Specific Subunits of Heterotrimeric G Proteins Play Important Roles during Nodulation in Soybean[1][W][OA]

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Heterotrimeric G proteins comprising Gα, Gβ, and Gγ subunits regulate many fundamental growth and development processes in all eukaryotes. Plants possess a relatively limited number of G-protein components compared with mammalian systems, and their detailed functional characterization has been performed mostly in Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*). However, the presence of single Gα and Gβ proteins in both these species has significantly undermined the complexity and specificity of response regulation in plant G-protein signaling. There is ample pharmacological evidence for the role of G proteins in regulation of legume-specific processes such as nodulation, but the lack of genetic data from a leguminous species has restricted its direct assessment. Our recent identification and characterization of an elaborate G-protein family in soybean (*Glycine max*) and the availability of appropriate molecular-genetic resources have allowed us to directly evaluate the role of G-protein subunits during nodulation. We demonstrate that all G-protein genes are expressed in nodules and exhibit significant changes in their expression in response to *Bradyrhizobium japonicum* infection and in representative supernodulating and nonnodulating soybean mutants. RNA interference suppression and overexpression of specific G-protein components results in lower and higher nodule numbers, respectively, validating their roles as positive regulators of nodule formation. Our data further show preferential usage of distinct G-protein subunits in the presence of an additional signal during nodulation. Interestingly, the Gα proteins directly interact with the soybean nodulation factor receptors NFR1α and NFR1β, suggesting that the plant G proteins may couple with receptors other than the canonical heptahelical receptors common in metazoans to modulate signaling.

Heterotrimeric G proteins (hereafter, G proteins) consisting of α-, β-, and γ-subunits are key signaling intermediates in all eukaryotes (Cabrera-Vera et al., 2003). In metazoans, the importance of G proteins in regulating fundamental signaling pathways involved in sensory perception, neurotransmission, hormone perception, and immunity-related cues has prompted their in-depth characterization (Cabrera-Vera et al., 2003; Offermanns, 2003). Such studies have revealed an elegant mechanism where a signal-dependent exchange of GTP for GDP on Gα protein leads to the dissociation of inactive GDP·Gαβγ heterotrimer into active GTP·Gα and Gβγ dimer. Both these freed entities can interact with a variety of different effector proteins to transduce the signal. The inherent GTPase activity of Gα generates GDP·Gα, which reassociates with the Gβγ dimer, and the proteins return to the inactive GDP·Gαβγ trimeric conformation. Depending on the regulation of a particular signaling pathway by Gα and Gβγ proteins, individually or in combination, different outputs can be predicted (Pandey et al., 2010). In addition, in some cases, alternative signaling mechanisms, such as signaling only via Gβγ with no input from Gα protein or signaling by a nondissociated heterotrimer, have also been reported (Adjobo-Hermans et al., 2006; Pandey et al., 2010).

In plants, the structure/function information on G proteins is mostly limited to Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*), where their roles have been established in regulation of a multitude of signaling pathways, such as those regulated by multiple phytohormones, sugar, light, and pathogens (Fujisawa et al., 1999; Ueguchi-Tanaka et al., 2000; Ullah et al., 2001; Wang et al., 2001; Pandey and Assmann, 2004; Oki et al., 2005; Pandey et al., 2006; Trusov et al., 2006; Temple and Jones, 2007; Warpeha et al., 2007; Nilson and Assmann, 2010; Chakravorty et al., 2011; Utsunomiya et al., 2011). However, compared with the presence of multiple G-protein subunits in mammals (e.g. 23 Gα, 5 Gβ, and 12 Gγ in humans), Arabidopsis contains only 1 Gα, 1 Gβ, and 3 Gγ proteins. Similarly, humans have approximately 1,000 G-protein coupled receptors (GPCRs), whereas only a few divergent GPCRs have been identified in Arabidopsis (Gookin et al., 2008). Therefore, the diversity that is present in the mammalian G-protein signaling pathways due to the existence of signal-specific combinations of receptors and G-protein subunits remains underestimated in plants. Furthermore, the identity of receptors that couple with Gα proteins also remains elusive for many G-protein-regulated signaling pathways in plants (Gookin et al., 2008; Pandey et al., 2009).

Multiple pharmacological and biochemical experiments have provided indirect evidence for heterotrimeric G proteins' involvement in control of nodule formation in many leguminous species. For example, the G-protein

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agonist mastoparan mimics Nod factor-induced gene expression in *Medicago truncatula* root hair cells during symbiosis (Sun et al., 2007) and root hair deformation in *Vigna unguiculata*, which is inhibited by the G-protein antagonist pertussis toxin (Kelly and Irving, 2003). Several established downstream components of G-protein signaling, such as phospholipase C and D (De Los Santos-Briones et al., 2009), phosphatidic acid, diacylglycerol pyrophosphate, and G-protein related phosphoinositoid 3-kinase have been shown to be involved in regulation of nodulation (Peleg-Grossman et al., 2007). However, the lack of information on well-characterized G-protein components from any leguminous species till very recently and the relatively limited availability of molecular-genetic resources have restricted a targeted evaluation of the role of G proteins during nodulation.

We have recently identified an elaborate G-protein family in soybean (*Glycine max*), consisting of 4 Gα, 4 Gβ, and 10 Gγ proteins (Bisht et al., 2010; Choudhury et al., 2011), which has greatly expanded the plant G-protein networks. Detailed analysis of these proteins has started to reveal properties reminiscent of mammalian G proteins such as subunit-specific expression patterns, interaction specificity between different protein subunits, and distinct biochemical activities of individual Gα proteins (Choudhury et al., 2012); however, the functional significance of such differences remains unclear.

We have now used the hairy root transformation system (Govindarajulu et al., 2009) to generate transgenic soybean lines expressing altered levels of specific G-protein gene families to directly evaluate their role in regulation of nodulation. Moreover, using nodulation as the readout of a complex biological phenomenon, we have also assessed some of the fundamental questions related to the specificity of G-protein signaling in plants. Specifically, we have asked if there is preferential usage of particular subunits and whether duplicated genes have undergone any subfunctionalization. In addition, we have also explored the extent to which the subunit specificity and usage changes in the presence of an additional signal during nodulation. Furthermore, given the general position of G proteins as integrators of receptor and effector proteins during signaling, we evaluated whether G proteins interact with known Nod factor receptors (NFRs). Our data demonstrates that heterotrimeric G proteins are positive regulators of nodule formation and Gα proteins directly interact with NFR1 proteins. We also show that subunit-specific and signal-dependent G-protein heterotrimers regulate nodule formation, underscoring the intricacy of plant G-protein networks.

RESULTS

Specific G-Protein Genes Are Up-Regulated in Response to *Bradyrhizobium japonicum* Infection and Exhibit Altered Expression in Supernodulating and Nonnodulating Mutants of Soybean

The soybean G proteins have been divided in different subfamilies (e.g. Gα I and II, Gβ I and II, and Gγ I, II, and III) based on their sequence similarities and biochemical properties (Bisht et al., 2010; Choudhury et al., 2011). Many of the G-protein genes are expressed in the hairy roots and nodules of soybean (Bisht et al., 2010; Choudhury et al., 2011); however, it is not known whether their expression is affected by infection with compatible bacteria. We evaluated the expression levels of the four *Gα*, four *Gβ*, and 10 *Gγ* genes in soybean roots (cv Williams 82) in response to *B. japonicum* infection. The group I Gα genes *Gα1* and *Gα4* showed a sharp increase in transcript level compared with their expression in noninfected control root, whereas the expression of the group II Gα genes *Gα2* and *Gα3* remained mostly unchanged (Fig. 1; Supplemental Fig. S1). Two Gβ genes, *Gβ3* and *Gβ4*, also showed modest (approximately 5-fold) but significant increase in expression level in response to *B. japonicum* infection (Fig. 1). Changes in expression were also observed for the specific members of group I and group III *Gγ* genes, ranging from 4- to 20-fold. Interestingly, the group II *Gγ* genes, though expressed in nodules, did not exhibit a significant change in their transcript level in response to *B. japonicum* infection (Fig. 1).

To evaluate whether the differential expression of G-protein transcripts in response to *B. japonicum* infection is biologically relevant, we analyzed their expression in a supernodulating *nitrate tolerant symbiotic382* (*nts382*) and a nonnodulating *nod49* (Carroll et al., 1985a, 1985b; Mathews et al., 1989) mutant of soybean 'Bragg' following treatment with *B. japonicum*. Interestingly, the expression of all but one of the 18 genes analyzed was significantly lower in the *nod49* mutant compared with wild-type roots (Fig. 2). The *nod49* gene encodes for NFR1 and is directly involved in perceiving the Nod factors produced by rhizobia to initiate nodulation (Mathews et al., 1989; Indrasumunar et al., 2011). By contrast, the expression of G-protein genes in *nts382* plants, which harbor a mutation in a nodulation autoregulation receptor kinase (Carroll et al., 1985a, 1985b; Miyahara et al., 2008), was either similar to or higher than their expression in wild-type roots (Fig. 2). A clear specificity was observed in the cases of two Gα subfamily genes, where, similar to *B. japonicum*-dependent up-regulation, the transcript levels of group I, but not group II, *Gα* genes were higher in *nts382* mutants compared with the wild-type Bragg cultivar roots. Overall, an increase in the transcript level of different G-protein genes both in response to *B. japonicum* infection and in a supernodulating mutant, and a significantly lower expression in a nonnodulating mutant, strongly suggests their role during nodulation.

Heterotrimeric G Proteins Are Positive Regulators of Nodule Formation

To directly assess the role of G proteins in regulation of nodulation, a knockdown approach was pursued. Because multiple copies of G-protein genes have resulted from recent genome duplication events in soybean (Bisht et al., 2010; Schmutz et al., 2010; Choudhury et al., 2011),

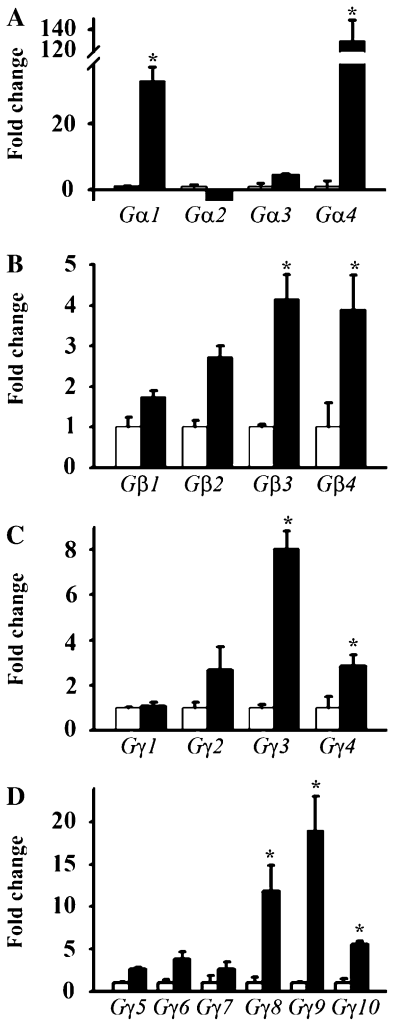

Figure 1. Expression analysis of G-protein genes in response to *B. japonicum* infection. Expression of Gα genes (*GmGα1–GmGα4*; A), Gβ genes (*GmGβ1–GmGβ4*; B), group I Gγ genes (*GmGγ1–GmGγ4*; C), and group II (*GmGγ5–GmGγ7*; D) and group III Gγ genes (*GmGγ8–GmGγ10*; D) in soybean roots at 32 d after *B. japonicum* infection. Fold change represents expression level of the genes (black bars) compared with their expression in nontreated roots (white bars) grown under identical conditions. Quantitative reverse transcription-PCR amplification experiments were performed using two biological replicates with three technical replicates each, and data were averaged. The expression values across different samples were normalized against soybean *Actin* gene expression, which was set at 1. Error bars represent the SE of means (*$P < 0.05$, Student's *t* test).

the coding sequences, as well as the 3′ and 5′ untranslated region sequences of specific G-protein subunits, are highly conserved. Therefore, RNA interference (RNAi) constructs were generated to target the four *Gα* family genes (Gα-RNAi), four *Gβ* family genes (Gβ-RNAi), four group I *Gγ* family genes (group I Gγ-RNAi), three group II *Gγ* family genes (group II Gγ-RNAi), and three group III *Gγ* family genes (group III Gγ-RNAi) under the control of Figwort Mosaic Virus (FMV) promoter as detailed in Table I. Corresponding complementary DNAs (cDNAs) were amplified from soybean roots and cloned in binary vector CGT11017A that also expresses a GFP reporter gene (Libault et al., 2009) to aid visual selection of transgenic roots. The constructs, along with a vector-only control construct (empty vector [EV]), were transformed into soybean by hairy root transformation (Govindarajulu et al., 2009; Libault et al., 2009). The transcript levels of individual G-protein genes were measured in RNAi-silenced roots to confirm the genes' decreased expression (Supplemental Fig. S2). Transgenic hairy roots were evaluated for their nodulation phenotype in response to

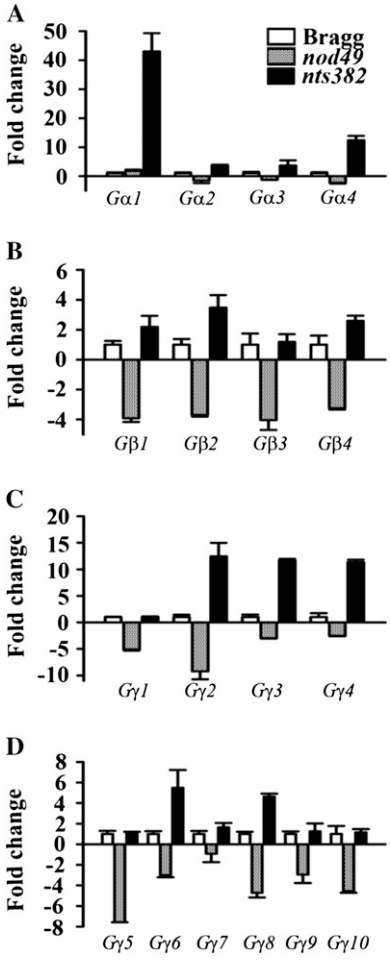

Figure 2. Expression levels of different G-protein genes in supernodulating (*nts382*) and nonnodulating (*nod49*) soybean mutants compared with wild-type cv Bragg. Expression of Gα genes (*GmGα1–GmGα4*; A), Gβ genes (*GmGβ1–GmGβ4*; B), group I Gγ genes (*GmGγ1–GmGγ4*; C), and group II (*GmGγ5–GmGγ7*; D) and group III Gγ genes (*GmGγ8–GmGγ10*; D) in wild-type and mutant soybean roots at 32 d after *B. japonicum* infection. Fold change represents expression level of the genes in mutant roots compared with their expression in wild-type roots grown under identical conditions. Quantitative reverse transcription-PCR amplification experiments were performed using two biological replicates with three technical replicates each, and data were averaged. The expression values across different samples were normalized against soybean *Actin* gene expression, which was set at 1. Error bars represent the SE of means.

B. japonicum infection. At 32 d post infection (dpi), the Gα-RNAi lines had similar numbers of nodules as the EV control lines, whereas the Gβ-RNAi and Gγ-RNAi lines exhibited significantly lower nodule number per root (Fig. 3). The strongest effect was seen in group I Gγ-RNAi lines, where only three to four nodules developed per transgenic root, compared with an average of 20 nodules per transgenic root in EV lines (Fig. 3C). Approximately 50% reduction in nodule number was recorded for the Gβ- RNAi and group II and group III Gγ-RNAi lines. Significant differences were also observed in nodule sizes in different RNAi lines. To quantify, the nodules were divided into small (<0.5 mm in diameter), medium (0.5–2 mm in diameter), and large (>2 mm in diameter) categories (Tu, 1975). The Gβ-RNAi and Gγ-RNAi lines had a significantly larger percentage of small nodules compared with the EV control lines (Fig. 3A). Most nodules of group I Gγ-RNAi transgenic roots fell into the small or immature nodule category (Fig. 3, A and C). The occasional large nodules formed on these roots were pale in color, probably due to lack of leghemoglobin.

To further confirm the role of G proteins in regulation of nodulation and evaluate any specificity between individual family members, we took a gain-of-function approach to evaluate the role of specific members of the Gβ and Gγ gene families. Transgenic lines were generated using constructs driven by a nodule-specific Early Nodulin40 (ENOD40) promoter for all four *Gβ* genes and for representatives of each of the Gγ family genes (*Gγ4*, *Gγ5*, and *Gγ8* from group I, group II, and group III Gγ families, respectively; Table I). The transcript levels of individual G-protein genes were measured in ENOD40 promoter-driven roots to ascertain the genes' higher expression levels (Supplemental Fig. S3). The transgenic hairy roots were evaluated for nodulation phenotypes and compared with the EV control roots. Interestingly, the two Gβ subfamilies exhibited a clear difference in nodulation phenotype; ENOD40 promoter-driven expression of group II Gβ (*Gβ3* and *Gβ4*), but not group I Gβ (*Gβ1* and *Gβ2*), led to a significant increase (approximately 50%) in nodule number compared with the EV control lines (Fig. 4). These data are in accordance with the altered expression level analysis of the specific *Gβ* genes (Fig. 1). Of the three representative Gγ lines, only *Gγ4* (representative of group I Gγ family) expression driven by the ENOD40 promoter led to an increase in nodule number. This corroborates the results obtained with the RNAi suppression, where group I Gγ-RNAi had the most significant effect on nodule number and nodule morphology. Similar results were obtained with Cassava vein mosaic virus (CvMV) promoter-driven constructs with individual Gβ genes (Supplemental Fig. S4). These data imply a direct and predominant role for group II Gβ and group I Gγ proteins during nodulation and a possible subfunctionalization of the recently duplicated G-protein genes.

Because G proteins are known to control cell division in Arabidopsis (Ullah et al., 2001), we compared the cross sections of similar-sized nodules from different RNAi lines with EV lines to evaluate whether alteration in nodule morphology was due to an effect on cell division. No major changes were observed in cell size; however, the cross sections revealed a poorly developed cortex in the Gβ-RNAi and group I Gγ-RNAi nodules (Fig. 5). Furthermore, a significant lack of bacteroids was observed in different G-protein RNAi lines compared with the EV lines that had cells packed with bacteroids (Supplemental Fig. S5). The most pronounced differences were seen in the Gβ-RNAi and group I Gγ-RNAi nodule sections, where most cells were devoid of any bacteroids, corroborating the lower numbers of functional nodules observed in these lines. These data demonstrate that the lower expression of specific G-protein genes interferes with the process of bacteroid formation during nodule development.

Nodulation Marker Genes Exhibit Altered Expression in G-Protein RNAi Lines

A large number of genes exhibit changes in their expression during nodulation (Libault et al., 2009). To

Table I. *Details of constructs used for the nodulation experiments*

Construct Name	Type	Description
FMV::Gα RNAi	RNAi	Targeting *GmGα1*, *GmGα2*, *GmGα3*, and *GmGα4*
FMV::Gβ RNAi	RNAi	Targeting *GmGβ1*, *GmGβ2*, *GmGβ3*, and *GmGβ4*
FMV::1Gγ RNAi	RNAi	Targeting *GmGγ1*, *GmGγ2*, *GmGγ3*, and *GmGγ4*
FMV::2Gγ RNAi	RNAi	Targeting *GmGγ5*, *GmGγ6*, and *GmGγ7*
FMV::3Gγ RNAi	RNAi	Targeting *GmGγ8*, *GmGγ9*, and *GmGγ10*
ENOD::GmGβ1	OE	Expression of *GmGβ1* driven by ENOD40 promoter
ENOD::GmGβ2	OE	Expression of *GmGβ2* driven by ENOD40 promoter
ENOD::GmGβ3	OE	Expression of *GmGβ3* driven by ENOD40 promoter
ENOD::GmGβ4	OE	Expression of *GmGβ4* driven by ENOD40 promoter
ENOD::GmGγ4	OE	Expression of *GmGγ4* (a representative of Group I Gγ) driven by ENOD40 promoter
ENOD::GmGγ5	OE	Expression of *GmGγ5* (a representative of Group II Gγ) driven by ENOD40 promoter
ENOD::GmGγ8	OE	Expression of *GmGγ8* (a representative of Group III Gγ) driven by ENOD40 promoter
CvMV::GmGβ1	OE	Overexpression of *GmGβ1* driven by CvMV promoter
CvMV::GmGβ2	OE	Overexpression of *GmGβ2* driven by CvMV promoter
CvMV::GmGβ3	OE	Overexpression of *GmGβ3* driven by CvMV promoter
CvMV::GmGβ4	OE	Overexpression of *GmGβ4* driven by CvMV promoter

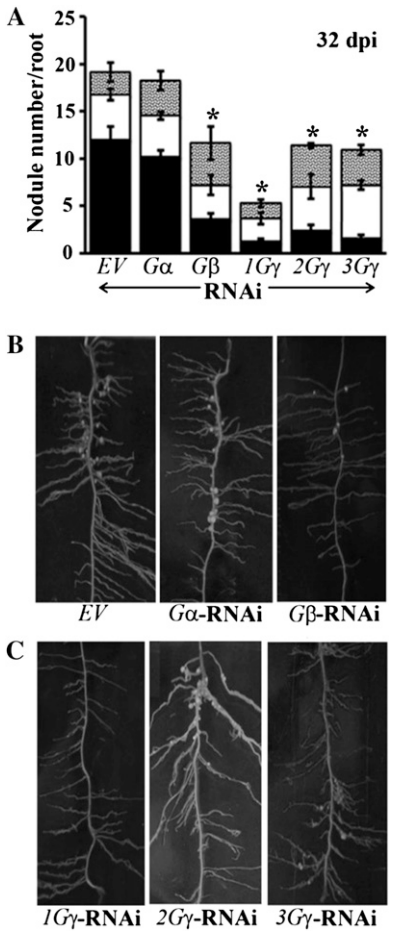

Figure 3. Regulation of nodule formation by heterotrimeric G proteins. A, Comparison of total nodule numbers per transgenic root and nodule sizes in different G-protein RNAi lines to the EV control lines at 32 dpi. Black, white, and hatched sections of the bars represent large (>2 mm in diameter), medium (0.5–2 mm in diameter), and small/immature (<0.5 mm in diameter) nodules, respectively. B and C, Nodulation phenotypes of representative transgenic hairy roots of individual RNAi lines. 1Gγ, 2Gγ, and 3Gγ represent group I, group II, and group III Gγ-RNAi lines, respectively. The data are average of three biological replicates. Each replicate consisted of 40 to 50 root samples per construct. Error bars represent the SE of means. Asterisks indicate statistically significant differences compared with EV control (*$P < 0.05$, Student's *t* test).

evaluate the expression changes in nodulation marker genes in the context of G-protein signaling, we analyzed the transcript levels of six representative genes in different G-protein RNAi lines and compared it to the EV control lines at 32 dpi (Fig. 6). Clear differences in the gene expression patterns were observed in different G-protein RNAi lines. Specifically, the expression of each of the genes tested was significantly down-regulated in the Gβ-RNAi lines and in group I Gγ-RNAi lines. Some of the nodulation marker genes, such as *ENOD40* and *Nodulin35*, did not show a difference in their expression in the Gα-RNAi lines and in the group III Gγ-RNAi lines, whereas additional genes proposed to be involved during the later stages of nodule development and in signaling during nodulation, such as *lectin*, *cytokinin oxidase*, a *MYB transcription factor*, and a *substilin-like protease*, exhibited significant changes in the Gα-RNAi, Gβ-RNAi, and group I and group III Gγ-RNAi lines. No differences in the expression levels were observed for any of the genes in group II Gγ-RNAi lines. It should also be noted that the group II *Gγ* genes did not show transcript level changes in response to *B. japonicum* infection (Fig. 1C). A stark difference in transcript level changes and nodulation phenotypes between group I and group II *Gγ* genes, which are very similar except for the lack of a prenylation signal in group II *Gγ* genes (Choudhury et al., 2011), underscores the importance of subunit-specific G-protein heterotrimers in control of important physiological processes.

ABA Affects Nodule Formation Using a Distinct Set of G-Protein Components

We further explored whether the presence of an additional signal during nodule formation would have any effect on the usage of specific G-protein subunits. Abscisic acid (ABA) was chosen as an additional signal for these experiments because it is an established regulator of G-protein signaling in plants (Pandey et al., 2006, 2010). Furthermore, ABA also affects nodule formation, although some conflicting information exists on its role in different species (Ding et al., 2008; Biswas et al., 2009). Treatment of EV control and different RNAi lines with 5 μM ABA following *B. japonicum* infection led to a significant decrease in the number of nodules formed in

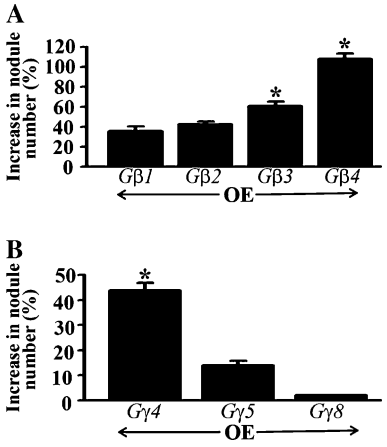

Figure 4. The effect of ENOD40-driven expression of specific G-protein genes on nodule formation. Percentage increase in nodule number due to the *Enod40* promoter-driven expression of Gβ genes (*GmGβ1–GmGβ4*; A) and a representative group I Gγ gene (*GmGγ4*; B), a group II Gγ gene (*GmGγ5*; B), and a group III Gγ gene (*GmGγ8*; B) in soybean roots at 32 dpi compared with EV control lines. The data are average of three biological replicates. Each replicate consisted of 40 to 50 root samples per construct. Error bars represent the SE of means. Asterisks indicate statistically significant differences compared with EV control (*$P < 0.05$, Student's *t* test).

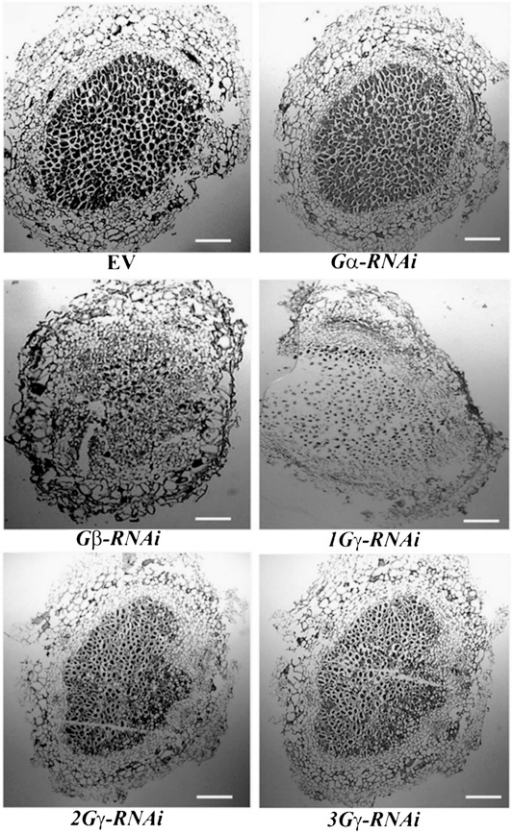

Figure 5. Cross-sectional view of nodules developed on G-protein knockdown hairy roots infected with *B. japonicum*. Mature nodules (32 dpi with *B. japonicum*) developed on EV, Gα-RNAi, Gβ-RNAi, group I Gγ-RNAi (1Gγ), group II Gγ-RNAi (2Gγ), and group III Gγ-RNAi (3Gγ) lines were used for microscopic studies. At least eight to 10 similar size nodules of three independent experiments were used for sectioning, and a representative image is shown for each line. Nodules developed on EV, Gα-RNAi, group II Gγ-RNAi, and group III Gγ-RNAi have a well-developed cortex, which is not present in the nodules developed in Gβ-RNAi and especially group I Gγ-RNAi lines. The infected cells in Gβ-RNAi and especially group I Gγ-RNAi lines are mostly dead, lack bacteroids, and contain only infection thread remnants. Bars = 200 μm.

soybean roots. The EV control plants exhibited approximately 45% reduction in nodule number per plant compared with non-ABA treated controls (Fig. 7). The effect of ABA on various G-protein RNAi lines was dependent on the suppression of specific G-protein subunits. In contrast to nodulation in the absence of ABA, where Gα-RNAi roots do not exhibit any obvious effect on nodule number, both Gα-RNAi and Gβ-RNAi lines displayed a hypersensitivity to ABA, with up to 70% reduction in nodule number (Fig. 7). Interestingly, the group I Gγ-RNAi lines that exhibited the strongest phenotypes in terms of nodule number were affected by ABA to a similar extent as the EV control lines. Similar results were observed for the group II Gγ-RNAi plants. The group III Gγ-RNAi plants, however, were highly sensitive to ABA and showed 70% to 75% reduction in nodule number compared with non-ABA controls (Fig. 7). Incidentally, the inhibition of lateral root formation by ABA was also predominantly affected in the group III Gγ-RNAi plants (Supplemental Fig. S6). These data demonstrate that the Gα and group III Gγ proteins play a more predominant role during ABA suppression of nodulation, together with Gβ proteins further corroborating a subunit-specific regulation of G-protein signaling.

Soybean Gα Proteins Interact with NFRs

The role of G proteins as positive regulators of nodule formation, the general position of Gα proteins in the signaling pathways as direct interactors of receptor proteins, and the significantly compromised expression of G-protein genes in NFR1α mutant (*nod49*) background (Fig. 2) prompted us to evaluate whether Gα proteins directly interact with NFRs in soybean. The NFR proteins NFR1 and NFR5 are the most acknowledged receptors for signaling during nodulation (Indrasumunar et al., 2011). The soybean genome encodes two copies of NFR1 and NFR5 proteins: NFR1α and NFR1β, and NFR5α and NFR5β (Indrasumunar et al., 2011). We cloned full-length *NFR1α*, *NFR1β*, *NFR5α*, and *NFR5β* genes from soybean nodule cDNA and tested their interaction with the four Gα proteins in a split ubiquitin-based interaction system. For these interactions, the NFR proteins were expressed as C-terminal ubiquitin (CUb) fusions and the Gα proteins were expressed as N-terminal ubiquitin (NUb) fusions in both orientations (NUb-Gα and Gα-NUb). All four Gα proteins interacted with NFR1α and NFR1β, but not with NFR5α and NFR5β, as evaluated by yeast (*Saccharomyces cerevisiae*) growth on media lacking Leu, Trp, His, and adenine, in the presence of 1 mM Met (Fig. 8A; Supplemental Fig. S7). The interaction between Gα and NFR proteins was further confirmed by plant-based bimolecular fluorescence complementation (BiFC) analysis. Coexpression of Gα and NFR1 as fusion proteins with split halves of yellow fluorescent protein (YFP) resulted in reconstitution of YFP fluorescence in tobacco (*Nicotiana tabacum*) epidermis, confirming in planta interaction (Fig. 8B; Supplemental Fig. S8). The C-terminal half of NFR1α and NFR1β proteins exhibited similar efficiency of interaction with the Gα proteins as the full-length proteins in both yeast- and BiFC-based assays, whereas no interaction was observed with the N-terminal half of the NFR1 proteins (Supplemental Figs. S9 and S10).

The role of classic GPCRs is to facilitate GDP/GTP exchange on Gα proteins during G-protein signaling. To evaluate whether the interaction with NFR1 proteins has any effect on the biochemical properties of Gα proteins, a real-time fluorescence-based GTP-binding and hydrolysis activity of Gα proteins (Pandey et al., 2009) was measured in the presence of purified C-terminal domain of the NFR1α protein. In this preliminary in vitro assay, NFR1α protein had no effect on either the GTP-binding or the GTPase activity of Gα proteins (Supplemental Fig. S11). Furthermore, a constitutively

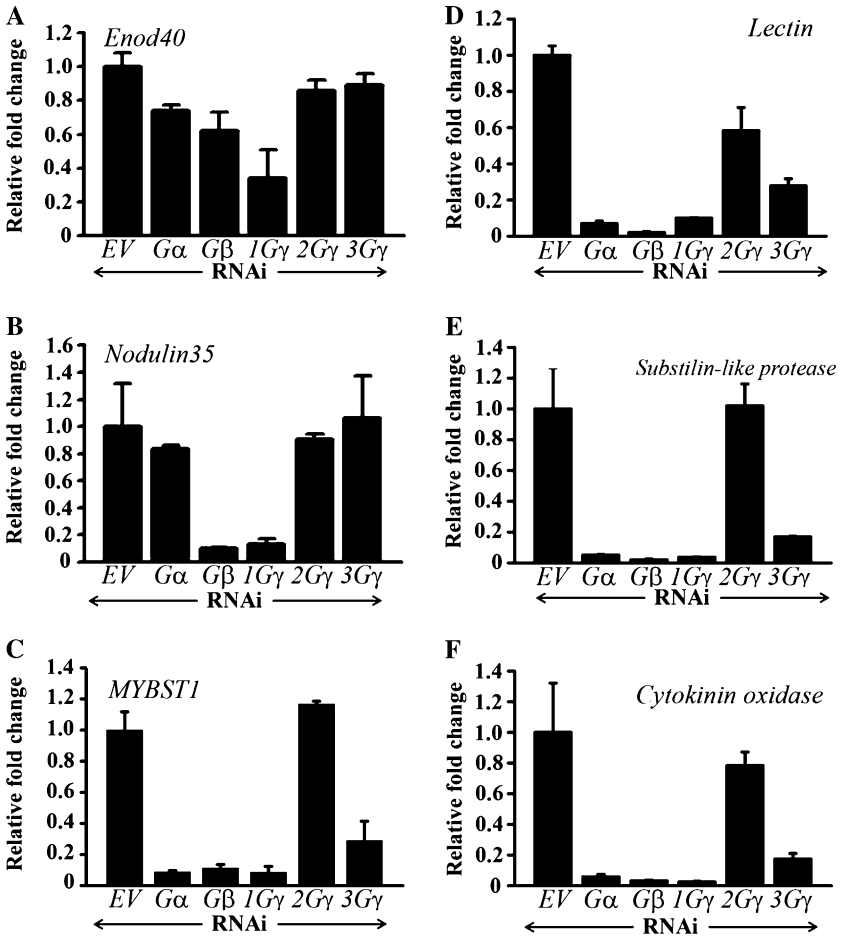

Figure 6. Relative expression of nodulation-related genes in different G-protein RNAi lines. Gene-specific primers were used to amplify and quantify the transcript levels of indicated genes. Two biological replicates with three technical replicates each were used for expression analysis, and data were averaged. The expression values across different samples were normalized against soybean *Actin* gene expression. Expression in EV lines was set at 1. Error bars represent the SE of means.

active version of Gα proteins (Gα Q222L), which locks it in its GTP-bound conformation, and NFR1 proteins interact with similar strength as the native Gα proteins in split ubiquitin-based system (Supplemental Fig. S12), suggesting that NFR proteins are most likely not involved in regulating the GTP-binding and hydrolysis activities of Gα proteins and that alternative, yet undiscovered mechanisms may characterize this interaction.

DISCUSSION

Heterotrimeric G Proteins Regulate Nodulation in Soybean

Heterotrimeric G proteins control many aspects of plant growth and development. Studies done with Arabidopsis and rice plants expressing altered levels of G-protein subunits suggest that the regulation of signaling pathways by this ubiquitously expressed group of proteins is fairly complex and diverse (Temple and Jones, 2007). The results reported in this study establish the role of G proteins signaling during nodulation. Higher expression of specific G-protein subunits in response to *B. japonicum* infection (Fig. 1) and differential expression levels of G-protein subunits in a supernodulating and a nonnodulating mutant background compared with wild-type plants (Fig. 2) suggested that the expression level of G-protein genes is tightly regulated during nodule development and is biologically relevant. This observation was corroborated by the use of RNAi and ENOD40 promoter-driven expression-based transgenic approaches (Figs. 3 and 4). Plants expressing

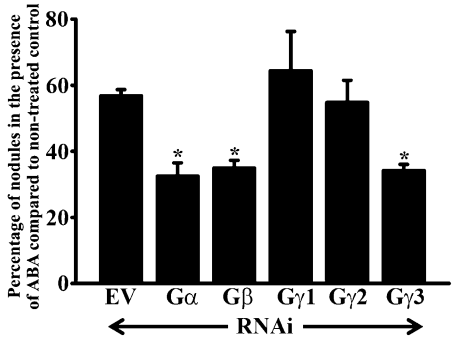

Figure 7. The effect of ABA on nodulation. Nodule number in EV and different G-protein RNAi lines in the ABA-treated versus mock-treated hairy roots. The data were recorded at 32 dpi and represent average of three biological replicates. Each replicate consisted of 40 to 50 root samples per construct. Error bars represent the SE of means (*$P < 0.05$, Student's *t* test).

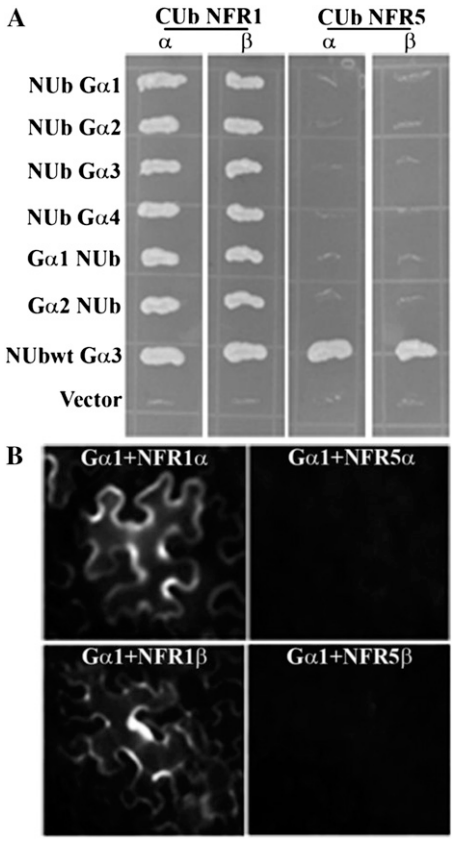

Figure 8. Interaction of Gα proteins with NFR proteins. A, Interaction between NFR (NFR1α, NFR1β, NFR5α, and NFR5β) and Gα proteins using split-ubiquitin-based interaction assay. The picture shows yeast growth on selective media with 1 mM Met. In all cases, Gα proteins were used as NUb fusions in both orientations (NUb-Gα denoting NUb fused to the N terminus of Gα and Gα-NUb denoting NUb fused to the C-terminus of Gα) and NFR proteins as CUb fusions. N-terminal wild-type ubiquitin (NUbwt) fusion constructs were used as positive controls for interaction, and NUb vectors were used as negative controls. Two biological replicates of the experiment were performed with identical results. B, Interaction between NFR (NFR1α, NFR1β, NFR5α, and NFR5β) in 77-nEYFP-N1 and Gα1 in 78-cEYFP-N1 proteins using bimolecular complementation assay. *Agrobacterium tumefaciens* containing different combinations of NFR and Gα were infiltrated in tobacco leaves, and reconstitution of YFP fluorescence due to protein-protein interaction was visualized with a microscope. At least four independent infiltrations were performed for each protein combination with similar results. Data with NFR1 and additional Gα proteins are shown in Supplemental Figures S7 and S8.

lower levels of G-protein genes, especially the Gβ-RNAi and group I Gγ-RNAi lines, exhibited a significantly-reduced nodule number compared with EV control lines, whereas higher nodule numbers were observed by overexpression of specific Gβ and Gγ genes. These data thus provide the direct molecular-genetic evidence for the involvement of G-protein signaling during nodulation. The cross sections of nodules form different RNAi lines (Fig. 5), and the expression profiling of various nodulation marker genes (Fig. 6) suggest that a lack of G-protein genes interferes with bacteroids formation in the infected cells and may affect early signal perception as well as later developmental and signaling events during nodulation.

Specific Subunits of G Proteins Regulate Nodule Development

The presence of multiple subunits for each of the G-protein genes in soybean (Bisht et al., 2010; Choudhury et al., 2011) offered an opportunity to assess the specificity of subunit usage and response regulation during plant G-protein signaling. Expression analysis shows that only group I Gα and group II Gβ are regulated at the transcript level in response to *B. japonicum* infection and in *nts382* mutants (Figs. 1 and 2; Supplemental Fig. S1). Interestingly, we have previously shown that these two groups of proteins, group I Gα and group II Gβ, also exhibit specific interaction in split ubiquitin-based assays (Bisht et al., 2010), suggesting that such interactions are physiologically relevant.

A clear correlation was observed between transcript levels (Fig. 1) and nodulation phenotypes in roots expressing altered levels of *Gβ* genes (Figs. 3 and 4), but not in the roots expressing altered levels of *Gα* genes. It is possible that a very high expression level of *Gα* genes during nodulation (Fig. 1; Supplemental Fig. S1) and an incomplete silencing of *Gα* genes in Gα-RNAi plants (Supplemental Fig. S2) allows for the presence of enough residual Gα to transduce the signal and mask the phenotype. However, alternative signaling mechanisms discussed later that do not require a direct input of Gα proteins cannot be ruled out at this stage.

The results with Gγ proteins present a relatively complex picture. It is clear that the group I Gγ proteins are predominantly involved during nodulation under normal growth conditions. These data are supported by their transcript analysis (Figs. 1 and 2), nodule cross sections (Fig. 5), gene expression analysis (Fig. 6), and the phenotypes of group I Gγ-RNAi and ENOD40 promoter-driven Gγ4 expression lines (Figs. 3 and 4). The homologs of group I Gγ proteins in Arabidopsis are involved in defense response against bacterial and fungal pathogens (Trusov et al., 2006). This group of Gγ proteins may therefore be generally involved in G-protein signaling during plant-microbe interactions.

The role of group III Gγ proteins seems to be obvious during nodulation in the presence of ABA (Fig. 7). The group III Gγ proteins are recently identified, unique, plant-specific Gγ proteins that have been shown to be involved in regulation of ABA signaling in Arabidopsis (Chakravorty et al., 2011). This suggests that the role of these unique proteins in ABA signaling is conserved between different species even though the rice homologs of these proteins are known to be involved in regulation of grain size and number (Huang et al., 2009; Wang et al., 2011). Whether rice Gγ proteins with homology to group III Gγ have any role in regulation of ABA response remains unknown, and it would be interesting to evaluate whether the function of these proteins is conserved in monocot versus dicot plants given some other differences

that exist in G-protein signaling in Arabidopsis versus rice (Perfus-Barbeoch et al., 2004).

In contrast to the group I and group III Gγ proteins, the group II Gγ proteins do not seem to have a significant role during nodulation based on the gene expression (Figs. 1 and 2) and ENOD40 promoter-driven expression data (Fig. 4), even though the RNAi lines of each of the Gγ proteins exhibited partial suppression of the nodulation phenotype. Because the Gγ proteins are obligate interactors of Gβ proteins and do not act independently, some of the effects that are observed in Gγ-RNAi lines could be due to the changes in stoichiometry or localization of specific Gβ-Gγ complexes. A homolog of group II Gγ proteins is not present in Arabidopsis, and their function in plants remains unevaluated at the moment. It should be noted that the group II Gγ proteins differ from the group I Gγ proteins mostly due to the lack of a prenylation motif at their C terminus. The genes seem to have evolved from the substitution of the CWIL motif in group I Gγ to the RWI motif in group II Gγ because most of the other amino acid sequence, as well as the intron-exon organization between these two groups of proteins, are highly conserved (Choudhury et al., 2011). A clear difference in the nodule phenotype of group I and group II Gγ-RNAi lines therefore suggests regulation of specific signaling pathways by these two groups of proteins, potentially through their interaction with different downstream targets. These data further corroborate the significant roles played by plant Gγ proteins in defining signal response coupling.

Our data also confirms that ABA significantly effects nodule formation, resulting in almost 45% reduction in nodule number compared with non-ABA-treated control plants (Fig. 7). From a physiological standpoint, it showcases a major impact of drought and other abiotic stresses on nodule formation and nitrogen fixation. Different G-protein RNAi lines show hypersensitivity to ABA during nodule formation, which is similar to what has been observed for Arabidopsis seedlings during germination and early seedling growth and development (Pandey et al., 2006) and is opposite of ABA's effect during stomatal aperture regulation, where Arabidopsis mutants lacking G-protein genes show less sensitivity to ABA (Wang et al., 2001).

Overall, these results support the hypothesis that specific combinations of heterotrimeric G proteins are involved in signal transduction during nodulation. Further experiments with altered levels of additional components of G-protein signaling, cell biology, and proteomics-based approaches to confirm the presence of subunit-specific, signal-dependent heterotrimers and identification of additional downstream components would help to shed light on the exact mechanisms of G-protein signaling during nodulation.

Soybean Gα Proteins Interact with NFR1

The importance of G-protein signaling in regulating nodulation is also underscored by Gα proteins' interaction with the nodulation receptor NFR1. We have shown both by yeast-based and in planta BiFC assays that soybean Gα proteins interact specifically with NFR1 (Fig. 8; Supplemental Figs S7–S10 and S12). In metazoan systems, the guanine nucleotide exchange factor activity of GPCRs is required for the activation of Gα proteins and onset of signaling. Plants have a very limited repertoire of GPCRs, and even though their interactions with Gα proteins have been shown in several cases, a ligand for a GPCR-like protein coupled to Gα is known only for the divergent GPCR-type G proteins (Pandey et al., 2009). It has been proposed that plant Gα proteins act independent of GPCR activation and may be self-activated in some cases (Temple and Jones, 2007; Urano et al., 2012). Plants have a large number of receptor kinases, and there is some indirect evidence for their interaction with Gα protein-regulated pathways (Llorente et al., 2005; Gao et al., 2008; Oki et al., 2009; Liu et al., 2013; Torres et al., 2013). Given the involvement of Gα proteins in regulating a multitude of signaling processes, it is conceivable that receptors other than canonical heptahelical proteins may act as GPCRs in plants. This study supports the interaction of Gα protein with one such receptor family, opening up the exciting possibility of Gα interaction with additional plant receptor protein families. Interestingly, the constitutively active Gα proteins (Gα Q222L) interact with NFR1 with similar strength as the native proteins (Supplemental Fig. S12), and in an in vitro assay, the purified C-terminal domain of NFR1 protein had no effect on either the GTP-binding or hydrolysis activity of Gα proteins (Supplemental Fig. S11). Therefore, the mechanisms by which divergent receptors may activate or regulate Gα proteins remain unexplored at this point. G-protein subunits have been shown to be associated with large macromolecular complexes in Arabidopsis and rice (Kato et al., 2004; Wang et al., 2008), and their interaction with NFR1 proteins may only be relevant in the context of a higher order signaling complex formation. Future experiments examining NFR-mediated regulation of G proteins will be able to shed light on these alternative signaling mechanisms.

Possible Mechanisms of G-Protein Signaling during Nodulation

Our results also offered the opportunity to speculate on the existence of distinct G-protein signaling mechanisms that might be operative during nodule formation. As described previously, both classical and nonclassical modes of G-protein signaling operate in plants during regulation of various growth and development pathways (Pandey et al., 2010). In the classical mode, both Gα and Gβγ proteins are involved in regulating the signal, either by direct interaction with the effectors or by helping correct targeting of Gα subunits. In this situation, loss of either Gα or Gβγ will result in a similar phenotype. This mode is exemplified by the rounded leaf morphology of Arabidopsis mutants lacking Gα (*gpa1*)

or Gβ (*agb1*) genes (Pandey et al., 2010). On the contrary, in the classical II mechanism, only Gβγ is required for signal transduction. If this mechanism is operative, loss of Gβγ will result in signal inhibition, whereas loss of Gα will allow for the availability of free Gβγ and lead to signal enhancement and thereby opposite phenotype. Lateral root formation in Arabidopsis, where *gpa1* has fewer and *agb1* has more lateral roots than wild-type plants (Ullah et al., 2003), is an example of classical II mechanism. Additional novel mechanisms exist in plants and other nonmammalian organisms where only Gβγ is involved in signaling with no effect whatsoever from the presence of Gα, for example, during root-waving response in Arabidopsis G-protein mutants or in primary root length of Arabidopsis G-protein mutants (Chen et al., 2006; Pandey et al., 2008), or signaling via intact heterotrimer (Adjobo-Hermans et al., 2006).

It is clear that during nodulation in the presence of ABA, both Gα and Gβγ entities are involved, as lower expression of either of the subunits results in hypersensitivity to ABA, suggesting the involvement of the classical I mechanism. However, during nodule development without ABA, we only observe a phenotype, due to lower expression of Gβγ, not Gα. This suggests the possible involvement of the nonclassical mechanism, where the signaling is regulated only by Gβγ proteins with no input from Gα proteins. However, the high expression of specific *Gα* genes in response to *B. japonicum* infection and in *nts382* mutants, and its specific interaction with NFR1 proteins, argues against this mechanism. An alternative explanation would suggest that Gα is involved in signaling during nodulation together with Gβγ proteins, either as a positive (classical I) or negative (classical II) regulator, but its effects are masked due to very high expression levels and an incomplete silencing of *Gα* genes in Gα-RNAi plants (Supplemental Fig. S2). Further experiments with plants exhibiting more effective silencing of *Gα* genes, overexpression of individual, constitutively active *Gα* genes, or manipulation of Regulator of G-protein signaling genes will help delineate the exact role of Gα proteins in regulating signaling during nodulation.

In conclusion, the results presented in this study not only establish the role of heterotrimeric G proteins in regulation of a physiologically and economically important process, but also provide novel information on the roles and mechanism of G-protein signaling in plants. Previous work in Arabidopsis has established the involvement of G proteins in control of processes such as cell division, ion channel activities, phospholipase D signaling, phosphorylation profiles, and calcium and reactive oxygen species signaling (Temple and Jones, 2007). Interestingly, all these processes and pathways are involved in control of nodule formation (Oldroyd and Downie, 2008) and deserve further exploration in the context of regulation by G proteins. Recent work has highlighted the importance of G proteins as regulators of optimum plant growth and development under fluctuating environmental conditions as well as major determinants of yield (Assmann, 2004; Botella, 2012). A comprehensive understanding of the role of G proteins in regulating nodule formation has the potential to majorly influence the agricultural economy of the future.

MATERIALS AND METHODS

Plant Growth and Hairy Root Transformation

Soybean (*Glycine max* 'Williams 82' and 'Bragg') plants were grown at 16-h light/8-h dark at 25°C for 12 d. Hairy root transformation was performed essentially as described previously (Govindarajulu et al., 2009; Libault et al., 2009). The transgenic roots were collected at different time points based on detection of GFP fluorescence with the Nikon Eclipse E800 microscope with epifluorescence module. Three biological replicates were used for each construct with at least 40 to 50 root samples in each individual experiment for each construct. Nodule number and sizes were recorded manually from all biological replicates, and data were averaged. To examine the effects of ABA during nodulation, roots were treated with ABA (5 μM) for 32 d after *Bradyrhizobium japonicum* (USDA110) infection.

RNA Isolation and Real-Time Quantitative PCR

Total RNA was isolated from *B. japonicum*-inoculated or noninoculated hairy roots of specified plants using TRIzol reagent (Invitrogen). cDNA synthesis and real-time PCRs were performed as previously described (Bisht et al., 2010). The primers used for PCR are listed in Supplemental Table S1.

Generation of Constructs for RNAi and Overexpression

The RNAi constructs were generated using CGT11017A vector as described previously (Govindarajulu et al., 2008). The gene fragments for RNAi constructs (Gα, Gβ, and group I, group II, and group III Gγ) were cloned into the pCR8/GW vector (Invitrogen) and confirmed by sequencing. The constructs were transferred into CGT11017A vector using LR Clonase (Invitrogen). For overexpression, full-length soybean cDNAs corresponding to four Gβ and three Gγ proteins (representative of each group) were cloned into the pCR8/GW vector, followed by a transfer to binary vectors pCAMGFP-CvMV-GWi (CvMV promoter) and pCAMGFP-GmENOD40-2p:GW (ENOD40 promoter) using LR Clonase. Sequence-verified constructs and respective EVs (used as controls) were transformed into *Agrobacterium rhizogenes* strain K599 (Kereszt et al., 2007) and used for transformation.

Preparation of Nodule Sections for Light Microscopy

Nodule sections were prepared using a protocol modified from Madsen et al. (2010). Mature nodules from RNAi and EV-containing transgenic roots were fixed in 2% (w/v) glutaraldehyde containing 0.1 M PIPES buffer (pH 6.8) for 2 h. The samples were dehydrated with graded water/ethanol series followed by infiltration with Histoclear:wax (1:1) mixture for 8 h at 56°C. Semithin (5-μm) sections, obtained using a microtome, were observed under a light microscope.

Protein-Protein Interaction Assays

The mating-based yeast (*Saccharomyces cerevisiae*) split-ubiquitin system was used to study the interaction between the Gα and NFR proteins, essentially according to (Pandey and Assmann, 2004). BiFC assay was performed with Gα:nYFP (in 77 nEYFP-N1 vector; Citovsky et al., 2006) and NFR1:cYFP (in 78 cEYFP-N1 vector; Citovsky et al., 2006). The constructs were transferred into *Agrobacterium tumefaciens* strain GV3101 by electroporation and coinfiltrated in the abaxial side of tobacco (*Nicotiana tabacum*) leaves. The pictures were imaged with the Nikon Eclipse E800 microscope with epifluorescence module for YFP fluorescence detection as described previously (Choudhury et al., 2012). At least four independent transformations were performed for each construct.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers GmGα1 (Glyma04g045960.1), GmGα2 (Glyma14g11140.1), GmGα3 (Glyma06g05960.1), GmGα4 (Glyma17g34450.1), GmGβ1

(Glyma11g12600.1), GmGβ2 (Glyma12g04810.1), GmGβ3 (Glyma06g01510.1), GmGβ4 (Glyma04g01460.1), GmGγ1 (Glyma10g03610.1), GmGγ2 (Glyma02g16190.1), GmGγ3 (Glyma20g33390.1), GmGγ4 (Glyma10g32215.1), GmGγ5 (Glyma11g18050.1), GmGγ6 (Glyma14g17060.1), GmGγ7 (Glyma17g29590.1), GmGγ8 (Glyma15g196300.1), GmGγ9 (Glyma17g05640.1), and GmGγ10 (Glyma07g04510.1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Absolute quantities of Gα and Gβ transcripts in control and *B. japonicum*-infected hairy roots of soybean at 32 dpi.

Supplemental Figure S2. Expression of G-protein genes in soybean G-protein-silenced hairy roots.

Supplemental Figure S3. Relative transcript levels of specific *Gβ* and *Gγ* genes in ENOD40 promoter-driven transgenic hairy roots.

Supplemental Figure S4. Nodule formation on transgenic soybean hairy roots overexpressing G-protein genes under the control of *CvMV* promoter.

Supplemental Figure S5. Cross-sectional view of nodules developed on G-protein knockdown hairy roots infected with *B. japonicum*.

Supplemental Figure S6. Effect of ABA on lateral root number in soybean G-protein-silenced hairy roots.

Supplemental Figure S7. Interaction between GmGα proteins with GmNFR proteins using split ubiquitin-based interaction assay.

Supplemental Figure S8. Interaction between GmGα (in 77-nEYFP-N1) and GmNFR (in 78-cEYFP-N1) proteins using bimolecular complementation assay.

Supplemental Figure S9. Interaction between GmGα proteins with the N-terminal and C-terminal halves of GmNFR1α and GmNFR1β proteins using split ubiquitin-based interaction assay.

Supplemental Figure S10. Interaction between GmGα (in 77-nEYFP-N1) and C-terminal half of GmNFR1 (in 78-cEYFP-N1) proteins using bimolecular complementation assay.

Supplemental Figure S11. GTPase activity of GmGα1 and GmGα2 in the presence of NFR1.

Supplemental Figure S12. Interaction between constitutively active GmGα1 (CAGα1, Q223L) protein with GmNFR proteins using split ubiquitin-based interaction assay.

Supplemental Table S1. List of primers used.

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