Conventional and Novel \( \gamma \) Protein Families Constitute the Heterotrimeric G-Protein Signaling Network in Soybean

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Abstract

Heterotrimeric G-proteins comprised of \( \alpha \), \( \beta \) and \( \gamma \) proteins are important signal transducers in all eukaryotes. The \( \gamma \) protein of the G-protein heterotrimer is crucial for its proper targeting at the plasma membrane and correct functioning. \( \gamma \) proteins are significantly smaller and more diverse than the \( \alpha \) and \( \beta \) proteins. In model plants Arabidopsis and rice that have a single \( \alpha \) and \( \beta \) protein, the presence of two canonical \( \gamma \) proteins provide some diversity to the possible heterotrimeric combinations. Our recent analysis of the latest version of the soybean genome has identified ten \( \gamma \) proteins which belong to three distinct families based on their C-termini. We amplified the full length cDNAs, analyzed their detailed expression profile by quantitative PCR, assessed their localization and performed yeast-based interaction analysis to evaluate interaction specificity with different \( \beta \) proteins. Our results show that ten \( \gamma \) genes are retained in the soybean genome and have interesting expression profiles across different developmental stages. Six of the newly identified proteins belong to two plant-specific \( \gamma \) protein families. Yeast-based interaction analyses predict some degree of interaction specificity between different \( \beta \) and \( \gamma \) proteins. This research thus identifies a highly diverse G-protein network from a plant species. Homologs of these novel proteins have been previously identified as QTLs for grain size and yield in rice.

Introduction

Heterotrimeric G-proteins comprised of three dissimilar subunits \( \alpha \), \( \beta \), and \( \gamma \) are important signaling intermediates in all eukaryotes [1–3]. The \( \alpha \) subunit, due to its ability to switch between the GDP-bound inactive form and GTP-bound active form, defines the status of signal transduction. Ligand binding to the GPCR causes a change in its conformation allowing an exchange of GTP for GDP on the \( \alpha \) subunit [4]. The GTP-bound \( \alpha \) dissociates from the \( \gamma \beta \) subunits and the released \( \alpha \)-GTP and \( \gamma \beta \) dimer interact with a variety of effector proteins to transduce the signal. The intrinsic GTPase activity of \( \alpha \) causes GTP hydrolysis, converting it back to its GDP-bound state. The \( \alpha \)-GDP reassociates with the \( \gamma \beta \) dimer and the proteins return back to trimeric conformation [4,5].

A wide range of fundamental signal transduction pathways are mediated via G-proteins in both plants and animals [4,6]. In non-plant systems the multiplicity of each of the G-protein subunits, together with almost one thousand GPCRs, tissue-specific expression and signal-dependent heterotrimer formation, provides for the specificity of response [7,8]. In plants the repertoire of G-protein components is relatively simple; the two most investigated species Arabidopsis and rice have only a single \( \alpha \), a single \( \beta \) and two canonical \( \gamma \) proteins [9]. Given the presence of a single \( \alpha \) and \( \beta \), the specificity in Arabidopsis and rice G-protein signaling is provided solely by the multiplicity of \( \gamma \) proteins.

We recently carried out an analysis of the soybean genome to assess if this paucity of G-protein components in plants is the norm and whether structural and functional diversity exists within the multiple copies of a gene present in highly duplicated genomes. Our analysis revealed a much more diverse plant G-protein family with the soybean genome encoding for four \( \alpha \), four \( \beta \) and two \( \gamma \) proteins [10]. The number of \( \alpha \) and \( \beta \) proteins in the soybean genome corresponds well to the two recent genome duplication events [11] resulting in four copies of each gene. Interestingly both \( \alpha \) and \( \beta \) proteins exhibit some degree of interaction specificity between them. Moreover, based on the GTP-binding and GTPase activity, four \( \alpha \) proteins form two distinct subgroups. These data thus revealed that the G-protein signaling in plants is significantly more diverse and complex than what was predicted based on the studies in Arabidopsis and rice [10].

The presence of only two \( \gamma \) proteins in the soybean genome however did not correspond to what was expected based on the genome duplication events. Additionally two of the \( \beta \) proteins GmGb1 and GmGb2 did not show any interaction with the...
Proteins. The presence of three different families of G-protein signaling mechanisms, distinct from what is known in mammals, is present at its C termini [17]. The function of plant G-protein has an extra 57 amino acid extension at its N terminus [17]. The two reported pea G-proteins AGG1 and AGG2 proteins are highly homologous to the Arabidopsis AGG1 protein and contain all the conserved features and motifs of G-proteins. The rice RGG1 and soybean GmG1 and GmG2 proteins are highly homologous to the AGG1 protein and contain all the conserved features and motifs of G-proteins. The rice RGG2 protein is relatively diverse as this protein has an extra 57 amino acid extension at its N terminus (compared to RGG1) and does not contain the signature prenylation motif. The two reported pea G-proteins PG1 and PG2 are somewhat unusual as they do not contain the highly conserved DPLL/I motif even though a possible prenylation motif is present at its C termini [17]. The function of plant G-proteins has been evaluated only in Arabidopsis where the proteins participate in known Gα and/or Gβ mediated signaling pathways. Molecular-genetic analysis of knockout mutants in AGG1 and AGG2 reveals that the proteins are involved in regulating response to fungal pathogens [16,18].

With the availability of a newer version of the soybean genome assembly [phytozone.net v7] and the use of a series of careful genome annotation programs, we queried the soybean genome to identify additional Gγ protein sequences. Our analysis identified two more canonical Gγ proteins that are present on the not yet annotated genome regions as well as six additional, novel Gγ proteins. The proteins display a great degree of diversity and can be grouped into three distinct families based on sequence features: the archetypal Gγ proteins, the prenylation-less Gγ proteins and the cysteine-rich Gγ proteins. This study describes the identification of these three families of Gγ proteins from soybean, details its expression profile in comparison to the expression profile of the GmGβ genes and evaluates its interaction with the specific GmGβ proteins. The presence of three different families of Gγ proteins in a single plant species supports a highly elaborate and diverse G-protein signaling network as well as provides clues to plant-specific G-protein signaling mechanisms, distinct from what is known based on mammalian systems.

Results

Identification of additional canonical and novel Gγ proteins from the soybean genome

Our previous analysis of the soybean genome had identified only two Gγ proteins [10]. We performed a careful search with the newer version of the soybean genome and identified eight additional Gγ proteins. The presence of ten Gγ proteins together with four Gα and four Gβ proteins thus corresponds to a total of one hundred and sixty possible heterotrimeric combinations.

Two of the newly identified Gγ proteins are highly homologous to the previously identified GmGγ1 and GmGγ2 and show high sequence homology between them (Table 1). We named these proteins GmGγ3 and GmGγ4, respectively. Both these proteins are present on the regions of the soybean chromosomes that have not yet been annotated. GmGγ5 is present on the chromosome 20 between the protein coding regions Glyma20g33300.1 and Glyma20g33320.1. We subsequently annotated the locus for GmGγ3 as Glyma20g33510.1. The open reading frame of this gene along with the positions of introns is reported in Figure S1. GmGγ4 is present on the chromosome 10 between the protein coding regions Glyma10g32210.1 and Glyma10g32220.1. We annotated the locus for GmGγ4 as Glyma10g32215.1. Figure S2 details the sequence of this newly annotated gene with its exons and intron.

These four GmGγ proteins Gγ1, 2, 3 and 4 have all the features of canonical Gγ proteins, namely the coiled-coil domain in the middle with the conserved DPLL/I motif at position 66-69 and conserved L30, E40 and S51 (amino acid numbers according to GmGγ1). These conserved features are important for the interaction of Gγ proteins with the Gβ proteins [19]. GmGγ3 and GmGγ4 also contain the CWIL motif at its C termini. This is the most common isoprenylation motif present in all plant Gγ proteins. We assigned these four prototypical Gγ proteins to group I. Figure 1 shows the protein sequence and conserved motifs of these Gγ proteins.

BLAST analysis [20] of the soybean genome using the coiled-coil region of the Gγ proteins identified three additional Gγ-like proteins on loci Glyma1g18050.1, Glyma1g17060.1 and Glyma1g29590.1. We named these proteins GmGγ5, GmGγ6 and GmGγ7, respectively. These three proteins share a high degree of sequence similarity with each other (Table 1). Based on its unique features, we assigned these three proteins to group II. Compared to the group I proteins, the group II proteins have an extra N terminal extension of 20-25 amino acids. The middle coiled-coil region of these proteins is highly similar to the group I proteins (Figure 1). Sequence features predicted to be involved in Gβ-Gγ interaction are conserved in group II Gγ proteins [19]. The most distinct feature of these group II proteins is the lack of C-terminal isoprenylation motif. The proteins end in a RWI sequence instead of a CWIL sequence. The group II proteins are thus somewhat similar to the rice RGG2 proteins as RGG2 also has an N terminal extension [ albeit longer, 57 aa] and lacks the prenylation motif [21]. The group II Gγ proteins are also small in size; GmGγ5 and GmGγ6 are encoded by 131 amino acids and GmGγ7 is encoded by 126 amino acids, similar to canonical Gγ proteins [5]. Surprisingly a prenylation motif-less Gγ protein is not present in Arabidopsis.

The GmGγ5 gene sequence is mis-annotated in the current version of the soybean genome. The predicted protein based on the genome annotation is much smaller and does not have the first exon as identified in our study. The genomic arrangement and experimental validation support the presented version of GmGγ5 as the correct version. The correct sequence of the gene is detailed in Figure S3.

We identified three additional Gγ-like novel proteins on loci Glyma15g19630.1, Glyma17g05640.1 and Glyma07g04510.1. We named these proteins GmGγ8, GmGγ9 and GmGγ10, respectively and assigned them to group III based on its distinctive features. The group III proteins are significantly larger than conventional Gγ proteins as GmGγ8, GmGγ9 and GmGγ10 are encoded by 213, 228 and 159 amino acids, respectively. The N terminal of group III proteins is similar to group I proteins and the middle coiled-coil domain is highly conserved (Figure 1). GmGγ8 and GmGγ9 have all the sequence features required for Gβ interaction. GmGγ10 is the only protein identified in our analysis that does not have the conserved DPLL/I motif, instead this protein contains a similar DPFT motif (Figure 1). It is interesting to note that a highly conserved sequence in mammalian Gγ proteins N62 P63, F64 (numbers based on human Gγ1) is not conserved in the
plant Gγ proteins. This region is required for the GPCR-dependent conformational change in Gγ [22].

In addition to large size, there are other features that are unique to group III Gγ proteins. The proteins are predicted to have a TNFR (tumor necrosis growth factor receptor) signature and a long cysteine rich C-terminal region which is not found in any other known Gγ proteins to date. The C terminus of these proteins is quite variable. Counting from the conserved DPLL (DPFT in case of GmGγ10) motif, the C terminal of GmGγ8, GmGγ9 and GmGγ10 consists of 130, 145 and 74 amino acids, respectively. This variable region of group III proteins is unusually high in cysteine content: GmGγ8 contains 30% Cys (39 out of 130 amino acids), GmGγ9 contains 33% Cys (49 out of 145 amino acids) and GmGγ10 contains 26% Cys (19 out of 74 amino acids).

The sequence of GmGγ8 and GmGγ10 is mis-annotated in the current version of the soybean genome assembly. The predicted genes could never be amplified in our analysis. We manually annotated these genes and amplified the full length product. Based

|        | GmGγ1 | GmGγ2 | GmGγ3 | GmGγ4 | GmGγ5 | GmGγ6 | GmGγ7 | GmGγ8 | GmGγ9 | GmGγ10 |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| GmGγ1  | ***   | 92.7  | 50.9  | 50.9  | 29.4  | 29.4  | 29.4  | 20.2  | 19.3  | 19.3   |
| GmGγ2  | ***   | 51.9  | 51.9  | 31.2  | 31.2  | 31.2  | 19.3  | 18.3  | 18.3  |        |
| GmGγ3  | ***   | 99.1  | 32.1  | 32.1  | 32.1  | 23.6  | 24.5  | 18.9  |        |        |
| GmGγ4  | ***   | 32.1  | 32.1  | 32.1  | 32.1  | 23.6  | 24.5  | 18.9  |        |        |
| GmGγ5  | ***   | 99.2  | 93.7  | 20.6  | 19.8  | 19.1  |        |        |        |        |
| GmGγ6  | ***   | 94.4  | 20.6  | 19.8  | 19.1  |        |        |        |        |        |
| GmGγ7  | ***   | 21.4  | 19.0  | 20.6  |        |        |        |        |        |        |
| GmGγ8  | ***   | 57.3  | 27.0  |        |        |        |        |        |        |        |
| GmGγ9  | ***   |       | 26.4  |        |        |        |        |        |        |        |
| GmGγ10 | ***   |       |       |        |        |        |        |        |        |        |

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Figure 1. Amino acid sequence alignment of GmGγ proteins. The sequence alignment of Arabidopsis AGG1, AGG2 and AtSG20635, rice RGG1 and RGG2, and the ten GmGγ was performed using Clustal W (www.clustal.org). Consensus sequences for interaction with Gβ proteins are marked with asterisks. Box represents the highly conserved DPLL/I motif in Gγ proteins. The amino acid positions are numbered in accordance with the GmGγ1.

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on the experimentally obtained cDNAs, we marked the correct exon-intron boundaries of GmG\(_{c}\)8 (Figure S4) and GmG\(_{c}\)10 (Figure S5).

We also identified a homolog of GmG\(_{c}\)9 in the Arabidopsis genome at locus At5g20635. This gene has been recently described as a \(G^c\) protein in Arabidopsis [23]. Homology of group III proteins are present in all plant species. The proteins also show some homology to a keratin associated protein present in mammals. Interestingly the homology of group III proteins in rice which are named DEPI and GS3 have been recently identified as major QTLs for grain size and yield determination [24,25].

**Genome organization and phylogenetic relationship analysis of soybean \(G^c\) proteins**

The availability of multiple \(G^c\) protein sequences in soybean with seemingly variable sequence features raised the question whether these protein families originated from the duplication of a single gene. We analyzed the chromosomal location of all ten GmG\(_{c}\) genes and the organization of exon and introns (Figure 2). Group I and group II GmG\(_{c}\) genes have four exons each, whereas five exons are present in the group III genes. The length of the second and third exons is highly conserved between all ten GmG\(_{c}\) genes. The second exon is 32 bp in group I and II and 53 bp in group III. Similarly, the third exon is 45 bp long in group I and II and 44 bp long in group III GmG\(_{c}\) genes. These two exons code for the highly conserved, middle coiled-coil domain of GmG\(_{c}\) proteins. This extreme conservation of the exon organization suggests that the proteins originated from this core sequence and acquired variable N- and C-terminal sequences. The group I and group II GmG\(_{c}\) genes also show a highly conserved fourth exon. It is also interesting to note that despite similarity in the size of cDNAs and proteins of the group I and group II GmG\(_{c}\)s, the group I genes are encoded by large genomic regions (~4–5 kb) and have a very long first intron ranging from 3.5 to 4.5 kb. This feature is not present in the group II GmG\(_{c}\) genes. The group III GmG\(_{c}\) genes show relatively less conserved genome organization even within the group. The last two exons of this group encode for the cysteine rich region of the proteins and display variability in size (Figure 2).

Phylogenetic analysis of all soybean \(G^c\) proteins, along with the Arabidopsis and rice sequences, groups them in three expected subgroups with the two Arabidopsis proteins, the rice RGG1 and the GmG\(_{c}\)1-4 in one subgroup, the GmG\(_{c}\) group II proteins and rice RGG2 in another subgroup and the GmG\(_{c}\) group III proteins, the Arabidopsis At5g20635 and rice DEP1 and GS3 in the third subgroup (Figure S6). Analysis of chromosome location of GmG\(_{c}\) genes suggests that the four group I genes have resulted from two genome duplication events with the first event resulting in highly homologous GmG\(_{c}\)1 and GmG\(_{c}\)2 forming one pair and GmG\(_{c}\)3 and GmG\(_{c}\)4 forming another. Similarly within group II, the genes GmG\(_{c}\)5 and GmG\(_{c}\)6 form a duplicated gene pair; and within group III, the genes GmG\(_{c}\)8 and GmG\(_{c}\)9 form a duplicated gene pair. We did not identify duplicated gene pairs corresponding to GmG\(_{c}\)7 and GmG\(_{c}\)10. These might have been lost during evolution.

![Figure 2. Exon-intron organization of the genomic regions coding for GmG\(_{c}\) proteins. Boxes represent exons and lines represent introns. The introns are not drawn to scale. Numbers denote the length of respective exons and introns.](doi:10.1371/journal.pone.0023361.g002)
Tissue-dependent expression analysis of soybean Gγ genes

In mammalian systems where multiple isoforms of Gγ proteins are present, a high degree of tissue specificity is observed for expression. Similarly the Arabidopsis Gγ proteins also show distinct expression patterns [16]. We assessed the expression profile of the ten GmGγ genes by real-time quantitative PCR to evaluate whether all ten GmGγ genes are expressed and the comparative expression levels. We quantified the absolute expression of each gene by a 100 fold serial dilution of cloned plasmid DNA and ascertained the specificity and efficiency of the individual primer pairs (Table S2, Figure S7). A linear correlation coefficient (R²) of 0.98-0.99 was observed over a 100,000 fold dilution. Interestingly unlike Gα and Gβ γ genes that are expressed at relatively similar levels in different tissue types [10], a range of variable expression levels were observed for the GmGγ genes.

We analyzed the tissue specific expression of the three families of GmGγ genes in vegetative tissues and reproductive tissues. Additionally given the possible role of G-protein dependent signaling during nodulation [26–30], we also analyzed the expression of GmGγ genes in this legume-specific tissue. Of the group I genes, GmGγ4 exhibits overall high expression compared with the other members of this group (Figure 3A). In general, all four genes are expressed in all tissue types tested; however, the expression of GmGγ4 is comparatively lower in roots than in aerial tissues whereas GmGγ3 is most highly expressed in nodules and at a very low level in developing seeds (S4). The expression data of the genes GmGγ1 and GmGγ2 are presented for comparing relative expression level to the GmGγ3 and GmGγ4 genes.

Within the group II genes GmGγ6 is expressed at a relatively low level compared to the other two members of this group (Figure 3B). GmGγ7 in general has lower expression in leaves at all stages of development. All three genes are expressed at a moderate level in nodules. Additionally the group II genes are expressed at a relatively lower level in reproductive tissues i.e. the inflorescence apex, flowers and at an almost non-detectable level in seeds compared to the vegetative tissue (Figure 3B).

The group III genes show maximum variability in tissue specific expression. In this group GmGγ5 is expressed at an extremely high level when comparing within this group or with any of the other GmGγ genes; whereas GmGγ10 is expressed at a very low level. Noticeably this group of genes shows poor expression in nodules and seeds (Figure 3C).

Expression of GmGγ genes during seed development and germination

Our previous gene expression analysis with the soybean Gα and Gβ γ genes showed interesting expression patterns during seed development and germination [10]. We analyzed the expression pattern of the newly discovered GmGγ genes during seed development and germination and compared it to the expression of GmGβ γ genes.

Specific patterns of gene expression were observed for different GmGγ genes. For example GmGγ3, GmGγ6 and GmGγ7 do not show any change in expression during seed development (Figure 4) similar to GmGβ2 and GmGβ4 [10]; whereas GmGγ1, GmGγ2 and GmGγ9 show moderate down-regulation during seed maturation (Figure 4). Conversely, GmGγ4, GmGγ5 and GmGγ8 exhibit significant up-regulation of expression during seed maturation (Figure 4). These genes thus show expression profiles similar to GmGβ2 and GmGβ3. It is also noticeable that the expression of these three Gγ genes, GmGγ4, GmGγ5 and GmGγ8, is maintained at a high level in dry seeds whereas the expression of GmGβ2 and GmGβ3 genes returns back to basal levels in dry seeds [10].

![Figure 3. Expression of soybean GmGγ genes in different tissue types](https://example.com/figure3.png)

**Figure 3. Expression of soybean GmGγ genes in different tissue types.** The stages are defined as VC: cotyledon; V1-R: primary root at stage V1 (appearance of the first set of unfolded trifoliate leaves); V1-S: primary stem at stage V1; V1-TF: first trifoliate leaf; Vn-R: mature root; Vn-S: mature stem; Vn-L: mature leaves; Apex: inflorescence apex; Vn-F: flower; seeds: developing seeds at stage S4. qRT-PCR amplifications were performed thrice independently for each target, and the data were averaged. The expression values across different tissue types were normalized against soybean Actin gene expression [59], which was set at 100. Error bars represent the standard error of the mean. (A) Relative expression of the group I GmGγ genes. (B) Relative expression of the group II GmGγ genes. (C) Relative expression of the group III GmGγ genes. doi:10.1371/journal.pone.0023361.g003
Diversity of G\textgamma Proteins in Soybean

G-proteins are involved in the regulation of seed germination in Arabidopsis [31,32] and the soybean GmG\textalpha and GmG\textbeta genes show distinct patterns of expression during different stages of seed germination [10]. GmG\textgamma1 and GmG\textgamma4 follow the similar pattern as GmG\textbeta3 and GmG\textbeta4 with higher expression starting 6 h after imbibition, maximizing at 12 h followed by a decrease in expression (Figure 5). The expression of GmG\textgamma5 and GmG\textgamma9 is similar to the expression of GmG\textbeta1 and GmG\textbeta2 with expression peaking at 6 h after imbibition followed by a decrease in expression (Figure 5). The genes GmG\textgamma6 and GmG\textgamma10 are expressed at a significantly low level in dry seeds and do not show any change in expression during germination (Figure 5); GmG\textgamma7 is also expressed at a very low level in dry seeds (Figure 4C); however, the expression of this gene is significantly up-regulated during germination. The genes show higher expression starting 6 h post-imbibition and maximum expression is observed at 24 h post imbibition followed by a gradual decrease. In contrast GmG\textgamma8 which is expressed at a high level in dry seeds shows a significant down-regulation in its expression following 6 h post imbibition.

We also tested the expression of different GmG\textgamma genes in response to various stresses. No significant differences were observed in the expression of any of the genes under the conditions where a stress-marker gene GmRab18 was expressed at significantly higher level (data not shown). These data are similar to what we earlier observed for the GmG\textalpha and GmG\textbeta genes [10].

Localizaton of soybean G\textgamma proteins

Canonical G\textgamma proteins are localized to the plasma membrane via the isoprenylation of C terminal sequence [33,34]. The presence of three G\textgamma protein groups with distinctly variable C terminal sequences allowed us to assess whether the three groups of proteins exhibit any differences in localization. We transiently transformed tobacco leaves via agrobacterium-mediated transformation with YFP (yellow fluorescent protein) fused with respective GmG\textgamma genes at N termini. The transformed leaves were visualized with confocal microscopy. The group I fusion proteins YFP-GmG\textgamma1, YFP-GmG\textgamma2, YFP-GmG\textgamma3 and YFP-GmG\textgamma4 showed fluorescence localized to the periphery of the cells as expected based on the presence of a canonical isoprenylation motif at the C terminal of these proteins (Figure 6). Similarly the group II fusion proteins YFP-GmG\textgamma5, YFP-GmG\textgamma6 and YFP-GmG\textgamma7 also showed fluorescence restricted to the periphery which suggests the predominantly plasma membrane localization for these proteins (Figure 6). This is intriguing as these proteins lack a canonical prenylation motif and do not have any cysteine residues in the vicinity for such modifications. This is similar to the localization of rice RGG2 protein to the plasma membrane despite lacking a prenylation motif at its C terminal [21]. GmG\textgamma8 and GmG\textgamma9 have a conserved C at position 4 and all three group III genes have a conserved cysteine at position 6 from C-terminal in addition to multiple other cysteine residues in the vicinity. These cysteines qualify for the possible lipid modification by farnesyl transferase or by geranylgeranyl transferases [33]. However, the localization of this group of proteins is interesting as in addition to most of the protein being present at the periphery, fluorescence was also observed as clear puncta especially in the case of YFP-GmG\textgamma8 (Figure 6). Such patterns are indicative of either protein aggregate formation or endosomal localizations [34]. Further studies with stably transformed plants will be required to critically assess the localization of group III GmG\textgamma proteins.

Protein-protein interaction between GmG\textbeta and GmG\textgamma proteins

We assessed the protein-protein interaction specificity of the three groups of G\textgamma proteins with all four G\textbeta proteins of soybean using the ProQuest yeast-2-hybrid system. The group I proteins GmG\textbeta3 and GmG\textgamma4 showed strong and specific interactions with GmG\textbeta2 and GmG\textbeta4 but not with GmG\textbeta1 and GmG\textbeta3 proteins.
Figure 5. Expression of GmGγ genes during seed germination.
Seed germination was followed starting from dry seeds (0 h) up to 30 h when an obvious radical had protruded. Seed samples were collected at every 6 h following imbibition. qRT-PCR amplification experiments were performed thrice independently for each target, and the data were averaged. The expression values across different seed germination stages were normalized against soybean Actin gene expression. Error bars represent the standard error of the mean. (A) Relative expression of the group I GmGγ genes during different stages of seed development. (B) Relative expression of the group II GmGγ genes. (C) Relative expression of the group III GmGγ genes.

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Figure 6. Localization of GmGγ proteins.
Localization of YFP:GmGγ 1-10 genes in transiently transformed tobacco leaves using confocal microscopy. At least three independent transformations were performed. The figure shows representative picture from each transformation.

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(Figure 7A) as is also the case with GmGγ1 and GmGγ2 [10]. The group II GmGγ proteins exhibit relatively weaker interaction with the GmGb proteins compared to the group I proteins except the interaction between GmGγ3 and GmGb4. However, specific interactions of group II proteins were observed with all four GmGb proteins (Figure 7B).

The establishment of interaction with Gβ proteins was an utmost requirement to classify the group III proteins as novel Gγ proteins. As shown in Figure 7C, the group III GmGγ proteins showed very strong and specific interaction with GmGb proteins. Similar to the group I proteins, the group III proteins also interacted strongly with GmGb2 and GmGb4. However, distinct from the group I proteins, where no interaction was seen with GmGb1 and GmGb3, the group III proteins clearly show weak but specific interaction with GmGb1 and GmGb3 proteins (Figure 7C). We also tested whether the N terminus itself was sufficient for this interaction by making deletion constructs of proteins that either expressed the N-terminal half (tilt DPLL/DPTF motif) or the C-terminal half (protein sequence following the DPLL/DPTF motif). Significantly weaker interaction was detected using either of the truncated proteins (Figure S8), suggesting a full length protein is required for such interactions.

The presence of long cysteine rich C-terminal regions on the group III GmGγ proteins prompted us to test whether these proteins interact with one another. We tested interaction between GmGγ8, GmGγ9 and GmGγ10 in all nine possible combinations. The proteins exhibit strong interaction with each other as shown in Figure 8.

**Discussion**

**Identification of three distinct Gγ protein families in soybean**

Gγ proteins are an integral part of the G-protein heterotrimer. In mammalian systems, these proteins are relatively small (7–8.5 kDa) and are the most diverse of the three subunits with only ~50% sequence homology between different isoforms. The proteins also show a high degree of tissue specific expression and isoform-specific interactions with Gβ proteins. In both plants and animals, the Gγ proteins are required for proper targeting of the Gβ subunit and of the intact heterotrimer to the plasma membrane [5,36–38]. Identification of diverse Gγ proteins from soybean (Figure 1) along with the presence of multiple Gα and Gβ proteins expands the number of possible heterotrimeric combinations in soybean in addition to identifying novel, plant specific components of G-protein signaling.

The C-terminus of Gγ proteins is the basis of our classification of the proteins into three distinct groups. Group I is comprised of canonical Gγ protein that have all the conserved features of Gγ proteins as described based on the mammalian paradigm. Homologs of this family are present in all plant species including gymnosperms and mosses. Most of the reported plant Gγ proteins to date, AGG1, AGG2, RGG1 and GmGγ1-4, are members of this group (Figure 1). The group II proteins consisting of Gγ5, Gγ6 and Gγ7 differ from the family I protein mainly due to the absence of conserved cysteines in the C-terminal region. The genes seem to have evolved from a single amino acid substitution of the CWIL motif to the RVI motif (the most common C-terminal motif present in dicot plants) as most of the protein sequence and exon-intron organization of family I and family II proteins is highly conserved (Figure 2). The rice RGG2 protein is a member of this family although the protein ends in a KGDFS sequence which also seems to be conserved in other monocot species. Lack of cysteine residues in group II proteins precludes the possibility of

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**Figure 7. Interaction between soybean G-protein β and γ subunits.** Interaction between GmGb1 (in pDEST32) and GmGγ (in pDEST22) proteins was determined using yeast-2 hybrid-based colorimetric assay. The assays were performed in triplicates and data were averaged. Error bars represent the standard error of the mean. Two biological replicates of the experiment were performed with similar results. (A) Interaction between GmGb1 proteins and group I GmGγ proteins. (B) Interaction between GmGb1 proteins and group II GmGγ proteins. (C) Interaction between GmGb1 proteins and group III GmGγ proteins. Strong, weak and -ve refer to the interaction strength between RalGDS-wt-pDEST32 with Krev1-pDEST22, RalGDS-m1-pDEST32 with Krev1-pDEST22 and RalGDS-m2-pDEST32 with Krev1-pDEST22, respectively. The controls are provided with the ProQuest two hybrid system (Invitrogen). doi:10.1371/journal.pone.0023361.g007
prenylation; however, the proteins do seem to localize at the plasma membrane (Figure 6) as has also been reported for the rice RGG2 [21]. A single cysteine present in the middle of these proteins could be a potential target for palmitoylation which might assist in its anchoring to the plasma membrane [21]. Additionally, the proteins have a high number of positively charged and aromatic amino acids at the C-terminus (7 out of 10) which may assist in its anchoring to the plasma membrane by the formation of an α helix [39]. In mammals, lack of prenylation either by mutation of the conserved cysteine residue or by chemical inhibition has been shown to result in localization of specific Gβγ proteins to the nucleus of the cells and its possible role in regulating transcription, a function not typically associated with G-proteins [40]. Lack of a prenylation-less gene in the Arabidopsis genome has limited the functional characterization of this family of Gγ in plants. The availability of an insertional mutant line in RGG2 might be able to resolve the issue of whether such proteins play any unconventional roles in plants.

The group III proteins constitute a novel Gγ family, specific to plants. Homologs of these proteins are present in both angiosperm and gymnosperm families. We applied the following criteria to establish the group III proteins as authentic Gγ proteins. The coiled-coil domain of group III GmGγ proteins is highly similar to the conventional Gγ proteins with full conservation of amino acid residues involved in the interaction with Gβ proteins (Figure 1). The size of the second and third exons of these proteins is very similar to group I and group II Gγ proteins (Figure 2). Additionally, a homology modeling-based analysis of three-dimensional protein structures using a fold recognition server Phyre (Protein Homology/analogY Recognition Engine, http://www.sbg.bio.ic.ac.uk/~phyre/) predicted these proteins to be Gγ proteins with 40–55% precision. Finally, the proteins showed strong and specific interaction with the GmGβ proteins. The Arabidopsis homolog of group III family protein Atg9g20635 has recently been identified as a novel Gγ protein [23].

Rice has two proteins that show homology to group III proteins, DEP1 (dense and erect panicle 1) and GS3 (grain size 3), that have been isolated as major QTLs for seed size and yield [24,25]. Interestingly, the rice Gαi protein RCA1 is also involved in regulation of seed size [41]. The rice DEP1 and GS3 proteins have been described as novel proteins containing a TNFR-motif, a transmembrane domain and proteins with homology to human keratin-associated protein. We also identified a TNFR motif in the soybean group III family proteins; however, using multiple transmembrane prediction programs including TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), HMMTOP (http://www.enzim.hu/hmmtop/) and DAS (http://www.sbc.su.se/~milkos/DAS/), we did not identify any transmembrane domains in the GmGγ8, GmGγ9 or GmGγ10 proteins. Rice and Arabidopsis group III proteins are predicted to have a single transmembrane domain using DAS, but not with TMHMM and HMMTOP. Experimental verification of the presence of a transmembrane domain and any possible role it might play in localization and/or positioning of these at the plasma membrane will be needed to evaluate its importance. Interestingly, YFP-fused group III GmGγ proteins, in addition to the peripheral YFP fluorescence, also showed small vesicle like structures which were very evident in GmGγ8. These proteins might be localized to endosomes structures in addition to the plasma membrane. However, since these proteins are highly cysteine rich such structures could also be due to protein aggregate formation or self-interaction (Figure 8). Our data at this time cannot differentiate between these possibilities. Expression of proteins with native promoters in a protein null background will help decipher correct localization.

Expression profile of soybean Gγ proteins and possible correlation with Gβ proteins

The analysis of the complete repertoire of the GmGγ genes and its comparison with the expression pattern of GmGα and GmGβ genes began to display specific expression patterns related to particular genes or gene families. Moreover, when comparing the absolute expression levels within different subunits, a wide range of expression levels were observed for the GmGγ genes (e.g. Figure 3, GmGγ9 versus GmGγ6), whereas all GmGα and GmGβ genes were expressed at a relatively similar level to each other. Additionally, the duplicated gene pairs of GmGα or GmGβ typically showed similar expression patterns, a trend not observed between duplicated gene pairs of GmGγ genes. GmGγ9 was the most highly expressed gene, whereas its duplicated gene GmGγ8 was expressed at the moderate level. Likewise, GmGγ4 was a highly expressed gene but its duplicated gene pair GmGγ3 was relatively poorly expressed.

Some tissue specificity of gene expression was also evident while comparing the expression of multiple Gγ genes such as low expression of group II genes in reproductive organs or lower expression of group III genes in nodules (Figure 3). Additionally, during seed development and germination, specific expression profiles were observed for individual genes which in some cases corresponded well to the expression of GmGβ genes. These observations suggest that developmental stage-specific or tissue-specific expression of particular genes may lead to specific Gγ/β combinations, similar to what is observed in the mammalian systems [42,43].

Since the two rice homologs of group III genes DEP1 and GS3 are involved in grain size determination and yield, we focused on the expression pattern of soybean group III GmGγ genes during...
seed development. Our data showed that of the three group III genes in soybean, *GmGy10* shows the most interesting expression pattern during seed development and germination (Figure 4, 5). The expression of this gene was highly up-regulated when seed is undergoing maturation (stages S7 onwards), whereas a sharp decrease was observed during seed germination. This gene could be a true functional homolog of the rice DEP1 or G33 gene. Additionally, the expression of this gene in seeds could be dependent on the endogenous ABA and/or GA concentration as the levels of both these hormones change significantly during seed maturation and germination. Interestingly, *GmGy5* also followed a similar expression profile where the expression was up-regulated during seed maturation and generally down-regulated during germination. These genes could be potential targets for manipulation to regulate soybean seed development. It was also obvious that this group of genes is highly variable functionally as *GmGy10* shows very little expression in seeds at all developmental stages and exhibits no change in expression profile during germination.

**Interaction specificity of GmGb and GmGy proteins**

Specific mammalian Gb and Gγ proteins form non-dissociable dimers and interact very strongly under a variety of in vitro and in vivo conditions. The data presented in this study suggest that there is specificity of interaction between different GmGb and Gγ proteins. It is especially intriguing that the GmGb1 and GmGb3 are in general weaker interactors compared to GmGb2 and GmGb4 even though they have more than 90% sequence similarity at the protein level (Figure 7). Additionally the group II GmGb proteins also exhibited weak interactions compared to the group I and group III proteins even though they do interact with similar strengths with all four GmGb proteins. Similar differences in the interaction between mammalian Gb and Gγ proteins have also been observed. The human Gβ1-4 share 80–90% sequence identity; however, Gβ1 in general interacts with multiple Gγ isoforms, Gβ2 is more restricted in its interaction partners and Gβ3 displays significantly weaker interactions [44–46]. In most cases, however, the interaction data were based on in vitro assays and its relevance in the context of a specific cell type or a signal remains to be evaluated in both mammalian and plant systems.

An intriguing observation in our studies is the strong interaction between different members of the group III proteins themselves (Figure 8). It would be interesting to assess how the oligomerization of these proteins might affect interaction with GmGb proteins or other possible interactors. The unusual nature of these proteins does not preclude the possibility of its involvement in some plant-specific signaling mechanisms which are different from what is known from studies in mammalian systems. Plants do have several unconventional G-protein components such as the extra-large G-proteins that have a Gz domain [47–50]; the GbT proteins that have GTP-binding and hydrolysis activity of their own and are regulated by GPA1 [51]; and the RGS1 protein that has a 7TM GPCR-like structure fused to RGS domain [52]. Likewise, most of the known effector proteins of G-protein signaling in plants are also distinct from the conventional effector proteins of mammalian systems. For example, PRN1 (Pirin1) which is a member of an iron-containing subgroup of the cupin superfamily, PD1 [prephenate dehydratase1], a protein involved in phenylalanine biosynthesis, and a NF-Y family transcription factor form a signaling complex during G-protein mediated light and ABA signaling pathways during early growth and development in *Arabidopsis* [53–55]. Similarly, a chloroplast-localized protein THF1 (thylakoid formation 1) is a GPA1 effector protein during sugar signaling [56]. Detailed study of specific pathways mediated by these unconventional proteins in the context of canonical heterotrimeric G-protein signaling is only in its infancy and future work may divulge additional signaling mechanisms specifically evolved in plants.

**Conclusion**

We have identified three distinct families of Gγ proteins including a novel, plant-specific Gγ protein family in the soybean genome. The elucidation of this complete repertoire of different G-protein subunits in soybean reveals a highly elaborate G-protein signaling network in plants. Our data also suggest the presence of subunit-specific and tissue-type or developmental stage-specific heteromeric combinations. Additionally the homology of the group III Gγ protein have been identified as major QTLs for grain size and yield in rice. Further work with the generation of RNAi and overexpression lines of soybean G-protein genes will help us decipher its signaling mechanisms as well as its use as potential targets for biotechnological applications.

**Materials and Methods**

**Plant material and growth conditions**

Soybean (*Glycine max* L.) cv. Jack seeds were grown in growth chamber (26/20°C day/night temperature, photoperiod of 14/10 h, 800 μmol m⁻² s⁻¹ light intensity, and 60% humidity). Different developmental stages of soybean plants were collected, immediately frozen in liquid nitrogen and stored at −80°C. Tissue for germination and stress-related experiments was prepared as described in [10].

**Cloning of soybean G-protein genes**

Soybean Gγ genes were identified by analysis of the latest the soybean genome assembly (www.phytozome.net/soybean) with *Arabidopsis* and rice full length and middle coiled-coil region Gγ protein sequences as queries. Full-length Gγ genes were amplified from soybean seedling cDNA using gene-specific primers (Table S1). The eight newly identified genes were cloned into the pENTR/D-TOPO vector and confirmed by sequencing.

**RNA isolation and qRT-PCR**

Total RNA was isolated from different tissues of soybean plants using Trizol reagent (Invitrogen) and qRT-PCR experiments were performed essentially according to [10]. The real-time PCR amplification was repeated three times and data were averaged. Sequencing and melt curve analysis of amplicons confirmed specificity. Standard curves for each of the genes were generated using the cloned plasmid DNA of each gene.

**Localization of GmGy proteins**

The ten GmGy genes were cloned into the pEarleyGate 104 [57] destination vector using LR clonase mix (Invitrogen). Sequence-confirmed recombinant plasmids containing the *YFP;GmGy1-10* were transformed into *A. tumefaciens* strain GV3101 for subsequent plant transformation.

Abaxial surface of tobacco leaves were infiltrated with a log-phase culture of *A. tumefaciens* containing either the gene of interest or an empty vector control according to [58]. Infiltrated plants were incubated in darkness for 36 h followed by 24 h in light. The leaves were imaged with the Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss) using a 45x water-immersion, 1.2 numerical aperture, C-Apochromat objective. The yellow fluorescent protein (YFP) was excited with the 458-nm line of the argon laser. At least three independent infiltrations were performed for each construct.
Protein-protein interaction assays
To determine the interaction between specific GmGb and GmGγ proteins, GATEWAY-based yeast-two-hybrid assay was performed (ProQuest Two Hybrid System, Invitrogen). Briefly, GmGb1-4 genes were cloned into pDEST32 bait vector (containing DNA-binding domain) and GmGγ1-10 genes (full length, N-terminal and C-terminal parts) were cloned into pDEST22 prey vector (containing DNA-activating domain). The constructs were co-transformed in yeast host strain MaV203 (Invitrogen) in specific combinations. Interaction was determined by growth of diploid yeast colonies on minimal media lacking leucine and tryptophan, containing 50 μM 3AT (3-Amino-1,2,4-triazole). The quantitative strength of interaction was determined by β-galactosidase (β-gal) expression assay using ONPG (o-nitrophenyl-β-D-galactopyranoside) as a substrate per manufacturer’s instructions.

Supporting Information
Table S1 Gene-specific primers used for expression analysis of GmGγ genes.

Table S2 Absolute copy number quantification and primer amplification efficiency test of GmGγ genes.

Figure S1 Genomic sequence of the newly annotated GmGγ3 on chromosome 20.

Figure S2 Genomic sequence of the newly annotated GmGγ4 on chromosome 10.

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