G-protein-coupled signaling in *Arabidopsis*

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With an essentially complete plant genome in hand, it is now possible to conclude that a single or possibly just two canonical heterotrimeric G-protein complexes are present in *Arabidopsis*. In stark contrast, more than one hundred such complexes are found in some metazoans. Nonetheless, it appears that heterotrimeric G-protein complexes couple or affect many different signaling pathways in plants. In addition, there are very few, if any, candidate G-protein-coupled receptors through which this single complex can couple to downstream effectors. Furthermore, some of the classical downstream effectors that are activated by heterotrimeric G proteins in metazoans are also lacking in plants. Thus, we are left with the urgent challenge to determine the novel mechanism of G-protein signaling in plant cells. Recent advances using reverse and molecular genetic approaches have re-opened this topic for plant biologists and the resulting tools will accelerate our progress.

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Abbreviations

ABA	abscissic acid
AGB1	*Arabidopsis* G PROTEIN, BETA SUBUNIT1
AGG1	*Arabidopsis* G PROTEIN, GAMMA SUBUNIT1
AGS1–3	ACTIVATOR OF G SIGNALING1–3
BR	brassinosteroid
bri1	*brassinosteroid insensitive1*
er	*erecta*
GA	gibberellic acid
GCR1	G-COUPLED RECEPTOR1
GPA1	Arabidopsis G PROTEIN, ALPHA SUBUNIT1
GPCR	G-protein-coupled receptor
PLD	phospholipase D

Introduction

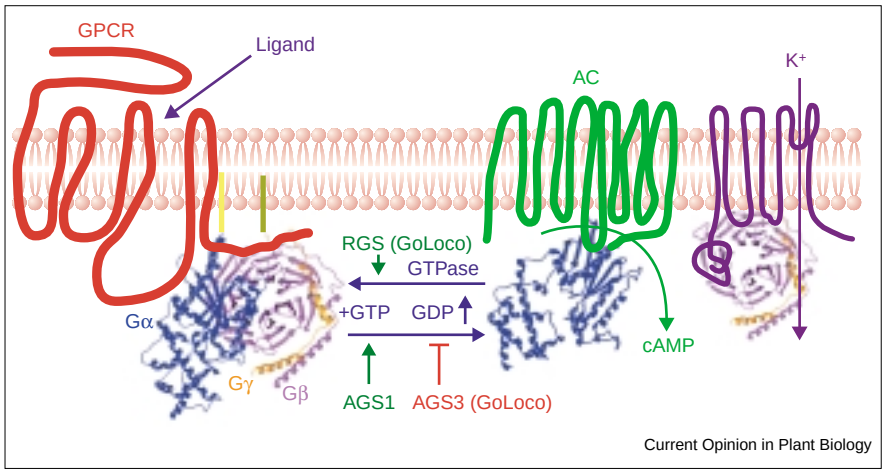
The largest gene family in animals encodes heptahelical transmembrane proteins that physically interact with a complex containing a GTPase, called a heterotrimeric G protein. These polytopic membrane proteins are collectively termed G-protein-coupled receptors (GPCRs) and their ligands are as diverse as is the GPCR family itself. Rhodopsin is a familiar example of a GPCR. It is known that light induces a photoisomerization of a *cis*-retinal moiety, which is covalently bound (within its helical core) to rhodopsin, to the all-*trans* retinal form. This photoisomerization is perceived intracellularly by a change in a cytoplasmic loop that is formed when two of the transmembrane helices shift position [1]. Similarly, small molecules such as serotonin, peptides such as somatostatin, and even large proteins such as thrombin bind to their cognate GPCRs and induce certain cytoplasmic conformations through shifts in helix positions that translate into specific loop conformations.

This is the 'apical step' in many signal transduction pathways. It works so well for so many signals because the consequence of receptor occupancy is a very simple output: a specific cytoplasmic protein conformation that can be recognized by a family of heterotrimeric G proteins that are able to couple this signal to downstream effectors (Figure 1). Heterotrimeric G proteins (henceforth referred to as G proteins or simply 'G' here) have an alpha subunit (Gα) that has two domains. One contains predominantly alpha helical secondary structure but little is known about the function of this domain. The other domain is called the ras domain and contains a GDP/GTP binding site, GTP hydrolase activity, and a covalently attached lipid that anchors this subunit to the bilayer. Also located within the ras domain are backbone loops, called switches, that position themselves depending on whether GDP or GTP occupies the nucleotide-binding site. When GDP is bound, the switches orient to permit tight association of Gα with the beta subunit (Gβ). Upon GTP binding, however, these switches re-orient such that the Gα/Gβ interaction is disrupted, permitting a slightly different interaction at the same interface with a membrane-localized enzyme.

These G-protein-activated enzymes are collectively called effectors. The nucleotide exchange is catalyzed by interaction of the Gα subunit with the activated conformation of the GPCR loop described above. This interaction between Gα and the cytoplasmic loop of the activated GPCR occurs at Gα's amino- and carboxy-terminal domains, which are distal to the switch regions. When Gα is released, the interaction between Gα and its cognate effector occurs along the same interface between Gα and Gβ. Gα undergoes a conformational change upon activation whereas Gβ does not. Whether sequestered by Gα or free, Gβ remains tightly bound to the gamma subunit (Gγ), which tethers the complex to the bilayer via lipid modification at its carboxy-terminus. Over time, and often assisted by accessory GTPase activating proteins (GAPs), GTP is hydrolyzed to GDP, thereby permitting the re-association of Gα with Gβγ and readying Gα for another cycle of activation by its cognate GPCR. Similar interaction cycles are repeated over and over for each of the thousands of signals using the GPCR pathway; obviously, mother nature knew a good thing when she saw it.

Such enormous multiplicity raises the central question: how can so many signals, each recognized independently by a separate GPCR, specifically couple to only a dozen or fewer effectors via G proteins? Specificity in signal

Figure 1



Classical model for G-protein-coupled signaling in animal cells. The binding of a ligand to its cognate receptor induces a conformational change that is perceived cytoplasmically. The ribbon structure of the three subunits of a heterotrimeric G-protein complex (B Temple, AM Jones, unpublished data) is shown to be associated with the receptor. The G protein is tethered to the bilayer and kept proximal to the receptor by lipid modification of its α and γ subunits (green and yellow bars). The activated receptor promotes GDP for GTP exchange, which dissociates the Gα from the Gβγ subunit. Both Gα and Gβγ can then activate downstream targets, such as adenyl cyclase (AC) and ion channels, to cause changes in the levels of secondary messengers. The effect of the G-protein subunits on their targets can be either positive or negative, although only stimulation is shown. The intrinsic GTPase of the Gα subunit hydrolyses GTP to GDP and thus returns the G-protein complex to its resting state. Regulators of G signaling (RGS) can facilitate the Gα GTPase (GAP) activity by interactions at their GoLoco domains. Some effectors exert GAP activity. Activities that alter the activated state of the G-protein complex independently of receptor activation are provided by AGS proteins. AGS1 facilitates GTP exchange whereas AGS3 acts as an inhibitor of GDP dissociation. AGS3 stimulates G-coupled pathways in which Gβγ is involved because AGS3 can displace the Gβγ subunit from the complex, enabling it to interact with its effectors. This model represents a highly simplified scheme because it does not convey that there are more than a thousand receptors, more than a hundred different G-protein complexes, and a dozen known effectors. Nor does it include de-sensitization mechanisms or modifications (e.g. phosphorylation, ubiquitinization and reversible myristilization) of the subunits.

coupling in metazoans is accomplished by two mechanisms. First, some G proteins are able to recognize a specific GPCR and a specific effector. Second, promiscuous G proteins are sequestered in signaling rafts that contain a specific GPCR, the cognate effector and all of the other components that operate on a particular pathway. A recently discovered example of this is illustrated by the co-localization of the β-adrenergic receptor with the $Ca_V1.2$ calcium channel in a macromolecular complex that contains many other elements of the signaling apparatus from receptor to effector [2••].

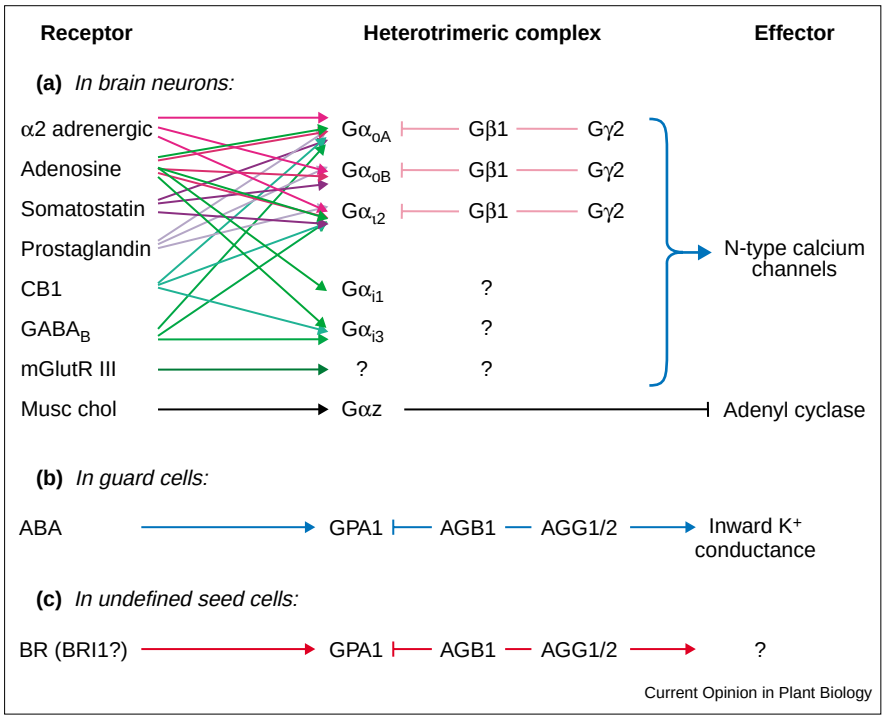
Animals have 23 different Gα, six Gβ, and 12 Gγ subunits, potentially assembling more than a thousand different G proteins. Given differences in the expression of G proteins among different cell types and the known exclusion of some subunit pairs, we can more conservatively estimate that more than a hundred heterotrimeric complexes exist in a cell. Gα forms four subfamilies, Gs, Gi, Gq, and G12, on the basis of their sequence. All except one member of the Gi subfamily can be covalently modified by ribosylation of a carboxy-terminal cysteine residue, catalyzed by pertusis toxin from *Bordetella pertusis*. Thus, Gα ribosylation is a diagnostic of subtype Gi. In general, Gs members stimulate adenyl cyclase (AC) whereas members of the Gi subfamily inhibit AC activity. Gq members typically stimulate phospholipase C-β (PLC) activity and G12 members operate via another class of GTP-binding proteins, the rho family. Gβγ subunits also can activate effectors such as channels and phospholipase A2 (PLA).

G proteins in *Arabidopsis*

In contrast to animals, *Arabidopsis* has single canonical Gα (*Arabidopsis* G PROTEIN, ALPHA SUBUNIT1 [GPA1]) and Gβ (*Arabidopsis* G PROTEIN, BETA SUBUNIT1 [AGB1]) subunits and possibly just two Gγ subunits (*Arabidopsis* G PROTEIN, GAMMA SUBUNIT1 [AGG1] and AGG2) [3–6]. The *Arabidopsis* Gα subunit is roughly 30% identical to mammalian Gα subunits of the Gi subfamily, and essentially all of this conservation lies in the few critical domains discussed above. GPA1 is most similar to an unusual member of the Gi subfamily called Gz. Like Gz, GPA1 lacks the carboxy-terminal cysteine that is targeted for ribosylation by pertusis toxin. GPA1 shares slightly more identity with Gz than with other members of the Gi subtype, and contains a Gz-specific myristolization motif. Gz plays a role in cell proliferation and death via its control of potassium channeling, thus it is possible that GPA1 operates in an analogous way.

Arabidopsis gpa1 mutants, which lack GPA1, have reduced cell division during hypocotyl and leaf formation [7•]. The overexpression of *GPA1* causes ectopic cell divisions, including massive overproliferation of meristem formation at high GPA1 expression levels. Overexpression of pea Gα stimulates cell division in yeast [8]. These observations suggest that GPA1 couples a signal that controls cell division. A likely candidate is auxin. However, auxin-induced cell division occurs in mutants that lack either Gα or Gβ, indicating that auxin cannot be coupled directly by a G protein (H Ullah *et al.*, unpublished data). However, although G-protein mutants respond to auxin, they have

Figure 2



A comparison of the potential complexity of G-coupled signaling in animal and plant cells. Interactions that have been confirmed *in vivo* in an individual cell type are indicated in color. **(a)** The interactions in brain neuronal cells as revealed mainly by the work of Straiker *et al.* [31•] (green) and of Jeong and Ikeda [32•] (pink). Gz coupling in these cells has been identified in a number of studies using indirect approaches but has not been confirmed *in vivo*. The receptors shown are only a fraction of those expressed in neurons, and the effectors indicated are only two of several types of G-coupled outputs that are possible. Thus, the promiscuity of G proteins in neurons is grossly underestimated in this figure. **(b)** In contrast to animal cells, G coupling in plant cells is simple because of the presence of a single α and a single β subunit, and possibly just two gamma subunits (AGG1 and AGG2). Wang *et al.* [9••] have shown that the *Arabidopsis* G-protein complex couples an unidentified ABA receptor to potassium and anion channels (blue) in guard cells. Ullah *et al.* [10•] proposed that a brassinosteroid receptor, possibly BRI1, is coupled to an unidentified effector (red) that regulates GA sensitivity in germination by an unknown mechanism. In the latter case, the specific seed cell type in which this signaling mechanism occurs has not been identified, but on the basis of other evidence, it probably takes place in the cells comprising the aleurone. CB1, cannabinoid receptor; GABA$_B$, γ-amino butyric acid B; mGlutR III, group III metabotropic glutamate; musc chol, muscarinic cholinergic.

dramatically altered auxin sensitivity. It is therefore possible that some other, as yet unknown, G protein-coupled pathway interacts with auxin signaling in a way that controls auxin sensitivity.

Unlike auxin signaling, an abscisic acid (ABA) signaling pathway appears to be directly coupled by a G protein. Wang *et al.* [9••] demonstrated that ABA inhibition of light-induced stomatal opening is completely lacking in *gpa1* mutants. Consistent with this loss of ABA responsiveness, ABA does not inhibit inward K^+ channels or activate pH-independent anion channels in *gpa1* mutants (Figure 2). Interestingly, ABA-induced stomatal closure that is mediated by a pH change remains unaffected by the loss of GPA1 function, indicating that there are ABA pathways that are independent of G protein in guard cells [9••].

Not only can a specific cell type contain multiple signaling mechanisms for one hormone, such as ABA, but different cell types can also have different mechanisms for the same hormone [10•]. For example, although the guard cells of *gpa1* mutants are ABA insensitive, *gpa1* seeds have wildtype sensitivity to ABA but are 100-fold less sensitive to gibberellic acid (GA) and completely insensitive to brassinosteroid (BR). Seeds that overexpress *GPA1* are a million-fold more sensitive to GA than wildtype seeds but still require GA for germination. One interpretation of these loss- and gain-of-function results is that GA signaling in seed germination is not directly coupled by G, but rather that some other G-coupled pathway crosstalks in a way that controls GA sensitivity. This indirect effect on a pathway via control of sensitivity is a re-occurring theme. Because it is known that BR regulates GA sensitivity, and that seeds that have reduced GA levels will fully germinate when treated with BR, it is possible that a BR pathway coupled by a G protein is the sought after pathway that controls GA sensitivity (Figure 2). Consistent with this, Ullah *et al.* [10•] have shown that BR synthesis and response mutants have the same reduced GA sensitivity as *gpa1* mutants, and that BR was completely ineffective at rescuing the germination of *gpa1* seeds when GA levels were reduced.

What is upstream of G in plants?

To date, no plant receptor has been shown to be directly coupled by G. Furthermore, in contrast to the thousand or more heptahelical transmembrane proteins in animals, *Arabidopsis* has only a few candidates with just one plant protein actually confirmed to be heptahelical to date [11]. This protein, called MLO1, confers resistance to powdery

mildew when present in its recessive form, but the mechanism of resistance is unknown. Recent evidence indicates that disease resistance conferred by *mlo* is not dependent on a G protein [8]. However, the possibility remains that MLO is coupled by a G protein in another signaling pathway because the function of this putative orphan receptor is unknown.

G-COUPLED RECEPTOR1 (GCR1) is a protein that shares some sequence identity to animal GPCRs of the rhodopsin/serotonin family [12]. GCR1 has a predicted heptahelical structure but this has not yet been confirmed by direct analyses. Overexpression of GCR1 modifies the cell cycle in a manner that is difficult to interpret [13]. Specifically, M phase appears to be uncoupled from S (see Figure 3 of [13]). On the other hand, *gcr1* loss-of-function mutants do not share any of the G-protein mutant phenotypes (AM Jones, unpublished results), suggesting either that GCR1 is not coupled by GPA1 or that the GCR1 function is redundant.

Receptor-independent, G-protein signaling occurs in animals. Using a functional screen in yeast, Lanier's group [14•] found three proteins (ACTIVATOR OF G SIGNALING1–3 [AGS1–3]) that are capable of activating G-protein signaling in the absence of a cognate receptor. Perhaps the most interesting of these is AGS3, which has subsequently been shown to be a guanine dissociation inhibitor (GDI) [15•]. AGS3 binds the GDP-bound form of Gα to release Gβγ via a protein interaction involving a GoLoco motif [16•,17]. In yeast, this interaction directly activates a mitogen-activated protein (MAP) kinase pathway [18]. However, it is not known if AGS3 homologs or GoLoco-containing proteins are found in plants.

Thus, we are left with only three possible conclusions: in contrast to animals, plants couple only one or a few heptahelical receptors by a G protein to downstream effectors; and/or receptor-independent G-protein signaling is the primary mechanism in plants; and/or plants couple non-heptahelical receptors. Although the jury is still out, some interesting facts shed light on this problem. First, the carboxy-terminal domain of all plant G protein orthologs is nearly 100% conserved, whereas in animals this region is poorly conserved due to the diversity of Gα–receptor interactions. Complete conservation in sequence among plant Gα carboxy-terminal domains suggests that there is a single or only a few receptors with which plant Gα can interact. Second, indirect observations are consistent with G coupling to nontraditional receptors. For example, as discussed above, Ullah *et al.* [10•] found that the germination of *gpa1* seeds is insensitive to BR. They also showed that *bri1* seeds have the same BR-insensitive phenotype as *gpa1* seeds. *BRI1* encodes a putative BR receptor-like kinase. Lease and coworkers [19] found that a putative protein-null allele of *AGB1*, encoding the single candidate Gβ subunit, shares many fruit phenotypes with the receptor-like kinase mutant *erecta* (*er*). Interestingly, double *er agb1* mutants have complex phenotypes. Depending on the trait, *AGB1* is epistatic to *ER* or *vice versa*, suggesting either that multiple parallel pathways are operating or that AGB1 and ER interact. These recent observations raise the exciting possibility that G couples one or more of the more than 400 receptor-like kinases in plants [20].

What is downstream of G in plants?

Phospholipase D (PLD), and potassium and calcium channels are presently the only three candidate effectors implicated in G-protein signaling in plants. In rice aleurone, GA-induced α-amylase secretion is greatly reduced in a Gα mutant, *d1* [21•], suggesting that a signal that crosstalks to the GA/ABA pathway is coupled by G. Using isolated aleurone membrane extracts, Ritchie and Gilroy [22] showed that GTPγS alters PLD activity, consistent with PLD residing directly downstream of an activated G protein. Lein and Saalbach [23] provide *in vitro* evidence for Gα and PLD interaction. Wang *et al.* [9••] showed that the *gpa1* mutant, which likely has excessive Gβγ, lacks ABA inhibition of K^+ influx, suggesting either that GPA1 is required for channel activation (possibly via PLD) or that, unlike in animals, Gβγ blocks channel activation. Another phospholipase activity, namely PLA2, rapidly increases activity after the application of auxin [24]. However, it is still not known if this occurs via coupling of auxin to PLA2 by G. The lipid by-products of PLA2 have been shown to activate a K^+ channel in animals [25]. In this case, PLA2 activation probably occurs by interaction with the released Gβγ [26]. The available mutants now make this easily testable. Finally, there is evidence that a heterotrimeric G protein may also activate calcium conductance in plant cells. Aharon *et al.* [27] used single-channel recording to measure cation influx in the presence of recombinant Gα. They found that Gα increased the mean probability of channel opening, suggesting regulation by G. It is not known yet if this regulation is direct as in animal cells [28].

Conclusions

Assmann [29] summarized the evidence for the G coupling of many signals in plants, leaving us to wonder how only one or two G-protein complexes in *Arabidopsis* can provide specificity in coupling a particular signal to a particular effect. Obviously, one mechanism is to control the constellation of receptors and effectors that are expressed in a particular cell type; in other words, by a temporal control of the up- and downstream components of G protein signaling in a cell. A second mechanism is to physically sequester receptors and effectors with G and other signaling components in a type of signaling raft, as described in the introduction for β-adrenergic signaling. Not surprisingly, signaling rafts are found in plants [30], although none have yet been shown to include a heterotrimeric G-protein component.

Some of the many signals proposed to be coupled directly by G in plants may actually lie on pathways that are only indirectly regulated by G proteins. Thus, as we re-visit this

complexity in the immediate future with our new genetic tools, we may find that it gets simpler for a brief while before becoming complicated again.

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