Title: Emerging themes in heterotrimeric G-protein signaling in plants

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Abstract

Heterotrimeric G-proteins are key signaling components involved during the regulation of a multitude of growth and developmental pathways in all eukaryotes. Although the core proteins (Gα, Gβ, Gγ subunits) and their basic biochemistries are conserved between plants and non-plant systems, seemingly different inherent properties of specific components, altered wirings of G-protein network architectures, and the presence of novel receptors and effector proteins make plant G-protein signaling mechanisms somewhat distinct from the well-established animal paradigm. G-protein research in plants is getting a lot of attention recently due to the emerging roles of these proteins in controlling many agronomically important traits. New findings on both canonical and novel G-protein components and their conserved and unique signaling mechanisms are expected to improve our understanding of this important module in affecting critical plant growth and development pathways and eventually their utilization to produce plants for the future needs. In this review, we briefly summarize what is currently known in plant G-protein research, describe new findings and how they are changing our perceptions of the field, and discuss important issues that still need to be addressed.

1. The heterotrimeric G-protein signaling cycle

The heterotrimeric G-protein complex plays a vital role in regulating multiple signaling pathways in all eukaryotes. The core G-protein heterotrimeric complex is made of one Gα, one Gβ and one Gγ protein. As per the classical paradigm, this plasma membrane-localized protein complex switches between the inactive and active states depending on the nucleotide-bound form of Gα [1-5]. During resting phase, the Gα is GDP-bound and remains associated with a Gβγ dimer. Activation occurs when ligand-binding or signal perception causes in a change in the conformation of a G-protein coupled receptor (GPCR). Activated GPCR acts as a guanine nucleotide exchange factor (GEF), catalyzing the GDP to GTP exchange on the Gα protein (Fig. 1). GTP bound Gα dissociates from the Gβγ dimer. Both the GTP-Gα monomer and the Gβγ dimer then interact with a variety of downstream effectors to transduce signals for distinct cellular and physiological functions, representing the active state of signaling [1-4]. Deactivation occurs via the inherent GTPase activity of Gα, which causes hydrolysis of bound GTP, regenerating its GDP-bound form (Fig. 1). GDP-Gα associates with Gβγ restoring the trimeric complex, ready to be activated for the next round of signaling [3, 6]. Because of the cyclic nature of G-protein signaling, both the activation and deactivation steps have to be synchronized for effective and continuous signaling [7]. However, the inherent rate of GTP-hydrolysis by Gα is significantly slower than the GDP/GTP-exchange rate [6, 8], necessitating help from proteins that can accelerate the GTPase activity of Gα. The GTPase activity accelerating proteins (GAPs), as the name suggests, interact with the Gα proteins and increase its rate of GTP-hydrolysis, facilitating effective deactivation and consequently continuation of the cycle. The Regulator of G-protein Signaling (RGS) proteins are the most well characterized GAPs of Gα proteins [5-7].

Multiple studies have reported on the structural and functional roles of the members of the G-protein signaling complex during regulation of various physiological functions in humans. For example, dysregulation of GPCR activity and the downstream circuits were reported in many prevalent disease conditions such as schizophrenia, Alzheimer's, cancer, vision impairment, obesity, hypertension, diabetes and olfaction [9-11] . Not surprisingly, due to the extensive roles of GPCRs in sensing various signals, the G-protein signaling pathways are an important target for the pharmaceutical industry and their effectors/regulators constitute a majority of commercially available therapeutic drugs [5, 12-17].

The core of the heterotrimeric G-proteins i.e. the Gα, Gβ and Gγ subunits, and their basic biochemistries such as the GTP- versus GDP-bound states dictating the monomeric, active versus trimeric, inactive Gα, respectively; the slow inherent GTPase activity of Gα; or the non-dissociability of the Gβγ dimer are fully conserved in all organisms. However, differences emerge when comparing the numbers of each of the subunits or their regulatory mechanisms. For example, the human genome encodes 23 Gα, 5 Gβ, 12 Gγ, 37 RGS and more than 800 GPCRs [1-3, 18]. Compared to this, the repertoire of G-proteins is extremely limited in plants such as Arabidopsis which possess only 1 canonical Gα, 1 Gβ, 2 canonical Gγ, 1 unique RGS and no GEF activity possessing GPCR; although additional non-canonical, plant-specific proteins exist and function together with the G-protein cycle [19-21]. Furthermore, comparative *in vitro* biochemical studies suggest that the regulation of the G-protein cycle itself may differ between the established models mostly derived from mammalian systems versus those that exist in plants [22-25]. The current hypothesis is that although the core G-protein complex is conserved among eukaryotes, it may be wired distinctly in different organisms depending on their specific needs. The receptors, regulators, and effectors may differ, especially between plants and non-plant systems, and novel mechanisms beyond what is known from the mammalian systems remain to be explored. In the next sections, we will discuss the heterotrimeric G-protein signaling in plants – beginning with what is known based on the studies in *Arabidopsis thaliana* (Arabidopsis, hereafter), where it is most well characterized, and then include information from other plant species and how it is changing our understanding of this important signaling paradigm.

2. G-protein signaling in plants

Several pharmacological studies in the late eighties and early nineties underlined the importance of G-protein signaling in plants [26-33]. However, our knowledge about the molecular genetic details of plant G-protein signaling originated from studies in Arabidopsis, where the proteins were first identified by gene cloning and expression analysis [34-37]. Further studies using gene knockout and overexpression lines of each of the genes of the G-protein complex established their pivotal roles in regulating a multitude of plant growth, development and physiological processes. Arabidopsis has one canonical Gα (GPA1), one Gβ (AGB1) and three Gγ (AGG1, AGG2, AGG3) proteins (Table 1). Using loss-of-function mutants in each of these genes or their combinations, the roles of Gα, Gβ and Gγ proteins have been demonstrated in seed germination, seedling development, cell division and patterning, ion channel regulation, stomatal

development and physiology, defense response, stress response, hormone signaling, sugar sensing, ROS mediated signaling, light sensing and response and yield improvement, encompassing almost every aspect of plants' life [38-60]. The detailed phenotypic analyses also uncovered certain novel aspects of G-protein signaling in plants. For example, certain responses were regulated by classical modes where knocking-out either Gα or Gβ led to similar phenotypes; whereas others were more complex. Some of the plant phenotypes such as root mass, stomatal density or defense responses, were oppositely regulated by Gα or Gβ proteins. Still others were independently regulated by one of the proteins (e.g. silique shape by Gβ) while a subset such as leaf shape, hypocotyl lengths and abscisic acid response showed quantitative differences in their regulation [61-63].

Biochemical characterization of the Arabidopsis Gα protein also suggested an altered mechanism of its activation compared to the mammalian models. Because an authentic GEF activity possessing GPCR has yet not been identified in plants, and because the Arabidopsis GPA1 exhibits an extremely high rate of GTP/GDP exchange coupled with a very slow rate of GTP hydrolysis; it has been proposed that the plant Gα proteins are self-activating GTPases. Moreover the key, rate-limiting regulatory step of plant G-protein cycle is its deactivation, which is facilitated by an unusual RGS protein [22-25, 64, 65]. Studies of G-protein signaling in rice confirmed some of these observations. Similar to Arabidopsis the repertoire of G-proteins in rice is also limited with one canonical Gα (RGA1), one Gβ (RGB1) and few Gγ proteins (Table 1) and they regulate critical growth, development and defense responses [66-77]. However, few differences became obvious as well, such as the rice Gα mutants are severely dwarf- a phenotype not observed in Arabidopsis Gα mutants [66, 75, 78]. Furthermore, a homolog of the RGS protein, which is thought to be critically important for the regulation of plant G-protein signaling, is missing from the rice genome [20, 79]. Overall, by the year 2010, a general consensus had emerged in the field suggesting that (i) the repertoire of G-proteins in plants is extremely limited compared to the mammalian systems, (ii) G-proteins are missing from the basal plant lineages, (iii) G-proteins are non-essential for plant survival (iv) the signaling mechanisms pertaining to the activation/deactivation of Gα are distinct for plant G-protein cycle and (v) monocots with one or two exceptions, have lost the regulatory RGS proteins [20, 80]. While these studies in the selected model species facilitated establishment of the G-protein core components and the regulation of multiple plant phenotypes by them, recent studies encompassing multiple species have significantly expanded our understanding of this important signaling pathway.

3. Plants possess an extensive repertoire of heterotrimeric G-proteins consisting of both canonical and novel proteins

One of the most obvious contrasts between the plant and non-plant G-protein signaling is the number of each of the subunits of the core complex. Upon sequencing of Arabidopsis and rice genomes it became clear that these plants contain a single Gα and Gβ protein, compared to 23 Gα and 5 Gβ proteins in humans (and similar expansion in other mammalian species) [81]. The plant G-proteins are also widely expressed, which suggested that the diversity in plant G-protein signaling potentially arose from the diversity in the Gγ proteins [67, 80, 82]. However, two recent developments have changed this perception. One, polyploid plants or many plants with diplodized genomes have retained multiple G-protein subunits. For example, the soybean genome, which is an allotetraploid, encodes 4 Gα, 4 Gβ and 10 Gγ proteins [83-85]. The genome of *Camelina sativa,* a highly undifferentiated hexaploid species of Brassicaceae family, codes for 3 Gα, 3 Gβ and potentially 8 Gγ proteins [86, 87]. Because more than 70% of the plants are polyploid and many others possess diplodized genomes, it is expected that these will reveal the presence of multiple copies of each of the G-protein subunits. Another, even more significant development is the identification and characterization of additional, plant-specific G-protein components. Some of these have been known for a while, such as the extra-large Gα (XLG) proteins (Table 1). The XLG proteins are double the size of the classical Gα proteins and possess a Gα like domain at their C-terminal region, with an N-terminal domain of unknown function [88-90]. Most diploid angiosperms contain three copies of XLG proteins, with higher numbers in polyploid plants. The XLG proteins were initially thought to work independently of the G-proteins due to the limited sequence similarities in their Gα domain with canonical Gα proteins and also due to the absence of certain key amino acid residues in their active site. However, recent studies have confirmed that the XLG proteins are indeed a part of the functional G-protein heterotrimer in plants. The proteins do interact with the Gβ proteins and regulate critical growth and developmental pathways in Arabidopsis [45, 89-93].

The studies of G-protein in the moss *Physcomitrella patens* further confirmed the role of XLG proteins as a part of functional G-protein complex. *P. patens* is unique among plants as it does not possess a canonical Gα protein but does have an XLG protein homolog as well as two canonical Gβ proteins. Therefore, this species presented an opportunity to evaluate the role of XLG proteins without the confounding effects of the presence of Gα. Loss of function of either *PpXLG* or *PpGβ2*

gene resulted in identical phenotypes with mutants showing slower growth, smaller, less elongated gametophytes and the inability to form a sporophyte. Furthermore, all these phenotypes can be fully complemented by introducing the Arabidopsis *XLG2* or *AGB1* genes in the corresponding moss mutants, confirming that the moss genes are their true functional orthologs and that the XLG and Gβ proteins work in the same genetic pathways [94].

Some unique, plant-specific Gγ proteins exemplify additional, novel G-protein components. . Sequence homology-based searches have identified three types of Gγ proteins in plants [67, 82, 85] (Table 1). The group I (or type A) are the canonical Gγ proteins found in all eukaryotes. These are small (100-120 aa) proteins, with a conserved DPLL/I motif, which together with few additional conserved amino acids in the middle coiled-coil region is required for their interaction with the Gβ proteins; and a C-terminal CAAX prenylation motif, which is required for their targeting to the plasma membrane. The originally identified AGG1 and AGG2 proteins of Arabidopsis, RGG1 of rice and Gγ1, Gγ2, Gγ3 and Gγ4 of soybean, all belong to this group [67, 82, 85]. Most plants (e.g. except for the members of Brassicaceae) also possess another variation of these proteins, which is almost identical to the group I Gγ except for the absence of prenylation motif at their C-terminal region. These have been named group II or type B Gγ proteins [85]. This is an interesting variation as the mutations that alter the prenylation sites in mammalian Gγ proteins usually result in severely altered phenotypes because the proteins can no longer be targeted to the plasma membrane [95-98]. The group II Gγ proteins (e.g. RGG2 of rice), despite lacking the prenylation motif, seem to be targeted to the plant plasma membrane and have been shown to work together with the Gβ proteins [99, 100]. The group III or type C Gγ proteins are unique both in terms of their size and domain architecture. These proteins are unusually long with an N-terminal Gγ domain, which is highly similar to the group I or II proteins, and a 100-400 aa C-terminal extension [67, 85, 101, 102]. This extended C-terminal region of group III Gγ proteins is extremely rich in amino acid cysteine, which may constitute up to 40% of the total amino acids in this region. The group III Gγ proteins are exemplified by AGG3 in Arabidopsis, DEP1, GS3 and GCG2 in rice and GmGγ8, GmGγ9 and GmGγ10 in soybean (Table 1). The homologs of group III Gγ proteins are missing from basal plants, but are present in all gymnosperms and angiosperms analyzed, to date. The proteins regulate critical growth and development pathways and have been shown to work together with the Gβ proteins [40, 85, 101-105].

These findings confirm that plants do possess an extensive network of G-proteins. Even a simple system such as Arabidopsis has 4 Gα (1 canonical and 3 XLGs), 1 Gβ and 3 Gγ proteins resulting in 12 possible G-protein heterotrimers; whereas plants such as soybean have 12 Gα, 4 Gβ and 10 Gγ proteins, with even more elaborate networks possible in plants with more complex genomes [83, 85]. The proteins are expected to interact in specific combinations, depending on the signal, expression patterns, tissue types or developmental stages, to expand the G-protein signaling and regulatory networks.

4. G-protein genes are present in the entire plant (Viridiplantae) lineage

Earlier sequence analyses identified the presence of G-protein genes in all the sequenced plant genomes, except in the green algae. The fully sequenced genomes of the green algae such as *Volvox carteri, Chlamydomonas reinhardtii, Coccomyxa subellipsoidea C-169, Micromonas pusilla CCMP1545, M. pusilla RCC299,* and *Ostreococcus lucimarinus* exhibited no genes with significant sequence homology to G-protein genes [80]. Furthermore, the absence of a canonical Gα protein in the moss *P. patens* genome was also intriguing. It was presumed that the G-proteins do not exist in basal plants and were acquired when the plants became land-bound, with *P. patens* representing a transition state (possessing only a subset of the G-proteins). However, the identification of the complete functional G-protein complex genes in *Chara braunii* and in many other Charophyaceae algae changed this perception [106, 107]. The *Chara* genome codes for Gα, Gβ, Gγ as well as regulatory RGS proteins, all of which show high sequence similarity with the Arabidopsis proteins. The biochemical properties of *Chara* and Arabidopsis G-proteins are similar, suggesting that these proteins are indeed functional G-proteins [108]. Furthermore, a recent study has identified a *C. reinhardtii* gene *CGA1* as a heterotrimeric Gα protein subunit. The gene is functional as the knock-down mutant of *CGA1* exhibited higher survival rate in response to heat and osmotic stress [109]. This along with our demonstration that the XLG protein of the moss *P. patens* is a functional Gα protein [94] confirms that the G-proteins are present in and are functional along the entire plant lineage.

5. Both conserved and novel (plant-specific) signaling and regulatory mechanisms operate during plant G-protein signaling

As described in the previous sections, plant genomes possess both conserved and unique G-protein components and the proteins regulate critical growth and development pathways.

Moreover, the basic biochemistry of the G-protein components is similar to what is known for the metazoan G-proteins: the Gα binds and hydrolyzes GTP and the binding dictates its active versus inactive status. However, while the classic GPCRs are required for GDP to GTP exchange and activation of Gα proteins in all metazoans, these proteins are intriguingly missing from the plant genomes. This suggests that the regulation of G-protein cycle in plants is potentially different from what is known based on the metazoan systems.

5.1. G-protein activation mechanisms in plants

Several proteins that have sequence features similar to the mammalian GPCRs have been identified in plants [110-113]. Many of these interact with Gα and participate in signaling pathways regulated by G-proteins [55, 111, 114, 115]. However, none of these receptor-like proteins have been shown to possess the GEF activity *i.e.* the ability to facilitate the exchange of GTP for GDP on Gα. How might the G-protein cycle be activated in plants? There are two possible scenarios, each with some supporting evidence. One, because the Arabidopsis Gα protein has an extremely high rate of GTP-binding, coupled with a very slow GTP hydrolysis rate, it has been proposed to be able to spontaneously exchange GTP for GDP, without the requirement of a receptor's GEF activity [23, 25, 64]. *In vitro* experiments with Arabidopsis GPA1 and to some extent with the soybean Gα proteins confirm their unusual biochemical characteristics [21, 23, 25, 64, 116-118]. In such a situation, the role of a GAP such as RGS protein becomes central to the regulation of G-protein cycle (Fig. 1). However, the breadth of such a mechanism for plants in general is not known at this point. Even the four highly similar, canonical soybean Gα proteins differ in their rates of GTP-binding and hydrolysis [116, 119]. It is expected that different G-proteins from other plant species would also exhibit changes in their biochemical properties. Do all plant Gα proteins fall within that range of high GTP-binding and slow GTP-hydrolysis rates that would make them a spontaneous GTP/GDP exchanger or are there other possible alternatives? In case of soybean Gα proteins where the proteins share more than 90% sequence identity, small differences in their biochemical properties lead to differences in the regulation of plant processes by them. For example, when used for complementing the Arabidopsis *gpa1* mutant, two of the proteins GmGα2 and GmGα3 could fully complement each of the mutant phenotypes, whereas the other two proteins GmGα1 and GmGα4 could complement only a subset of those [120]. Interestingly, the soybean Gα proteins also exhibit differences when introduced in the yeast *gpa1* mutant. In yeast, GmGα1 and GmGα4 could fully restore all the growth and pheromone signaling phenotypes of

the yeast *gpa1* mutants whereas the GmGα2 and GmGα3 could only partially complement them [116]. Because yeast possesses a classic GPCR-dependent GDP/GTP-exchange based Gα activation, it implies that at least a subset of the plant Gα proteins can be activated by GPCRs in a heterologous system, regardless of their ability to be self-activated. Conversely, another set of Gα proteins, despite the ability to be self-activated, are not fully functional in the yeast system. It may be that the degree or rate of self-activation of plant Gα proteins varies and influences their ability during response regulation. It also suggests that alternative mechanisms may exist that facilitate Gα activation in plants, which could be the other possible scenario for G-protein cycle regulation [121]. There is mounting evidence that such regulation might be achieved via the interaction of receptor-like kinases (RLKs) with the G-protein cycle [39, 54, 122-124]. This is exciting, as plants possess a large number of RLKs (~600 in Arabidopsis) responsible for sensing a wide variety of signals.

The first evidence of the interaction of a G-protein component with an RLK was obtained during a genetic screen when the Gβ proteins (AGB1) was identified as an interactor of ERECTA (an RLK) during silique development in Arabidopsis [54, 125]. Several studies related to defense-related signaling also provided evidence for the involvement of G-protein subunits with different RLKs where both direct physical interactions and functional/genetic interactions have been identified. Specific Arabidopsis G-protein subunits directly interact with important defense- or development-related RLKs such as chitin elicitor receptor kinase 1 (CERK1), BRI1-associated receptor kinase 1 (BAK1) and BAK1-interacting receptor 1 (BIR1), the key immune receptor flagellin-sensitive 2 (FLS2), ERECTA, zygotic arrest 1 (ZAR1) and receptor-like protein kinase 2 (RPK2) [39, 54, 122-124, 126-128]. More definitive results came from the identification of the maize Gα protein as an interactor of Fea2 (CLAVATA-2) which is a receptor like protein of CLAVATA (an RLK) pathway [129]. Direct biochemical evidence for the regulation of G-protein cycle by an RLK was demonstrated during nodule formation in soybean. The Nod factor receptors (NFRs), a class of LysM containing RLKs, perceive the Nod factors secreted by rhizobia to promote nodule formation in legumes [130]. The soybean NFRs interact with both Gα and RGS proteins and phosphorylate the RGS proteins. Phosphorylated RGS exhibits higher GAP activity towards the Gα protein, implying that NFR-mediated phosphorylation of RGS leads to faster termination of the G-protein cycle. Because the introduction of phosphomimic versions of RGS protein in a soybean mutant lacking the active receptor (*nod49*) resulted in partial restoration of

the nodule formation, it confirmed that at least one of the roles of activated NFR1 is to phosphorylate RGS proteins for the regulation of G-protein cycle [116]. Given the involvement of G-proteins and RGS proteins in a multitude of pathways regulated by RLKs, this could potentially be a general, yet unexplored regulatory mechanism.

The activation mechanism of XLG proteins have not been explored at the biochemical level and whether these proteins have similar kinetics as the canonical Gα is not known. However, XLG proteins also interact with various RLKs. It has been demonstrated that the XLG2 and XLG3 proteins of Arabidopsis interact with the FLS2-BIK1 receptor complex during flg22 dependent defense response. It has been proposed that the ligand (flg22)-dependent activation of the FLS2 receptor results in dissociation of XLG protein from its trimeric complex with Gβγ, similar to what is known for the dissociation of metazoan Gα proteins upon GPCR-mediated activation [128]. The freed XLG protein is then phosphorylated by a key cytoplasmic kinase BIK1 to transduce the signal. While the detailed characterization of the activation/deactivation mechanisms of XLG containing G-protein trimeric complexes remains unknown, this study presents an exciting possibility that a plant trimeric G-protein complex can be directly activated by ligand binding to an RLK [128]. If such a mechanism holds true or is more widespread, it will certainly expand the network of G-proteins with a variety of RLKs potentially affecting the activity or availability of Gα proteins.

5.2. G-protein deactivation mechanisms in plants

While the exact details of the activation mechanisms of plant Gα proteins are still being explored, relatively more is known about their deactivation mechanisms. The Gα proteins, being GTPases possess the inherent ability to hydrolyze bound GTP to generate the GDP-bound Gα, which reconstitutes the trimeric complex [5]. However, the GTPase activity of Gα proteins in general, and the plant Gα proteins in particular, is extremely slow. To keep the G-protein activation and deactivation synchronous and enable continuous signaling, several proteins with the GAP activity are required for effective deactivation of the cycle [7]. RGS proteins are the most well established GAPs in all organisms. In metazoans, wide variety of proteins possess the conserved RGS domain, which makes close contact with the Gα protein to increase its GTPase activity [2, 6, 131]. In plants, all RGS proteins discovered to date are characterized by the presence of a seven transmembrane (7TM) domain linked to the RGS domain [108, 132]. The presence of a 7TM domain, which is typical of GPCRs, is intriguing but not unprecedented. Several other basal

organisms also possess 7TM containing RGS proteins although none of the 37 known RGS proteins from humans possesses this domain [81]. In Arabidopsis, the 7TM domain seems to be involved in the tethering of the RGS protein to the plasma membrane. Genetic and biochemical evidence confirm that the RGS proteins act together with the Gα proteins [22, 25, 118, 119, 133]. The GTPase activity of plant Gα proteins is increased by at least an order of magnitude in the presence of an RGS protein and many of the phenotypes of plants lacking Gα protein are similar to the plants overexpressing an RGS protein [46, 134].

Despite the fact that the RGS proteins are functionally important and are required for effective signaling via G-proteins, it was astonishing to notice their absence from the genomes of many grasses such as rice, *Brachypodium*, sorghum, maize etc. [108]. It was previously suggested that the majority of the monocots have lost the RGS protein due to an adaptive change corresponding to a particular amino acid in their Gα protein [23, 79]. However, deeper analysis of a wide range of monocots confirmed that this is not the case. Even though all eudicots, most monocots, basal angiosperms such as Amborella, gymnosperms, lycophytes and green algae have RGS protein coding genes in their genomes, the gene is lost randomly in some monocot orders [108]. Why there is a relaxed selection on this important signaling protein in one specific plant lineage remains unknown at this time. However, regardless of the presence of an inherent RGS protein, the Gα proteins of all plant species exhibit similar biochemical properties and maintain the ability to be affected by RGS proteins from heterologous species e.g. the GTPase activity of a Gα protein from rice or *Brachypodium* is increased significantly in the presence of an RGS protein from Arabidopsis or soybean [108]. Furthermore, the interaction interface between the RGS:Gα protein pairs is conserved through evolution, extending as far as between plants and humans [108].

This leads to the question whether there are other proteins, in addition to the RGS proteins, which can also accelerate GTP-hydrolysis by Gα. Our recent work in Arabidopsis demonstrates that phospholipase Dα1 (PLDα1) is one such protein, corroborating some previously published biochemical data [135]. The idea of phospholipases acting as GAPs is well established in mammalian systems, where phospholipase Cβ (PLCβ) isoforms act both as GAPs and as effectors of Gα proteins [7, 136-138]. However, plants lack classical PLCβ homologs precluding such a possibility. Our results suggest that this role is likely fulfilled by phospholipase D (PLD) proteins in plants. Genetic and biochemical analyses confirm that in Arabidopsis both RGS1 and PLDα1 accelerate the GTPase activity of Gα [121, 139-142]. Additionally, these two proteins interact with

each other as well as with the core G-proteins to form higher-order protein complexes *in vivo*. Furthermore, RGS1 and PLDα1 regulate the activity of each other in a double negative regulatory loop. The net outcome of such complex interactions may be an exquisitely controlled level and duration of active Gα, modulating the specificity of response regulation [120, 139].

The loss of RGS is tolerated in certain monocots can be explained by two alternative scenarios. First, it is possible that in plant species without an RGS, the PLD proteins have taken over the role of classical GAPs, without an additional regulatory loop, which is normally contributed by RGS. Alternatively, other proteins might exist that have similar biochemical properties to RGS, even though they lack sequence similarity. One such example could be the COLD1 protein in rice, which is reported to increase the GTPase activity of RGA1 [143] although its homologs in Arabidopsis, GTG1 and GTG2 proteins do not exhibit such an activity [114]. The deactivation mechanisms of XLG proteins are not known at this time, although it is conceivable that because the proteins are acting together with the canonical Gβγ proteins and going through the process of trimeric versus monomeric stages, a GTPase activity regulatory step would be an inherent part of the signaling cycle involving the XLG proteins.

5.3. G-protein effectors and downstream components

To perform such diverse functions, the G-proteins must be interacting with various effector (or target) proteins. The well-studied effectors in animal models are adenylyl cyclase and phospholipase Cβ, both of which are missing in plants and therefore, the identity of different effectors and downstream targets remains limited. An interactomics-based study identified several proteins that might interact with different G-protein subunits [144]. Few genetic and biochemical studies have also identified potential proteins acting downstream of G-proteins [50, 145-156]. However, a clear connection between a G-protein, its effector and a target protein, leading to a response regulation remains unknown at this point. Further studies targeted to specific pathways regulated by G-proteins in precise developmental and signaling context are required to identify any potential effectors.

6. G-proteins can be essential for plant growth and development

Plant G-proteins are involved in regulation of almost every aspect of growth, development, response to environmental and hormonal signals, biotic and abiotic stresses. The proteins are also known to regulate many fundamental aspects of plant biology, such as control of cell division and

regulation of ion channel activity. Despite this, the single and higher order Arabidopsis G-protein mutants are relatively normal and complete their life cycle without any major disadvantages. This has been a fundamental conundrum in the field, i.e. if the G-proteins are so important why the loss of them is tolerated in plants. The general consensus is that the plant G-proteins have evolved to suit the sedentary life-style and are involved in regulating the optimum plant response under any given condition, rather than being essential for one specific pathway or signal [157]. Overall, the idea still holds true. However, studies in plants other than Arabidopsis have started to uncover essential roles of plant G-proteins for growth and survival.

The first example is from rice mutans lacking the Gβ protein. Although the rice Gα mutants are severely dwarf and bushy, they do complete the life cycle [78, 158]. However, a complete Gβ null mutant of rice could never be obtained. RNAi-mediated knock-down of rice *Gβ* gene confirmed that while the partial suppression of the gene resulted in plants with severe defects in growth and development, the complete gene knock-outs are possibly seedling lethal [66]. Because the inventory of G-protein components is still being explored and expanded, it is possible that additional mutant combinations such as the lack of *XLG* genes or *XLG* genes together with the Gα gene would also lead to lethality. However it is noteworthy that the Arabidopsis plants lacking all three *XLG* genes are fairly normal under controlled growth conditions and the plants lacking all three *XLG* genes and the canonical *Gα* gene also survive to complete their life cycle [90, 159].

The situation is however different in the basal plant *P. patens* where plants lacking either the *XLG* gene or the *Gβ* gene can no longer form any sporophyte and therefore are unable to complete the life cycle [94]. Further studies with additional basal plants will uncover if the G-proteins were essential early during plant evolution and became non-essential later due to the development of overlapping regulatory circuits in higher plants. Alternatively, it is also possible that the G-proteins are non-essential in the dicot plant lineage, whereas basal plants or monocots require their complete repertoire for a successful life cycle. If this is the case, it will be interesting to uncover the specific regulatory pathways that differ between these two major plant subgroups.

7. G-proteins regulate important agronomic traits

In the earlier days of G-protein signaling in plants, especially with Arabidopsis, it seemed that not only the protein complex was non-essential for plants but also not agronomically relevant. This was a striking difference from the mammalian systems where the G-protein signaling pathways are a target of major pharmaceutical drugs. The *d1* dwarf mutant of rice, which is due to the lack

of a functional Gα protein, though promising, was not very useful for breeding purposes as it resulted in several unwanted phenotypes related to reduced yield [78]. Recent studies have changed this perception and identified a direct role of G-proteins in regulating several yield traits.

The newly identified group III Gγ genes have long been known as major quantitative trait loci (QTL) for important agronomic traits such as seed size and panicle architecture, long before their characterization as Gγ proteins. Two major yield QTL in rice, Grain size 3 (GS3) and dense and erect panicle 1 (DEP1), correspond to the group III Gγ proteins [68, 103, 160-163]. Similarly, the Arabidopsis AGG3 (another group III Gγ protein) was identified as an organ size regulator in a genetic screen. Overexpression of *AGG3* results in bigger flowers, fruits and seeds in Arabidopsis and in Camelina [40, 86, 87]. Interestingly, while in both dicot species where it has been studied in detail, the protein expression level is directly correlated with bigger organ size and higher yield; the situation seems to be more complex in monocots. Different alleles of *DEP1* or *GS3* result in distinct, sometime opposite, phenotypes depending on the position of the mutation or specific genetic background [68, 103, 104, 160, 164-168]. A long field-based study with the overexpression of the barley homolog of *DEP1* gene concluded that the effect of this gene is highly dependent on the environment and genetic background and may result in increased or decreased yield upon overexpression [169]. Our recent results by overexpressing the *AGG3* gene in a model monocot *Setaria viridis* also suggest only a subset of yield traits are directly correlated with the gene expression levels [170]. Incidentally, the same *DEP1* gene was also identified as a major QTL for nitrogen use efficiency in rice [171]. In this case, the protein has been shown to work together with the G-protein α and β subunits. These data suggest that the G-protein complex genes are potential targets for improved yield, thus deciphering their mode of action will be pivotal to map the regions or domains involved as well as for precision breeding.

8. Perspectives and future direction

Research in the field of plant G-protein signaling has entered an exciting phase where the majority, if not all, of the components have been discovered. Multiple well-established and novel mechanisms are being uncovered, and the potential for their use in solving real-world agronomic problems is being explored. There are still many unknowns such as what are the receptors upstream of G-proteins, what lies downstream of G-proteins, how the proteins connect to the established modules of hormone, defense or stress-related signaling. In addition, the question arises if the expanse of canonical and novel G-protein components and regulatory proteins and their mode of

action have been fully explored. Studies in multiple plant species have already highlighted the implicit variability in the numbers and pathways regulated by these proteins and their action mechanisms. Future targeted studies will certainly answer these questions and help manipulate the true agronomic potential of these proteins.

Acknowledgements

We sincerely thank several colleagues for multiple rounds of discussion during the writing of this review article. We also apologize to the colleagues whose work could not be cited sue to space constraint. Research in the Pandey lab is supported by NIFA/AFRI (2015-67013-22964) and NSF (IOS-1157942 and MCB-1714693) grants to SP.

Figure legend

Figure 1. Schematic diagram of G-protein cycle in mammalian versus plant systems. Only the core components of mammalian cycle are shown. Asterisk represents plant-specific components. Protein names in plant system are for Arabidopsis proteins. The activation/deactivation mechanisms of XLG proteins are not yet known, but they have been shown to function with canonical Gβ proteins and therefore represented in the G-protein cycle. The RGS protein is missing in many monocot plants.

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