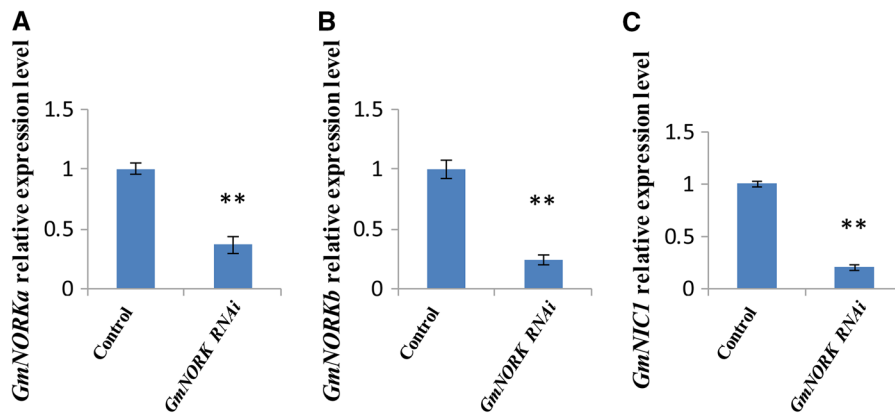
**Fig. 3** Phylogenetic analysis using the Maximum Likelihood method. Monocot and dicot NORKs group into distinct sub-clades, light blue and light green color sub-clades, respectively. In each dicot sub-clades, legume NORKs also arrange in specific clusters (light yellow color). (Color figure online)



**Fig. 4** RNA silencing of *GmNORK* results in reduced nodule number. **a** Representative transgenic root and nodules expressing *GUS RNAi*, *EV RNAi* and *GmNORK RNAi* constructs. Scale bars represent 1 mm. **b** Stained micrographs of nodule derived from *RNAi* transgenic roots at 21 days after inoculation with *B. japonicum*. Scale bars represent 1 mm. **c** Stained micrographs of nodule sections derived from *RNAi* transgenic roots at 21 days after inoculation with *B. japonicum*. The nodule morphology was similar among the nodules formed on roots expressing the *GUS*

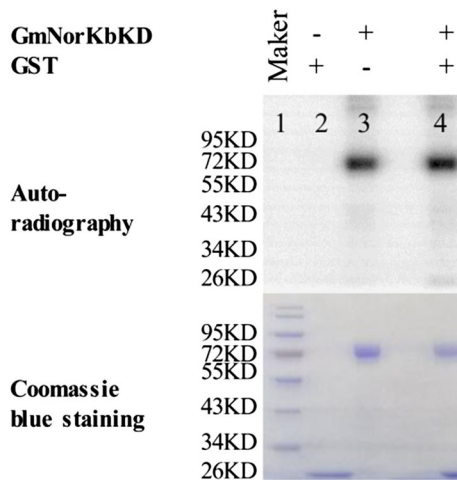
*RNAi*, *Empty vector (EV)*, and *GmNORK RNAi* constructs. Scale bars represent 1 mm. *EV RNAi* indicates *Empty vector RNAi*. **d** Nodulation was measured as nodule number per transgenic root. The nodule numbers per root in *GmNORK RNAi* transgenic lines decreased significantly compared to which in *GUS RNAi* and *Empty vector* transgenic lines. \*\* denotes significantly different at  $P < 0.01$ . Bars indicate standard error of the mean from 30 plants





**Fig. 5** The expression of *GmNORK1a*, *GmNORK1b* and *GmNIC1* in soybean roots was significantly reduced in *GmNORK RNAi* lines. **a** *GmNORK1a* relative expression levels in roots of soybean transformed with negative control vector, *GmNORK RNAi* plasmids. **b** *GmNORK1b* relative expression levels in roots of soybean transformed with negative control

vector, *GmNORK RNAi* plasmids. **c** *GmNIC1* relative expression levels in roots of soybean transformed with negative control vector, *GmNORK RNAi* plasmids. \*\* denotes significantly different at  $P < 0.01$ . Bars indicate standard error of the mean from 3 plants. Control indicates geometric mean of *GUS RNAi* and *Empty vector RNAi*



**Fig. 6** *GmNORK* has in vitro kinase activity. Upper panel is Autoradiography picture. Lower panel is Coomassie blue staining picture. Lane1 is maker. Lane2 is GST input. Lane3 is *GmNORK1b* kinase domain input. Lane4 is *GmNORK1b* kinase domain and GST inputs. (Color figure online)

**Acknowledgements** This work was supported by the PhD Start-up Fund of Natural Science Foundation under Grant No. 801100010121; Open fund project of State Key laboratory of Crop Genetics and Germplasm Enhancement under Grant No. ZW201706; Open fund project of oil crops biology and genetics and breeding key laboratory of Ministry of Agriculture under Grant No. 2016006. Open funds of State Key Laboratory of Agricultural Microbiology under Grant No. AMLKF201608.

**Authors’ contributions** LW, YW, YJ, YC, LD, and XB contributed equally to this work.

**References**

Antolin-Llovera M, Ried MK, Binder A, Parniske M (2012) Receptor kinase signaling pathways in plant–microbe interactions. *Annu Rev Phytopathol* 50:451–473

Arrighi JF, Barre A, Ben Amor B, Bersoult A, Soriano LC, Mirabella R, de Carvalho-Niebel F, Journet EP, Gherardi M, Huguet T, Geurts R, Dénarié J, Rougé P, Gough C (2006) The *Medicago truncatula* lysin [corrected] motif-receptor-like kinase gene family includes NFP and new nodule-expressed genes. *Plant Physiol* 142:265–279

Broughton WJ, Jabbouri S, Perret X (2000) Keys to symbiotic harmony. *J Bacteriol* 182:5641–5652

Capoen W, Goormachtig S, De Rycke R, Schroeyers K, Holsters M (2005) SrSymRK, a plant receptor essential for symbiosome formation. *Proc Natl Acad Sci USA* 102:10369–10374

Cole MA, Elkan GH (1973) Transmissible resistance to penicillin G, neomycin, and chloramphenicol in *Rhizobium japonicum*. *Antimicrob Agents Chemother* 4:248–253

Emerich DW, Krishnan HB (2014) Symbiosomes: temporary moonlighting organelles. *Biochem J* 460:1–11

Endre G, Kereszt A, Kevei Z, Mihacea S, Kalo P, Kiss GB (2002) A receptor kinase gene regulating symbiotic nodule development. *Nature* 417:962–966

Fahraeus G (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *J Gen Microbiol* 16:374–381

- Gibson KE, Kobayashi H, Walker GC (2008) Molecular determinants of a symbiotic chronic infection. *Annu Rev Genet* 42:413–441
- Gonzalez-Guerrero M, Matthiades A, Saez AN, Long TA (2014) Fixating on metals: new insights into the role of metals in nodulation and symbiotic nitrogen fixation. *Front Plant Sci* 5:45
- Govindarajulu M, Kim SY, Libault M, Berg RH, Tanaka K, Stacey G, Taylor CG (2009) GS52 ecto-apyrase plays a critical role during soybean nodulation. *Plant Physiol* 149:994–1004
- Halverson LJ, Stacey G (1986) Effect of lectin on nodulation by wild-type *Bradyrhizobium japonicum* and a nodulation-defective mutant. *Appl Environ Microbiol* 51:753–760
- Hogg BV, Cullimore JV, Ranjeva R, Bono JJ (2006) The DMI1 and DMI2 early symbiotic genes of medicago truncatula are required for a high-affinity nodulation factor-binding site associated to a particulate fraction of roots. *Plant Physiol* 140:365–373
- Indrasumunar A, Kereszt A, Searle I, Miyagi M, Li D, Nguyen CD, Men A, Carroll BJ, Gresshoff PM (2010) Inactivation of duplicated nod factor receptor 5 (NFR5) genes in recessive loss-of-function non-nodulation mutants of allotetraploid soybean (*Glycine max* L. Merr.). *Plant Cell Physiol* 51:201–214
- Indrasumunar A, Wilde J, Hayashi S, Li D, Gresshoff PM (2015) Functional analysis of duplicated Symbiosis Receptor Kinase (SymRK) genes during nodulation and mycorrhizal infection in soybean (*Glycine max*). *J Plant Physiol* 176:157–168
- Jedy C, Ruffel S, Freixes S, Tillard P, Santoni AL, Morel S, Journet EP, Duc G, Gojon A, Lepetit M, Salon C (2010) Adaptation of *Medicago truncatula* to nitrogen limitation is modulated via local and systemic nodule developmental responses. *New Phytol* 185:817–828
- Kevei Z, Lougnon G, Mergaert P, Horvath GV, Kereszt A, Jayaraman D, Zaman N, Marcel F, Regulski K, Kiss GB, Kondorosi A, Endre G, Kondorosi E, Ane JM (2007) 3-Hydroxy-3-methylglutaryl coenzyme a reductase 1 interacts with NORK and is crucial for nodulation in *Medicago truncatula*. *Plant Cell* 19:3974–3989
- Libault M, Zhang XC, Govindarajulu M, Qiu J, Ong YT, Brechenmacher L, Berg RH, Hurley-Sommer A, Taylor CG, Stacey G (2010) A member of the highly conserved FWL (tomato FW2.2-like) gene family is essential for soybean nodule organogenesis. *Plant J* 62:852–864
- Limpens E, Mirabella R, Fedorova E, Franken C, Franssen H, Bisseling T, Geurts R (2005) Formation of organelle-like N<sub>2</sub>-fixing symbiosomes in legume root nodules is controlled by DMI2. *Proc Natl Acad Sci USA* 102:10375–10380
- Loh JT, Yuen-Tsai JP, Stacey MG, Lohar D, Welborn A, Stacey G (2001) Population density-dependent regulation of the *Bradyrhizobium japonicum* nodulation genes. *Mol Microbiol* 42:37–46
- Madsen EB, Madsen LH, Radutoiu S, Olbryt M, Rakwalska M, Szczyglowski K, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J (2003) A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. *Nature* 425:637–640
- Madsen LH, Tirichine L, Jurkiewicz A, Sullivan JT, Heckmann AB, Bek AS, Ronson CW, James EK, Stougaard J (2010) The molecular network governing nodule organogenesis and infection in the model legume *Lotus japonicus*. *Nat Commun* 1:10
- Mitra RM, Gleason CA, Edwards A, Hadfield J, Downie JA, Oldroyd GE, Long SR (2004) A Ca<sup>2+</sup>/calmodulin-dependent protein kinase required for symbiotic nodule development: gene identification by transcript-based cloning. *Proc Natl Acad Sci USA* 101:4701–4705
- Oldroyd GE, Murray JD, Poole PS, Downie JA (2011) The rules of engagement in the legume-rhizobial symbiosis. *Annu Rev Genet* 45:119–144
- Reid DE, Ferguson BJ, Gresshoff PM (2011) Inoculation- and nitrate-induced CLE peptides of soybean control NARK-dependent nodule formation. *Mol Plant Microbe Interact* 24:606–618
- Riely BK, Ane JM, Penmetza RV, Cook DR (2004) Genetic and genomic analysis in model legumes bring Nod-factor signaling to center stage. *Curr Opin Plant Biol* 7:408–413
- Samaddar S, Dutta A, Sinharoy S, Paul A, Bhattacharya A, Saha S, Chien KY, Goshe MB, DasGupta M (2013) Autophosphorylation of gatekeeper tyrosine by symbiosis receptor kinase. *FEBS Lett* 587:2972–2979
- Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J, Szczyglowski K, Parniske M (2002) A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature* 417:959–962
- Toth K, Stratil TF, Madsen EB, Ye J, Popp C, Antolin-Llovera M, Grossmann C, Jensen ON, Schussler A, Parniske M, Ott T (2012) Functional domain analysis of the Remorin protein LjSYMREM1 in *Lotus japonicus*. *PLoS ONE* 7:e30817
- Udvardi M, Poole PS (2013) Transport and metabolism in legume-rhizobia symbioses. *Annu Rev Plant Biol* 64:781–805
- Yoshida S, Parniske M (2005) Regulation of plant symbiosis receptor kinase through serine and threonine phosphorylation. *J Biol Chem* 280:9203–9209

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.