Phosphorylation-Dependent Regulation of G-Protein Cycle during Nodule Formation in Soybean

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INTRODUCTION

Biological nitrogen fixation has a major effect on the global nitrogen cycle. Leguminous plants such as soybean (Glycine max) develop specialized organs, the nodules, on their roots to host symbiotic rhizobia for the fixation of atmospheric nitrogen to ammonia. Nodule formation is an energetically demanding process and is therefore precisely controlled by the host plants. Several of these regulatory events, from Nod factor perception to downstream changes in gene expression, have been characterized in multiple leguminous plants (Cullimore et al., 2001; Kouchi et al., 2004; Mitra et al., 2004; Smit et al., 2005; Libault et al., 2009). It has been confirmed that the lysM (lysine) motif family of receptor-like kinases (NFR1 and NFR5) present at the plasma membrane of the epidermal cells directly bind Nod factors to initiate nodule formation (Madsen et al., 2003; Radutoiu et al., 2003, 2007; Broghammer et al., 2012). One of the important downstream events involves changes in calcium spiking in and around the cell nucleus; these changes are sensed by a calcium/calmodulin-dependent protein kinase (CCaMK). Activation of CCaMK is central to the regulation of nodule development as its constitutive activation leads to spontaneous nodule formation (Tirichine et al., 2006; Hayashi et al., 2010; Liao et al., 2012; Takeda et al., 2012; Routray et al., 2013). Active CCaMK phosphorylates transcriptional activator CYCLOPS, which transactivates NODULE INCEPTION to initiate nodule development (Marsh et al., 2007; Singh et al., 2014). CCaMK also induces transcription factors of the GRAS domain family, such as NSP1 and NSP2, which bind to the promoters of early nodulation (Enod) genes to regulate root hair deformation and nodule formation (Udvardi and Scheible, 2005; Gleason et al., 2006; Hirsch et al., 2009). Additional proteins involved in actin rearrangement and protein degradation, as well as hormone perception and signaling, are also involved in nodule development. Proteins of the nuclear pore complex (NEA), an ankyrin protein Vapyrin, an ARID domain-containing protein (SIP1), and HMGR1, have been shown to act in conjunction with CCaMK and have a role in nodule formation in Lotus japonicus and Medicago truncatula (Kevei et al., 2007; Zhu et al., 2008; Groth et al., 2010; Hayashi et al., 2010; Murray et al., 2011).

Nodule formation is an energetically demanding process and is therefore precisely controlled by the host plants. Several of these regulatory events, from Nod factor perception to downstream changes in gene expression, have been characterized in multiple leguminous plants (Cullimore et al., 2001; Kouchi et al., 2004; Mitra et al., 2004; Smit et al., 2005; Libault et al., 2009). It has been confirmed that the lysM (lysine) motif family of receptor-like kinases (NFR1 and NFR5) present at the plasma membrane of the epidermal cells directly bind Nod factors to initiate nodule formation (Madsen et al., 2003; Radutoiu et al., 2003, 2007; Broghammer et al., 2012). One of the important downstream events involves changes in calcium spiking in and around the cell nucleus; these changes are sensed by a calcium/calmodulin-dependent protein kinase (CCaMK). Activation of CCaMK is central to the regulation of nodule development as its constitutive activation leads to spontaneous nodule formation (Tirichine et al., 2006; Hayashi et al., 2010; Liao et al., 2012; Takeda et al., 2012; Routray et al., 2013). Active CCaMK phosphorylates transcriptional activator CYCLOPS, which transactivates NODULE INCEPTION to initiate nodule development (Marsh et al., 2007; Singh et al., 2014). CCaMK also induces transcription factors of the GRAS domain family, such as NSP1 and NSP2, which bind to the promoters of early nodulation (Enod) genes to regulate root hair deformation and nodule formation (Udvardi and Scheible, 2005; Gleason et al., 2006; Hirsch et al., 2009). Additional proteins involved in actin rearrangement and protein degradation, as well as hormone perception and signaling, are also involved in nodule development. Proteins of the nuclear pore complex (NEA), an ankyrin protein Vapyrin, an ARID domain-containing protein (SIP1), and HMGR1, have been shown to act in conjunction with CCaMK and have a role in nodule formation in Lotus japonicus and Medicago truncatula (Kevei et al., 2007; Zhu et al., 2008; Groth et al., 2010; Hayashi et al., 2010; Murray et al., 2011).

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While the events following the activation of CCaMK have been explored relatively extensively, how the signal perception at the plasma membrane is transduced to changes in the nucleus remains poorly defined. Specifically, the identity of proteins acting directly downstream of the receptors remains unknown. Biochemical, pharmacological, and genetic approaches have identified several possible candidates that can act as secondary messengers connecting events at the plasma membrane to nuclear responses. These include phospholipase C and D proteins, which can generate lipid secondary messengers (den Hartog et al., 2001; Munnik, 2001). It has been proposed that the lipid secondary messengers directly affect the calcium channels present at the nuclear membrane, resulting in the activation of CaMK (Delmas et al., 2005; Oldroyd and Downie, 2006; Downie, 2014). Additional signaling proteins that have been shown to affect nodulation include members of the mitogen-activated protein kinase cascade, 14-3-3 proteins, monomeric GTPases of Rab and Rac family, and the heterotrimeric GTP binding proteins (Fernandez-Pascual et al., 2006; Bianco et al., 2009; Chen et al., 2012; Ke et al., 2012; Radwan et al., 2012; Choudhury and Pandey, 2013). Of these, the components of heterotrimeric G-protein complex are especially interesting as these are traditionally known to interact with the receptors at the plasma membrane and relay the information to intracellular targets in a wide range of signaling pathways in all eukaryotes. Furthermore, heterotrimeric G-protein signaling has been linked to changes in calcium signature, mitogen-activated protein kinase activity, regulation of monomeric GTPases, and phospholipase C- and D-mediated signaling, all of which are involved during nodule development (Park et al., 1993; Zhu and Birnbaumer, 1996; Lopez-Illasaca, 1998; Perfus-Barbeoch et al., 2004; Qi and Elion, 2005; Currie, 2010).

The heterotrimeric G-protein complex is composed of α, β, and γ subunits and the regulator of G-protein signaling (RGS) protein. In the classical signaling paradigm, GDP-bound Gα interacts with GTP and is associated with a cell surface G-protein-coupled receptor (GPCR), representing its inactive stage. Signal perception by GPCR leads to the exchange of GTP for GDP on Gα, which results in the generation of active Gα·GTP and freed GTPγ, both of which can interact with different effectors to propagate the signal. The intrinsic GTPase activity of Gα returns it to its inactive form (Cabrera-Vera et al., 2003; Offemanns, 2003). RGS proteins are one of the key regulators of the G-protein cycle. These act as GTPase activating proteins (GAPs) by enhancing the rate of GTP hydrolysis by Gα. The G-protein cycle can be modified, genetically or biochemically, to favor the presence of active or inactive states (McCudden et al., 2005; Siderovski and Willard, 2005; Lamb et al., 2010). While the basic G-protein components and their overall biochemical activities are conserved between plant and mammalian systems, the plant G-protein cycle seems to be regulated differently. The plant Gα proteins are relatively slower GTPases in comparison to the mammalian Gα proteins and are thought to be constitutively active (Urano et al., 2012b). The RGS protein-mediated acceleration of GTP hydrolysis has been proposed to be the key regulatory step of plant G-protein signaling in contrast to mammalian systems where the GDP/GTP exchange mediated by GPCRs is the rate-limiting step of the G-protein cycle (Johnston et al., 2007; Urano et al., 2012a).

Plants also possess relatively fewer G-protein subunits when compared with the mammalian systems (Chen et al., 2003; Perfus-Barbeoch et al., 2004; Chakraborty et al., 2011). The most elaborate plant G-protein network identified to date is present in soybean where recent genome duplication has led to existence of 4 Gα, 4 Gβ, 12 Gγ, and 2 RGS proteins (Bisht et al., 2011; Choudhury et al., 2011, 2012). Detailed characterization of G-proteins from soybean has offered the opportunity to test their direct role in signaling during nodulation. We previously reported that decreased expression of Gβ and group I Gγ genes leads to a significant decrease in nodule number, whereas the converse is true for the overexpression of specific Gβ and Gγ genes. We have also shown that the Gα proteins interact with the Nod factor receptors NFR1α and NFR1β, even though we did not see an effect of decrease in Gα level on nodule development (Choudhury and Pandey, 2013). Our data suggested two likely possibilities: Either the GTPγ proteins are directly involved in regulation of nodulation with no input from the Gα proteins, or the RNAi-mediated suppression of Gα proteins was not sufficient to result in change in nodulation phenotype.

In this work, we used a combination of genetics and biochemistry to uncover the mechanism of G-protein cycle-dependent regulation of nodulation. Our data show that the active Gα proteins are negative regulators of nodulation in soybean. Changing the availability of free, active Gα proteins by modulating the level of the regulatory RGS proteins results in significantly altered nodule numbers. We further demonstrate that the RGS proteins directly interact with, and are phosphorylated by, the NFR1 proteins. Phosphorylation of Gα proteins has important physiological consequences as overexpression of phospho-dead or phospho-mimic versions of RGS proteins results in a significant effect on nodule formation. Our data support a model where the components of heterotrimeric G-proteins and its regulator act downstream of NFR1 to control nodulation.

RESULTS

Active Gα Proteins Are Negative Regulators of Nodule Formation

The inherent nature of the G-protein cycle entails specific modes of regulation where active Gα and freed GTPγ proteins can either transduce the signal individually with no input or effect on the other subunit or in combination where the availability or proper localization of the subunits is dependent on each other. Less common possibilities, such as signaling by an intact heterotrimer, also exist. These regulatory modes allow for an extremely high degree of plasticity in signal response coupling by G-proteins (Pandey et al., 2010).

In our previous experiments, we observed no significant effect of lower expression of Gα genes on nodulation phenotypes of plants (Choudhury and Pandey, 2013). This could be due to two possible scenarios: One, the signaling is mediated exclusively by freed Gβγ subunits with no input from Gα proteins, as has been observed during regulation of primary root growth in Arabidopsis thaliana (Chen et al., 2006); or two, Gα proteins are in fact involved during this process, but their effect is not obvious because of
incomplete suppression of their expression, which is significantly high in nodules and in hairy roots (Choudhury and Pandey, 2013). To address these two possibilities, we generated additional Gα-RNAi constructs driven by a Figwort mosaic virus (FMV) promoter targeting the highly conserved switch region that is required for its activity, or the C-terminal region (Supplemental Figure 1), and evaluated their effect on nodule development in a soybean hairy root transformation system. Transcript levels of each of the four Gα genes were significantly decreased in both these RNAi lines (Supplemental Figures 2A and 2B). We recorded the number of nodules formed on the hairy roots 32 d post-inoculation (dpi) with Bradyrhizobium japonicum. Interestingly, a significant increase in nodule number was observed for the Gα-RNAi lines compared with the empty vector (EV) containing control lines using both these RNAi constructs (Figures 1A and 1B). On an average −17 nodules were formed per hairy root in EV control lines compared with −23 nodules per hairy root in Gα-RNAi plants (Figures 1A and 1B). These results implied that the effective suppression of Gα gene expression promotes nodulation. This phenotype is opposite of what we had observed previously in the Gβ- and Gγ-RNAi lines (Choudhury and Pandey, 2013), where the suppression of Gβ or Gγ genes resulted in the formation of fewer nodules on transgenic roots. Such opposite regulatory mechanisms by different G-protein subunits have also been seen in Arabidopsis Gα or Gβ mutants during the lateral root formation (Chen et al., 2006) or stomatal development (Zhang et al., 2008), although in both these cases, the Gβ protein acts as a negative regulator of signaling.

To corroborate the role of Gα proteins in regulation of nodule development, we took a gain of function approach. We generated Gα overexpression lines where expression of each of the four Gα genes was driven by a constitutively active Cassava vein mosaic virus (CvMV) promoter or by a nodule-specific Enod40 promoter (Supplemental Figure 1). The transcript level of each Gα gene in overexpression lines was higher compared with their respective EV control lines (Supplemental Figure 3A). The nodulation phenotype of transformed hairy roots was observed after 32 dpi with B. japonicum. Overexpression of individual Gα genes led to a decrease in nodule number per plant (Figure 1C). The phenotype was more pronounced upon overexpression of group II Gα genes (Gα 2 and 3) compared with what was observed with group I Gα genes (Gα 1 and 4). On average, −13 and −8 nodules were observed per hairy root upon overexpression of group I and group II Gα, respectively, compared with −19 nodules per hairy root in EV controls (Figure 1C). In additional experiments, we made use of known point mutant versions of Gα proteins that have distinct effects on their activity. A mutation in a conserved glutamine residue, demonstrated to be important for the GTPase activity of Gα proteins (Q223L in Gα1), results in a GTPase activity-lacking, constitutively active protein (Roy Choudhury et al., 2014). Similarly, we have previously shown that a glycine-to-serine mutation in Gα protein (G196S in soybean Gα1; corresponds to G302S of yeast Gpa1) makes it nearly incapable of being deactivated by RGS protein (Roy Choudhury et al., 2014). Overexpression of Gα1Q223L and Gα1G196S, as confirmed by evaluating the transcript level of the transformed genes (Supplemental Figure 3B), also resulted in a significant decrease in nodule number per transformed root (Supplemental Figure 3C), similar to what was observed with the overexpression of Gα1. These results confirmed a negative role of active Gα proteins during soybean nodulation.

RGS Proteins Affect Nodulation

The role of RGS proteins as key regulators of the G-protein cycle is well established. Since the inherent GTPase activity of plant Gα proteins is thought to be slow, RGS proteins have been proposed to be absolutely required for the generation of GDP-bound Gα proteins and consequently the formation of heterotrimer (Urano et al., 2012a). We have previously shown that both RGS genes of soybean are expressed in roots, hairy roots, and in nodules (Choudhury et al., 2012). We evaluated the transcript levels of RGS1 and RGS2 in response to B. japonicum infection at different time points as well as their expression levels in non-nodulating nod49 and in supernodulating nts382 soybean mutants. In wild-type roots, the transcript levels of both RGS genes increased significantly after B. japonicum infection compared with the noninfected roots at each of the time points tested (Figure 2A). The infected roots also had noticeably more RGS protein compared with its level in noninfected roots (Figure 2A, inset). Furthermore, compared with the wild-type Bragg variety, RGS1 and RGS2 exhibited lower transcript levels in nod49 mutant roots (Supplemental Figures 4A and 4B). Conversely, in nts382 mutant...
roots, higher transcript levels of both RGS genes were observed at each of the time points tested (Supplemental Figures 4A and 4B).

To test the effect of altered expression of RGS proteins during nodule development, RNAi and overexpression approaches were followed. Due to the high sequence identity between two RGS genes, a single construct driven by the FMV promoter was used for RNAi-mediated silencing. The transcript levels of both RGS genes were significantly reduced in RGS-RNAi lines compared with the EV control lines (Supplemental Figure 5A). RGS-RNAi roots displayed significantly less root hair deformation (Supplemental Figure 5B) and lower number of nodules from early on. Approximately 40% reduction in nodule number compared with EV containing roots was observed at 32 dpi (Figures 2B and 2C). Moreover, significantly lower numbers of large, mature nodules were observed on RGS-RNAi hairy roots and ~35% of the nodules were small, pale, and immature (Figure 2D).

**Both Early Perception and Late Developmental Events Are Affected by the Lower Expression of Ga and RGS Genes**

A complex series of signaling and developmental events leads to nodule formation. To evaluate whether the altered expression of Ga and RGS genes affects the early signaling and perception events, or only the later developmental stages resulting in altered nodule numbers, we analyzed a set of early events during nodule formation. We determined the expression levels of two early nodulation marker genes, Enod40 (Figure 3A) and cytokinin oxidase (Figure 3B), in the hairy roots of Ga-RNAi and RGS-RNAi lines at 6, 12, and 24 h postinoculation with *B. japonicum* and compared it to their expression levels in control hairy roots containing empty vectors. Both these genes displayed the expected increase in transcript levels in response to *B. japonicum* infection at each of the time points tested in the EV control plants. Enod40 exhibited overall higher expression in Ga-RNAi and lower expression in RGS-RNAi lines compared with its expression in the EV control plants. The expression of cytokinin oxidase increased to a similar level in both EV control lines and in the Ga-RNAi lines, whereas its expression was significantly lower in the RGS-RNAi lines than that of the EV control at each of the time points tested. These data suggest that the early gene expression changes in response to *B. japonicum* infection are affected by the altered expression of Ga and RGS genes.

We also evaluated the number of deformed root hairs in both Ga-RNAi and RGS-RNAi hairy roots at 4 dpi with *B. japonicum*. Ga-RNAi exhibited significantly higher whereas RGS-RNAi exhibited significantly lower root hair deformation, respectively, compared with the EV control hairy roots (Figure 3C). A similar trend was observed with the developing nodule primordia, where higher and lower numbers of nodule primordia were observed due to lower expression of Ga and RGS genes, respectively, at 6 dpi with *B. japonicum* (Figure 3D) compared with roots containing EV constructs. Cross-sectional views of the similar sized nodules from EV and RGS-RNAi roots exhibited a severe reduction of bacterial infection and bacteroids in the nodules formed on RGS-RNAi roots (Figures 4A to 4C). No additional effect on overall root length or lateral root formation was seen in Ga-RNAi or RGS-RNAi roots (Supplemental Figures 6C and 6D).

**RGS Proteins Are Positive Regulators of Nodule Formation**

To confirm the positive regulation of nodule formation by RGS proteins, we generated RGS1 and RGS2 overexpression lines using constructs driven by the CvMV and Enod40 promoters. Transcript levels of both genes were analyzed in the transformed hairy roots to ascertain their higher expression (Supplemental Figure 7A). Clear differences in nodule numbers were observed, as overexpression of RGS1 and RGS2 led to ~20 and 40% more nodules, respectively (Figures 5A and 5B).
Figure 3. Quantification of Early Changes in Response to *B. japonicum* Infection in *Gα*-RNAi and RGS-RNAi Lines.

(A) and (B) Relative expression of early nodulation marker genes in soybean *Gα*-RNAi and RGS-RNAi hairy roots. Gene-specific primers were used to amplify and quantify the transcript levels of *Enod40* (Glyma01g03470.1) (A) and *Cytokinin oxidase* (Glyma.17g054500.1) (B) at 6, 12, and 24 h post-inoculation with *B. japonicum*. 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 to *Actin* expression. Error bars represent the SE of the mean. Asterisks indicate statistically significant differences compared with EV control (*P* < 0.05; Student’s *t* test).

(C) Quantification of deformed root hairs/cm transgenic roots in *Gα*-RNAi and RGS-RNAi lines.

(D) Quantification of nodule primordia/cm transgenic roots in *Gα*-RNAi and RGS-RNAi lines. The data in (C) and (D) are average values from three independent experiments (n = 10 to 12 plant each replicate). Error bars represent the SE. Asterisks indicate statistically significant differences compared with EV control (*P* < 0.05; Mann-Whitney *U* test).

Plant RGS proteins are chimeric as they contain an N-terminal seven-transmembrane domain that is fused with the C-terminal RGS box-containing domain. This C-terminal domain is responsible for the GAP activity of RGS proteins and for their interaction with *Gα* protein (Chen et al., 2003). To test whether the nodulation-related effects of altered expression of RGS is related to its biochemical activity and consequently its influence on the regulation of G-protein cycle, we overexpressed the N-terminal (1 to 250 amino acids) and C-terminal domains (251 to 464 amino acids) of RGS2 protein in soybean hairy roots. Overexpression of the C-terminal region resulted in a significant increase in nodule number per hairy root, similar to the full-length RGS proteins, whereas no statistically significant effect was seen due to the overexpression of the N-terminal region only (Figure 5C). This implies that the biochemically active domain of the RGS protein is responsible and sufficient for the regulation of nodule development. Finally, to determine whether this effect is linked to the modulation of G-protein cycle and not due to some yet undefined G-protein independent role of RGS proteins in plants, we made use of a GAP activity-dead version of RGS protein. A single point mutation in RGS protein that changes a conserved glutamate to glutamine, lysine, or alanine (E319K/Q/A) abrogates its GAP activity on *Gα* protein (Choudhury et al., 2012) (see also Figure 10). Overexpression of RGS2E319K had no effect on nodule development, confirming that the effect of RGS protein during nodule development is via its regulation of G-protein cycle (Figure 5C). The transcript levels of native and mutated versions of individual genes were tested in all overexpression lines to ascertain their higher expression levels (Supplemental Figure 7B).

Expression of a Subset of Nodulation Marker Genes Corresponds to the RGS-Dependent Regulation of G-Protein Cycle

A number of nodulation marker genes have been characterized in soybean (Bergmann et al., 1983; Yang et al., 1993; Govindarajulu

Figure 4. Altered Morphology of Nodules Formed on RGS-RNAi Hairy Roots.

(A) Light micrographs (4 ×) of soybean root node sections of EV and RGS-RNAi lines. Semithin (5 mm thickness) wax sections, obtained using a microtome, were observed under a light microscope. Bars = 200 μm.

(B) Light micrographs of soybean root nodule at 20 × magnification (partial sectional view). Bars = 100 μm.

(C) Nodule sections (0.5 mm thickness) from resin-embedded material were observed using a phase contrast light microscope at 60 × magnification. Bars = 15 μm.

Mature large nodules of EV and mature large and small nodules of RGS-RNAi lines (32 dpi with *B. japonicum*) were used for microscopic studies and representative images obtained using at least 8 to 10 nodules of three independent experiments are shown. The infected cells in EV-transformed nodules are heavily packed with bacteroids, while the cells in RGS-RNAi lines are only partially filled.
et al., 2009; Libault et al., 2009, 2010; Um et al., 2013). We have previously shown that the expression of many of these genes is altered in the Gβ- and Gγ-RNAi lines (Choudhury and Pandey, 2013).

Since the phenotype of Gα-RNAi roots is opposite to that of the RGS-RNAi roots, we assessed the transcript level of a set of nodulation marker genes in both these backgrounds to determine the extent to which their expression is affected by G-protein cycle. The transcript levels were analyzed at 32 dpi and were compared with the EV control roots. Clear differences in the gene expression patterns were observed. Specifically, the expression of each of the genes tested was significantly downregulated in the RGS-RNAi lines (Figure 6), similar to Gβ- and Gγ-RNAi lines (Choudhury and Pandey, 2013). This suggests that complex gene regulatory patterns exist during nodule formation and the expression of at least a subset of nodulation marker genes is regulated in a G-protein-dependent manner.

**RGS Proteins Interact with NFR1 Receptors**

In metazoan systems, Gα proteins are known to interact with GPCRs, whereas data from various plant species suggest that the plant Gα proteins may couple with additional cell surface-localized receptors such as LRR family receptor kinases (Bommert et al., 2013; Liu et al., 2013; Ishida et al., 2014). Toward this, we have previously shown that the soybean Gα proteins interact with the NFR1 receptors (Choudhury and Pandey, 2013). However, the...
mechanism by which the receptor might affect Gα activity or its interaction with other proteins remains unknown at this time. Since G-proteins and their regulatory proteins usually exist in large macromolecular complexes and the plant RGS proteins are plasma membrane localized, we assessed the direct interaction of RGS proteins with the NFR receptors. The soybean genome encodes two copies of NFR1 and NFR5 proteins, NFR1α and NFR1β, and NFR5α and NFR5β. These are lysM (lysine) motif receptor kinases, with one or two transmembrane domains (Supplemental Figure 8). We cloned full-length NFR1α, NFR1β,

Figure 7. Soybean RGS Proteins Interact with NFR1 Receptors.

(A) Interaction between RGS and NFR using a split ubiquitin-based interaction assay. The picture shows yeast growth on selective media with 200 μM methionine. In all cases, full-length RGS proteins, the N-terminal seven-transmembrane region (7TM), and the C-terminal RGS domain containing RGS proteins were used as NUb fusions in both orientations (NUb-RGS denoting NUb fused to the N terminus of RGS and RGS-NUb denoting NUb fused to the C terminus of RGS). NFR1α was used for the CUB fusion. NUbwt and NUb-vector fusion constructs were used as positive and negative controls, respectively. Two biological replicates of the experiment were performed with identical results.

(B) Interaction between RGS (in 77-nEYFP-N1) and NFR1 (in 78-cEYFP-N1) proteins using BiFC assay. Agrobacteria containing different combinations of RGS1 and RGS2 and NFR1α were infiltrated in tobacco leaves, and reconstitution of YFP fluorescence due to protein-protein interaction was visualized under a Nikon Eclipse E800 microscope with epifluorescence modules. At least four independent infiltrations were performed for each protein combination with similar results.

(C) Interaction between RGS and NFR1α protein using an in vivo co-IP assay. Anti-Myc antibody can pull down HA-tagged RGS2 from total protein extracts of plants expressing 35S:HA-RGS2 and 35S:Myc-NFR1α (lane 1) but not from total protein extracts from plants expressing 35S:HA-RGS2 and Myc-tagged EV (lane 2) or 35S:Myc-NFR1α and HA-tagged EV (lane 3).

(D) The N-terminal seven-transmembrane domain of RGS2 did not interact with NFR1α in a similar in vivo co-IP assay.
NFR5α, and NFR5β genes from soybean nodule cDNA and tested their interaction with the RGS proteins in a split ubiquitin-based interaction system. For these interactions, the full-length or the kinase domain containing the C-terminal region of NFR proteins was expressed as CUB fusions and the RGS proteins were expressed as NUb fusions in both orientations (NUb-RGS and RGS-NUb). NUb fusions were made with full-length, N-terminal, and C-terminal regions of RGS proteins. RGS proteins (full-length as well as C-terminal domain) interacted with NFR1α (Figure 7A) and NFR1β (Supplemental Figure 9A), but not with NFR5α and NFR5β (data not shown), as evaluated by yeast growth on media lacking Leu, Trp, His, and Ade, in the presence of 200 µM Met. The N-terminal region of RGS protein (RGS 7TM) did not interact with NFR1α protein (Figure 7A; Supplemental Figure 9A). Interaction between the two proteins was also assessed by bimolecular fluorescence complementation (BiFC) analysis. Each RGS protein was expressed as an N-terminal fusion to the C terminus of YFP (RGS-cYFP), and NFR1 was expressed as an N-terminal fusion to the N terminus of YFP (NFR1-nYFP). The interaction was confirmed by reconstitution of YFP fluorescence in tobacco (Nicotiana tabacum) leaves coinfiltated with both the expression constructs. Strong YFP fluorescence was observed in all four possible combinations indicating that both soybean RGS proteins can interact with NFR1α and NFR1β proteins (Figure 7B; Supplemental Figure 10A). Similar to yeast based assays, the N-terminal region of RGS2 did not interact with NFR1α, whereas the C-terminal region of RGS2 interacted with NFR1α with similar efficiency as wild-type RGS2 (Figure 7B). The interaction between RGS and NFR1 was further confirmed by in vivo communoprecipitation (co-IP) assay. Tobacco leaves expressing HA-tagged RGS2 and Myc-tagged NFR1α were immunoprecipitated with anti-Myc antibodies. Immunoblotting with anti-HA antibodies showed the presence of RGS2 from plants expressing Myc-tagged NFR1α but not from plants expressing empty vectors (Figure 7C). No interaction was observed when the N-terminal region of RGS2 was used in co-IP assay under identical conditions (Figure 7D). In additional assays, the C-terminal region of NFR1 interacted with the C-terminal region of RGS with similar efficiency as the full-length proteins, in both yeast-based assays (Supplemental Figures 9B to 9E) and in BiFC assays (Supplemental Figure 10B). The N-terminal region of NFR1 did not interact with RGS proteins (Supplemental Figure 10B).

We have previously shown that NFR1 interacts with Gα. Moreover, Gα protein also interacts with RGS. All three proteins are expressed in nodules and in hairy roots (Figure 8A; Supplemental Figure 11). To evaluate the possibility that a tripartite complex might exist between NFR1, Gα, and RGS proteins, we performed additional co-IP assays. Flag-tagged Gα1, HA-tagged RGS2, and Myc-tagged NFR1α proteins were infiltrated in tobacco leaves. The proteins were immunoprecipitated with anti-Myc antibodies. Immunoblotting with Myc, HA, and Flag antibodies showed the protein bands corresponding to the tagged versions of Gα1, RGS2, and NFR1α (Figure 8B), suggesting that the three proteins likely exist as a complex in vivo.

**NFR1 Receptors Phosphorylate RGS Proteins**

NFR1 receptors are active kinases (Madsen et al., 2011; Wang et al., 2014a) and phosphorylate NFR5 coreceptors, but whether there are additional phosphorylation substrates in vivo is not known. The interaction between RGS proteins and the Gα proteins with NFR1 receptors prompted us to evaluate if either of these proteins could be the in vivo substrates of NFR1 receptors. In the Arabidopsis sugar signaling pathway, WNK8 kinase phosphorylates RGS1 and a phosphatase inhibitor calcineurin A increases RGS1 phosphorylation, suggesting that RGS1 can undergo steady state phosphorylation and dephosphorylation (Urano et al., 2012b). Based on sequence identity with Arabidopsis RGS1, soybean RGS proteins are predicted to have 50 potential phosphorylation sites at their C-terminal region. Multiple potential phospho-sites are predicted in Gα proteins as well, based on its homology with the Arabidopsis Gα protein.

We performed in vitro phosphorylation assays using recombinant Gα protein or C-terminal RGS proteins as substrates.
and the C-terminal domain of NFR1α protein as a kinase. NFR1α was able to effectively phosphorylate RGS1 and RGS2 (Figure 9A). No phosphorylation was observed when Goα was used as substrate either in its native or constitutively active form (Figure 9A). NFR1 was also autophosphorylated under these assay conditions (Figure 9A).

A single tyrosine residue present in the activation loop of the L. japonicus homolog of NFR1 has been shown to be important for its kinase activity (Madsen et al., 2011). This amino acid corresponds to Tyr-473 in soybean NFR1α. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis identified 14 phosphorylation sites at minimum localization threshold of 99% within the C-terminal region of phosphorylated NFR1α. Tyr-473 was identified as one of these sites (Supplemental Figure 12). To confirm the specificity of phosphorylation of RGS proteins by NFR1 receptors, we generated its kinase-dead version, NFR1αT473A, and assessed its ability to phosphorylate RGS. No phosphorylation of RGS was observed with the NFR1αT473A even when significantly higher protein quantities were used (Figure 9B). However, in contrast to the mutation in L. japonicus protein, which results in abrogation of its autophosphorylation activity as well as its substrate phosphorylation activity, the soybean NFR1αT473A mutation had no effect on its autophosphorylation activity. Incidentally, the autophosphorylated NFR1αT473A migrates faster on gels compared with the native NFR1α (Figure 9B).

LC-MS/MS analysis identified five phosphorylation sites in NFR1α phosphorylated RGS2 at a minimum localization threshold of 99%: Thr-267, Ser-269, Ser-277, Ser-405, and Thr-428. An additional site, Ser-437, was identified when the minimum localization threshold was decreased to 80% (Supplemental Figures 13 and 14). The Ser-437 phosphosite has been shown to be critical during the RGS1-dependent regulation of the Arabidopsis sugar signaling pathway (Urano et al., 2012b). To validate LC-MS/MS data and to identify the exact residue(s)

![Figure 9. NFR1α Autophosphorylates and Transphosphorylates RGS Proteins.](image-url)

**A** The indicated proteins (recombinant, purified NFR1α C-terminal, RGS1 C-terminal, RGS2 C-terminal, Goα, and GoαQ223L) were subjected to in vitro phosphorylation assay either alone or in combination.

**B** Effect of a point mutation in the active site of NFR1α (NFR1αT473A) on RGS protein phosphorylation.

**C** Phosphorylation assay using different single point mutant versions of RGS2 proteins (based on the information from LC-MS/MS data).


Recombinant purified proteins were incubated with NFR1α protein for in vitro phosphorylation assay and gel image was visualized after Pro-Q Diamond phosphoprotein gel staining. 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. The bottom panel of **C** and **D** shows the quantification of band intensity of phosphorylated RGS2 protein by Image J. Asterisks indicate statistically significant differences compared with native RGS2 control (*P < 0.05; Student’s t test).
Constitutive Expression of Phospho-Mimic RGS Proteins Results in Significant Increases in Nodule Formation

To determine the in planta effect of RGS phosphorylation on nodule formation in soybean, the phospho-mimic versions of RGS were cloned into overexpression vector (driven by the CvMV promoter) and transformed into soybean hairy roots. All transgenic roots were tested for increased transcript accumulation (Supplemental Figure 18). Clear differences in nodule numbers were observed when roots were transformed with native versus phospho amino acid variant constructs. Overexpression of phospho-deficient mutant RGS2S405A or RGS2hexaA resulted in a decrease in nodule number compared with the native RGS2 overexpression. Importantly, nodule number significantly increased in the presence of phospho-mimic mutants, RGS2S405D and RGS2hexaD, compared with what was observed with the overexpression of native RGS2 (Figure 11). Similar results were obtained when using only the C-terminal regions of native and mutant RGS proteins driven by CvMV or Enod40 promoters (Supplemental Figure 19). These data suggest that phosphorylation of RGS proteins by NFR1 receptors positively regulates nodule formation in soybean.

We further assessed the ability of RGS proteins to restore nodule formation in the non-nodulating nod49 mutant of soybean, which lacks a functional NFR1α. Expression of native NFR1α gene (used as a positive control) partially restored nodulation (Figure 12, Table 1). Overexpression of RGS2 and its phospho-mimic versions RGS2S405D and RGS2hexaD also resulted in development of few nodules on nod49 mutant hairy roots (Figure 12A), whereas no nodules were observed on roots transformed with EV or phospho-dead version of RGS2 (RGS2S405A and RGS2hexaA). The nodules that developed on the RGS2hexaD transformed nod49 roots exhibited normal morphology, similar to the nodules formed on wild-type roots (Figure 12B). However, the nodule numbers in all these cases were significantly lower than the nodules formed on a normal hairy root (Figure 12, Table 1).

**DISCUSSION**

The Role of G-Protein Complex during Nodule Formation in Soybean

Heterotrimeric G-proteins are involved in regulation of a range of physiological processes in plants, similar to what has been discovered in yeast and metazoans. The simplicity of G-protein complex system in Arabidopsis, together with the ease of performing direct genetic analysis of loss-of-function mutants, has enabled the identification of a multitude of processes that involve G-proteins. Phenotypic analysis combined with large-scale transcriptomic analyses have also identified several modes of G-protein function, some similar to what exists in other systems and others specific to plants (Pandey et al., 2010; Urano and Jones, 2014).
Native RGS2 gene, single mutant phospho-dead and phospho-mimic versions (RGS2S405A and RGS2S405D), and hexa mutant phospho-dead and phospho-mimic versions (RGS2HexaA and RGS2HexaD) of RGS2 genes driven by the CvmV promoter were used for hairy root transformation. Nodules developed on overexpression roots were counted at 32 dpi with B. japonicum and compared with the EV-containing hairy roots. The data represent average of three biological replicates (40 to 50 individual plants/biological replicate) containing transgenic nodulated roots. Different letters indicate significant differences (Dunn’s multiple comparisons test, P < 0.05) between samples.

The inherent nature of the G-protein cycle predicts different regulatory modes. Activation of the G-protein cycle results in generation of Gβγ and freed Gβγ subunits and depending on the involvement of both these entities or only one, the modes are defined as classical modes I and II, respectively. In classical mode Ia, both Gα and Gβγ interact with downstream effectors. The phenotypes of mutants lacking either of these subunits are similar, as has been demonstrated during G-protein-regulated leaf shape and abscisic acid responses in Arabidopsis (Perfus-Barbeoch et al., 2004). In classical mode Ib, only Gα interacts with the downstream effectors, but the Gβγ subunits are required for its correct localization. In this case too, the phenotypes of mutants lacking one or both subunits are similar (Pandey et al., 2010). In classical mode II, only the Gβγ dimer interacts with the downstream effectors. Pathways controlled by this regulatory mode exhibit opposite phenotypes due to lack of Gα or Gβγ, as has been seen during the development of lateral roots or stomata. In such cases, the lack of Gβγ results in abrogation of signal transduction, whereas the lack of Gα results in constitutive signaling by Gβγ since it can no longer be sequestered in its inactive conformation (Chen et al., 2006; Pandey et al., 2010). Our previous results with G-protein regulation of nodule development suggested the involvement of Gβγ subunits, but the effect of Gα subunits remained inconclusive (Choudhury and Pandey, 2013). Furthermore, additional regulatory steps such as the involvement of RGS protein and its effects on the activation/deactivation of G-protein cycle during nodule formation were also not known.

The data presented here clearly show a distinct role of Gα proteins and regulation of their activity by RGS proteins during nodule formation in soybean. Effective silencing of Gα proteins resulted in higher nodule numbers compared with the EV-containing hairy roots, which is opposite of the effect of Gβ and Gγ silencing proteins showing relatively high expression (Choudhury and Pandey, 2013). It may be that the effective increase in group II Gα proteins is much higher due to their lower basal expression in hairy roots and nodules, thereby resulting in stronger suppression of nodule formation. Alternatively, there might be inherent differences between these proteins, as we have shown previously during the complementation of yeast Gpa1 mutants in pheromone response pathways (Roy Choudhury et al., 2014). However, it is clear that Gα proteins are negative regulators of nodule formation in soybean (Figures 1A and 1B). Moreover, overexpression of individual proteins, using two different promoters, resulted in lower nodule numbers, corroborating the data obtained by RNAi approaches (Figure 1C). Furthermore, there were differences between the group I Gα proteins (Gα1 and Gα4) versus group II Gα proteins (Gα2 and Gα3), with group II proteins showing stronger phenotypes (Figure 1C). These two subgroups also exhibit different expression levels during nodule development, with group I proteins showing relatively high expression (Choudhury and Pandey, 2013). It may be that the effective increase in group II Gα proteins is much higher due to their lower basal expression in hairy roots and nodules, thereby resulting in stronger suppression of nodule formation. Alternatively, there might be inherent differences between these proteins, as we have shown previously during the complementation of yeast Gpa1 mutants in pheromone response pathways (Roy Choudhury et al., 2014). However, it is clear that Gα proteins are negative regulators of nodule formation in soybean.

**Figure 11.** Nodule Formation on Transgenic Soybean Hairy Roots Overexpressing Phospho-Dead and Phospho-Mimic Versions of RGS Protein.

[Graph showing nodule formation on transgenic soybean hairy roots overexpressing phospho-dead and phospho-mimic versions of RGS protein.]
reverse is true for lower RGS levels. A lower percentage of nodules formed on the infected roots had a significantly higher number per plant (Figures 2A, 3A, and 6). These genes displayed a nodule development-dependent increase in their expression and the B. japonicum-infected roots had a significantly higher level of RGS proteins compared with the noninfected roots (Figure 2A). Furthermore, altering the level of RGS proteins by RNAi-mediated silencing or by overexpression resulted in lower and higher nodule number per root, respectively (Figures 2 and 5). Both the early events during nodulation such as expression of marker genes, root hair deformation, and formation of nodule primordia (Figure 3), and the later stages of nodule development (Figures 2F and 4) were affected by altered expression of G-protein complex genes. A higher percentage of nodules formed on the RGS-RNAi hairy roots were not fully developed (Figures 2F and 4).

Also, the role of RGS proteins during regulation of nodule formation is not independent of the G-protein cycle per se. Higher expression of the C-terminal RGS domain-containing region has the same effects as the full-length proteins, and an inactive RGS protein, RGS2S405D, which has no effect on the GTPase activity of Gα protein (Figure 10), does not affect nodule formation (Figure 5C). Gene expression analysis suggests that at least a subset of nodulation marker genes are oppositely regulated in Gα-RNAi lines versus RGS RNAi/lines (Figures 3A, 3B, and 6). These genes could be direct downstream targets of regulation via G-protein-mediated pathways. Future large-scale analysis of gene expression changes in different G-protein mutants will shed light on such targets. However, these data do suggest that the role of RGS proteins during nodule development is to keep Gα in its inactive, trimeric conformation. Overexpression of RGS proteins leads to lower levels of free Gα, resulting in more nodules, whereas the reverse is true for lower RGS levels.

Table 1. The Effect of Overexpression of GmNFR1α and Different Phospho-Mimic and Phospho-Deficient Version of GmRGS2 Gene on Soybean Nodulation

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<th>Construct</th>
<th>No. of Plants</th>
<th>Total No. of Nodulated Plants</th>
<th>Nodules/Nodulated Plants</th>
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<td>0</td>
</tr>
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<td>18</td>
<td>2.17 ± 0.26</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
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<td>57</td>
<td>42</td>
<td>3.14 ± 0.20</td>
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The root system of nod49 was transformed with Agrobacterium rhizogenes strain K599 carrying GmNFR1α and different GmRGS2 cDNAs driven by the CvMV promoter. Nodulation is expressed as nodule number per plant (± se). Nodule numbers were recorded 32 dpi with B. japonicum.

Phosphorylation of RGS Proteins by NFR1

The paucity of classical G-protein coupled receptors has posed interesting questions about the activation of G-protein cycle in plants. Very few proteins which may act upstream of G-proteins have been identified. These include several receptor-like kinases (RLKs) that have been shown to directly interact with Gα proteins (Bommert et al., 2013; Choudhury and Pandey, 2013). Similarly, many established pathways that are controlled by well-defined RLKs have been shown to involve members of G-protein complex (Llorente et al., 2005; Bommert et al., 2013; Liu et al., 2013; Ishida et al., 2014). However, in most cases, the mechanism by which the receptors regulate the G-protein cycle remains unknown. We have previously shown that the soybean Gα proteins interact with the NFR1 receptors, but there was no effect of this interaction on Gα activity (Choudhury and Pandey, 2013). Our current work shows that the Gα proteins are not phosphorylated by NFR1. The precise effect of such an interaction remains unknown; however it is possible that interaction with NFR1 changes the availability of Gα proteins for additional effectors or interacting partners.

Interestingly, RGS proteins can also interact with the NFR1 receptors (Figure 7), and the three proteins likely form a tripartite complex in vivo (Figure 8). This is exciting as plant RGS proteins are plasma membrane localized due to the presence of the seven-transmembrane domain and are likely present in close proximity to the plasma membrane-localized NFR1 receptors and Gα proteins. However, in contrast to Gα, the RGS proteins are efficiently phosphorylated by NFR1 receptors (Figure 9A). NFR1 proteins are well-established kinases and are known to phosphorylate the NFR5 coreceptors, but the extent to which they phosphorylate additional proteins in cells was not known (Madsen et al., 2011). Similarly, RGS proteins are well-known phosphorylation substrates in metazoan signaling systems (Garrison et al., 1999; Hollinger et al., 2003; Sokal et al., 2003; Huang et al., 2007; Moroi et al., 2007; Xie et al., 2010; Kach et al., 2012) and during sugar signaling in Arabidopsis, but their phosphorylation by a receptor-like kinase may suggest a novel regulatory mode of G-protein signaling in Arabidopsis.
signaling pathways in plants. Phosphorylation of RGS proteins affects their biochemical activity (Figure 10), and this effect is clearly biologically relevant because overexpression of the phospho-mimic versions of the proteins results in significantly higher nodule numbers in wild-type soybean hairy roots (Figure 11) and partial restoration of the nodulation phenotype in nod49 mutant roots (Figure 12, Table 1). Incidentally, during sugar signaling pathway in Arabidopsis, RGS1 is phosphorylated by a WNK family kinase, and this phosphorylation alters its plasma membrane localization, thereby making it unavailable to accelerate G-protein hydrolysis (Urano et al., 2012b). In comparison, RGS phosphorylation by NFR1, which occurs at different sites than that by WNK family kinases, affects its activity, but not localization (Supplemental Figure 20). It may be that during the regulation of signaling pathways that require an active Gα, RGS protein is removed from the vicinity of G-proteins to maintain them in an active conformation. On the contrary, pathways that are negatively regulated by an active Gα protein may require active RGS at the plasma membrane to maintain the G-proteins in their trimeric conformation. These data can also be expanded to other signaling systems where the involvement of G-proteins has been shown in conjunction with various RLKs. It is likely that other RLKs can also phosphorylate RGS, thereby regulating G-protein-mediated responses. Further analyses of additional RLKs in the context of their effect on RGS activity and/or localization may shed light on these mechanistic details and possibly identify novel, plant-specific regulatory mechanisms.

Mechanism of G-Protein Regulation of Nodule Formation

Based on these data, we propose the following model for G-protein complex-dependent regulation of nodulation in soybean (Figure 13). During nodule development, free (active) Gα proteins act as negative regulators of signaling. Nod factor binding activates NFR1 receptors, which phosphorylate RGS, thereby activating them. Active, phosphorylated RGS maintains Gα proteins in their trimeric, inactive conformation, thus allowing for nodulation. Higher expression of Gβγ proteins sequesters the available pool of Gα in trimeric conformation, resulting in higher nodule numbers. Additionally, an independent role of GRS proteins or freed Gβγ proteins in directly interacting with downstream effectors to regulate nodulation cannot be ruled out at this stage (Figure 13). These data, together with previously identified roles of RGS in the Arabidopsis sugar signaling pathway, suggest that plants possibly use the RGS-based regulation of G-protein deactivation as a predominant control mechanism, compared with metazoans, which essentially use a GPCR-based regulation of G-protein activation as a key mechanism. However, the prevalence of such a regulatory mechanism is notable also in the context of plants such as rice (Oryza sativa), Brachypodium distachyon, and many other grasses that do not have a canonical RGS homolog in their genomes. Whether novel, yet unidentified proteins fulfill such a regulatory role, or the G-protein cycle is regulated differently in these plants remains to be investigated.

Our model also opens up several questions, most important being: What is downstream of the G-protein cycle during nodulation? It is well established that the nuclear-localized CCaMK proteins are central to nodule development (Gleason et al., 2006; Tirichine et al., 2006; Takeda et al., 2012). How might the chain of events started at the level of plasma membrane-localized G-proteins be continued to the nuclear proteins? As we have mentioned previously, multiple downstream components proposed to be important for successful nodulation have been linked to G-protein components in other organisms. Future studies targeted toward dissecting the individual signaling components will certainly unravel many of these pathways. Another important question is whether there are proteins or signaling complex(es) in addition to G-proteins, which are involved in transducing the signal from plasma membrane to intracellular components. Nodulation is an important, high energy-demanding event and plants must have evolved multiple layers of regulation to intricately balance it under any given environment. What these additional components might be and how they might interact with the G-protein-regulated processes will be an active area of future research.

METHODS

Plant Material and Construction of Vectors

Soybean (Glycine max) wild-type (‘Williams 82’ and ‘Bragg’) and mutant (super-nodulating nitrate tolerant symbiotic382 ‘nts382’ and a non-nodulating ‘nod40’) seeds were grown on Pro-Mix BX soil (Premier Horticulture) in the greenhouse (16 h light/8 h dark) at 25°C.

Mutant versions of Gα1 and RGS2 proteins were prepared by site-directed mutagenesis using the QuikChange PCR method (Agilent). For overexpression constructs, native and site-directed mutant versions of genes were cloned into the plR8/GW vector (Invitrogen) and confirmed by sequencing. Each overexpression construct was transfected by Gateway-based cloning into pCMV promoter and Enod40 promoter containing binary vectors (pCAMGFp-CMV-GWi and pCAMGFp-GmEnod40-2p:GW) using LR clonase (Choudhury and Pandey, 2013). The RNAi constructs were expressed under the control of the FMV promoter in GGT11017A vector (Govindaraju et al., 2008). After sequence verification, all constructs including empty vectors (used as controls), were transformed into Agrobacterium rhizogenes strain K599.

Hairy Root Transformation and Evaluation of Nodulation Phenotypes

Shoot apices with a single fully expanded trifoliate leaf from 2-week-old soybean plants were used for hairy root transformation. The hairy root transformation of soybean was performed essentially as described previously (Govindaraju et al., 2009; Choudhury and Pandey, 2013). After transformation, the plants were maintained in nitrogen-free media. To assay the deformed root hairs and the nodule primordia, plants were infected with Bradyrhizobium japonicum (strain USDA110). Root segments 3 cm below the root-hypocotyl junction were cut and harvested at 4 and 6 dpi, respectively. Roots were fixed with ethanol/glacial acetic acid (3:1) for 2 h and stained with 0.01% methylene blue for 15 min (Wang et al., 2014b). The stained transgenic roots were observed by light microscopy to detect the deformed root hairs and nodule primordia in three different biological replicates each with n = 10 to 12 roots. Root hairs with curly/wavy growth direction, bulged tips, or branching at 4 dpi with B. japonicum were considered as deformed root hairs (Supplemental Figure 6A). Nodule primordia were counted at 6 dpi with B. japonicum. Early nodule primordia were differentiated from the lateral root primordia by their cell division patterns. Nodule primordia typically have a higher frequency of cell division
RNA Isolation and Real-Time Quantitative PCR

The soybean hairy roots were collected at different time points after *B. japonicum* infection. Total RNA was isolated from uninoculated and inoculated hairy roots, using Trizol reagent (Invitrogen). RNA samples were treated with DNase I to remove the genomic DNA. First-strand cDNA was synthesized using the Superscript III RT kit (Invitrogen). Real-time quantitative PCRs were performed as previously described (Bisht et al., 2011) with a StepOne Plus real-time PCR system (Applied Biosciences) using the SYBR advantage qPCR premix (Clontech). The oligonucleotide primers used for PCR are listed in Supplemental Table 1. Two different biological replicates with three technical replicates each were performed for each experiment. The data were averaged and statistically significant values were determined by Mann-Whitney U test.

Microscopy

For analysis of the nodule cross sections, mature nodules from RNAi- and empty vector-containing transgenic roots were fixed in 2% glutaraldehyde containing 0.1 M PIPES buffer (pH 6.8) for 2 h. Sample preparation and microscopic observation were performed as described previously (Choudhury and Pandey, 2013). For phase contrast microscopy, nodules from RGS-RNAI and EV-transformed lines were fixed by high-pressure freezing and packed in 0.1 M PIPES buffer (pH 6.8) plus 300 mM sucrose. Samples were freeze substituted for 5 d at −80°C in 2% osmium tetroxide in acetone, then slowly thawed to room temperature, rinsed in acetone, and embedded in Spurr’s resin. Nodule sections (0.5 μm thick) were imaged using Nikon Eclipse 800 wide-field microscope.

For the reporter gene expression analysis, putative promoter regions of *Gm1f* (−2 kb), *Gm2* (−1.5 kb), and *NFR1a* (−1.5 kb) were amplified from cv Williams 82 genomic DNA and cloned in pcr8GW vector (Invitrogen). Sequence confirmed clones were then introduced into pYXT1 or pYXT2 destination vectors carrying the GUS and GFP reporter genes, respectively (Xiao et al., 2005). These constructs were transformed using the hairy root transformation system and transgenic roots were inoculated with *B. japonicum*. The detection of GFP fluorescence and GUS assay were performed 10 d after *B. japonicum* infection in hairy roots and different days after infection in nodules. To observe the cellular level expression, 28-d-old mature nodules were sectioned followed by GUS staining.

Protein-Protein Interaction Assays and Immunoblotting

Interaction assays between *Gm*, RGS, and NFR proteins using split ubiquitin-based and BIFC-based assays were performed essentially as previously described (Bisht et al., 2011; Choudhury et al., 2012). At least two independent transformations for the split ubiquitin-based assays and four independent transformations for BIFC-based assays were performed. For co-IP assays, 3- to 4-week-old tobacco (*Nicotiana tabacum*) plants were used for *Agrobacterium tumefaciens*-mediated transient expression of soybean RGS2 fused to HA tag, soybean NFR1a fused to Myc tag, and soybean Gm1 protein fused to the Flag tag. After 3 to 4 d, proteins were extracted from inoculated leaves in the extraction buffer containing 250 mM sucrose, 25 mM HEPES-KOH, pH 7.5, 10 mM MgCl2, 1 mM PMSF, 1 mM DTT, and 1% Triton X-100 with protease inhibitor cocktail (Sigma-Aldrich). The homogenate was centrifuged at 8000g for 10 min to remove debris. For each immunoprecipitation experiment, ~200 μg of protein extracts was incubated with anti-Myc antibody in the presence of binding buffer (25 mM HEPES-KOH, pH 7.5, 100 mM NaCl, 3 mM MgCl2, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail). The immunocomplex was adsorbed into binding buffer equilibrated with protein A agarose according to the manufacturer’s protocol (Sigma-Aldrich). The mixture was incubated overnight at 4°C with gentle rotation. Protein A agarose beads were washed three times with 1 mL of binding buffer by gentle shaking followed by centrifugation. Lastly, pulled-down proteins were separated on SDS-PAGE and analyzed by immunoblotting with anti-HA, anti-Myc, and anti-Flag antibodies (Sigma-Aldrich). To detect RGS protein levels, microsomal protein fractions isolated from *B. japonicum* inoculated or noninoculated hairy roots were used for immunoblotting with RGS antibodies, essentially according to Bisht et al. (2011).

Recombinant Protein Purification and in Vitro GTPase Activity Assay

Native and mutant versions of *Gm*, C-terminal region of RGS, and C-terminal region of NFR1α proteins were cloned into pET28a vector (Novagen), and the proteins were expressed and purified as described previously (Bisht et al., 2011). GAP activity of native and mutant versions RGS proteins was assayed using the ENZcheq phosphate assay kit (Invitrogen) essentially according to Choudhury et al. (2012). Real-time fluorescence-based GTP binding and GTP hydrolysis assays were performed using BODIPY-GTP FL (Choudhury et al., 2013).

Accession Numbers

Sequence data from this article can be found in the SoyKB (http://soykb.org/) under the following accession numbers: *Gm1f* (Glyma04g05960.1), *Gm2* (Glyma17g34450.1), *Gm3* (Glyma14g11140.1), *Gm4* (Glyma06g05960.1), *RGS1* (Glyma18g01490.1), *RGS2* (Glyma11g37540.1), *Enod40* (Glyma01g03470), *Nodulin35* (Glyma10g23790), *Apyrase GS52* (Glyma16g04750), and *Calmodulin* (CaM)-like protein (Glyma02g06680).

Supplemental Data

Supplemental Figure 1. Constructs used for RNAi-mediated silencing of soybean *Gm* and *RGS* driven by FMV promoter and for overexpression of *Gm* and *RGS* driven by constitutive (*CvMV*) and nodule-specific (*Enod40*) promoter.

Supplemental Figure 2. Transcript levels of soybean *Gm* genes in *Gm*-RNAi hairy roots and nodulation phenotypes.

Supplemental Figure 3. Transcript levels of soybean *Gm* genes in *Gm*-overexpressing hairy roots and nodulation phenotypes.

Supplemental Figure 4. *B. japonicum*-induced expression of soybean RGS1 and RGS2 in wild-type, non-nodulating, and supernodulating soybean hairy roots at different time points.

Supplemental Figure 5. Study of expression levels of RGS genes and root hair deformation in RGS-RNAi silenced transgenic hairy roots.

Supplemental Figure 6. Phenotypes of root hairs, lateral root primordia, nodule primordia, and roots in *Gm*-RNAi and RGS-RNAi transgenic lines.

Supplemental Figure 7. Expression levels of RGS genes in RGS-overexpressing transgenic hairy roots.

Supplemental Figure 8. Domain architecture of soybean NFR1α.
Supplemental Figure 9. Interaction between soybean RGS1 and RGS2 with NFR1α and NFR1β using split ubiquitin-based interaction assay.

Supplemental Figure 10. Interaction between RGS (in 77-cEYFP-N1) and NFR1 (in 78-cEYFP-N1) using bimolecular fluorescence complementation assay.

Supplemental Figure 11. Analysis of promoter of Gα1, RGS2, and NFR1α.

Supplemental Figure 12. Detection of phosphorylated amino acid residues in NFR1α C-terminal kinase domain by LC-MS/MS after in vitro phosphorylation assay.

Supplemental Figure 13. Detection of phosphorylated amino acid residues in RGS2 C-terminal region by LC-MS/MS after in vitro phosphorylation assay.

Supplemental Figure 14. Detection of phosphorylation sites within soybean RGS2 C-terminal region by LC-MS/MS after in vitro phosphorylation assay.

Supplemental Figure 15. Changes of GTPase activity of Gα1 in the presence of different mutant versions of RGS2.

Supplemental Figure 16. Interaction between Gα1 with wild-type and mutant RGS2 and NFR1α using split ubiquitin-based interaction assay.

Supplemental Figure 17. Myc-tagged wild-type NFR1α and mutant NFR1αT473A associate with soybean RGS2 in vivo.

Supplemental Figure 18. Transcript level of soybean RGS2 in native and mutant RGS-overexpressing hairy roots.

Supplemental Figure 19. Nodule formation on transgenic soybean and mutant RGS1 using bimolecular fluorescence complementation assay.

Supplemental Figure 20. Localization of native and mutant RGS2.

Supplemental Table 1. Primers used in experiments described in the article.

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AUTHOR CONTRIBUTIONS

S.P. conceived, directed, and supervised the study. S.R.C. conducted all of the experimental work. Both authors contributed to designing experiments, interpreting results, and writing the article.

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Phosphorylation-Dependent Regulation of G-Protein Cycle during Nodule Formation in Soybean
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