

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

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Heterotrimeric G proteins comprising $G\alpha$, $G\beta$, and $G\gamma$ 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 $G\alpha$ and $G\beta$ 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 $G\alpha$ proteins directly interact with the soybean nodulation factor receptors NFR1 α and NFR1 β , 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 $G\alpha$ protein leads to the dissociation of inactive GDP· $G\alpha\beta\gamma$ heterotrimer into active GTP· $G\alpha$ and $G\beta\gamma$ dimer. Both these freed entities can interact with a variety of different effector proteins to transduce the signal. The inherent GTPase activity of $G\alpha$ generates GDP· $G\alpha$, which reassociates with the $G\beta\gamma$ dimer, and the proteins return to the inactive GDP· $G\alpha\beta\gamma$ trimeric conformation. Depending on the regulation of a particular signaling pathway by $G\alpha$ and $G\beta\gamma$ 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 $G\beta\gamma$ with no input from $G\alpha$ 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 $G\alpha$, 5 $G\beta$, and 12 $G\gamma$ in humans), *Arabidopsis* contains only 1 $G\alpha$, 1 $G\beta$, and 3 $G\gamma$ 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 $G\alpha$ 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 $G\alpha$, 4 $G\beta$, and 10 $G\gamma$ 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 $G\alpha$ 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 $G\alpha$ 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. $G\alpha$ I and II, $G\beta$ I and II, and $G\gamma$ 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 $G\alpha$, four $G\beta$, and 10 $G\gamma$ genes in soybean roots (cv Williams 82) in response to *B. japonicum* infection. The group I $G\alpha$ genes $G\alpha 1$ and $G\alpha 4$ showed a sharp increase in transcript level compared with their expression in noninfected control root, whereas the expression of the group II $G\alpha$ genes $G\alpha 2$ and $G\alpha 3$ remained mostly unchanged (Fig. 1; Supplemental Fig. S1). Two $G\beta$ genes, $G\beta 3$ and $G\beta 4$, 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 $G\gamma$ genes, ranging from 4- to 20-fold. Interestingly, the group II $G\gamma$ 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 $G\alpha$ subfamily genes, where, similar to *B. japonicum*-dependent up-regulation, the transcript levels of group I, but not group II, $G\alpha$ 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),

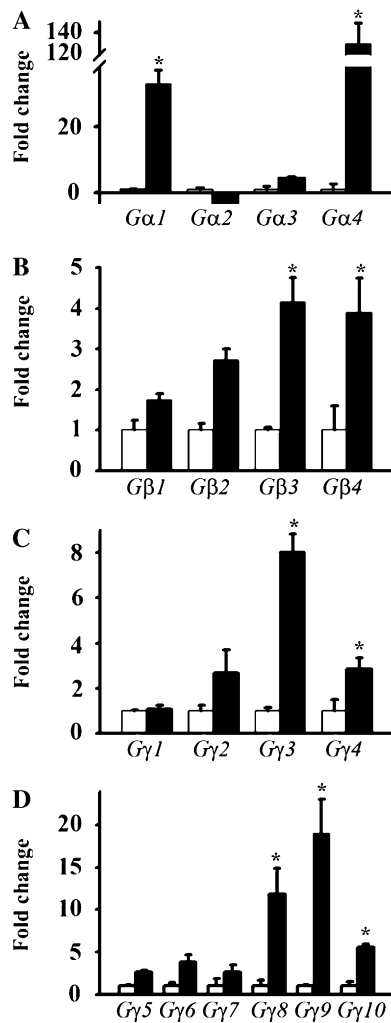


Figure 1. Expression analysis of G-protein genes in response to *B. japonicum* infection. Expression of $G\alpha$ genes (*GmGα1*–*GmGα4*; A), $G\beta$ genes (*GmGβ1*–*GmGβ4*; B), group I $G\gamma$ genes (*GmGγ1*–*GmGγ4*; C), and group II (*GmGγ5*–*GmGγ7*; D) and group III $G\gamma$ genes (*GmGγ8*–*GmGγ10*; 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 $G\alpha$ family genes ($G\alpha$ -RNAi), four $G\beta$ family genes ($G\beta$ -RNAi), four group I $G\gamma$ family genes (group I $G\gamma$ -RNAi), three group II $G\gamma$ family genes (group II $G\gamma$ -RNAi), and three group III $G\gamma$ family genes (group III $G\gamma$ -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

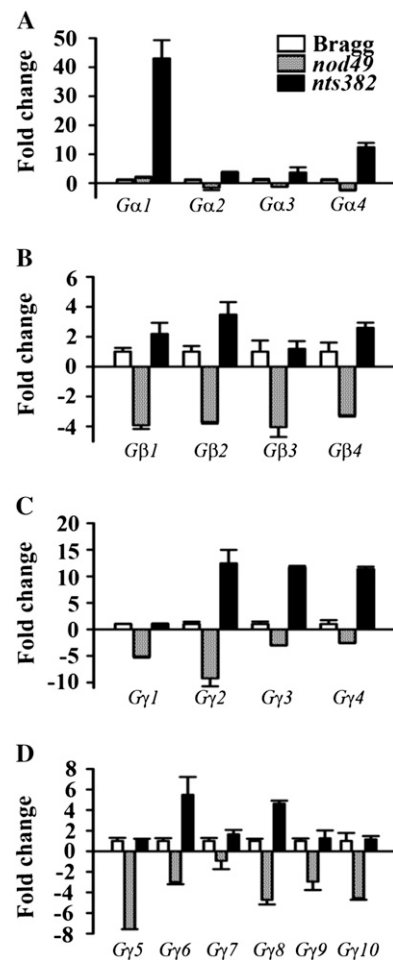


Figure 2. Expression levels of different G-protein genes in super-nodulating (*nts382*) and nonnodulating (*nod49*) soybean mutants compared with wild-type cv Bragg. Expression of $G\alpha$ genes (*GmGα1*–*GmGα4*; A), $G\beta$ genes (*GmGβ1*–*GmGβ4*; B), group I $G\gamma$ genes (*GmGγ1*–*GmGγ4*; C), and group II (*GmGγ5*–*GmGγ7*; D) and group III $G\gamma$ 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 $G\alpha$ -RNAi lines had similar numbers of nodules as the EV control lines, whereas the $G\beta$ -RNAi and $G\gamma$ -RNAi lines exhibited significantly lower nodule number per root (Fig. 3). The strongest effect was seen in group I $G\gamma$ -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 $G\beta$ -RNAi and group II and group III $G\gamma$ -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 $G\beta$ -RNAi and $G\gamma$ -RNAi lines had a significantly larger percentage of small nodules compared with the EV control lines (Fig. 3A). Most nodules of group I $G\gamma$ -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 $G\beta$ and $G\gamma$ gene families. Transgenic lines were generated using constructs driven by a nodule-specific Early Nodulin40 (ENOD40) promoter for all four $G\beta$ genes and for representatives of each of the $G\gamma$ family genes ($G\gamma 4$, $G\gamma 5$, and $G\gamma 8$ from group I, group II, and group III $G\gamma$ 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 $G\beta$ subfamilies exhibited a clear difference in nodulation phenotype; ENOD40 promoter-driven expression of group II $G\beta$ ($G\beta 3$ and $G\beta 4$), but not group I $G\beta$ ($G\beta 1$ and $G\beta 2$), 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 $G\beta$ genes (Fig. 1). Of the three representative $G\gamma$ lines, only $G\gamma 4$ (representative of group I $G\gamma$ 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 $G\gamma$ -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 $G\beta$ genes (Supplemental Fig. S4). These data imply a direct and predominant role for group II $G\beta$ and group I $G\gamma$ 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 $G\beta$ -RNAi and group I $G\gamma$ -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 $G\beta$ -RNAi and group I $G\gamma$ -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::G α RNAi	RNAi	Targeting <i>GmGα1</i> , <i>GmGα2</i> , <i>GmGα3</i> , and <i>GmGα4</i>
FMV::G β RNAi	RNAi	Targeting <i>GmGβ1</i> , <i>GmGβ2</i> , <i>GmGβ3</i> , and <i>GmGβ4</i>
FMV::1G γ RNAi	RNAi	Targeting <i>GmGγ1</i> , <i>GmGγ2</i> , <i>GmGγ3</i> , and <i>GmGγ4</i>
FMV::2G γ RNAi	RNAi	Targeting <i>GmGγ5</i> , <i>GmGγ6</i> , and <i>GmGγ7</i>
FMV::3G γ RNAi	RNAi	Targeting <i>GmGγ8</i> , <i>GmGγ9</i> , and <i>GmGγ10</i>
ENOD::GmG β 1	OE	Expression of <i>GmGβ1</i> driven by ENOD40 promoter
ENOD::GmG β 2	OE	Expression of <i>GmGβ2</i> driven by ENOD40 promoter
ENOD::GmG β 3	OE	Expression of <i>GmGβ3</i> driven by ENOD40 promoter
ENOD::GmG β 4	OE	Expression of <i>GmGβ4</i> driven by ENOD40 promoter
ENOD::GmG γ 4	OE	Expression of <i>GmGγ4</i> (a representative of Group I G γ) driven by ENOD40 promoter
ENOD::GmG γ 5	OE	Expression of <i>GmGγ5</i> (a representative of Group II G γ) driven by ENOD40 promoter
ENOD::GmG γ 8	OE	Expression of <i>GmGγ8</i> (a representative of Group III G γ) driven by ENOD40 promoter
CvMV::GmG β 1	OE	Overexpression of <i>GmGβ1</i> driven by CvMV promoter
CvMV::GmG β 2	OE	Overexpression of <i>GmGβ2</i> driven by CvMV promoter
CvMV::GmG β 3	OE	Overexpression of <i>GmGβ3</i> driven by CvMV promoter
CvMV::GmG β 4	OE	Overexpression of <i>GmGβ4</i> driven by CvMV promoter

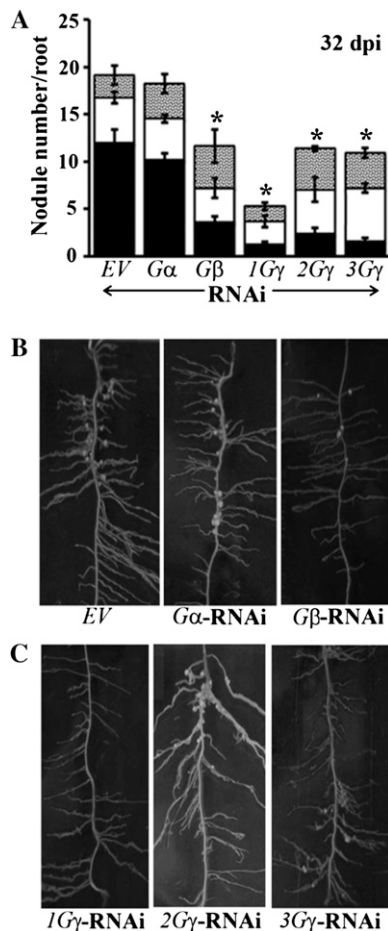


Figure 3. Regulation of nodule formation by heterotrimeric G proteins. A, Comparison of total nodule numbers per transgenic root and nodule sizes in different G-protein RNAi lines to the EV control lines at 32 dpi. Black, white, and hatched sections of the bars represent large (>2 mm in diameter), medium (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. 1G γ , 2G γ , and 3G γ represent group I, group II, and group III G γ -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 G β -RNAi lines and in group I G γ -RNAi lines. Some of the nodulation marker genes, such as *ENOD40* and *Nodulin35*, did not show a difference in their expression in the G α -RNAi lines and in the group III G γ -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 *subtilisin-like protease*, exhibited significant changes in the G α -RNAi, G β -RNAi, and group I and group III G γ -RNAi lines. No differences in the expression levels were observed for any of the genes in group II G γ -RNAi lines. It should also be noted that the group II G γ 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 G γ genes, which are very similar except for the lack of a prenylation signal in group II G γ 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 μ M ABA following *B. japonicum* infection led to a significant decrease in the number of nodules formed in

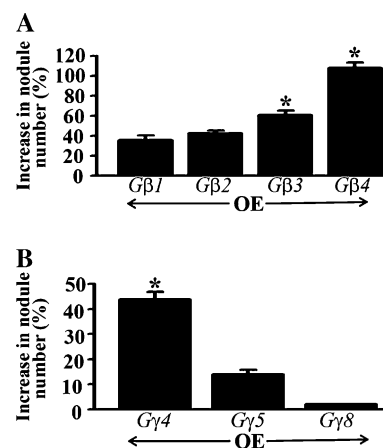


Figure 4. The effect of ENOD40-driven expression of specific G-protein genes on nodule formation. Percentage increase in nodule number due to the *Enod40* promoter-driven expression of G β genes (*GmGβ1–GmGβ4*; A) and a representative group I G γ gene (*GmGγ4*; B), a group II G γ gene (*GmGγ5*; B), and a group III G γ 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).

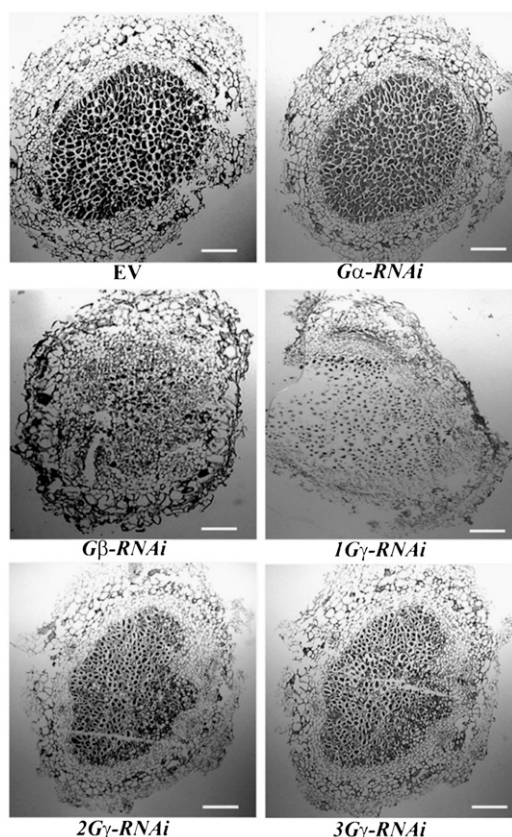


Figure 5. Cross-sectional view of nodules developed on G-protein knockdown hairy roots infected with *B. japonicum*. Mature nodules (32 dpi with *B. japonicum*) developed on EV, $G\alpha$ -RNAi, $G\beta$ -RNAi, group I $G\gamma$ -RNAi (1 $G\gamma$), group II $G\gamma$ -RNAi (2 $G\gamma$), and group III $G\gamma$ -RNAi (3 $G\gamma$) 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, $G\alpha$ -RNAi, group II $G\gamma$ -RNAi, and group III $G\gamma$ -RNAi have a well-developed cortex, which is not present in the nodules developed in $G\beta$ -RNAi and especially group I $G\gamma$ -RNAi lines. The infected cells in $G\beta$ -RNAi and especially group I $G\gamma$ -RNAi lines are mostly dead, lack bacteroids, and contain only infection thread remnants. Bars = 200 μ m.

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 $G\alpha$ -RNAi roots do not exhibit any obvious effect on nodule number, both $G\alpha$ -RNAi and $G\beta$ -RNAi lines displayed a hypersensitivity to ABA, with up to 70% reduction in nodule number (Fig. 7). Interestingly, the group I $G\gamma$ -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 $G\gamma$ -RNAi plants. The group III $G\gamma$ -RNAi plants, however, were highly sensitive to ABA and showed 70% to 75% reduction in

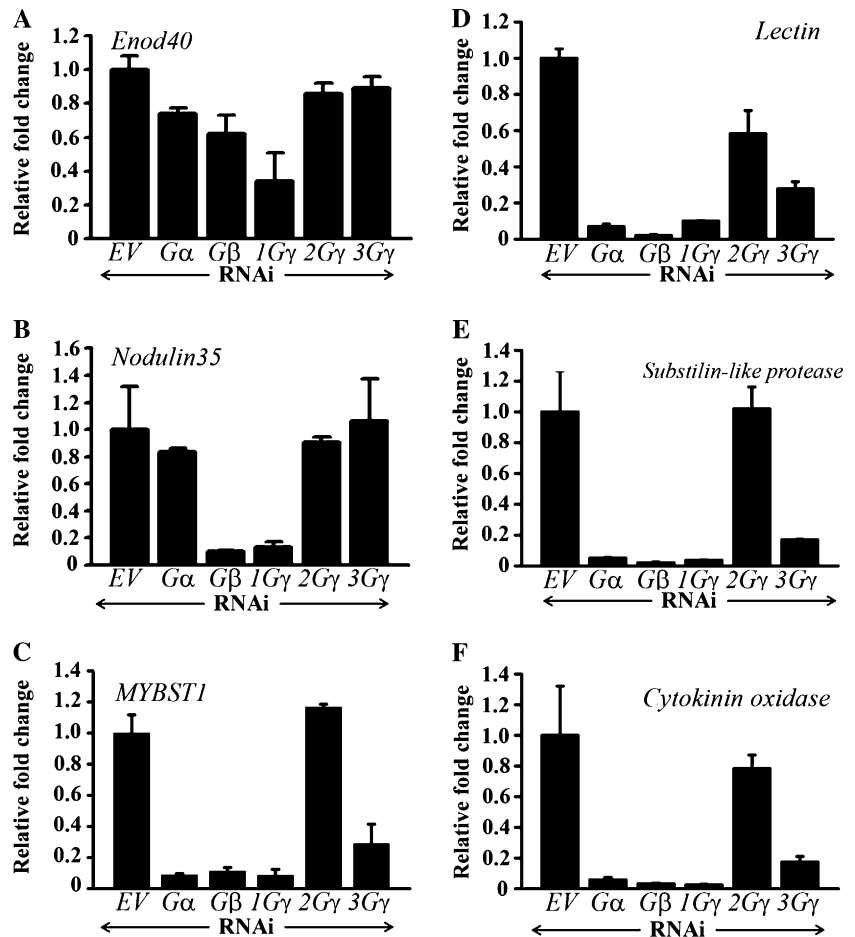
nodule number compared with non-ABA controls (Fig. 7). Incidentally, the inhibition of lateral root formation by ABA was also predominantly affected in the group III $G\gamma$ -RNAi plants (Supplemental Fig. S6). These data demonstrate that the $G\alpha$ and group III $G\gamma$ proteins play a more predominant role during ABA suppression of nodulation, together with $G\beta$ proteins further corroborating a subunit-specific regulation of G-protein signaling.

Soybean $G\alpha$ Proteins Interact with NFRs

The role of G proteins as positive regulators of nodule formation, the general position of $G\alpha$ proteins in the signaling pathways as direct interactors of receptor proteins, and the significantly compromised expression of G-protein genes in NFR1 α mutant (*nod49*) background (Fig. 2) prompted us to evaluate whether $G\alpha$ 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: NFR1 α and NFR1 β , and NFR5 α and NFR5 β (Indrasumunar et al., 2011). We cloned full-length NFR1 α , NFR1 β , NFR5 α , and NFR5 β genes from soybean nodule cDNA and tested their interaction with the four $G\alpha$ proteins in a split ubiquitin-based interaction system. For these interactions, the NFR proteins were expressed as C-terminal ubiquitin (CUB) fusions and the $G\alpha$ proteins were expressed as N-terminal ubiquitin (NUB) fusions in both orientations (NUB- $G\alpha$ and $G\alpha$ -NUB). All four $G\alpha$ proteins interacted with NFR1 α and NFR1 β , but not with NFR5 α and NFR5 β , 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 $G\alpha$ and NFR proteins was further confirmed by plant-based bimolecular fluorescence complementation (BiFC) analysis. Coexpression of $G\alpha$ 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 NFR1 α and NFR1 β proteins exhibited similar efficiency of interaction with the $G\alpha$ 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 $G\alpha$ proteins during G-protein signaling. To evaluate whether the interaction with NFR1 proteins has any effect on the biochemical properties of $G\alpha$ proteins, a real-time fluorescence-based GTP-binding and hydrolysis activity of $G\alpha$ proteins (Pandey et al., 2009) was measured in the presence of purified C-terminal domain of the NFR1 α protein. In this preliminary in vitro assay, NFR1 α protein had no effect on either the GTP-binding or the GTPase activity of $G\alpha$ 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

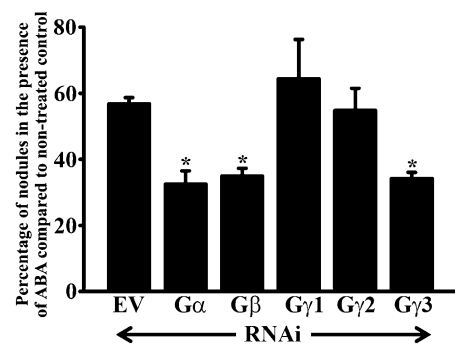


Figure 7. The effect of ABA on nodulation. Nodule number in EV and different G-protein RNAi lines in the ABA-treated versus mock-treated hairy roots. The data were recorded at 32 dpi and represent average of three biological replicates. Each replicate consisted of 40 to 50 root samples per construct.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

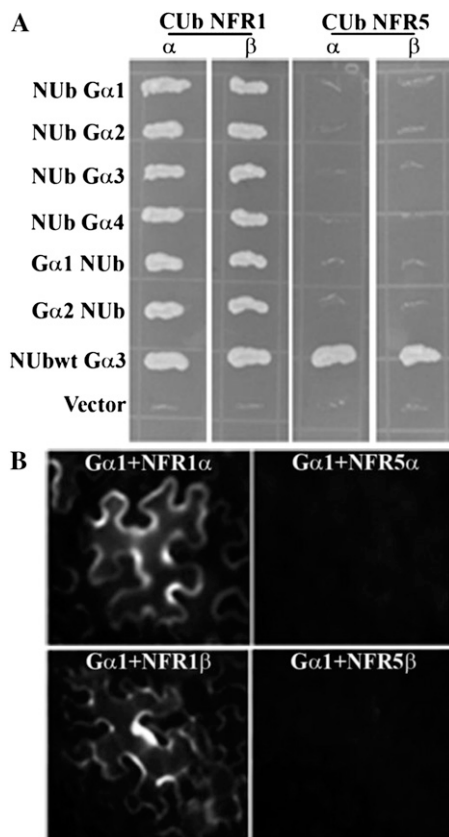


Figure 8. Interaction of $G\alpha$ proteins with NFR proteins. **A**, Interaction between NFR (NFR1 α , NFR1 β , NFR5 α , and NFR5 β) and $G\alpha$ proteins using split-ubiquitin-based interaction assay. The picture shows yeast growth on selective media with 1 mM Met. In all cases, $G\alpha$ proteins were used as NUb fusions in both orientations (NUb- $G\alpha$ denoting NUb fused to the N terminus of $G\alpha$ and $G\alpha$ -NUb denoting NUb fused to the C-terminus of $G\alpha$) 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 (NFR1 α , NFR1 β , NFR5 α , and NFR5 β) in 77-nEYFP-N1 and $G\alpha$ 1 in 78-cEYFP-N1 proteins using bimolecular complementation assay. *Agrobacterium tumefaciens* containing different combinations of NFR and $G\alpha$ 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 $G\alpha$ proteins are shown in Supplemental Figures S7 and S8.

lower levels of G-protein genes, especially the $G\beta$ -RNAi and group I $G\gamma$ -RNAi lines, exhibited a significantly-reduced nodule number compared with EV control lines, whereas higher nodule numbers were observed by overexpression of specific $G\beta$ and $G\gamma$ genes. These data thus provide the direct molecular-genetic evidence for the involvement of G-protein signaling during nodulation. The cross sections of nodules from 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 $G\alpha$ and group II $G\beta$ 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 $G\alpha$ and group II $G\beta$, 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 $G\beta$ genes (Figs. 3 and 4), but not in the roots expressing altered levels of $G\alpha$ genes. It is possible that a very high expression level of $G\alpha$ genes during nodulation (Fig. 1; Supplemental Fig. S1) and an incomplete silencing of $G\alpha$ genes in $G\alpha$ -RNAi plants (Supplemental Fig. S2) allows for the presence of enough residual $G\alpha$ to transduce the signal and mask the phenotype. However, alternative signaling mechanisms discussed later that do not require a direct input of $G\alpha$ proteins cannot be ruled out at this stage.

The results with $G\gamma$ proteins present a relatively complex picture. It is clear that the group I $G\gamma$ 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 $G\gamma$ -RNAi and ENOD40 promoter-driven $G\gamma$ 4 expression lines (Figs. 3 and 4). The homologs of group I $G\gamma$ proteins in Arabidopsis are involved in defense response against bacterial and fungal pathogens (Trusov et al., 2006). This group of $G\gamma$ proteins may therefore be generally involved in G-protein signaling during plant-microbe interactions.

The role of group III $G\gamma$ proteins seems to be obvious during nodulation in the presence of ABA (Fig. 7). The group III $G\gamma$ proteins are recently identified, unique, plant-specific $G\gamma$ 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 $G\gamma$ proteins with homology to group III $G\gamma$ 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 $G\gamma$ proteins, the group II $G\gamma$ 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 $G\gamma$ proteins exhibited partial suppression of the nodulation phenotype. Because the $G\gamma$ proteins are obligate interactors of $G\beta$ proteins and do not act independently, some of the effects that are observed in $G\gamma$ -RNAi lines could be due to the changes in stoichiometry or localization of specific $G\beta$ - $G\gamma$ complexes. A homolog of group II $G\gamma$ proteins is not present in Arabidopsis, and their function in plants remains unevaluated at the moment. It should be noted that the group II $G\gamma$ proteins differ from the group I $G\gamma$ 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 $G\gamma$ to the RWI motif in group II $G\gamma$ 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 $G\gamma$ -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 $G\gamma$ 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 $G\alpha$ Proteins Interact with NFR1

The importance of G-protein signaling in regulating nodulation is also underscored by $G\alpha$ proteins' interaction

with the nodulation receptor NFR1. We have shown both by yeast-based and in planta BiFC assays that soybean $G\alpha$ proteins interact specifically with NFR1 (Fig. 8; Supplemental Figs S7–S10 and S12). In meta-zoan systems, the guanine nucleotide exchange factor activity of GPCRs is required for the activation of $G\alpha$ proteins and onset of signaling. Plants have a very limited repertoire of GPCRs, and even though their interactions with $G\alpha$ proteins have been shown in several cases, a ligand for a GPCR-like protein coupled to $G\alpha$ is known only for the divergent GPCR-type G proteins (Pandey et al., 2009). It has been proposed that plant $G\alpha$ 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 $G\alpha$ 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 $G\alpha$ 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 $G\alpha$ protein with one such receptor family, opening up the exciting possibility of $G\alpha$ interaction with additional plant receptor protein families. Interestingly, the constitutively active $G\alpha$ proteins ($G\alpha$ 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 $G\alpha$ proteins (Supplemental Fig. S11). Therefore, the mechanisms by which divergent receptors may activate or regulate $G\alpha$ 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 $G\alpha$ and $G\beta\gamma$ proteins are involved in regulating the signal, either by direct interaction with the effectors or by helping correct targeting of $G\alpha$ subunits. In this situation, loss of either $G\alpha$ or $G\beta\gamma$ will result in a similar phenotype. This mode is exemplified by the rounded leaf morphology of Arabidopsis mutants lacking $G\alpha$ (*gpa1*)

or $G\beta$ (*agb1*) genes (Pandey et al., 2010). On the contrary, in the classical II mechanism, only $G\beta\gamma$ is required for signal transduction. If this mechanism is operative, loss of $G\beta\gamma$ will result in signal inhibition, whereas loss of $G\alpha$ will allow for the availability of free $G\beta\gamma$ 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 $G\beta\gamma$ is involved in signaling with no effect whatsoever from the presence of $G\alpha$, 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 $G\alpha$ and $G\beta\gamma$ 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 $G\beta\gamma$, not $G\alpha$. This suggests the possible involvement of the nonclassical mechanism, where the signaling is regulated only by $G\beta\gamma$ proteins with no input from $G\alpha$ proteins. However, the high expression of specific $G\alpha$ 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 $G\alpha$ is involved in signaling during nodulation together with $G\beta\gamma$ 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 $G\alpha$ genes in $G\alpha$ -RNAi plants (Supplemental Fig. S2). Further experiments with plants exhibiting more effective silencing of $G\alpha$ genes, overexpression of individual, constitutively active $G\alpha$ genes, or manipulation of Regulator of G-protein signaling genes will help delineate the exact role of $G\alpha$ 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 μ M) 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 ($G\alpha$, $G\beta$, and group I, group II, and group III $G\gamma$) 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 $G\beta$ and three $G\gamma$ 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- μ m) 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 $G\alpha$ and NFR proteins, essentially according to (Pandey and Assmann, 2004). BiFC assay was performed with $G\alpha$: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 Gm $G\alpha$ 1 (Glyma04g045960.1), Gm $G\alpha$ 2 (Glyma14g11140.1), Gm $G\alpha$ 3 (Glyma06g05960.1), Gm $G\alpha$ 4 (Glyma17g34450.1), Gm $G\beta$ 1

(Glyma11g12600.1), GmG β 2 (Glyma12g04810.1), GmG β 3 (Glyma06g01510.1), GmG β 4 (Glyma04g01460.1), GmG γ 1 (Glyma10g03610.1), GmG γ 2 (Glyma02g16190.1), GmG γ 3 (Glyma20g33390.1), GmG γ 4 (Glyma10g32215.1), GmG γ 5 (Glyma11g18050.1), GmG γ 6 (Glyma14g17060.1), GmG γ 7 (Glyma17g29590.1), GmG γ 8 (Glyma15g196300.1), GmG γ 9 (Glyma17g05640.1), and GmG γ 10 (Glyma07g04510.1).

Supplemental Data

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

Supplemental Figure S1. Absolute quantities of G α and G β 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 G β and G γ 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 GmG α proteins with GmNFR proteins using split ubiquitin-based interaction assay.

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

Supplemental Figure S9. Interaction between GmG α proteins with the N-terminal and C-terminal halves of GmNFR1 α and GmNFR1 β proteins using split ubiquitin-based interaction assay.

Supplemental Figure S10. Interaction between GmG α (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 GmG α 1 and GmG α 2 in the presence of NFR1.

Supplemental Figure S12. Interaction between constitutively active GmG α 1 (CAG α 1, Q223L) protein with GmNFR proteins using split ubiquitin-based interaction assay.

Supplemental Table S1. List of primers used.

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