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SymRK Regulates G-Protein Signaling During Nodulation in Soybean (*Glycine max*) by Modifying RGS Phosphorylation and Activity

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Molecular interspecies dialogue between leguminous plants and nitrogen-fixing rhizobia results in the development of symbiotic root nodules. This is initiated by several nodulation-related receptors present on the surface of root hair epidermal cells. We have shown previously that specific subunits of heterotrimeric G-proteins and their associated regulator of G-protein signaling (RGS) proteins act as molecular links between the receptors and downstream components during nodule formation in soybeans. Nod factor receptor 1 (NFR1) interacts with and phosphorylates RGS proteins to regulate the G-protein cycle. Symbiosis receptor-like kinases (SymRK) phosphorylate Gα to make it inactive and unavailable for Gβγ. We now show that like NFR1, SymRK also interacts with the RGS proteins to phosphorylate them. Phosphorylated RGS has higher activity for accelerating guanosine triphosphate (GTP) hydrolysis by Gα, which favors conversion of active Gα to its inactive form. Phosphorylation of RGS proteins is physiologically relevant, as overexpression of a phospho-mimic version of the RGS protein enhances nodule formation in soybean. These results reveal an intricate fine-tuning of the G-protein signaling during nodulation, where a negative regulator (Gα) is effectively deactivated by RGS due to the concerted efforts of several receptor proteins to ensure adequate nodulation.

Keywords: heterotrimeric G-proteins, nodulation, protein-protein interaction, receptor-mediated phosphorylation, regulator of G-protein signaling, RGS proteins, RLK, soybean (*Glycine max*), symbiosis-related receptor-like kinase, SymRK

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Root nodule symbiosis (RNS) is a well-established mutualism between nitrogen-fixing rhizobia and legume roots, where rhizobia enable the conversion of atmospheric nitrogen into a form that is directly accessible to plants (Yamazaki et al. 2022). This endosymbiotic relationship contributes almost one-fifth of all nitrogen input (about 35 to 44 million tons) into agriculture worldwide (Verma et al. 2020). A complex genetic and molecular program governs a series of physiological and developmental changes in roots during nodule formation (Oldroyd 2013; Oldroyd et al. 2011). Upon perception of aromatic signals (flavonoids) from legume roots, compatible rhizobia release nodulation factors (NF), which are perceived by the host-plant cell surface localized receptors to initiate a series of signaling and developmental events. The rhizobia enter the epidermal cells of the host intracellularly or intercellularly. Concurrent division of the root cortical cells allows rhizobia to penetrate the root cortex tissue from infection threads or root epidermal cells. Rhizobia colonize the host cortex cells usually within a specialized organelle-like compartment termed "symbiosomes", where they differentiate into their nitrogen-fixating forms called bacteroids. The multiplication of bacteroids within the dividing cortical space and subsequent developmental events result in the formation of a mature nodule (Desbrosses and Stougaard 2011; Oldroyd et al. 2011).

Similar to many plant-developmental programs, RNS is regulated by receptor-like kinase (RLK) complexes (De Smet et al. 2009; Desbrosses and Stougaard 2011; Ghantasala and Roy Choudhury 2022; Ried et al. 2014). At the early infection stage, rhizobia-derived NF activates plasma membrane-localized Lysin-motif (LysM) RLKs, known as nod factor receptors 1 (NFR1) and 5 (NFR5) (Madsen et al. 2003; Radutoiu et al. 2003). In addition, a malectin-like domain leucine-rich repeat receptor kinase (MLD-LRR-RK), named symbiosis receptor-like kinase (SymRK), acts downstream of NFRs and contributes to signaling events that lead to early and later stages of symbiotic interactions (Endre et al. 2002; Gherbi et al. 2008; Holsters 2008; Stracke et al. 2002), whereas the nodule autoregulation receptor kinases (NARKs) control systemic signaling during nodule development (Kinkema and Gresshoff 2008).

SymRK from *Lotus japonicus* and its homologs DOES NOT MAKE INFECTIONS 2 (DMI2) (Abel et al. 2024) in *Medicago truncatula*, nodulation receptor kinase (NORK/SymRK) in *Glycine max*, and SYM19 in *Pisum sativum* encode a protein with three LRR domains in their predicted extracellular region, a transmembrane domain, and a protein kinase domain (KD) facing the cytoplasm (Endre et al. 2002; Holsters 2008; Stracke et al. 2002). The MLD-LRR domains in SymRK are probably indispensable for protein-ligand and protein-protein interactions,

whereas the intracellular KD is active in its autophosphorylated form (Abel et al. 2024; Bhattacharya et al. 2019; Saha et al. 2016; Yoshida and Parniske 2005). Despite their crucial roles in the regulation of nodulation (and the common symbiotic pathway [CSP]), the mechanistic details of SymRK are relatively unclear. To date, multiple SymRK-interacting partners have been identified in different plants. For example, in *Medicago*, 3-hydroxy-3-methylglutaryl CoA reductase1 (HMGR1), a key enzyme that regulates the mevalonate pathway, promotes the production of cytokinins and steroids to modulate the cell division during nodule organogenesis by interacting with the KD of DMI2 (Kevei et al. 2007). A plant-specific scaffolding protein, remorin1 (SymREM1) interacts with nodulation receptor kinases (NFR1/LYK3, NFR5/NFP, and SymRK/DMI2) to perceive bacterial signaling molecules, which are essential at the pre-infection stage of nodule formation (Lefebvre et al. 2010). An E3 ubiquitin ligase PUB1 also interacts with DMI2, although it does not affect the stability of DMI2. Most likely, phosphorylation of PUB1 by DMI2 leads to downstream signaling events that negatively regulate the rhizobial and arbuscular mycorrhizal symbioses (Vernié et al. 2016). On the other hand, SymRK-interacting E3 ubiquitin ligase (SIE3) binds to and ubiquitinates SymRK to positively modulate nodulation (Yuan et al. 2012). SEVEN IN ABSENTIA 4 (SINA4), another E3 ubiquitin ligase, binds to and destabilizes SymRK to negatively affect the rhizobial infection process (Den Herder et al. 2012). Two other positive regulators of nodulation, an AT-rich interaction domain (ARID) containing SymRK-interacting protein 1 (SIP1) and a member of plant mitogen-activated protein kinase kinase (MAPKK) family, SIP2, are also identified as interacting partners of SymRK (Chen et al. 2012; Wang et al. 2013; Zhu et al. 2008). Interestingly, SymRK shows a specific inhibitory effect on the kinase activity of SIP2 toward the MPK6 substrate, which uncovers a signal transmission route via a MAPK-based pathway (Chen et al. 2012).

We have previously established the role of heterotrimeric G-proteins in regulating signaling during nodule development in soybeans (Roy Choudhury and Pandey 2013, 2015). G-proteins, comprising Gα, Gβ, and Gγ subunits, are key conduits for signal transduction in eukaryotes. In metazoan systems, ligand-bound, active G-protein-coupled receptors (GPCR) stimulate the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the Gα subunit of the trimeric complex, which results in its dissociation in two functional elements, GTP-Gα and Gβγ dimer. Both these entities facilitate signaling by independently interacting with multiple downstream effectors (Offermanns 2003; Siderovski and Willard 2005). The regulator of G-protein signaling (RGS) proteins act as GTPase-activity accelerating proteins (GAP) of Gα to efficiently deactivate them. This RGS-mediated deactivation has been shown to be critical for plant G-protein signaling, but the classical GPCR-mediated GDP/GTP exchange on the Gα for activation has not been observed to date (Mohanasundaram and Pandey 2023; Mohanasundaram et al. 2022; Pandey 2017, 2019; Pandey and Vijayakumar 2018). Instead, genetic and functional evidence suggest that plant G-proteins interact with RLK to transduce various signals to downstream effectors (Pandey 2020; Roy Choudhury and Pandey 2016). The RLK/G-protein module has been shown to regulate diverse biological processes, including sugar-responsive growth and development, zygote asymmetric cell division and daughter cell fate, shoot apical meristem (SAM) development, stem cell proliferation, stomatal development and movement, salinity response, and plant-microbe interactions (Bommert et al. 2013; Ishida et al. 2014; Liang et al. 2016, 2018; Liu et al. 2013; Pandey 2019, 2020; Roy Choudhury and Pandey 2016; Wu et al. 2020; Yu and Assmann 2018; Yu et al. 2016).

We have previously demonstrated the roles of NFR1 and SymRK in the regulation of different aspects of G-protein signaling during RNS in soybeans (Roy Choudhury and Pandey 2015, 2022). We showed that active Gα proteins are the negative regulators of nodule formation, and their lower and higher expression results in more or fewer nodule numbers, respectively (Roy Choudhury and Pandey 2013). The RGS protein deactivates Gα and is therefore expected to positively regulate nodulation, which we have confirmed in our previous studies. We established that the RNS receptor complex exerts a two-pronged control on G-protein signaling to constrain the negative regulator of nodulation: (i) the Gα proteins are directly phosphorylated by SymRK at their active site, which makes them completely inactive and (ii) the RGS proteins are phosphorylated by NFR1, which activates them for efficient deactivation of any active Gα pools (Roy Choudhury and Pandey 2015, 2022). We have also confirmed that these proteins, SymRK, NFR1, Gα, and RGS, exist as multi-protein complexes in planta. In this study, we show that SymRK also interacts with and phosphorylates RGS proteins. Phosphorylated RGS proteins have higher GAP activity, which accelerates the conversion of Gα from its active to inactive form, thereby enabling root nodule formation.

Results

Soybean SymRK interacts with RGS proteins

We have recently established the role of soybean SymRK in the regulation of G-protein signaling via Gα phosphorylation during nodulation (Roy Choudhury and Pandey 2022). As the RGS protein forms a complex with active Gα and all these proteins are colocalized at the plasma membrane, we evaluated the effect of SymRK on RGS protein. We first tested the interaction between RGS and SymRK proteins in a split ubiquitin-based yeast 2-hybrid interaction system. The soybean genome encodes two copies each of SymRK (SymRKα and SymRKβ) (Roy Choudhury and Pandey 2022) and RGS (RGS1 and RGS2) (Roy Choudhury et al. 2012), and these recently duplicated copies of proteins share over 95% sequence identity. To test interactions, the full-length N- and C-terminal domains of SymRKα and SymRKβ were expressed as bait proteins (CUb fusions), and the full-length N- and C-terminal domains of RGS proteins (RGS1 and RGS2) were expressed as prey proteins (NUb fusions). The full-length RGS proteins interacted with the full-length and C-terminal regions of SymRKα but not with the N-terminal region of SymRKα (Supplementary Fig. S1) as evaluated by yeast growth on selection media. Similarly, the C-terminal region of SymRKα and SymRKβ interacted with the full-length and the C-terminal region of the RGS proteins. No interactions were observed between the N-terminal region of SymRKα and the N-terminal region of the RGS proteins (Fig. 1A; Supplementary Fig. S1). These results suggested interactions between the cytosolic regions of SymRK and RGS proteins. To corroborate the yeast-based interaction data, the bimolecular fluorescence complementation (BiFC) assay was used to test the in planta interaction between SymRKα and RGS proteins in different combinations. For BiFC assays, RGS proteins were expressed as a fusion protein with nEYFP at their C-terminal end, and SymRKα was expressed as a fusion protein with cEYFP at its C-terminal end. A strong fluorescence signal was detected when both these constructs were co-expressed in tobacco leaves, confirming interactions between RGS and SymRK proteins (Fig. 1B). No interaction was detected when the N-terminal domain of RGS2 was used, while the C-terminal RGS2 domain interacted with SymRKα, which is similar to full-length RGS proteins. These data establish a physical interaction between SymRK and RGS proteins. Due to the extensive similarities between RGS1 and RGS2 and between SymRKα and SymRKβ, and because of no

obvious differences between their protein-protein interactions patterns, further experiments were performed with RGS2 and SymRKα as representative proteins.

SymRKα phosphorylates RGS proteins

RGS phosphorylation by RLKs is a predominant regulatory mechanism in plant G-protein signaling (Liang et al. 2018; Tunc-Ozdemir et al. 2016). The interaction between RGS and SymRK prompted us to evaluate whether RGS can be phosphorylated by SymRK. We performed in vitro phosphorylation assays using the recombinant C-terminal RGS2 protein (amino acids 251 to 464) as a substrate and the C-terminal domain of SymRKα (amino acids 551 to 981) as a kinase and established that SymRK can phosphorylate RGS (Fig. 2A). Liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis identified five potential phosphorylation sites in RGS2 at a minimum localization threshold of 99%: Ser (S)277; Ser(S)419; Ser(S)433; Ser(S)269; and Ser(S)307 (Supplementary Figs. S2 and S3). Notably, two of these sites, Ser(S)277 and Ser(S)269, are also phosphorylated NFR1α (Roy Choudhury and Pandey 2015). To validate the LC-MS/MS data, we created alanine substitutions at each of the five serine residues to change the corresponding phosphosites to phospho-deficient versions (S277A, S419A, S433A, S269A, and S307A) and tested the phosphorylation status of recombinant mutant RGS2 proteins in the presence of SymRKα using an in vitro phosphorylation assay. The phosphorylation status was marginally reduced in RGS2^{S269A} and RGS2^{S307A} compared with other single mutants (Supplementary Fig. S4). To further substantiate these data, we generated double (RGS2^{S277A,S419A}), triple (RGS2^{S277A,S419A,S433A}), quadruple (RGS2^{S277A,S419A,S433A,S269A}), and quintuple (RGS2^{S277A,S419A,S433A,S269A,S307A} or RGS2^{quinA}) phospho-deficient versions of RGS2 and performed a phosphorylation assay using mutant recombinant proteins in the presence of SymRKα. Each additional mutation led to reduced phosphorylation, and a negligible amount of phosphorylation was observed in the RGS2^{quinA} mutant, suggesting that SymRKα phosphorylates RGS at multiple serine residues (Fig. 2B).

A lysine residue, Lys(K)622, in the phosphotransfer motif is vital for the kinase activity of *L. japonicus* SymRK, and its substitution to Glu results in a kinase-dead version of the protein. Furthermore, as an active kinase, SymRK also autophosphorylates. The autophosphorylation at amino acids Thr(T)760 and Ser(S)754 in the activation loop and Thr(T)593 in the juxtamembrane region have previously been shown to be essential for SymRK kinase activity in *L. japonicus* (Yoshida and Parniske 2005). We identified the corresponding amino acid residues

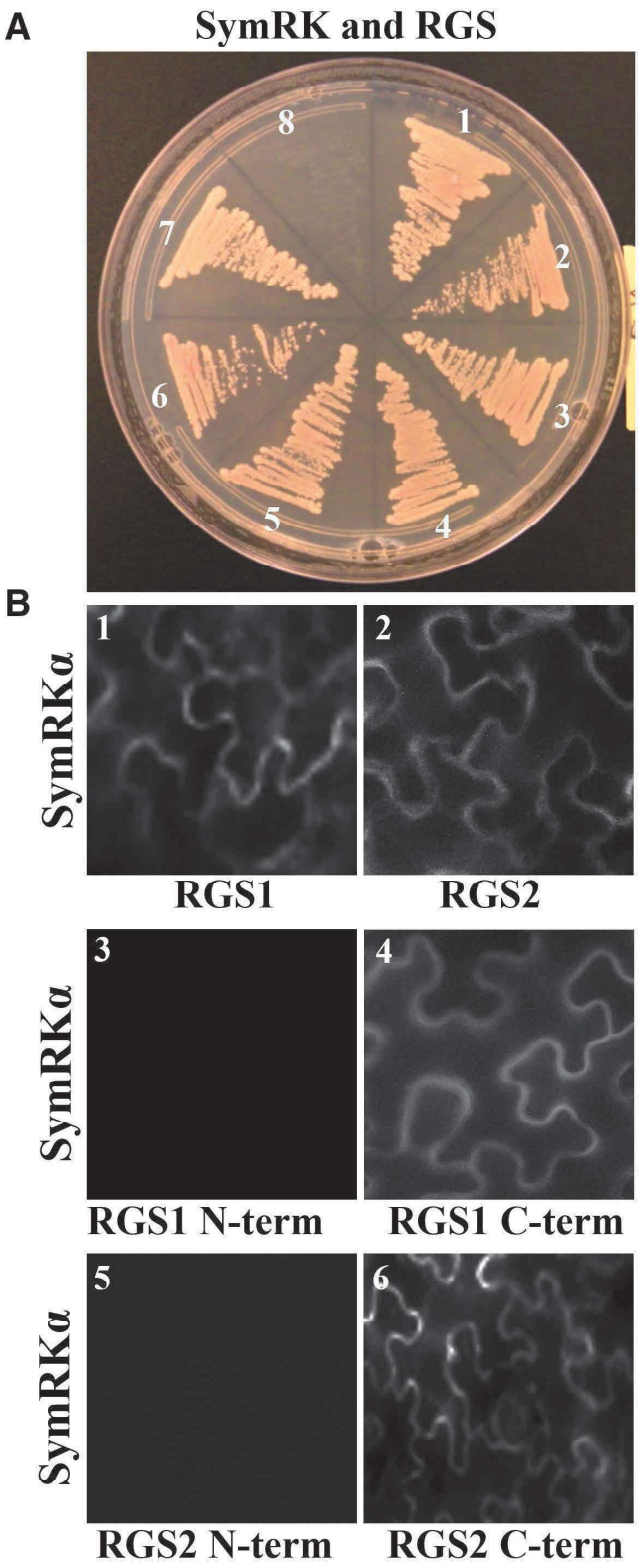


Fig. 1. Soybean regulator of G-protein signaling (RGS) proteins interact with symbiosis receptor-like kinases (SymRK). **A,** Interaction between RGS1 and RGS2 with C-terminal SymRKα and SymRKβ protein using split ubiquitin-based interaction assay. The picture shows yeast growth on selective media supplemented with 250 μM of methionine. For all combinations, RGS proteins were fused with the N-terminal half of ubiquitin (NUb fusion), and SymRK proteins were fused with the C-terminal half of ubiquitin (CUb fusion). NUb_{wt} fusion and $NUb\text{-}_{vector}$ fusion constructs were used as positive and negative controls, respectively. Three biological replicates of the experiment were performed with similar results, and a representative image is shown. The combinations are as follows: (1) SymRKα C-terminal-CUb + RGS1-Nub; (2) SymRKα C-terminal-CUb + RGS2-Nub; (3) SymRKβ C-terminal-CUb + RGS1-Nub; (4) SymRKβ C-terminal-CUb + RGS2-Nub; (5) SymRKα C-terminal-CUb + RGS2 C-terminal-Nub; (6) SymRKβ C-terminal-CUb + RGS2 C-terminal-Nub; (7) SymRKα C-terminal-CUb + RGS1-NUbwt (positive control); and (8) SymRKα C-terminal-CUb + NUb-vector (negative control). Additional interactions are shown in Supplementary Figure S1. **B,** Interaction between RGS1 and RGS2 with SymRKα using bimolecular fluorescence complementation (BiFC) assay. RGS proteins were fused with the N-terminal half of enhanced yellow fluorescent protein (EYFP) in 77-nEYFP-N1 vector, and SymRKα was fused with the C-terminal half of EYFP (in 78-cEYFP-N1 vector). Agrobacteria containing different combinations were infiltrated in tobacco leaves. The reconstitution of YFP fluorescence due to protein-protein interaction was visualized using a Nikon Eclipse E800 microscope with the epi-fluorescence module. At least five independent infiltrations were performed for each protein combination with similar results. A representative image is shown. The combinations are as follows: (1) RGS1-SymRKα; (2) RGS2-SymRKα; (3) RGS1 N-terminal-SymRKα; (4) RGS1 C-terminal-SymRKα; (5) RGS2 N-terminal-SymRKα; and (6) RGS2 C-terminal-SymRKα. term, terminal.

in soybean SymRKα (Lys(K)617; Thr(T)755; Ser(S)749; and Thr(T)588, respectively) by multiple sequence alignment. To confirm the role of these amino acids in SymRK activity and RGS phosphorylation, we made mutant versions of the SymRKα protein and examined their ability to phosphorylate RGS. Both auto- and transphosphorylation activities of the kinase-dead version of SymRKα (SymRKα^{K617E}) were inhibited, as expected based on what was seen in *L. japonicus*. Of the other three mutants, SymRKα^{T755A} was unable to phosphorylate RGS2, whereas SymRKα^{T588A} and SymRKα^{T749A} phosphorylated RGS2 similar to native SymRKα (Fig. 2C). To verify whether phosphorylation of RGS affected its interaction with SymRKα, we tested the interaction between RGS2 with native and mutant SymRKα by a split ubiquitin-based interaction assay. Except for the kinase-dead version (SymRKα^{K617E}), all SymRKα variants (SymRKα^{T755A}, SymRKα^{T588A}, and SymRKα^{T749A}) interacted with RGS2 with similar efficiency as native SymRKα (Supplementary Fig. S5). These data confirm that under in vitro conditions, SymRKα^{T755A} interacts with RGS2 but is unable to phosphorylate it.

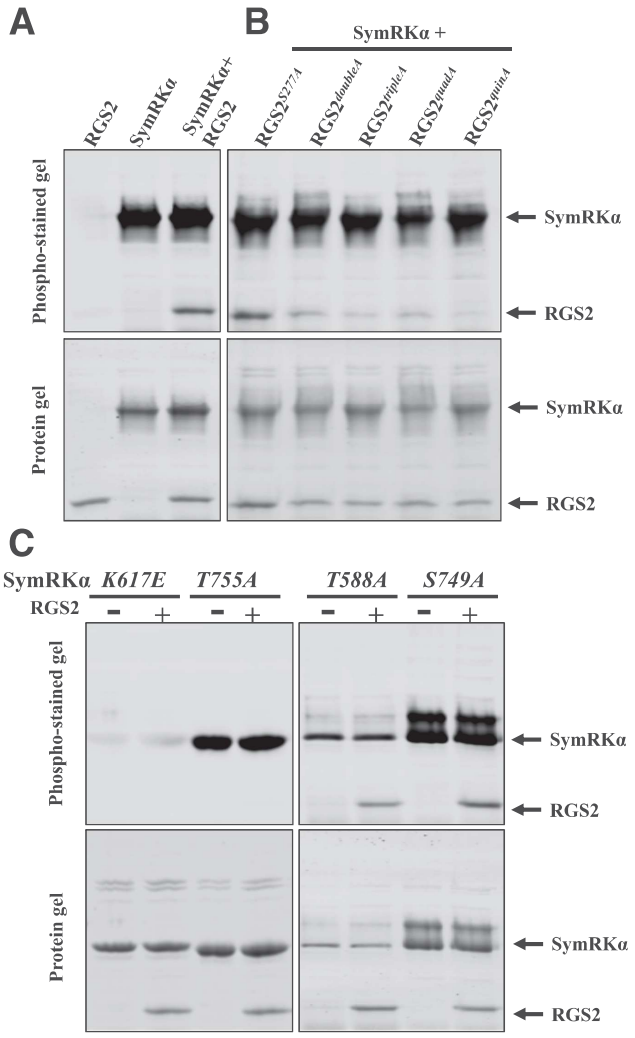


Fig. 2. Symbiosis receptor-like kinase α (SymRKα) phosphorylates the regulator of G-protein signaling 2 (RGS2) protein. **A,** SymRKα autophosphorylates and phosphorylates RGS2 protein under in vitro conditions. The C-terminal RGS2 protein was incubated with native SymRKα C-terminal protein for in vitro phosphorylation assay. **B,** Phosphorylation assay using single and higher order mutations in RGS2 protein (based on the information from liquid chromatography-mass spectrometry [LC-MS/MS] data). Recombinant purified proteins (single mutant: RGS2^{S277A}; higher order mutants: RGS2^{doubleA} = RGS2^{S277A,S419A}, RGS2^{tripleA} = RGS2^{S277A,S419A,S433A}, RGS2^{quadA} = RGS2^{S277A,S419A,S433A,S269A}, and RGS2^{quinA} = RGS2 ^{S277A,S419A,S433A,S269A,S307A}) were incubated with the SymRKα C-terminal protein for the in vitro phosphorylation assay. **C,** The C-terminal RGS2 protein was incubated with mutant SymRKα C-terminal proteins (SymRK^{K617E}, SymRK^{T755A}, SymRKα^{T588A}, and SymRKα^{S749A}) for in vitro the phosphorylation assay. In all cases, the upper panel represents Pro-Q Diamond-stained phosphoprotein gel, and the lower panel shows the same gel stained with Sypro Ruby to visualize protein profiles.

Phosphorylated RGS exhibits improved GAP activity on Gα

To evaluate the effect of phosphorylation on the activity of the RGS protein, we generated phospho-mimic versions of RGS2 by replacing serine residues with aspartic acid (D) (RGS2^{S277D,S419D,S433D,S269D,S307D} or RGS2^{quinD}). The effect of phosphorylation of RGS2 on its GAP activity was examined using a phosphate release assay with recombinant, purified proteins. The phosphate released from soybean Gα1 due to its inherent and RGS-accelerated GTPase activity was quantified in the presence of native, phospho-deficient (RGS2^{quinA}) and phospho-mimic (RGS2^{quinD}) versions of RGS2. All three versions of RGS2 were active and increased the GTPase activity of Gα1 over its basal level. However, the GAP activity of RGS2^{quinD} was significantly higher compared with native RGS2, while RGS2^{quinA} exhibited relatively reduced activity (Fig. 3). Overall, these results demonstrate that phosphorylation by SymRKα enhances the GAP activity of RGS2, which can promote faster deactivation of Gα.

Overexpression of phospho-mimic RGS increases the nodule number and the expression of nodulation marker genes

To determine the in planta effects of SymRKα-mediated phosphorylation of RGS, we overexpressed the RGS2^{quinA} and RGS2^{quinD} versions of RGS2 in soybean hairy roots. Transcript levels of *RGS* genes were tested to ascertain their higher expression (Supplementary Fig. S6). We evaluated the number of deformed root hairs at 4 days postinoculation (dpi) with *Bradyrhizobium japonicum*. The overexpression of native *RGS2* resulted in increased root hair deformation compared with empty vector (*EV*) controls, as reported previously (Roy Choudhury and Pandey 2015). In comparison, the expression of the phospho-mimic but not the phospho-deficient *RGS2* showed even higher (approximately 67%) root hair deformation (Fig. 4A). A sim-

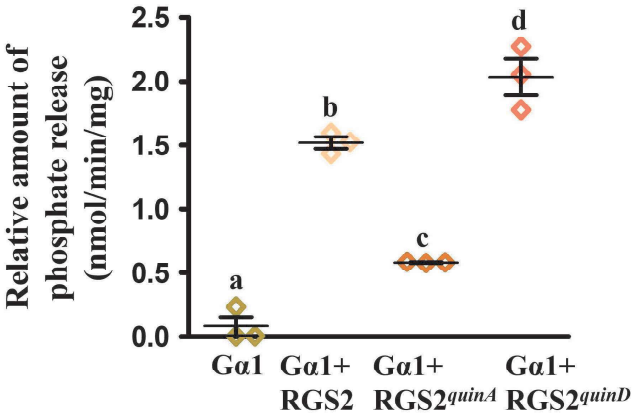


Fig. 3. Effect of regulator of G-protein signaling (RGS) phosphorylation on the GTPase activity of soybean Gα1. Rate of inorganic phosphate (Pi) released due to the GTPase activity of Gα1 in the presence of native, phospho-deficient (RGS2^{quinA} = RGS2^{S277A,S419A,S433A,S269A,S307A}), and phospho-mimic (RGS2^{quinD} = RGS2^{S277D,S419D,S433D,S269D,S307D}) versions of RGS2 proteins. The experiment was repeated three times, and data were averaged. Error bars represent the mean ± standard error (SE). The assays were performed using an Infinite M200 Pro Plate Reader (Tecan). Different letters indicate significant differences (Tukey's multiple-comparisons test, $P < 0.05$) between samples.

ilar trend was observed with the developing nodule primordia (Fig. 4B), with the phospho-mimic versions showing significantly higher numbers of developing nodule primordia compared with native *RGS2*, respectively. Clear differences in the final nodule numbers were also observed as overexpression of *RGS2* led to approximately 24% more nodules compared with *EV* transformed roots, whereas overexpression of $RGS2^{quinD}$ led to approximately 68% more nodules (Fig 4C and D). These data suggest that the phosphorylation of RGS positively regulates nodule formation in soybean.

To corroborate the role of SymRKα-mediated RGS2 phosphorylation in RNS, we further assessed the nodulation phenotypes of roots overexpressing native and mutant SymRKα proteins. The transcript levels of native and mutated versions of *SymRKα* were tested to ascertain their higher expression levels (Supplementary Fig. S7). As SymRK is a known positive regulator of nodulation (Abel et al. 2024; Dávila-Delgado et al. 2023; Ried et al. 2014), overexpression of native *SymRKα* led to an expected increase in nodule number. Compared with *EV* transformed roots, nodule number decreased in $SymRK\alpha^{K617E}$ and $SymRK\alpha^{T755A}$ overexpression lines. On the other hand, two other mutants ($SymRK\alpha^{T588A}$ and $SymRK\alpha^{T749A}$) that did not suppress SymRKα-mediated RGS2 phosphorylation showed nodule numbers similar to or marginally lower than what is observed with the overexpression of native *SymRKα* (Fig. 5A and B).

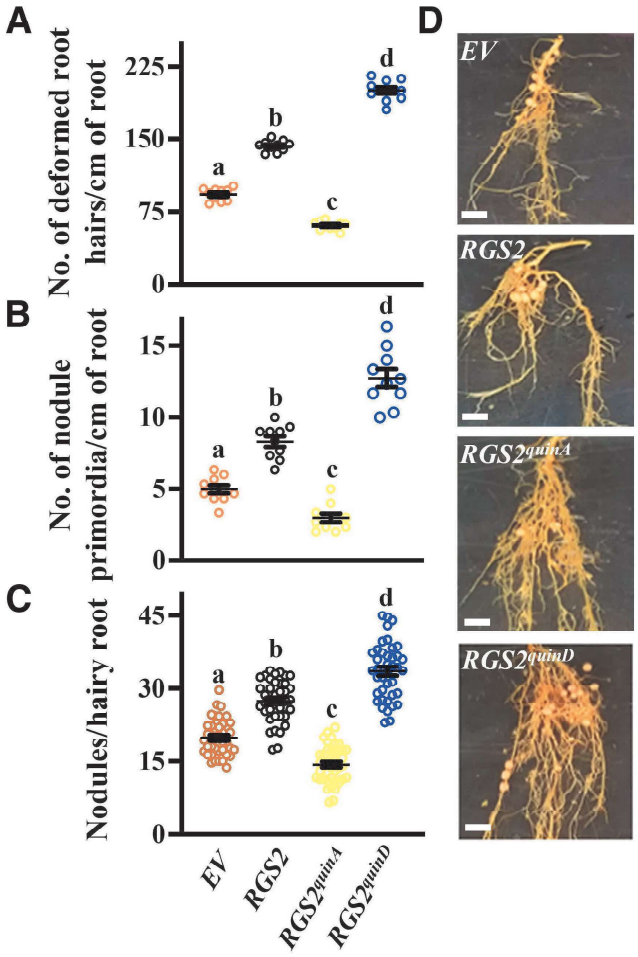


Fig. 4. The effect of overexpression of phospho-deficient and phospho-mimic versions of the regulator of G-protein signaling 2 gene (*RGS2*). **A,** Native, phospho-deficient ($RGS2^{quinA} = RGS2^{S277A,S419A,S433A,S269A,S307A}$), and phospho-mimic ($RGS2^{quinD} = RGS2^{S277D,S419D,S433D,S269D,S307D}$) genes driven by a *Cassava vein mosaic virus* (CvMV) promoter were used for hairy root transformation. Quantification of deformed root hairs per centimeter in overexpression roots compared with empty vector (*EV*) at 4 days postinoculation (dpi) with *Bradyrhizobium japonicum*. **B,** Quantification of nodule primordia per centimeter in overexpression roots compared with *EV* at 6 dpi with *B. japonicum*. The data represented in A and B are average values from three independent experiments ($n = 10$). **C,** Quantification of nodule number in overexpression hairy roots compared with *EV* at 32 dpi with *B. japonicum*. The data represent an average of three biological replicates (35 to 40 individual plants/biological replicate) containing transgenic nodulated roots. In all images, error bars represent ±standard error (SE). Different letters indicate significant differences (Tukey's multiple-comparisons test, $P < 0.0001$) between samples. **D,** Representative picture of transgenic hairy roots overexpressing native, $RGS2^{quinA}$, or $RGS2^{quinD}$ versions of *RGS2* compared with *EV* control transformed roots at 32 dpi with *B. japonicum*. Scale bars = 50 mm.

We have previously reported that the expression of a subset of nodulation marker genes such as *Enod40* and *Nodulin35* was regulated in a *Gα*- and *RGS*-dependent manner (Roy Choudhury and Pandey 2015), whereas additional nodulation-related marker genes (e.g., *Cytokinin oxidase*, *Nodulin 35*, *NSP1*, *NIN1*, and *NFYA1*) exhibit a SymRK-dependent regulation in expression levels (Roy Choudhury and Pandey 2022). To determine whether SymRK-mediated phosphorylation of RGS impacts the expression of these downstream nodulation marker genes, we performed quantitative real-time PCR (qRT-PCR) on 4-day-old transgenic hairy roots after inoculation with *B. japonicum* to check their transcript levels. The transcript level of each gene was significantly increased in both native and phospho-mimic versions of the *RGS2* ($RGS2^{quinD}$) overexpression lines compared with the *EV* expressing roots (Fig. 6). Furthermore, these five nodulation marker genes showed higher expression in *SymRKα* overexpressing lines and significantly lower or unchanged expression in $SymRK\alpha^{K617E}$ and $SymRK\alpha^{T755A}$ overexpression lines compared with *EV* expressing roots (control) (Supplementary Fig. S8). Together, these results provide evidence that these nodulation marker genes are potential downstream targets of the SymRK/G-protein-dependent pathway. We also evaluated whether phosphorylation of RGS has any effect on its localization. $RGS2^{quinD}$ and $RGS2^{quinA}$ were localized to the cell periphery, similar to native RGS2 (Supplementary Fig. S9).

Based on our previous and current results, we propose the following model for receptor complex-mediated regulation of G-protein signaling during nodulation (Fig. 7). Both NFR1 and SymRK can phosphorylate and enhance the GAP activity of RGS proteins. Active, phosphorylated RGS proteins deactivate any free Gα, which are negative regulators of nodule formation. We have also shown previously that SymRK phosphorylates Gα at its active site to deactivate it and make it unable to interact with Gβγ. The net effect of the concerted action of these receptors is to make Gα fully inactive, thereby attenuating its role as a negative regulator, and to allow the positive regulators, Gβγ, to signal freely (or with other proteins).

Discussion

Symbiotic N_2 fixation in the legume root nodules is an outcome of exquisite plant-biotic interactions, which require sophisticated control of the underlying signal transduction pathways. Nodule formation is an energy-intensive process and is therefore intricately regulated by many signaling modules, both at the plasma membrane and intracellular locations (Chaulagain and Frugoli 2021; Montiel et al. 2021; Popp and Ott 2011). Many molecular aspects of this plant-microbe communication, such as the identity of receptors, changes in plasma membrane depolarization after NF perception, nuclear calcium spiking, and gene expression changes, are well characterized, and key nuclear proteins and transcription factors that control nodule formation are known (Broghammer et al. 2012; Gough 2003; Haney et al. 2011; Indrasumunar et al. 2011; Kim et al. 2019; Laffont et al.

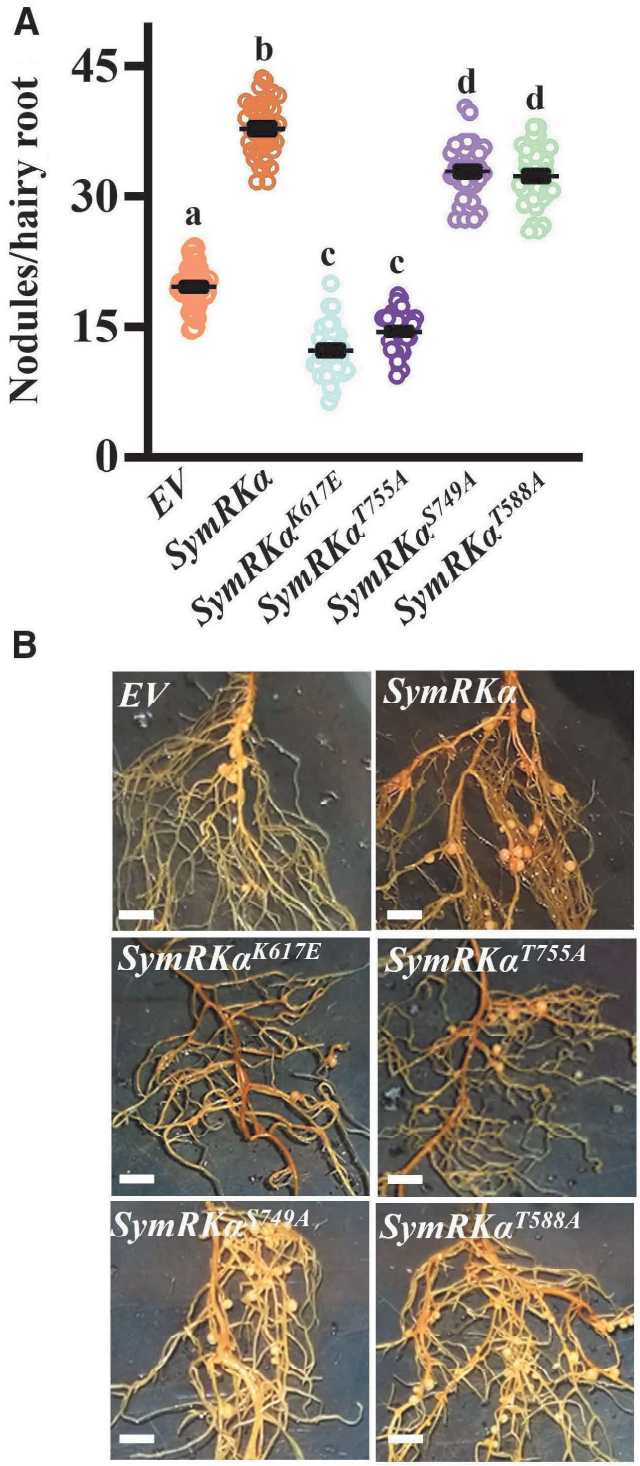
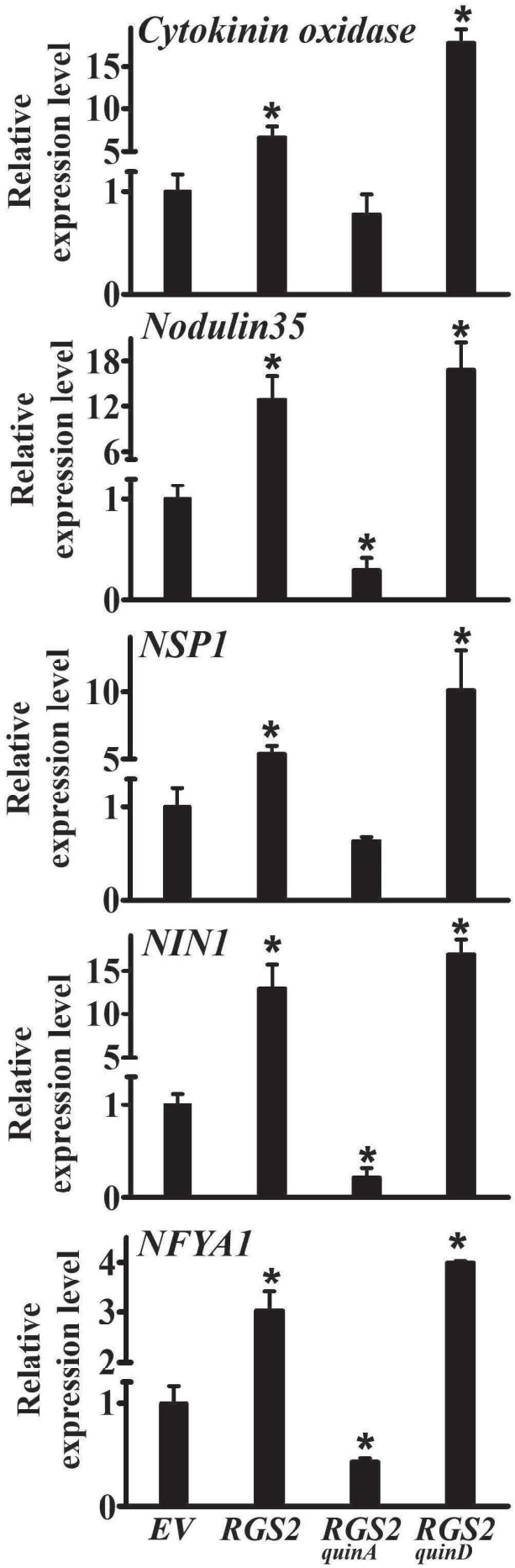


Fig. 5. Nodulation phenotypes in roots overexpressing native and mutant versions of *SymRKα*. **A,** Detection of nodule numbers in hairy roots overexpressing native and mutant *SymRKα* genes (*SymRKα*^{K617E}, *SymRKα*^{T755A}, *SymRKα*^{T588A}, and *SymRKα*^{S749A}) compared with empty vector (*EV*) at 32 days postinoculation (dpi) with *Bradyrhizobium japonicum*. The data represent average of three biological replicates (35 to 40 individual plants/biological replicate) containing transgenic nodulated roots. Error bars represent ± standard error (SE). Different letters indicate significant differences (Tukey's multiple-comparisons test, $P < 0.0001$) between samples. **B,** Representative picture of transgenic hairy roots overexpressing native and mutant versions of *SymRKα* compared with *EV* control lines at 32 dpi with *B. japonicum*. Scale bars = 50 mm.

2020; Lévy et al. 2004; Limpens et al. 2003; Madsen et al. 2003; Mbengue et al. 2010; Mergaert et al. 2020; Moling et al. 2014; Oldroyd and Downie 2006; Popp and Ott 2011; Vernié et al. 2015; Wong et al. 2019). However, events immediately following NF perception at the receptor protein complex and how this information is relayed to cause subsequent changes in the nucleus remain relatively unclear (Wong et al. 2019; Zhou et al. 2019). We have previously shown that heterotrimeric G-proteins mediate at least a part of this signal relay, as specific subunits of the G-protein complex interact with the NFR complexes directly. We have shown that during nodule formation in soybeans, NFR1 receptors interact with the Gα and RGS proteins and phosphorylate RGS proteins (Roy Choudhury and Pandey 2013, 2015). We have also shown that SymRK interacts with Gα proteins and phosphorylate them at their active site (Roy Choudhury and Pandey 2022). The data presented in this manuscript uncover an additional level of regulation of the G-protein cycle by SymRK.

SymRK are plasma membrane-localized, MLD-LRR receptor kinases that act downstream of NFRs and are the first member of the common symbiotic pathway (CSP), which is operative during symbiosis with rhizobia, arbuscular mycorrhizal fungi, and *Frankia*. SymRK are essential for nodule organogenesis (Gherbi et al. 2008; Holsters 2008; Ried et al. 2014). As with many signaling proteins, the intracellular KD of SymRK has been shown to interact with a variety of proteins (Chen et al. 2012; Den Herder et al. 2012; Yamazaki et al. 2022; Zhu et al. 2008), and we show its interaction with the intracellular domain of RGS proteins (Fig. 1). We propose that the proteins directly or indirectly involved in the perception of the NF (e.g., NFR1, NFR5, SymRK, EPR3, and RINKRK) exist as complexes and show multipartite interactions with the downstream signaling proteins such as the G-protein complex. RLKs are known to function as protein complexes, as has been shown during brassinosteroid signaling using the BRI1/BAK1/ BIN2 complex (Chinchilla et al. 2009; Delesalle et al. 2024; Peng et al. 2018), stomatal development (Herrmann and Torii 2021; Smit and Bergmann 2023), immune signaling using the FLS2/BAK1 protein complex (Albert et al. 2010; Chinchilla et al. 2009; Liang et al. 2016, 2018; Robatzek and Wirthmueller 2013), meristem development (Becraft 2002; Bommert et al. 2013; Cui et al. 2022), and others. Furthermore, specific members of the heterotrimeric G-proteins have been shown to be modulated via direct interactions with RLKs. In *Arabidopsis*, G-proteins interact with key RLKs such as FLS2, EFR1, CERK1, BAK1, BIR1, and ERECTA to mediate signaling triggered by a suite of bacterial and fungal pathogens (Cheng et al. 2015; Liang et al. 2016, 2018; Wang et al. 2018; Xue et al. 2020; Zhou et al. 2019). The FER and BRI1/BAK1 complex also regulates G-protein-dependent salinity- and sugar-sensitive growth, respectively (Peng et al. 2018; Yu and Assmann 2018). In maize, another RLK, CLV, genetically interacts with Gα to maintain SAM size (Bommert et al. 2013). These (and additional) studies support the hypothesis that G-proteins act as a converging point for several RLKs in plants (Aranda-Sicilia et al. 2015; Liang et al. 2018; Liu et al. 2013; Pandey 2020; Xue et al. 2020).

The intracellular KD of SymRK is involved in extensive auto- and transphosphorylations. Several phosphosites have been mapped to the KD (named alpha-1 motif in *L. japonicus* SymRK), which are required for its activity (Abel et al. 2024; Bhattacharya et al. 2019; Feng et al. 2021; Saha et al. 2014, 2016). For example, spontaneous nodule formation without rhizobia was observed after overexpression of the KD of *Arachis hypogaea* SymRK in *M. truncatula* (Bhattacharya et al. 2019; Saha et al. 2016). The KD of SymRK catalyzes autophosphorylation for its own activation, and active SymRK phosphorylates RGS proteins (Fig. 2) at multiple sites. Two of these phosphosites are the same as the ones phosphorylated by the NFR1 recep-



tors (Roy Choudhury and Pandey 2015). Furthermore, similar to NFR1 phosphorylation, SymRK phosphorylation also increases the GAP activity of RGS proteins (Fig. 3). The overall effect of this enhanced activity is more efficient deactivation of Gα, which is required for nodule development. This is also reflected in the nodulation phenotypes of plants expressing phospho-mimic or phospho-deficient versions of the RGS protein (Fig. 4). Additionally, the phosphorylation of SymRK is relevant because the overexpression of the phospho-deficient version of the protein fails to produce more nodules (Fig. 5).

Overall, these data are consistent with the model we have previously proposed and offer an additional layer of control. G-protein subunits Gα and Gβγ play antagonistic roles during root nodule symbiosis, acting as negative and positive regulators of nodulation, respectively. The receptor complex exerts a two-pronged control. On the one hand, different receptors such as NFR1 (Roy Choudhury and Pandey 2015) and SymRK (this study) phosphorylate and activate RGS proteins, which facilitates deactivation of any active Gα. Similar phosphorylation-dependent regulation of PUB1 has been reported in *Medicago*. PUB1 is a negative regulator of rhizobial and arbuscular mycorrhizal symbioses, and its ubiquitination is modulated by LYK3 (homolog of NFR1) and DMI2 (homologue of SymRK) in a phosphorylation-dependent manner, which calibrates the overall activity required for optimum symbiosis (Vernié et al. 2016). In the case of G-proteins, the conventional mechanism suggests that the deactivated Gα would bind with the Gβγ and sequester them from further signaling. However, direct phosphorylation of Gα by SymRK in its GTP-binding P loop abolishes its interaction with Gβγ, thus potentially setting them free for direct signaling or making them exclusively available for the XLGα proteins, whose role remains to be established during nodulation. We propose that the signaling output from nodulation receptor complexes is precisely controlled by multiple components and that G-proteins represent one such signaling module. It is also evident from the up or downregulation of a few nodulation marker genes in phospho-mimic or phospho-deficient RGS overexpressed lines, respectively. A comprehensive understanding of how the signal travels from the G-proteins to the control of gene expression is still lacking and warrants further research.

Materials and Methods

Plant material and cloning of native and mutant genes

Soybean (*G. max*) wild-type (Williams 82) seeds were used for all experiments. Total RNA was extracted from 5-day-old seedlings using Trizol, and cDNA was synthesized using a Superscript III cDNA synthesis kit (Invitrogen) following the manufacturer's instructions; 2 μg of cDNA was treated with DNAse I (Turbo DNA-free, Ambion) as described pre-

Fig. 6. Relative expression of nodulation-related genes in native, phospho-deficient, and phospho-mimic versions of the regulator of G-protein signaling 2 gene (*RGS2*) overexpression hairy roots. Relative expression of nodulation-related genes in soybean empty vector (*EV*) (control), native, phospho-deficient, and phospho-mimic versions of *RGS2* overexpression hairy roots at 4 days postinoculation (dpi) of *Bradyrhizobium japonicum*. Gene-specific primers were used to amplify and quantify the transcript levels of *Cytokinin oxidase* (*Glyma.17g054500.1*), *Nodulin35* (*Glyma10g23790.1*), *NSP1* (*Glyma07g04430*), *NIN1* (*Glyma14g00470*), and *NFYA1* (*Glyma10g10240*) in transgenic hairy roots. Two biological replicates with three technical replicates each were used for expression analysis, and data were averaged. The expression values across different samples are normalized against soybean *Actin* gene expression. Expression in *EV* roots was set at 1. Error bars represent ±standard error (SE). Asterisks (*) indicate statistically significant differences compared with *EV* control (* = $P < 0.05$; Student's *t* test).

viously (Romero-Castillo et al. 2015). Gene-specific primers (Supplementary Table S1) were used for the amplification of full-length genes. Sequences were verified after cloning in pCR8/GW/TOPO TA vector (Invitrogen). RGS2 single mutants with specific amino acid substitutions ($RGS2^{S277A}$, $RGS2^{S419A}$, $RGS2^{S433A}$, $RGS2^{S269A}$, and $RGS2^{S307A}$) and higher-order mutants with a combination of amino acid substitutions ($RGS2^{doubleA}$, $RGS2^{S277A,S419A}$; $RGS2^{tripleA}$, $RGS2^{S277A,S419A,S433A}$; $RGS2^{quadA}$, $RGS2^{S277A,S419A,S433A,S269A}$; $RGS2^{quinA}$, $RGS2^{S277A,S419A,S433A,S269A,S307A}$; $RGS2^{doubleD}$, $RGS2^{S277D,S419D}$; $RGS2^{tripleD}$, $RGS2^{S277D,S419D,S433D}$; $RGS2^{quadD}$, $RGS2^{S277D,S419D,S433D,S269D}$; and $RGS2^{quinD}$, $RGS2^{S277D,S419D,S433D,S269D,S307D}$) were generated by mutagenesis of native full-length and C-terminal *RGS2* using the QuikChange site-directed mutagenesis kit and Pfu Turbo DNA polymerase (Agilent) as described previously (Roy Choudhury and Pandey 2015). Similarly, $SymRK\alpha^{K617E}$, $SymRK\alpha^{T755A}$, $SymRK\alpha^{T588A}$, and $SymRK\alpha^{S749A}$ were generated by site-directed mutagenesis of native full-length and C-terminal *SymRKα*. Native and mutant full-length *RGS2* genes (*RGS2*, $RGS2^{quinA}$, and $RGS2^{quinD}$) and *SymRKα* genes (*SymRKα*, $SymRK\alpha^{K617E}$, $SymRK\alpha^{T755A}$, $SymRK\alpha^{T588A}$, and $SymRK\alpha^{S749A}$) were transferred into the pCAMGFP-CvMV-GWi (*CvMV* promoter) vector from pCR8/GW/TOPO TA vector (Invitrogen) using LR Clonase (Invitrogen) (Roy Choudhury and Pandey 2013). After sequence verification, all constructs and *EV* were transformed into *Agrobacterium rhizogenes* strain K599 (Kereszt et al. 2007) for hairy root transformation.

Hairy root transformation

Wild-type soybean seeds were grown in the greenhouse (16 h of light/8 h of dark) for 12 days at 25°C. Hairy root transformation was performed according to our previously published protocols (Roy Choudhury and Pandey 2013). Briefly, 2-week-old trifoliate leaves were cut using a razor blade and inoculated with *A. rhizogenes* (K599 strain containing binary plasmid) followed by inserting the leaves in rock wool cubes. After 3 weeks, plantlets containing hairy roots were transferred to SOILRITE (vermiculite/perlite/sand at a 3:1:1 ratio) and treated with actively growing (0.08 at OD_{600}) rhizobia (*B. japonicum*, USDA110). The transgenic green-fluorescent protein (GFP) roots were identified with the Nikon Eclipse E800 microscope with an epifluorescence module. Deformed root hairs and nodule primordia were quantified at 4 and 6 dpi, respectively, after *B. japonicum* treatment. Four weeks after the rhizobia infection, nodule numbers and sizes were quantified. For each construct, three biological replicates were performed, and at least 40 transgenic hairy roots were used in each individual experiment. Statistically significant values were estimated by Tukey's multiple-comparisons test ($P < 0.0001$) between samples.

qRT-PCR

The cDNA samples from different overexpression lines (roots) were diluted to 1:20 in sterile water. qRT-PCR was performed with transcript-specific primers. Two biological replicates were performed, and qRT-PCR was repeated three times for each biological replicate. All data were averaged as described previously (Roy Choudhury and Pandey 2022). The soybean *Actin* gene was used as a normalization control.

Yeast-based interaction

To study the interaction between full-length SymRKα or N-terminal SymRKα or C-terminal SymRKα/β with full-length RGS1/2 or N-terminal RGS1/2 or C-terminal RGS1/2, we used the mating-based yeast split-ubiquitin system (Roy Choudhury and Pandey 2015, 2022). *RGS* genes were used to generate NUb fusion constructs, and both *SymRKα* and *SymRKβ* genes were used to generate CUb fusion constructs. NUbwt fusion and EV fusion were used as positive and negative controls, respectively. All experiments were repeated three times, and a representative image is shown.

Bimolecular fluorescence complementation and localization

To study the interaction between full-length SymRKα with full-length RGS1/2 or N-terminal/C-terminal RGS2, both *RGS* genes were cloned into 77 nEYFP-N1 vectors (containing enhanced yellow fluorescent protein (EYFP) at the C-terminal end; Citovsky et al. 2006), and the *SymRKα* gene was cloned into 78cEYFP-N1vectors (containing cEYFP at the C-terminal end; Citovsky et al. 2006). After transforming into *A. tumefaciens* strain GV3101, all constructs were co-infiltrated in the abaxial side of tobacco leaves. After 72 h postinfiltration, tobacco leaves were imaged with the Nikon Eclipse E800 microscope with an epifluorescence module for YFP fluorescence detection. All experiments were repeated two times, and each time at least five independent infiltrations were performed for each construct. To study the localization of native and mutant RGS2,

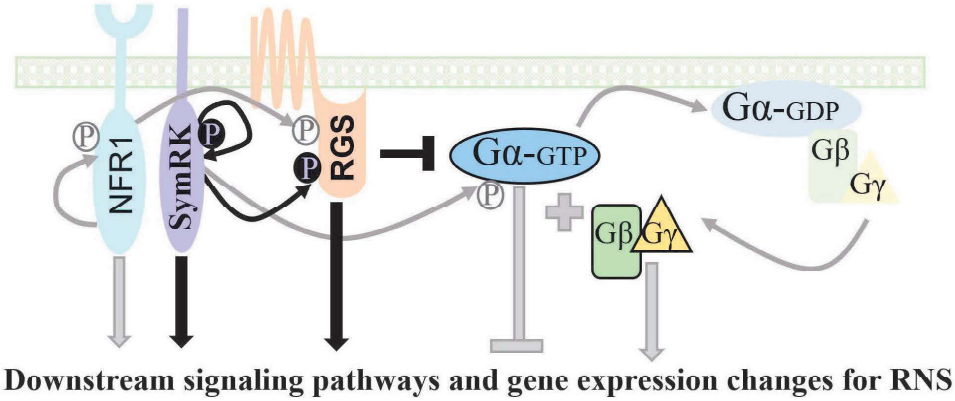


Fig. 7. Proposed model for G-protein-regulated nodule formation in soybean. Symbiosis receptor-like kinase (SymRK) phosphorylates the regulator of G-protein signaling (RGS) to accelerate the GTPase activity of the Gα protein. Phosphorylation of RGS proteins by Nod factor receptor 1 (NFR1) and SymRK increases its activity to efficiently decrease the amount of active Gα, which is a negative regulator of root nodule symbiosis (RNS). Inhibition of this negative regulator is required for successful nodulation. Black arrows and connections show data from the current work. Gray arrows and connections represent previously established pathways. Phosphorylation reactions are marked with an encircled P. Nodulation is also regulated by G-protein-independent signaling pathways (not shown).

native and mutant *RGS* genes were transferred into binary vector pEarlyGate101 by Gateway-based cloning (Invitrogen) followed by transformation into *A. tumefaciens* strain GV3101. For the localization of native and mutant full-length *RGS2* variants (RGS2^{quinA} and RGS2^{quinD}), the abaxial surface of tobacco leaves were infiltrated with *A. tumefaciens* containing the gene of interest. After 36 to 48 h of incubation in the dark, protein fluorescence was imaged with the Nikon Eclipse E800 microscope. At least four independent infiltrations were performed for each construct (Roy Choudhury and Pandey 2015).

Phosphate release assay, in vitro phosphorylation assay, and LC-MS/MS analysis

cDNA sequences corresponding to native and mutant C-terminal RGS2 (amino acids 251 to 464) and SymRKα (amino acids 551 to 918) were cloned into the pET-28a vector (Novagen, WI) and transformed into *Escherichia coli* Rosetta cells (Novagen). Protein expression and purification were performed using Ni^{2+} affinity chromatography. The pelleted bacterial cells were mixed in lysis buffer containing 50 mM Tris-HCl, pH 8.0; 500 mM NaCl; 5 mM $MgCl_2$; 20 mM imidazole; 10% glycerol (v/v); 1% Tween (v/v); and 1 mM phenylmethylsulfonyl fluoride (PMSF). After mechanical cell lysis using a sonicator (Sonic Dismembrator 550, Fisher Scientific), the supernatant was collected from the lysed cell mixture by centrifugation. After passing the supernatant through an Ni-NTA-agarose resin column (Qiagen), proteins bound to the column were collected at 4°C using elution buffer (50 mM Tris-HCl, pH 8.0; 500 mM NaCl; 5 mM $MgCl_2$; 250 mM imidazole; and 10% glycerol).

The GAP activity of native and mutant RGS recombinant proteins was assayed using the EnzChek Phosphate Assay Kit (Invitrogen). For these assays, 1 μM of recombinant, purified Gα1 protein was incubated with 1 mM GTP in the absence or presence of 0.5 μM RGS2 (wild-type or mutant) proteins. Phosphate (Pi) production was measured as a change in absorbance at 360 nm using Infinite M200 Pro (Tecan) for up to 30 min at 25°C (Roy Choudhury et al. 2013). Experiments were repeated at least three times, and data were averaged.

In vitro phosphorylation assays were performed using recombinant native and mutant C-terminal SymRKα proteins as kinases and recombinant native and mutant C-terminal RGS2 proteins as substrates. For these assays, 1.8 μg of kinase and 1 μg of substrate proteins were incubated in kinase assay buffer containing 25 mM Tris-Cl (pH 8.0), 15 mM $MgCl_2$, 2 mM $MnCl_2$, 7.5 mM NaCl, 0.05 mM EDTA, 0.05 mM DTT, 5% glycerol, and 75 μM adenosine triphosphate (ATP) at 27°C for 30 min. Subsequently, all reactions were analyzed by 10% SDS-PAGE. Proteins separated on SDS-PAGE, following fixation and multiple washing steps, were immersed in Pro-Q Diamond phosphoprotein gel stain (Invitrogen) before destaining. Following multiple washes, the gel was imaged using the Typhoon 9410 variable mode imager (Molecular Dynamics) with default settings. The same gel was then immersed in SYPRO ruby protein gel stain (Invitrogen) for total protein staining. After destaining (10% methanol and 10% acetic acid; 1 h) and washing twice with deionized water, the gel was again imaged using the Typhoon 9410 variable mode imager (Molecular Dynamics) with default settings as described previously (Roy Choudhury and Pandey 2015, 2022).

LC-MS with a Dionex RSLCnano HPLC coupled to an LTQ-Orbitrap Velos Pro (Thermo Scientific) mass spectrometer was used to analyze extracted peptides from the kinase-substrate protein solution (Roy Choudhury and Pandey 2015, 2022). The protein solution was reduced with 10 mM tris(2-carboxyethyl)phosphine (TCEP) and alkylated by treatment with 20 mM Iodoacetamide, followed by trypsin digestion, acidification with 1% trifluoro acetic acid (TFA), and cleaning up with a C18 tip. Extracted dried peptides were resuspended in 20 μl of a 5% acetonitrile/0.1% formic acid solution before analysis by LC-MS (Dionex RSLCnano HPLC coupled to an LTQ-Orbitrap Velos Pro mass spectrometer, Thermo Scientific).

All MS/MS sample analyses were performed by Mascot (Matrix Science, London, U.K.; version 2.5.1.0), where phosphorylation of serine, threonine, and tyrosine; oxidation of methionine; carbamidomethylation of cysteine; and acetylation of N-terminal of protein were specified. To validate MS/MS-based peptide and protein identifications, Scaffold (version Scaffold_4.5.3 Proteome Software Inc., Portland, OR) was used.

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