JIPB Journal of Integrative Plant Biology

Research Article
https://doi.org/10.1111/jipb.13436

A novel secreted protein, NISP1, is phosphorylated by soybean Nodulation Receptor Kinase to promote nodule symbiosis

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ABSTRACT

Nodulation Receptor Kinase (NORK) functions as a co-receptor of Nod factor receptors to mediate rhizobial symbiosis in legumes, but its direct phosphorylation substrates that positively mediate root nodulation remain to be fully identified. Here, we identified a GmNORK-Interacting Small Protein (GmNISP1) that functions as a phosphorylation target of GmNORK to promote soybean nodulation. GmNORKα directly interacted with and phosphorylated GmNISP1. Transcription of *GmNISP1* was strongly induced after rhizobial infection in soybean roots and nodules. *GmNISP1* encodes a peptide containing 90 amino acids with a "DY" consensus motif at its N-terminus. GmNISP1 protein was detected to be present in the apoplastic space. Phosphorylation of GmNISP1 by GmNORKα could enhance its secretion into the apoplast. Pretreatment with either purified GmNISP1 or phosphorylation-mimic GmNISP1^{12D} on the roots could significantly increase nodule numbers compared with the treatment with phosphorylation-inactive GmNISP1^{12A}. The data suggested a model that soybean GmNORK phosphorylates GmNISP1 to promote its secretion into the apoplast, which might function as a potential peptide hormone to promote root nodulation.

Keywords: legume-rhizobia symbiosis, Nodulation Receptor Kinase, protein phosphorylation, root nodule symbiosis, soybean

Fu, B., Xu, Z., Lei, Y., Dong, R., Wang, Y., Guo, X., Zhu, H., Cao, Y., and Yan, Z. (2023). A novel secreted protein, NISP1, is phosphorylated by soybean Nodulation Receptor Kinase to promote nodule symbiosis. J. Integr. Plant Biol. **00:** 1–16.

INTRODUCTION

The establishment of legume-rhizobial symbiosis involves reciprocal dialogs with exchange of signals between hosts and symbionts (Suzaki et al., 2015; Roy et al., 2020). Among all the dialogs, perception of rhizobial nodulation factors (NFs) by plant receptors serves as a determining one to activate the symbiotic signaling pathway that leads to rhizobial infection and nodule development for nitrogen fixation (Roy et al., 2020; Yang et al., 2022). It is clear that the heterocomplex of two lysin-motif receptor kinases from plants, such as Nod factor receptor1 (NFR1) and NFR5 in *Lotus japonicus* or *Glycine max*, recognizes rhizobial NFs to initiate symbiotic signaling in plant cells (reviewed in Roy et al., 2020; Yang et al., 2022). The component located downstream from NFRs was identified as another membrane-localized receptor kinase, that is, Nodulation Receptor Kinase (NORK) in soybean (also called Symbiosis Receptor Kinase (SYMRK) in *L. japonicus*, or Does not make infection2 (DMI2) in *Medicago truncatula* (Endre et al., 2002; Stracke et al., 2002; Indrasumunar et al., 2015).

Interestingly, NORK/SYMRK/DMI2 has been shown to be an essential kinase required for both rhizobial symbiosis and mycorrhizal symbiosis (Wang et al., 2022). With three receptor kinases identified as essential components in the symbiotic pathway, it has been theorized that phosphorylation plays an essential role regulating root nodulation (Chiu and Paszkowski, 2020). However, the direct phosphorylation target proteins of NORK that could positively transduce symbiotic signals to downstream components needs to be fully identified.

NORK/SYMRK/DMI2 is a protein containing an extracellular malectin-like domain (MLD), leucine-rich repeats (LRR) domain, a transmembrane domain, and an intracellular kinase domain that might undergo structural diversification during evolution (Endre et al., 2002; Markmann et al., 2008). Interestingly, the MLD of LjSYMRK could be cleavaged by unknown mechanisms to release a truncated LRR kinase, which was shown to physically interact with LjNFR5 in *L. japonicus* (Antolin-Llovera et al., 2014). Thus, SYMRK/NORK/DMI2 was proposed to serve as a co-receptor of NFRs to mediate symbiotic signaling transduction (Ried et al., 2014). Due to the key role of NORK/SYMRK/DMI2 in symbiosis, its biological function has been studied based on the several NORK/SYMRK/DMI2-interacting proteins identified from different plants. In *M. truncatula*, 3-hydroxy-3-methylglutaryl CoA reductase1 (HMGR1), a key enzyme in the mevalonate biosynthetic pathway was identified to interact with MtDMI2 (Kevei et al., 2007; Venkateshwaran et al., 2015), In *L. japonicus*, SYMRK-Interacting Protein1 (SIP1) and SIP2, which represent an ARID-type DNA-binding protein and a mitogen-activated protein kinase kinase (MAPKK), respectively, were shown to interact with LjSYMRK (Zhu et al., 2008; Chen et al., 2012), suggesting that gene expression regulation and MAPK cascade might be involved in the symbiotic pathway (Yin et al., 2019). In addition, NORK/SYMRK/DMI2 was also shown to interact with three E3 ligases, that is, SEVEN IN ABSENTIA4 (SINA4) and SymRK-Interacting E3 ligase (SIE3) in *L. japonicus* and MtPUB1 (Plant U–Box) in *M. truncatula*, suggesting protein degradation might be involved in regulating the SYMRK-mediated symbiosis pathway (Den Herder et al., 2012; Yuan et al., 2012; Vernie et al., 2016), but the above three E3 ligases were not shown to mediate the degradation of NORK/SYMRK/DMI2. *L. japonicus* SYMRK was also shown to have a role in suppressing plant immunity through directly inhibiting the kinase activity of LjBAK1, the homolog of which from different plant species serves as a co-receptor for multiple immune receptors in response to pathogens (Feng et al., 2021). Recent work identified that soybean SymRK (NORK) directly phosphorylates Gα protein to inhibit the interaction with Gβγ allowing a constitutive signaling by Gβγ (Roy Choudhury and Pandey, 2022). Among all the interacting proteins identified above, MtPUB1, a negative regulator of symbiosis, and Gα protein were shown to be directly phosphorylated by SYMRK/NORK/DMI2 (Vernie et al., 2016; Roy Choudhury and Pandey, 2022). Thus, with the essential role of SYMRK/NORK/DMI2 in symbiosis, it is of great interest to identify its phosphorylation target proteins to elucidate the mechanisms of symbiotic signaling transduction from plant membrane to downstream components *via* phosphorylation.

Nitrogen fixation in root nodules is an energy-costly process; hence, the competency to develop nodules and nodule numbers are strictly controlled by local and systemic endogenous signals in plants, such as small signaling peptide hormones (Djordjevic et al., 2015). Previous studies have identified several types of small peptide hormones (usually <100 amino acids) in plants (Czyzewicz et al., 2013; Wang et al., 2017; Yang et al., 2017). Some of the secreted peptides, such as the well-known cysteine-rich peptides (e.g., RAPID ALKALINIZATION FACTOR (RALF)/RALF-like, EPIDERMAL PATTERNING FACTOR), are processed by proteases after removal of the N-terminal secretion signal to function as peptide hormones regulating plant growth development and response to different stimuli (Czyzewicz et al., 2013; Matsubayashi, 2014; Murphy and De Smet, 2014). Other peptide hormones were characterized as proline-rich proteins, including CLEs, CEPs, ROOT GROWTH FACTORs (RGFs), and INFLORESCENCE DEFICIENT IN ABSCISSIONs (IDAs). Several relatively well-studied peptides, including CLEs, CEPs, ENOD40, and NODULE SPECIFIC CYSTEINE-RICHs (NCRs), play distinct yet coordinated roles in nodule development (Djordjevic et al., 2015). But the exact function of peptide hormones in regulating rhizobium–legume symbiosis is largely unclear.

Here, we studied the molecular basis of NORK protein regulating soybean nodulation. A novel secreted protein, GmNISP1 (GmNORK-Interacting Small Protein1) was characterized as a direct phosphorylation substrate of GmNORKα. GmNISP1 could be detected at the extracellular space and might function as a peptide hormone to promote nodulation in soybean. Pretreatment with purified NISP1 protein significantly increased nodule numbers on soybean roots. Interestingly, phosphorylation of GmNISP1 by GmNORK might enhance the secretion of NISP1 to the apoplast. Taken together, our data suggest an exciting model that GmNORK could interact with and phosphorylate GmNISP1, which then might function as a potential peptide hormone to promote soybean nodulation.

RESULTS

Soybean NORK interacts with GmNISP1

To identify the phosphorylation substrate of soybean NORK, we used the kinase domain (KD) of GmNORKα as the bait in a yeast two-hybrid (Y2H) screen of a complementary DNA (cDNA) library created from young nodules and roots of the soybean (Williams82). Interactions were tested in repeated experiments on the selective medium. One of the positive clones, encoding an unknown small protein that we named GmNISP1, was selected for further study. The full-length cDNA of *GmNISP1* (Glyma.17G084600) contains a 273-bp open reading frame encoding a protein of 90 amino acids.

A protein Basic Local Alignment Search Tool (BLASTp) search of the Phytozome database identified NISP1 homologs in both legumes and non-legumes (Figure S1A). Phylogenetic analysis showed that the GmNISP1 homologs from legumes and non-legumes are located in separate clades (Figure S1B). Two homologs of GmNISP1 in soybean, Gm04G188300 and Gm06G177500, were identified as GmNISP-L1 (GmNISP1-Like 1) and GmNISP-L2, respectively (Figure S1A, B). Sequence alignment revealed that the N-terminal region of GmNISP1 homologs is highly conserved and includes a DY consensus motif that is typically required for protein secretion and peptide tyrosine sulfation (Kaufmann and Sauter, 2019), while the C-terminal region of the GmNISP1 homologs is variable in legumes (Figure S1A).

The interaction between GmNISP1 and GmNORKα was further confirmed using Y2H assay. Yeast cells were transformed with a construct carrying the binding domain (BD) fused to full-length GmNISP1, along with constructs carrying the activation domain (AD) fused to the KDs of GmNORKα (GmNORKα^{KD}), GmNORKβ^{KD} (GmNORKβ^{KD}), kinase-dead variants of GmNORKα$^{KD\text{-}K618E}$ (Lysine-601 is the key residue in the adenosine triphosphate (ATP) binding loop), GmNORKβ$^{KD\text{-}K618E}$, GmNFR1^{KD}, and GmNFR5^{KD}. Based on the ability of the yeast cells to grow on quadruple dropout medium, GmNISP1 was shown to interact with the KDs of GmNORKα and GmNORKβ but not of GmNFR1 or GmNFR5 (Figures 1A, S1C, D). In addition, Y2H assay also showed that GmNISP1 could interact with *L. japonicus* LjSymRK^{KD} and the CDs (cytoplasmic domains) of GmNORKα and GmNORKβ (Figures 1B, S1C, D). Two homologs of GmNISP1, GmNISP-L1 and GmNISP-L2 were also shown to interact with GmNORKα in yeast cells (Figure S1E). The expression of GmNISP1, GmNORK^{KD}, GmNORK^{CD} proteins in yeast cells could be detected using immunoblot (Figure S1F). These data strongly indicated that GmNORK^{CD} interacts with GmNISP1 and its homologs in yeast cells.

The interaction between GmNISP1 and GmNORKα was further verified using an *in vitro* pull-down assay (Figure 1C). Strep-tagged NISP1 but not negative control mannose-binding protein (MBP)-Strep could be pulled down in the sample containing MBP-tagged GmNORKα^{CD}

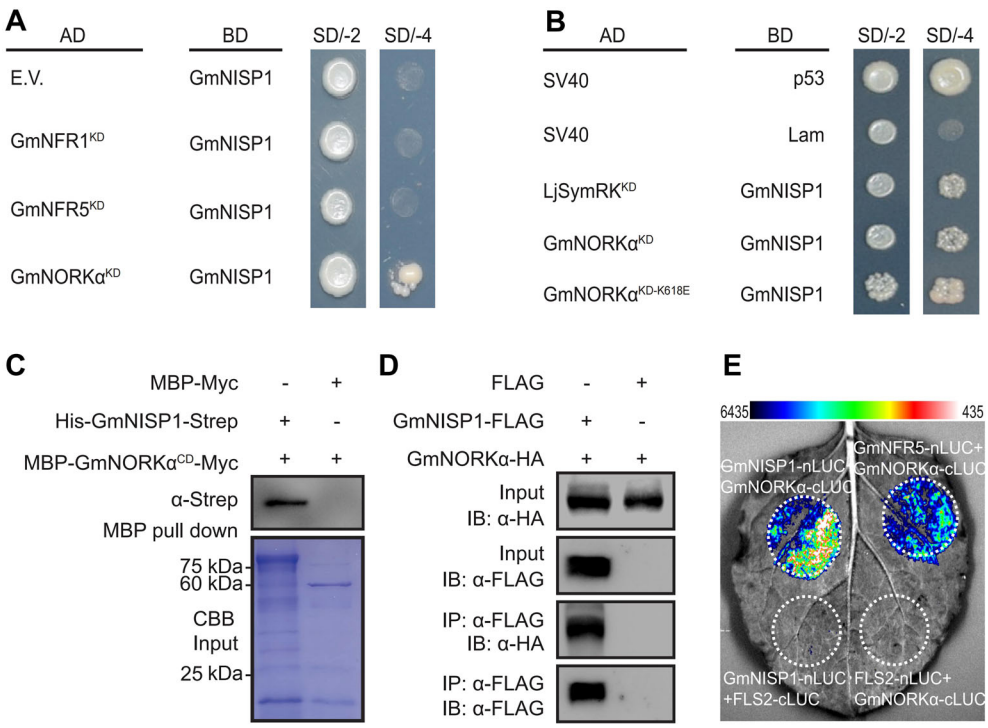

Figure 1. *Glycine max* Nodulation Receptor Kinase α (GmNORKα) interacts with GmNORK-Interacting Small Protein (GmNISP1) in both yeast and plant cells

(A) GmNORKα interacts with GmNISP1 in yeast two-hybrid (Y2H) assays. The kinase domains (KDs) of *Glycine max* Nod factor receptor1 (GmNFR1), GmNFR5, and GmNORKα were tested for interactions with GmNISP1 on synthetic defined (SD)/–4 (–Trp/–Leu/–His/–Ade) or SD/–2 (–Trp/–Leu) medium. AD, activation domain; BD, DNA-binding domain; EV, empty vector. **(B)** GmNISP1 interacts with GmNORKα or its homolog LjSymRK from *Lotus japonicus*. GmNORKα$^{KD\text{-}K618E}$ is a kinase-dead variant. The combinations p53/SV40 and Lam/SV40 served as positive and negative controls, respectively. **(C)** Mannose-binding protein (MBP)-GmNORKα physically associates with His-GmNISP1 in an *in vitro* pull-down assay. Pull-down was performed using amylose resin and detected with anti-Strep antibody. CBB, Coomassie brilliant blue staining. **(D)** GmNORKα-hemagglutinin (HA) interacts with GmNISP1-FLAG in a co-immunoprecipitation assay. Immunoprecipitation was performed using anti-FLAG M2 beads and analyzed using anti-FLAG and anti-HA antibodies. **(E)** Split-luciferase complementation assays of the interaction between GmNORKα and GmNISP1 in *Nicotiana benthamiana* leaves. All data in the figure are representative of three independent experiments.

(Figure 1C). To test whether the interaction between GmNISP1 and GmNORKα occurs *in vivo*, we used a co-immunoprecipitation (Co-IP) assay, in which 3× hemagglutinin (HA)-tagged GmNORKα was co-expressed with 3× FLAG-tagged GmNISP1 in *Nicotiana benthamiana* leaves. Proteins were precipitated using anti-FLAG beads and then probed with an anti-HA antibody. We found that GmNISP1 was co-immunoprecipitated with GmNORKα (Figure 1D). Furthermore, a split-luciferase assay performed in *N. benthamiana* leaves transiently co-expressing GmNISP1 fused to the N-terminal luciferase (GmNISP1-nLUC) and GmNORKα fused to the C-terminal luciferase (GmNORKα-cLUC) showed interaction between the two proteins (Figure 1E). The interaction between NISP1 and *Arabidopsis* FLS2, a negative control, was not detected in Split-LUC assay (Figure 1E). Taken together, these results strongly indicated that GmNISP1 is an interacting protein of GmNORKα.

Rhizobial treatment induces the expression of *GmNISP1*

To investigate the expression pattern of *GmNISP1* during plant growth and nodulation, total RNA was extracted from soybean stems, leaves, flowers, nodules, and root tissues inoculated with *Bradyrhizobium diazoefficiens* USDA 110. Real-time quantitative polymerase chain reaction (RT-qPCR) analysis detected high expression of *GmNISP1* in all the tissues except stems, but the levels of *GmNISP1* transcripts in roots and nodules was much higher than those in flowers and leaves (Figure 2A). Next, we examined the expression profile of *GmNISP1* during symbiotic development by analyzing the relative transcript levels of *GmNISP1* in soybean roots after inoculation with *B. diazoefficiens* USDA110 or with water as a mock control. As shown in Figure 2B, the expression of both *GmNISP1* and the nodulation marker gene *GmNIN1a* was significantly higher at all time points in inoculated

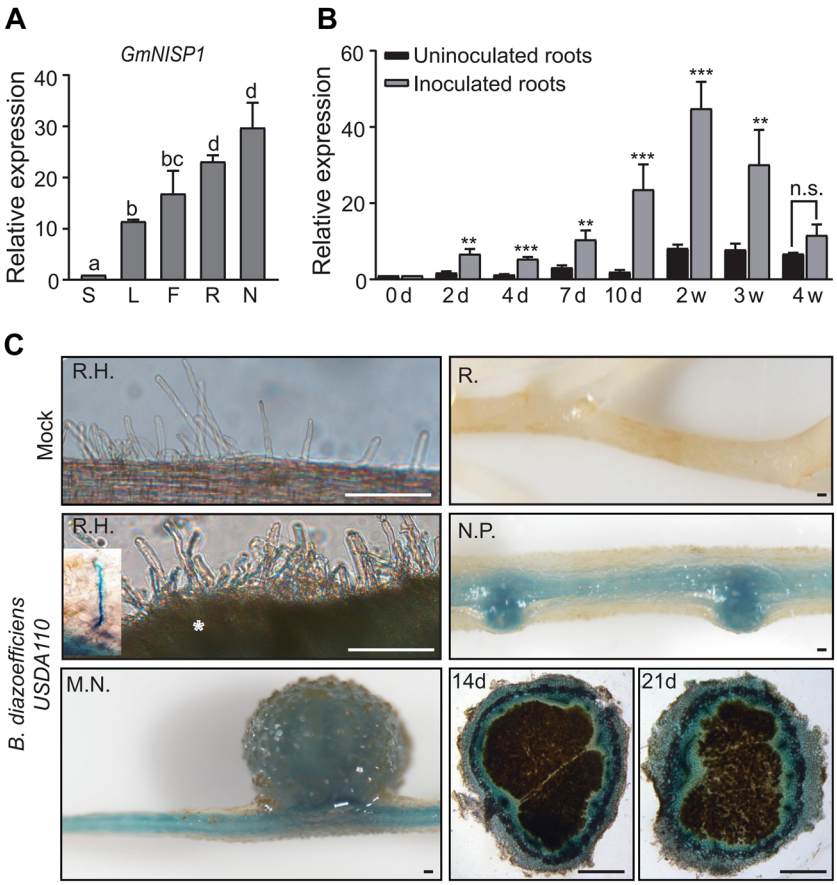

Figure 2. The expression pattern of *GmNISP1* in soybean

(A) *GmNISP1* transcript levels in different tissues, including stems (S), leaves (L), flowers (F), roots (R), and nodules (N), as measured by real-time quantitative polymerase chain reaction (RT-qPCR). The relative expression levels of *GmNISP1* were calculated by normalizing the transcription level of *GmATS1* (the same below). Data are mean ± *SE* ($n = 3$, Student's *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, $^{****}P < 0.0001$). **(B)** *GmNISP1* transcript levels in inoculated roots at 0, 2, 4, 7, and 10 d post-inoculation (dpi) and 2, 3, and 4 weeks post-inoculation (wpi) with rhizobia. Data are mean ± *SE* ($n = 3$, Student's *t*-test: $^{**}P < 0.01$; $^{***}P < 0.001$; n.s., no significance). **(C)** Promoter-GUS (β-glucuronidase) staining showing the pattern of *GmNISP1* expression in root hairs, nodule primordia, and nodules. Roots treated with water were used as the mock control. Root hairs and nodule primordia were observed 4 and 7 dpi, respectively. Root nodules were observed 14 and 21 dpi. R.H., root hair; R., root; N.P., nodule primordia; M.N., mature nodule. White asterisk indicates dividing cortical cells. Bar = 100 μm.

roots compared to uninoculated roots (Figure S2A). Moreover, *GmNISP1* transcript levels increased markedly during rhizobial infection and nodule development, with the highest expression level detected 2 weeks post-inoculation (Figure 2B). These data suggested that *GmNISP1* might be involved in rhizobial infection and the initiation of nodule organogenesis.

Next, we investigated the spatial expression patterns of *GmNISP1* in soybean roots. The 1.9-kb promoter of *GmNISP1* was fused with the *udiA* gene and expressed in transgenic hairy roots using *Agrobacterium rhizogenes*-mediated transformation (Tóth et al., 2016). After inoculation with *B. diazoefficiens* USDA 110, histochemical β-glucuronidase (GUS) staining indicated that *GmNISP1* was highly expressed in the root hairs compared to the mock controls (Figures 2C, S2B–H). Strong GUS signal driven by the *GmNISP1* promoter was also observed in nodule primordia, epidermal cells, and vascular bundles in mature nodules (Figure S2B–H). These results were consistent with the *GmNISP1* transcript levels observed in the RT-qPCR analysis of root tissues. Taken together, the data show that *GmNISP1* gene expression is enhanced during soybean–rhizobium interaction, suggesting that *GmNISP1* might function in soybean nodule development.

GmNISP1 regulates soybean nodulation

To gain further insight into the role of GmNISP1 in soybean–rhizobium symbiosis, we overexpressed *GmNISP1* under the control of the Cassava vein mosaic virus promoter (*OX-GmNISP1*) in transgenic soybean roots (Figure 3A, B). Real-time qPCR analysis confirmed that the expression of *GmNISP1* was more than 35-fold higher in the *OX-GmNISP1* transgenic roots than in roots transformed with the empty vector (EV) (Figure 3C). Next, we compared the nodulation phenotypes of the EV and *OX-GmNISP1* transgenic roots. As shown in Figure 3B, *OX-GmNISP1* roots had significantly

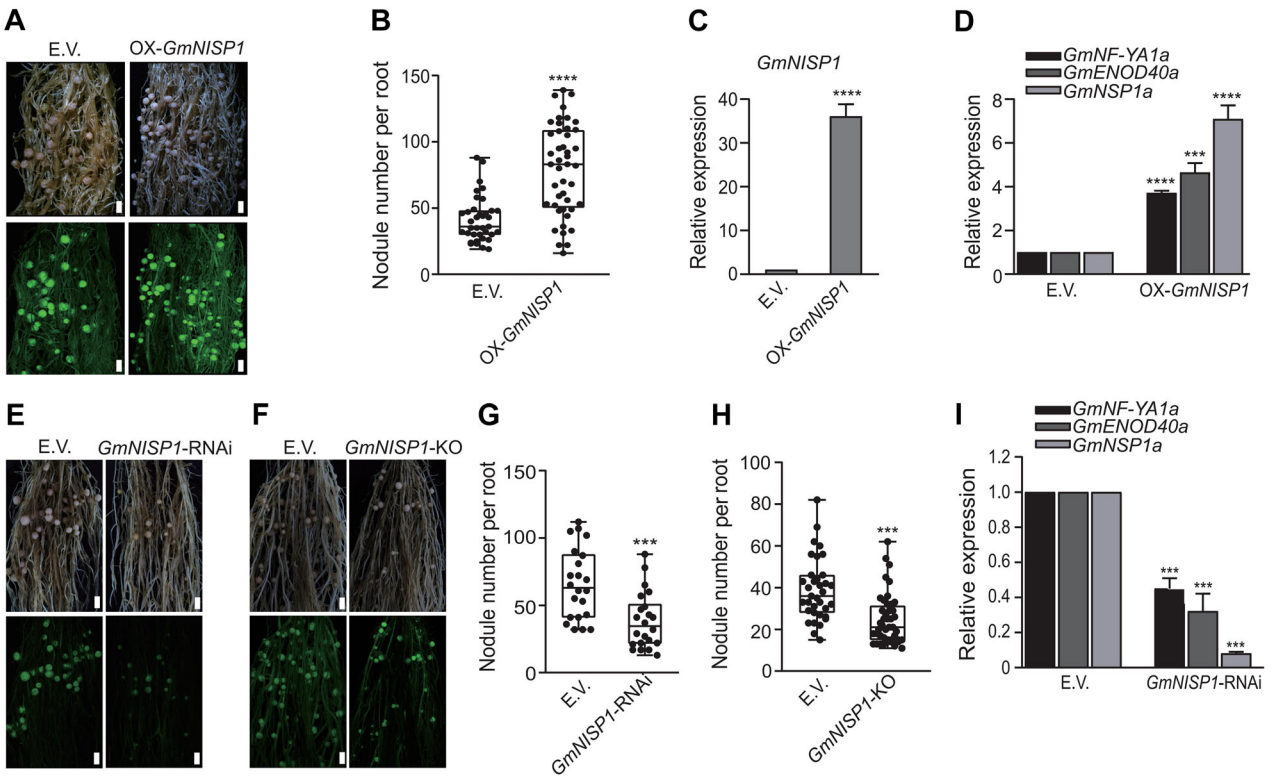

Figure 3. *Glycine max* Nodulation Receptor Kinase-Interacting Small Protein (GmNISP1) positively regulates nodule organogenesis in soybean

(A) Representative hairy roots expressing empty vector (EV) or the OX-*GmNISP1* construct 21 d post-inoculation (dpi) with rhizobia. Bar = 1 cm. **(B)** Nodule numbers per root 21 dpi in roots transformed with EV or OX-*GmNISP1*. Three biological replicates and at least 35 independent transgenic hairy roots per construct were performed and used for statistical analysis. Data are mean ± *SE* (Student's *t*-test: ****$P < 0.0001$). **(C)** *GmNISP1* transcript levels in EV and OX-*GmNISP1* transgenic roots. The relative expression was calculated by normalizing the transcription level of *GmATS1*. Data are mean ± *SE* ($n = 3$, Student's *t*-test: ****$P < 0.0001$). **(D)** Transcript levels of nodulation marker genes *GmNF-YA1a*, *GmENOD40a*, and *GmNSP1a* in EV and OX-*GmNISP1* transgenic roots. Data are mean ± *SE* ($n = 3$, Student's *t*-test: ***$P < 0.001$; ****$P < 0.0001$). **(E)** and **(F)** Representative hairy roots of *GmNISP1* knockdown (*GmNISP1*-RNA interference (RNAi)) and knock-out (*GmNISP1*-KO) transgenic plants 14 dpi. Bar = 1 cm. **(G)** and **(H)** Nodule numbers per root at 14 dpi for hairy roots transformed with *GmNISP1*-RNAi ($n > 20$) or *GmNISP1*-KO ($n > 35$). Data are mean ± *SE* from three biological replicates with at least 25 individual transgenic roots used in each repeat (Student's *t*-test: ***$P < 0.001$). **(I)** Transcript levels of nodulation marker genes *GmNF-YA1a*, *GmENOD40a*, *GmNSP1a* in EV and *GmNISP1*-RNAi transgenic roots. The relative expression was calculated by normalizing the transcription level of the examined gene against that of *GmATS1*. Data are mean ± *SE* ($n = 3$, Student's *t*-test, ***$P < 0.001$).

more nodules than control roots at 21 d post-inoculation (dpi) with *B. diazoefficiens* USDA 110. In the same plants, we also measured the expression levels of the symbiotic marker genes *GmNSP1a*, *GmENOD40a*, and *GmNF-YA1a*, all of which were markedly more up-regulated after inoculation in *OX-GmNISP1* hairy roots compared with the control (Figure 3D). However, overexpression of either *GmNISP-L1* or *GmNISP-L2*, and the homologs of GmNISP1, were not observed to develop altered nodule numbers compared with the control transgens (Figure S3A).

We also investigated the effect of RNA interference (RNAi) knockdown of *GmNISP1* in soybean using *A. rhizogenes*-mediated hairy root transformation. *GmNISP1* transcript levels were detected at low levels in the *GmNISP1*-RNAi roots compared to the control transgens (Figure S3B). More than 20 individual transgenic roots with decreased expression of *GmNISP1* were inoculated with rhizobia to examine their symbiotic phenotypes; at 14 dpi, all had significantly fewer nodules per root compared with the EV controls (Figure 3E, F). We then generated transgenic hairy roots with *GmNISP1* knocked out (*GmNISP1*-KO) using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology. Two different guide RNAs targeting *GmNISP1* were included in the same construct for transgenic experiments. We obtained more than 20 individual transgenic roots (*GmNISP1*-KO) in which editing of *GmNISP1* was confirmed by sequencing (Figure S4A). When these roots were inoculated, their nodule numbers were significantly reduced at 14 dpi compared with the controls (Figure 3G, H). The nodulation marker genes *GmNSP1a*, *GmENOD40a*, and *GmNF-YA1* were expressed at reduced levels in the *GmNISP1*-RNAi and *GmNISP1*-KO hairy roots compared with controls after inoculation with rhizobia (Figures 3I, S4B), indicating that GmNISP1 plays an important role regulating root nodulation in soybean.

GmNORK phosphorylates GmNISP1

NORK/SYMRK/DMI2 is a receptor kinase with strong auto- and trans-phosphorylation activity that is required for root nodule symbiosis (Yoshida and Parniske, 2005; Saha et al., 2016), which led us to ask whether GmNISP1 is a phosphorylation target of GmNORK. To test this hypothesis, a kinase assay was carried out in *Escherichia coli* cells using the modified Duet expression system (Han et al., 2017). For this experiment, we fused the CDs of both GmNORKα (GmNORKα^{CD}) and the kinase-dead version GmNORKα$^{CD\text{-}K618E}$ to Myc tag in a compatible Duet expression vector (Novagen). A recombinant protein in which GmNISP1 fused with hexahistidine (6× His) and Strep-tag at its N-terminus was expressed in *E. coli*. As shown in Figure 4A, co-expression of GmNISP1 and GmNORKα^{CD} but not GmNORKα$^{CD\text{-}K618E}$ in *E. coli* resulted in a strong band shift representing GmNISP1. The addition of lambda phosphatase, a reagent widely used to reverse phosphorylation, resulted in the loss of band shift representing phosphorylated GmNISP1 and GmNORKα (Figure 4A). These results indicate that GmNISP1 is directly phosphorylated by GmNORKα.

To confirm the phosphorylation of GmNISP1 by GmNORKα, an *in vitro* kinase assay in presence of $^{[\gamma 32P]}$ATP was carried out. MBP-tagged NORKα^{CD} and kinase-dead version of NORKα$^{CD\text{-}K618E}$, His-tagged NISP1 and NISP1 with different point mutations were all expressed and purified from *E. coli*. As shown in Figure S5A, NORKα^{CD} but not NORKα$^{CD\text{-}K618E}$ could directly phosphorylate NISP1 by making a clear band after autoradiography. These data confirmed that NORK could physically phosphorylate GmNISP1 *in vitro*.

Phosphorylation of GmNISP1 promotes nodule development

To determine which GmNISP1 residues are phosphorylated by GmNORKα, the shifted band representing phosphorylated GmNISP1 on sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) was analyzed by mass spectrometry. We identified 12 potential phosphorylation sites, which were located in the N-terminal domain (S4, Y7, S12, T17, S19, and T22) and the variable domain (S35, S39, T43, T45, T54, T55) (Table S1). We then purified a mutated version of GmNISP1 with all 12 potential phosphorylation sites substituted with alanines (GmNISP1^{12A}) and tested the mutant peptide in a kinase assay with GmNORKα. As shown in Figure 4B, the mobility shift of GmNISP1^{12A} was significantly reduced compared with wild-type GmNISP1. However, a significantly weak band shift was still detected for GmNISP1^{12A} in the presence of GmNORKα^{CD}, demonstrating that GmNISP1 may have other unidentified phosphorylation sites (Figure 4B). Taken together, the data suggested that the 12 residues of GmNISP1 are major phosphorylation sites of GmNORKα.

Phosphorylated peptides containing Ser-35 and Ser-39 were shown to be repeatedly detected during mass spectrometry. We then tested the phosphorylation of GmNISP1 by GmNORKα in an *in vitro* kinase assay. As shown in Figure S5B, substitution of Ser-35 but not Ser-39 with alanine of GmNISP1 was detected at low phosphorylated levels compared with GmNISP1, suggesting that Ser-35 of GmNISP1 is one of the phosphorylation sites by GmNORKα. The function of Ser-35 was tested in transgenic roots. Overexpression of both *GmNISP1^{S35A}* and *GmNISP1^{S35D}* induced more nodules generated on the transgenic roots compared with control (Figure S5C). However, transgenic roots expressing *GmNISP1^{S35D}* produced many more nodules than the roots expressing *GmNISP1^{S35A}* (Figure S5C). The data indicated that Ser-35 is one of the important residues phosphorylated by GmNORK.

To investigate whether the phosphorylation of GmNISP1 is related to its symbiotic function, we synthesized another version of *GmNISP1* (*GmNISP1^{12D}*) in which all 12 phosphorylation sites were changed to aspartates, acidic residues which are widely used to mimic phosphorylation. We generated transgenic soybean hairy roots overexpressing *GmNISP1^{12A}* and *GmNISP1^{12D}* and assessed their symbiotic

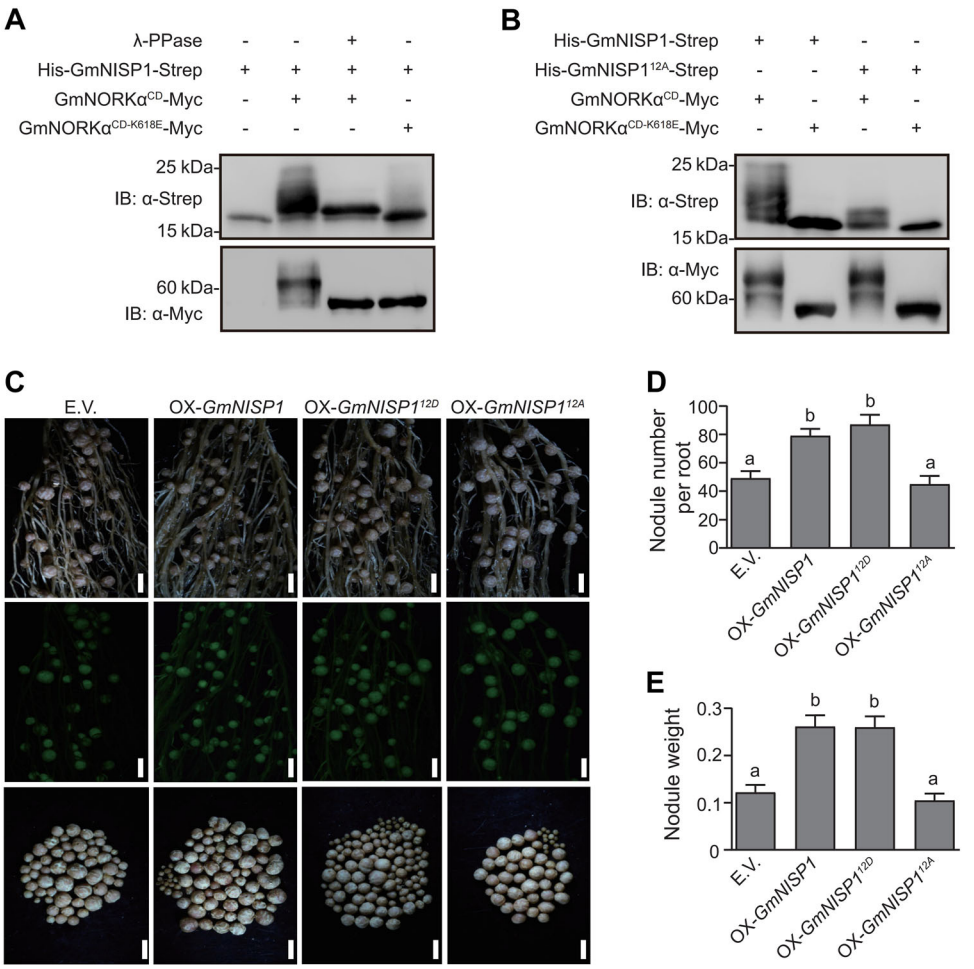

Figure 4. *Glycine max* Nodulation Receptor Kinase-Interacting Small Protein (GmNISP1) is phosphorylated by GmNORKα and promotes nodulation in its phosphorylated state

(A) GmNORKα phosphorylates GmNISP1. His-GmNISP1-Strep and GmNORKα^{CD} or GmNORKα$^{CD\text{-}K618E}$ expressed from *Escherichia coli* were separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblot with an anti-Strep antibody (upper panel) or an anti-Myc antibody (lower panel). Phosphorylation was blocked by adding Lambda Protein Phosphatase (λ-PPase) to the reaction mix. Data are representative of three independent experiments. **(B)** Phosphorylation of GmNISP1 by GmNORKα is prevented if the 12 potential phosphorylation sites of GmNISP1 are mutated to alanine (GmNISP1^{12A}). The recombinant proteins His-GmNISP1-Strep or His-GmNISP1^{12A}-Strep and Myc-GmNORKα^{CD} or Myc-GmNORKα$^{CD\text{-}K618E}$ were separated by SDS-PAGE and analyzed by Western blotting with an anti-Strep antibody (upper panel) or an anti-Myc antibody (lower panel). Data are representative of three independent experiments. **(C)** Nodules from transgenic roots overexpressing wild-type *GmNISP1*, *GmNISP1^{12D}* or *GmNISP1^{12A}* 21 d post-inoculation (dpi). EV, empty vector control. Bar = 1 cm. **(D)** and **(E)** Nodule numbers and nodule weight per root at 21 dpi from transgenic roots overexpressing *GmNISP1*, *GmNISP1^{12D}* or *GmNISP1^{12A}*. Data are mean ± *SE* from three biological replicates with at least 25 independent soybean roots used in each repeat (Student's *t*-test, $^{***}P < 0.001$).

phenotypes. Transgenic roots overexpressing *GmNISP1^{12D}* or wild-type *GmNISP1* produced increased nodule numbers and nodule weights compared with controls at 21 dpi (Figure 4C–E). However, no differences in the nodule number or nodule weight were observed in the transgenic roots overexpressing *GmNISP1^{12A}* compared to control roots (Figure 4C–E). These results suggested that phosphorylation of GmNISP1 plays an essential role in the establishment of symbiosis.

GmNISP1 localizes at the apoplast

To determine the subcellular localization of GmNISP1, a green fluorescent protein (GFP) fusion protein was expressed in *N. benthamiana* leaves. GmNISP1-GFP fluorescence was observed in the cytosol, nucleus, and plasma membrane (Figure 5A). To further pinpoint the subcellular localization of GmNISP1, a plasma membrane marker protein, At-CERK1^{K350N} (kinase-dead version), was fused with mCherry fluorescent protein and co-expressed with GmNISP1-GFP in *N. benthamiana* leaves. Cells were plasmolyzed to investigate the intra- and extracellular distribution of fluorescently labeled GmNISP1. As shown in Figure 5D, the fluorescence signals were observed in the extracellular matrix and at the plasma membrane in *N. benthamiana* leaves (Figure 5D). In epidermal cells transformed with GmNISP1-GFP, the intensity of the GFP fluorescence was greater outside than

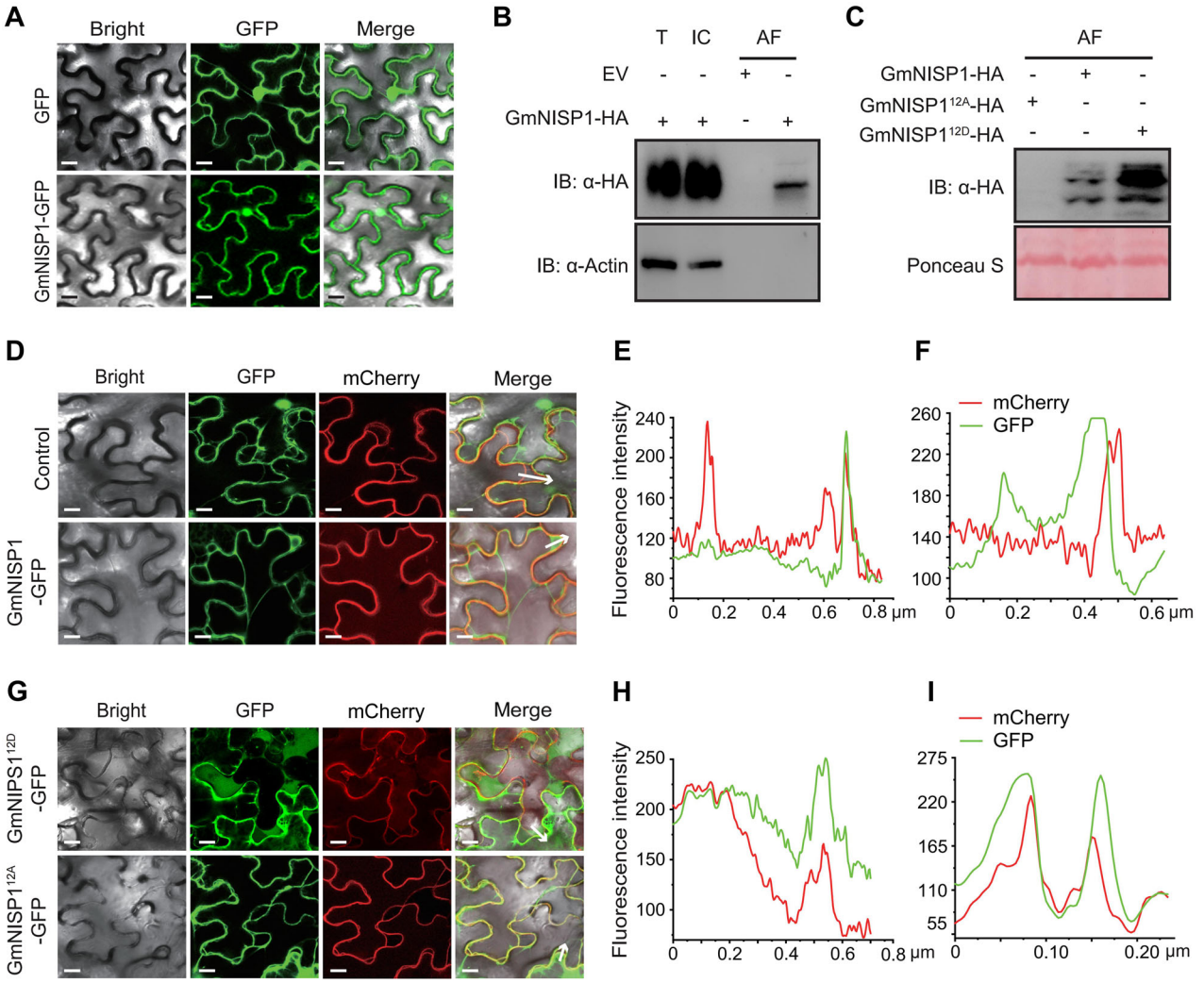

Figure 5. Subcellular localizations of *Glycine max* Nodulation Receptor Kinase α (GmNORKα) and GmNORK-Interacting Small Protein (GmNISP1)

(A) Subcellular localization of GmNISP1 in *Nicotiana benthamiana* leaf epidermal cells. Green fluorescence represents the signal from *N. benthamiana* leaves expressing GmNISP1-GFP or green fluorescent protein (GFP) control. Bar = 50 μm. **(B)** Immunoblot analysis of hemagglutinin (HA)-tagged GmNISP1 protein present in the apoplastic space of *N. benthamiana* leaves. Immunoblot analysis using anti-HA antibodies (upper panel) and anti-actin antibody (lower panel) shows NISP1 levels and equal loading for each lane, respectively. AF, apoplastic fluid; IC, intracellular protein; T, total protein. Data are representative of three independent experiments. **(C)** Immunoblot analysis of HA-tagged GmNISP1, GmNISP1^{12D}, and GmNISP1^{12A} protein present in the apoplastic space of *N. benthamiana* leaves. Immunoblot analysis using anti-HA antibodies shows NISP1 protein level (upper panel). The lower panel shows similar loading for each lane as indicated by Ponceau staining. **(D–F)** A plasma membrane-localized receptor (AtCERK1^{K350N}) fused with mCherry co-expressed with GmNISP1-GFP or GFP control in *N. benthamiana* leaves. The fluorescence images were taken using a confocal laser scanning microscope. *N. benthamiana* epidermal cells were treated with 1 mol/L NaCl for 3–5 min to induce plasmolysis before imaging. White arrows mark the vectors along which the distribution of fluorescence intensities was scanned. The fluorescence intensity was calculated by ImageJ. Bar = 50 μm. **(G–I)** AtCERK1^{K350N}-mCherry co-expressed with GmNISP1^{12D} or the GmNISP1^{12A} in *N. benthamiana* leaves. The fluorescence images were taken and calculated under the same condition shown above. Bar = 50 μm.

inside the cells (Figure 5E, F), indicating that GmNISP1 could be present in the extracellular matrix. To further test whether GmNISP1 is in the apoplast, the apoplastic fluid was collected by gentle centrifugation and analyzed by SDS-PAGE followed by immunoblotting to detect protein abundance. Although most of the GmNISP1 protein was present in the intracellular fraction, some was also observed in the apoplastic fluid (Figure 5B), which is consistent with the above observations that GmNISP1 protein is present in the apoplast. These results demonstrate that GmNISP1 might be a secretory protein that is transported into the extracellular space.

Phosphorylation of GmNISP1 increases its secretion in the apoplast

Given our finding that the phosphorylation of GmNISP1 positively regulates root nodule symbiosis in soybean, we next asked whether phosphorylation by GmNORKα could

regulate GmNISP1 protein secretion. We generated GmNISP1^{12A}-GFP and GmNISP1^{12D}-GFP fusion constructs for protein localization assays in *N. benthamiana* leaves. After plasmolysis of the transformed leaves, the GFP fluorescence signal in the apoplast was significantly higher for GmNISP1^{12D}-GFP than for GmNISP1^{12A}-GFP (Figure 5G). Consistent with this, the GmNISP1^{12A}-GFP fluorescence signal was more intense in the cytosol (Figure 5H, I). Immunoblot analysis also showed that GmNISP1^{12D}-HA was more abundant than either wild-type GmNISP1-HA or GmNISP1^{12A}-HA in the apoplast (Figure 5C). These results are consistent with the idea that the phosphorylation of GmNISP1 might enhance its secretion into the apoplast.

Exogenous treatment with purified GmNISP1 promotes soybean nodulation

To investigate whether GmNISP1 acts as a signal molecule to modulate soybean nodulation, we expressed and purified GST-tagged GmNISP1 protein from *E. coli* cells. We then applied different concentrations of the purified GmNISP1 protein to soybean roots before inoculation with rhizobia. At 14 and 21 dpi, the roots treated with purified GmNISP1-GST had more nodules than did roots treated with GST alone (Figures 6A, B, S6A, B). In addition, treatment with 100 nmol/L GmNISP1-GST enhanced the expression levels of several nodulation marker genes (i.e., *GmNIN1a*, *GmNISP1a*, *GmENOD40a*, and *GmNF-YA1a*) compared with rhizobium treatment alone in the soybean nodules (Figure 6C–F). Taken together, these data suggest that GmNISP1 might function as a peptide hormone promoting soybean root nodulation.

Given that GmNORK could phosphorylate GmNISP1, we then asked whether treatment with phosphorylated GmNISP1 could enhance soybean nodulation even more than non-phosphorylated GmNISP1. GmNISP1-GST was purified from *E. coli* with or without co-expression of NORKα^{CD}, yielding phosphorylated or non-phosphorylated GmNISP1-GST (GmNISP1^{P} and GmNISP1), respectively. GmNISP1^{12A}-GST and GmNISP1^{12D}-GST were also purified from *E. coli*, yielding GmNISP1^{12A} and GmNISP1^{12D}. These purified proteins were used to treat soybean plants prior to inoculation. Treatment of soybean roots with any of the purified proteins increased nodule numbers compared with the GST mock treatment. Treatment with GmNISP1^{12D} and GmNISP1^{P} resulted in a greater increase in nodule number

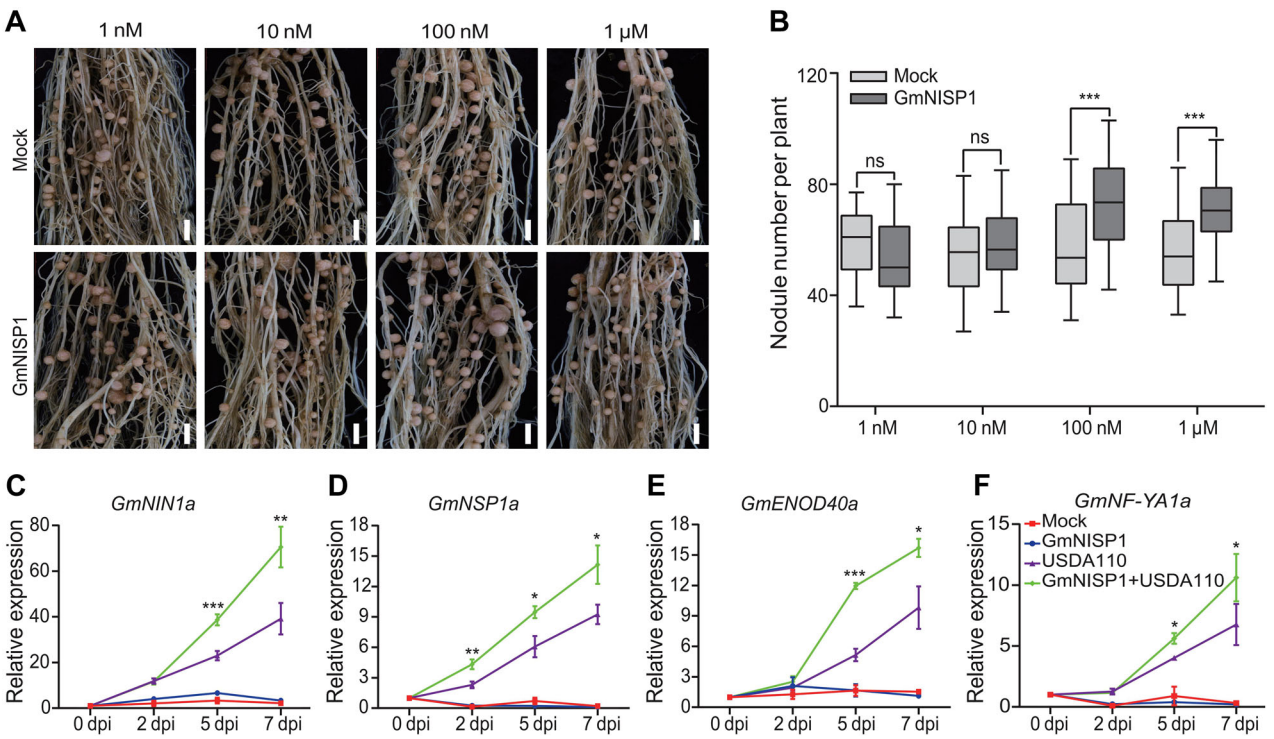

Figure 6. Treatment with purified *Glycine max* Nodulation Receptor Kinase-Interacting Small Protein (GmNISP1) protein increased soybean nodule number

(A) Representative soybean roots treated with different concentrations (1 nm, 10 nmol/L, 100 mol/L, 1 μmol/L) of glutathione-S-transferase (GST)-tagged GmNISP1 protein. Roots treated with GST served as the mock control. Bar = 1 cm. **(B)** Nodule numbers per plant at 14 d post-inoculation (dpi) after treatment with GST-GmNISP1 protein. Data are mean ± *SE* from three biological replicates with at least 35 independent soybean roots used in each repeat (Student's *t*-test, ***$P < 0.001$; n.s., not significant). **(C–F)** Transcript levels of nodulation marker genes *GmNIN1a* **(C)**, *GmNSP1a* **(D)**, *GmENOD40a* **(E)**, and *GmNF-YA1a* **(F)** in soybean roots treated with GST-GmNISP1 or GST control and inoculated with *Bradyrhizobium diazoefficiens* USDA 110 were analyzed using real-time quantitative polymerase chain reaction (RT-qPCR). Roots treated with water (uninoculated roots) were also harvested at the same time points and served as the mock controls. The relative expression was calculated by normalizing the transcription level of the examined gene against that of *GmATS1*. Data are mean ± *SE* ($n = 3$, Student's *t*-test: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

than treatment with the other proteins (Figures 7A–F, S6C). Consistent with this, expression of the nodulation marker genes *GmNIN1a*, *GmNISP1a*, *GmENOD40a*, and *GmNF-YA1a* was enhanced after application of 100 nmol/L GmNISP1 or GmNISP1^{12D} (Figure S7A–D). These data suggest that phosphorylation of GmNISP1 by GmNORKa could enhance root nodulation in soybean.

DISCUSSION

Legumes have evolved complex and precise signal transduction mechanisms to establish mutualisms with soil rhizobia (Ferguson et al., 2019; Chiu and Paszkowski, 2020). Accumulating evidence demonstrates that the formation of nitrogen-fixing root nodules is strictly regulated by both local and systemic endogenous signals (Okamoto et al., 2016; Nishida and Suzaki, 2018). The first interaction between rhizobia and host legumes is the recognition of rhizobial Nod factors by the plant's receptor-like kinase (Indrasumunar et al., 2010; Indrasumunar et al., 2011). NORK/SYMRK/DMI2 is another receptor kinase that functions as a co-receptor of NFRs to mediate symbiotic signal transduction leading to rhizobial endosymbiosis and nodule development (Ried et al., 2014). Hence, the phosphorylation process mediated by receptor kinases is required for signal transduction from plasma membrane to downstream components. Although several proteins were identified as interacting components of NORK/SYMRK/DMI2, most of them were not shown to be directly phosphorylated by NORK/SYMRK/DMI2. How NORK/SYMRK/DMI2 mediates signal transduction to the downstream components via direct phosphorylation is of great interest to be determined.

Here, we proposed an interesting model that soybean GmNORK interacts with and directly phosphorylates a secretory protein, GmNISP1, to promote nodulation in soybean (Figure 7G). The secretory process of GmNISP1 to the apoplast might be enhanced after phosphorylation by GmNORKα. Together with the fact that GmNISP1 is a small protein containing a "DY" consensus motif at its N-terminus which is a typical feature of peptide hormone (Doblas Verónica et al., 2017; Nakayama et al., 2017), we proposed that NORK phosphorylation might incorporate the peptide hormone signaling pathway to regulate nodulation in legumes. However, the mechanisms of phosphorylation-mediated proteolysis of GmNISP1 is of great interest to be determined.

Peptide hormones have a systemic function in modulating nodule number. For instance, the rhizobia-induced and

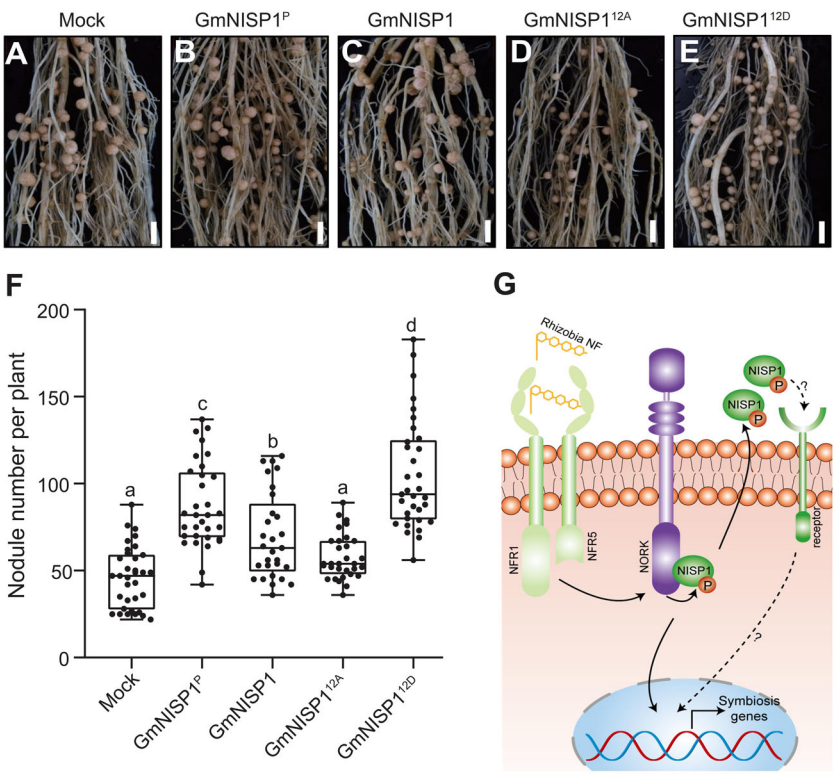

Figure 7. Phosphorylation of *Glycine max* Nodulation Receptor Kinase-Interacting Small Protein (GmNISP1) enhances nodulation
(A–G) Representative soybean roots treated with different forms of glutathione-S-transferase (GST)-GmNISP1 protein at 100 nmol/L. **(B)**, **(D)**, and **(F)** GST-GmNISP1, GST-GmNISP1-12D, and GST-GmNISP1-12A proteins were expressed and purified from *Escherichia coli* cells. GST-GmNISP1 was also purified from *E. coli* co-expressed with NORKα^{CD} to generated a phosphorylated version of NISP1^{P}. **(C)**, **(E)**, and **(G)** The same amount proteins purified from *E. coli* were set as the unphosphorylated forms. Roots treated without GmNISP1 protein served as the mock control. Bar = 1 cm.

nodule-associated CLE peptides are long-distance root-to-shoot signals that specifically interact with shoot receptors involved in autoregulation of nodulation (e.g., NODULE AUTOREGULATION RECEPTOR KINASE (NARK) in soybean (Searle et al., 2003; Miyahara et al., 2008); SUPER NUMERIC NODULES (SUNN) in *M. truncatula* (Schnabel et al., 2005); and HYPERNODULATION ABERRANT ROOT FORMATION (HAR1) in *L. japonicus* (Nishimura et al., 2002)). GmNISP1 similarly appears to play an integral role in regulating nodule organogenesis. Soybean roots treated with purified GmNISP1 protein formed significantly more nodules with increased symbiotic signaling responses activated than control nodules. Thus, GmNISP1 might function as another peptide hormone involved in root nodule symbiosis in soybean. The exact function of GmNISP1 as a peptide hormone will be elucidated with the identification of the receptor that recognizes GmNISP1 peptide.

With the essential role of NORK/SYMRK/DMI2 identified in symbiosis, several interacting proteins of NORK/SYMRK/DMI2 have been identified by different laboratories. Thus, the critical roles of NORK/SYMRK/DMI2 in regulating symbiosis were shown in different pathways, including mevalonate biosynthesis (Kevei et al., 2007), MAPK cascade In *L. japonicus* (Chen et al., 2012; Yin et al., 2019), *NIN* gene expression (Zhu et al., 2008), protein degradation-mediated by 26S proteasome (Vernie et al., 2016), and suppression of plant innate immunity (Feng et al., 2021). Among all these interacting proteins identified, the MtPUB1, a negative regulator mediating the degradation of MtDMI2, was shown to be phosphorylated by MtDMI2 in *M. truncatula* (Vernie et al., 2016). The negative role of MtPUB1 in symbiosis seems to function in a feed-back loop by inhibiting the overactivated signaling mediated by MtDMI2. Gα protein was shown to be a phosphorylation target of soybean NORK/SymRK to mediate root nodulation (Roy Choudhury and Pandey, 2022). But how NORK/SYMRK/DMI2 transduces symbiotic signal to downstream components *via* its kinase activity is of great interest to be characterized. GmNISP1 was identified as a new interacting protein of GmNORK. The important role of GmNISP1 in soybean nodulation and the direct phosphorylation of GmNISP1 by GmNROK provide insights into the symbiotic signaling pathway during legume–rhizobium symbiosis. Importantly, both function of GmNISP1 in promoting soybean nodulation, and the secretion of GmNISP1, seem to be facilitated after the phosphorylation by GmNORK. Thus, the central role of NORK/SYMRK/DMI2 in plant symbiosis was enriched and updated as integrating a potential peptide hormone pathway to promote nodulation in soybean.

In conclusion, this work shows that, in addition to its role in nodulation, GmNISP1 is also a novel secreted protein that acts as a phosphorylation substrate downstream of GmNORK. This finding provides a new perspective on how the receptor-like kinase of legumes activate signal transduction following perception of rhizobial signaling molecules in the establishment of symbiosis. However, more information is needed to elucidate the mode of action of GmNISP1 and whether there is a membrane-localized receptor to recognize secreted GmNISP1 in the apoplastic space to initiate nodulation signaling. Further experimentation is also required to determine whether the secretory pathway of GmNISP1 is enhanced after GmNORK phosphorylation during the initiation and progression of symbiosis. This work will help us understand how peptide hormones associate with the receptor-like kinase to mediate signaling cascades in endosymbiosis.

MATERIALS AND METHODS

Plant and rhizobium growth conditions

Soybean (*Glycine max*) cultivar Williams82 was used in this study. Soybean seeds were sterilized with chlorine gas for 14–16 h and germinated in vermiculite, and grown in a greenhouse under 16 h light/8 h dark at 25°C. Rhizobium *B. diazoefficiens* strain USDA 110 was cultured in a modified HM (HEPES-MES (2-[*N*-morpholino]ethanesulfonic acid)) medium composed of 1.3 g HEPES, 1.1 g MES, 0.25 g yeast extract, 1 g D-arabinose, 1 g sodium gluconate, 0.125 g Na_2HPO_4, 0.25 g Na_2SO_4, 0.32 g NH_4Cl, 0.18 g $MgSO_4{\cdot}7H_2O$, 0.004 g $FeCl_3$, and 0.001 g $CaCl_2$ per liter, adjusted to 6.6 pH with NaOH, and used for soybean root inoculation (optical density at 600 nm (OD_{600}) = 0.1). Nodule numbers were counted on soybean roots at 14 or 21 dpi with rhizobium.

Gene cloning and vector construction

The coding sequences of *GmNORKα* (Glyma.01G020100), *GmNORKβ* (Glyma.09G202300) and *GmNISP1* (Glyma.17G084600) were amplified from cDNA of Williams82 and cloned into the pEASY-Blunt cloning vector (TransGen Biotech, Beijing, China). For Y2H assay, the KDs (591–919 amino acids), the CD (539–919 amino acids) of GmNORKα, and GmNORKβ, the KD of GmNFR1 (Glyma.02G270800) andGmNFR5 (Glyma.11G063100), GmNISP1, GmNISP-L1 (NISP1-like 1; Gm04G18830), GmNISP-L2 (Gm06G177500) were cloned to pGADT7 or pGBKT7 using Gibson cloning method. For Co-IP assay, the full-length coding sequence of *GmNORKα* or *GmNISP1* without stop codon was cloned into the vector pUB-GFP-HA or pUB-GFP-FLAG using one step recombination reactions. For Split-LUC complementation assay, the full lengths of GmNORKα, GmNFR5 and GmNISP1 were cloned into engineered pGWB514-nLUC or pGWB514-cLUC. For pull-down assays, the CD of *GmNORKα* fused with Myc tag was cloned into the vector pMAL-c2X and the full length of *GmNISP1* was cloned into the vector pET-28a(+) in which the T7 tag was replaced by the Strep-tag. For *in vitro* protein expression and purification assay, the coding sequence of GmNISP1 was cloned into the pGEX-5X-1 vector. To analyze the expression pattern of *GmNISP1*, a 1.9 kb DNA fragment upstream of the translation start site was PCR-amplified and inserted into the *Bam*HI/*Hind*III site of DX2181-mCherry vector. Phosphorylation between GmNORKα and GmNISP1 was tested

by co-expression in *E. coli* cells in an *in vitro* phosphorylation assay. Briefly, *GmNISP1* and its point mutation genes were first cloned into the pET-28a(+) vector to generate C-terminal His fusions (Strep-GmNISP1-His). Next, GmNORKα or its kinase-dead mutation were fused with Myc tag and inserted between *Nde*I and *Xho*I in the MCS-II of pCDFDuet-1 to generate pCDFDuet-1:Myc-GmNORKα plasmid. The two plasmids, pET28-GmNISP1 and pCDFDuet-1:Myc-GmNORKα, were co-transformed into the *E. coli* strain BL21-CodonPlus RIL and protein expression was induced with 0.3 mmol/L isopropylthio-β-galactoside for 20 h at 18°C. For overexpression, the full length of GmNISP1 fused Myc tag was inserted in the modified pCAMBIA-CvMV vector (Graham et al., 2007). For RNAi silencing, the pG2RNAi2 plasmid (GenBank: KT954097) was used for vector construction. The full coding sequence of GmNISP1 was PCR-amplified using gene-specific primers containing *Asc*I/*Avr*II and *Swa*I/*Bam*HI sites in the forward and reverse primers, respectively. The PCR products were digested, purified, and ligated into the binary vector pG2RNAi2 under the control of the soybean *Ubiquitin* (*GmUbi*) promoter forming the hairpin construct. Then the recombinant plasmid was introduced into *A. rhizogenes* K599 for generating transgenic hairy roots. The CRISPR-Cas9 system was used to knock out *GmNISP1* in soybean. Single guide RNAs (sgRNAs) were designed using CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/), a web-based guided RNA design tool. The primer pairs as listed in Table S2, were annealed at 95°C for 5 min and then cool down to room temperature to form dimers, which were then integrated into the *Bbs*I-digested pBlueScript SK(+)-LjU6 vector. The resulting plasmid pBlueScript SK(+)-LjU6-sgRNA was digested with *Kpn*I and *Xba*I and ligated into pCAMBIA1300-sGFP-LjUBQpro-Cas9 for hairy root transformation. All primers used in this study are listed in Table S2.

Hairy root transformation

For overexpression, RNAi, CRISPR, and promoter-GUS assays, hairy root transformation was performed according to a previous report (Tóth et al., 2016). Soybean seeds lacking macroscopic signs of fungal or viral contaminations were surface-sterilized for 16–20 h in a desiccator jar with chlorine gas generated by adding 3.5 mL 12 N HCl into 100 mL household bleach (6% sodium hypochlorite). After germination, 5-d-old seedlings were cut slantwise above the hypocotyl, then the root was removed and co-cultured with *A. rhizogenes* strain K599 carrying the binary vector. Successful transformation was indicated by the presence of fluorescence from GFP or mCherry, which were used as visual markers. Only one healthy transgenic root was retained from each plant. Transgenic roots were inoculated with USDA 110 (OD_{600} = 0.1), and the nodules were counted at 14 or 21 dpi.

Histochemical staining

Positive transgenic roots harboring the promoter-GUS were soaked in a solution consisting of 0.5 mmol/L potassium ferricyanide, 0.5 mmol/L potassium ferrocyanide, 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 0.5 mg/mL X-Gluc, 0.1% SDS, 0.1% Triton X-100, and 0.1 mol/L sodium phosphate buffer (pH 7.0). After evacuation for 30 min, root samples were incubated in the staining solution overnight at 37°C in darkness. To stop the reaction and clear the roots, the samples were washed with 75% ethanol three times. The GUS-staining tissues were observed and photographed under a stereomicroscope.

Phylogenetic analysis

All the sequences were obtained from the Phytozome (https://phytozome-next.jgi.doe.gov/), then the amino acid sequences were aligned by ClustalX2; a phylogenetic tree of the alignment was generated with MEGA 5 software using the Neighbor-Joining method. Jalview software (University of Dundee) was used to display the multiple alignments.

RNA extraction and RT-PCR

Total RNA was extracted from soybean tissues with Biomarker Plant Total RNA Isolation Kit (Biomarker, Beijing, China). The RNA concentrations were quantified by using NanoDrop 2000 (Thermo-Fisher, Waltham, MA, US).

About 1 μg of total RNA was used for first-strand cDNA synthesis with ABScript II cDNA First Strand Synthesis Kit (Abclonal, Wuhan, China). The synthesized cDNA was then diluted 10 times for subsequent experiments. All RT-qPCR experiments were performed using an ABI ViiA 7 Real-Time PCR System under the standard cycling mode. The 10 μL reaction mixture was set up as follows: 2× qPCR SYBR Green Master Mix (11202ES03; Yeason, China), cDNA, and 10 μmol/L primers under the following conditions: 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 15 s. The integrity of the RT-qPCR products was confirmed by the melt curves, which only showed a single peak. Three biological replicates were used for all RT-qPCR reactions. *GmATS1* (Glyma.20G123600) served as a reference gene. All the primer sequences are listed in Table S2.

Yeast two-hybrid screen

Yeast two-hybrid assays were conducted according to the instruction in the yeast protocol handbook (Clontech, Palo Alto, CA, USA). GmNISP1, GmNISP-L1, GmNISP-L2 and the KDs and/or CDs of GmNORKα, GmNORKβ, GmNFR1α, and GmNFR5α were cloned into pGADT7 (for expression of activation domain-fused protein) and pGBKT7 (for expression of binding domain-fused protein) vectors. Different pairs of constructs were introduced into yeast strain AH109 and the cells were grown on synthetic defined (SD)/-2 synthetic dropout medium (-Trp/-Leu) and SD/-4 selective medium (-Trp/-Leu/-His/-Ade) and incubated at 28°C for an additional 4–5 d. Crude proteins from yeast cells expressing AD-GmNORKα^{CD}, AD-GmNORKα^{KD}, AD-GmNORKα$^{KD\text{-}K618E}$, AD-GmNORKβ^{CD}, AD-GmNORKβ^{KD}, AD-GmNORKβ$^{KD\text{-}K618E}$, and BD-GmNISP1 were extracted using Yeast Total Protein Extraction Kit (Sangon Biotech, C500013). Immunoblotting was performed using the anti-HA (12013819001; Sigma) and anti-Myc (626808; Bio-Legend) antibodies.

Split-LUC complementation assay

The plasmids including GmNISP1-nLUC, GmNFR5-nLUC,FLS2-nLUC, GmNORKα-cLUC, FLS2-cLUC, and GmNORKα-cLUC were introduced into *Agrobacterium tumefaciens* strain EHA105. After cultivation overnight and resuspension in infiltration buffer (10 mmol/L $MgCl_2$, 10 mmol/L MES, pH 5.7, and 200 μmol/L acetosyringone). The ODs of the cell suspensions in infiltration medium were adjusted to $OD_{600} \approx 0.4$ (final concentration for each strain in a mixture). Then, the mixture was co-infiltrated into *N. benthamiana* leaves with *A. tumefaciens* carrying P19 (35Spro:P19) for transient expression. *Arabidopsis* FLS2 (Flagellin Sensing 2), a LRR-RLK, was constructed as FLS2-cLUC and FLS2-nLUC and used as negative controls. Three days after infection, leaves were sprayed with 1 mmol/L luciferin (E1603; Promega) and the LUC activity was monitored using the Tanon 4600 Imaging system (Tanon Science and Technology Co. Ltd).

Co-immunoprecipitation

For Co-IP assays, both GmNORKα-HA and GmNISP1-FLAG were transformed into *A. tumefaciens* EHA105, and then co-injected into *N. benthamiana* leaves with *A. tumefaciens* EHA105 carrying P19 for transient expression. Total proteins were extracted from the *N. benthamiana* leaves in IP buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% TritonX-100, 5% glycerol) with protease inhibitor cocktail (S25910; Yuanye, Shanghai). Samples were incubated on ice for 30 min and centrifuged three times at 12,000 × *g* for 10 min at 4°C. The supernatant was added to 20 μL anti-Flag M2 agarose beads (A2220; Sigma) followed by end-to-end rotation at 4°C for 3 h. The beads were washed at least five times with washing buffer (50 mmol/L Tris-HCl, pH7.4, 150 mmol/L NaCl). Western blotting was performed using the anti-HA (clone 3F10; Sigma) and anti-FLAG (F1804; Sigma) antibodies.

Protein expression and purification

For prokaryotic protein expression, GST-, MBP- or His-tags fused recombinant proteins were expressed and purified from *E. coli* strain BL21-CodonPlus RIL cells. For *in vitro* pull-down assays, the whole CD of GmNORKα was fused to MBP-tag to generate MBP-GmNORKα^{CD}-Myc recombinant plasmid. The recombinant protein His-GmNISP1-Strep was incubated with MBP-Myc or MBP-GmNORKα^{CD}-Myc immobilized onto amylose-sepharose beads (New England Biolabs, Ipswich, MA, USA) in phosphate-buffered saline (PBS) at 4°C overnight. After washing 10 times with PBS buffer, proteins retained on the beads were resolved by SDS-PAGE and detected using an anti-Strep antibody (AB_2863792; ABclonal). The equal loading for each lane representing protein input was detected using Coomassie brilliant blue dye. Protein purification of the GST-tagged GmNISP1 was performed according to the manufacturer's instructions (Genscript, Nanjing, China).

Protein kinase assay

The phosphorylation reaction was carried out using a modified Duet vector system in *E. coli* cells (Han et al., 2017). The Western blotting was performed using the anti-Strep (AB_2863792; ABclonal) and anti-Myc (626808; Bio-Legend) antibodies. For phosphatase treatment, the reconstituted reaction mixture was added to Lambda Protein Phosphatase (λPP) (#P0753S; New England Biolabs) following the manufacturer's instruction at 30°C for 30 min. For tandem mass spectrometry analysis, all the samples were separated on SDS-PAGE gel and excised after staining with Coomassie brilliant blue dye.

In vitro kinase assay

About 0.5 μg of recombinant proteins purified from *E. coli* was used in an *in vitro* kinase assay in presence of [$^{\gamma-32}$P]ATP. In brief, a buffer containing 20 mmol/L HEPES (pH 7.4), 15 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, and 5 μCi [$^{\gamma-32}$P] ATP was used for phosphorylation assay at 25°C for 30 min. Kinase reaction was terminated by adding 5× SDS loading buffer. Samples were directly separated *via* SDS-PAGE and imaged by autoradiography using a phosphor screen and FUJI BAS2500 (Fujifilm, Tokyo, Japan). The same gel after autoradiography was then stained with Coomassie blue to indicate a control.

Confocal laser scanning microscopy

For subcellular localization, the *GmNISP1* coding region was cloned into the engineered pGWB505-GFP in which the CaMV 35S promoter was replaced with the *LjUbq1* promoter. After infiltration into *N. benthamiana* for 3 d, the leaves were examined under a confocal microscope (N-STORM; Nikon, Japan). The excitation wavelengths for GFP and mCherry were 488 and 561 nm, respectively, and emission wavelengths were detected at 500–530 nm for GFP signals and at 575–615 nm for mCherry signals.

Isolation of apoplastic fluid

The recombinant plasmid pUB-GFP-GmNISP1-HA and its phosphorylation mutatants were transformed into *A. tumefaciens* EHA105, respectively, and infiltrated into *N. benthamiana*. The leaves 2 dpi were vacuum-infiltrated with apoplastic fluid (AF) buffer (50 mmol/L phosphate buffer, pH 7.0) for 2 min following published methods (Kalde et al., 2007; Xia et al., 2020). AF was collected by centrifugation (20 min, 700 × *g*), mixed with 2 × SDS sample buffer, and subjected to SDS-PAGE analysis. The immunoblotting was performed using the anti-HA antibody (12013819001; Sigma).

Exogenous treatment with purified proteins

The GmNISP1 fused with GST tag was expressed and purified from *E. coli* strain BL21-CodonPlus RIL. The concentration was detected using a Bradford Protein Assay Kit (T9310A; TaKaRa). Purified GmNISP1-GST protein was diluted into four concentrations, that is, 1, 10, 100 nmol/L, and 1 μmol/L, and exogenously applied to soybean roots. For treatment, soybean seeds were infiltrated with different

proteins with different concentrations during germination. These purified proteins were then applied to the vermiculite after soybean plants were transplanted. Treatments with purified proteins in the vermiculite were repeatedly performed five times before nodulation assay.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (32090063 to Y.C.), the National Key R&D Program of China (2016YFD0100702 to Y.C.), the Natural Science Foundation of Hubei Province (2020CFA008 to Y.C.), and a Fundamental Research Fund for the Central Universities (2662022SKYJ002 to Y.C.).

CONFLICTS OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Z.Y. initiated the project working on NISP1. Y.C. and B.F. conceived and designed the project. Y.C. supervised the study. B.F. performed all the research and analyzed all the data with help from Z.X., Y.L., R.D., and Y.W. X.G. and H.Z. provided most reagents and conceptual insights into the article. B.F. and Y.C. drafted and wrote the MS. Y.Z. provided revisions in the text for resubmission. All authors read and approved of this manuscript.

Edited by: Fanjiang Kong, Guangzhou University, China

Received Sep. 9, 2022; **Accepted** Dec. 15, 2022; **Published** Dec. 19, 2022

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.13436/suppinfo

Figure S1. *Glycine max* Nodulation Receptor Kinase (GmNORK)-Interacting Small Protein (GmNISP1) interacts with GmNORK

Figure S2. The expression pattern of *GmNISP1* during nodulation development stages

Figure S3. The expression level of *GmNISP1* and several nodulation marker genes in *GmNISP1*-RNA interference and *GmNISP1*-Knock-out transgenic positive hairy roots inoculated with rhizobia

Figure S4. Knock-out of *Glycine max* Nodulation Receptor Kinase (GmNORK)-Interacting Small Protein (GmNISP1) reduces the expression of symbiotic genes

Figure S5. The cytoplasmic domain (CD) of Nodulation Receptor Kinase (NORK) phopshorylates NORK-Interacting Small Protein (NISP1) in an *in vitro* kinase assay

Figure S6. The nodule number of 21 d post-inoculation (dpi) after application of different concentrations of *Glycine max* Nodulation Receptor Kinase (GmNORK)-Interacting Small Protein (GmNISP1)

Figure S7. Transcript level of nodulation marker genes *GmNIN1a* **(A)**, *GmNSP1a* **(B)**, *GmENOD40a* **(C)**, *GmNF-YA1a* **(D)** treated with 100 nmol/L purified glutathione-S-transferase (GST)-tagged *Glycine max* Nodulation Receptor Kinase (GmNORK)-Interacting Small Protein (GmNISP1), phosphorylation-mimic $GmNISP1^{12D}$ and the phosphorylation-inactive $GmNISP1^{12A}$ protein after inoculation with *B. diazoefficiens* USDA110

Table S1. *Glycine max* Nodulation Receptor Kinase α (GmNORKα) phosphorylation sites of GmNORK-Interacting Small Protein (GmNISP1) identified by mass spectrometry

Table S2. Primers used in this study

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