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G protein subunit phosphorylation as a regulatory mechanism in heterotrimeric G protein signaling in mammals, yeast, and plants

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Abstract

Heterotrimeric G proteins composed of $G\alpha$, $G\beta$, and $G\gamma$ subunits are vital eukaryotic signaling elements that convey information from ligand-regulated G protein-coupled receptors (GPCRs) to cellular effectors. Heterotrimeric G protein-based signaling pathways are fundamental to human health [*Biochimica et Biophysica Acta* (2007) **1768**, 994–1005] and are the target of >30% of pharmaceuticals in clinical use [*Biotechnology Advances* (2013) **31**, 1676–1694; *Nature Reviews Drug Discovery* (2017) **16**, 829–842]. This review focuses on phosphorylation of G protein subunits as a regulatory mechanism in mammals, budding yeast, and plants. This is a re-emerging field, as evidence for phosphoregulation of mammalian G protein subunits from biochemical studies in the early 1990s can now be complemented with contemporary phosphoproteomics and genetic approaches applied to a diversity of model systems. In addition, new evidence implicates a family of plant kinases, the receptor-like kinases, which are monophyletic with the interleukin-1 receptor-associated kinase/Pelle kinases of metazoans, as possible GPCRs that signal via subunit phosphorylation. We describe early and modern observations on G protein subunit phosphorylation and its functional consequences in these three classes of organisms, and suggest future research directions.

‘Everything old is new again’ song lyric by Carole Bayer Sager

The canonical heterotrimeric G protein system as defined in mammalian systems

Heterotrimeric G proteins are particularly well understood in mammalian systems in part because of the many clinical manifestations of aberrant G protein signaling [1–3]. The basic design of the G protein heterotrimer consists of $G\alpha$, $G\beta$, and $G\gamma$ subunits and is conserved across eukaryotes. In the prototypical mammalian paradigm [4] (Figure 1), extracellular ligand binding by a seven-transmembrane domain (7TM) G protein-coupled receptor (GPCR) induces the $G\alpha$ subunit to exchange GDP for GTP, resulting in separation of $G\alpha$ from the non-dissociable $G\beta\gamma$ dimer [5]. Mammalian GPCRs thus act as guanine nucleotide exchange factors (GEFs), while $G\beta\gamma$ dimers act as guanine nucleotide dissociation

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inhibitors (GDIs). Mammals also have several classes of cytosolic GEFs and GDIs [6–8]. Upon heterotrimer dissociation, one or both of $G\alpha$ and $G\beta\gamma$ interact with downstream effector proteins to initiate downstream signaling. Termination of signaling occurs when the GTP is hydrolyzed to GDP by $G\alpha$ and the subunits re-associate in the heterotrimeric configuration [9]. GTP hydrolysis can be promoted by the regulator of G protein signaling (RGS) proteins with GTPase accelerating (GAP) activity [7,9,10]. The activation/deactivation cycle of the $G\alpha$ subunit utilizes five guanine nucleotide-binding motifs (G1–G5) that, in the folded protein, surround the nucleotide cofactor, co-ordinate Mg^{2+} binding [11,12] (Figure 2A,B), and are integral to GDP-/GTP-binding and GTP hydrolysis, as well as three flexible switch regions (switches I–III) that allow for alteration of conformation between inactive and active states [12,14] (Figure 2A,C).

The >800 members of the mammalian 7TM GPCR superfamily sense a vast array of signals, ranging from light and odorants to endogenous compounds such as neurotransmitters, hormones, and calcium ions, to exogenous ligands that constitute essential clinical drugs, including anti-hypertension and anti-Parkinson's drugs, anti-histamines and anti-asthmatics, anti-psychotics and antidepressants, and opioid analgesics [1,3,15]. Mammals have multiple genes encoding G protein subunits; for example, the human genome contains 16 $G\alpha$, 5 $G\beta$, and 12 $G\gamma$ genes (Table 1) [5], with additional complexity provided by splice variants. Mammalian $G\alpha$ subunits are historically divided into four classes (Table 1) based largely on sequence similarity: G_s (including G_{olf}), G_i (including G_z , G_o , and G_t sub-families), G_q (a class that also contains G_{11} , G_{14} , G_{15} , G_{16}), and $G_{12/13}$ [9,16]. G_s and G_i refer, respectively, to stimulatory or inhibitory effects on the enzyme adenylyl cyclase, which catalyzes the production of cAMP, a classic G protein-regulated secondary messenger in mammalian cells. G_q was first purified as the $G\alpha$ that activates phosphoinositide-dependent phospholipase C (PLC), which produces the second messengers diacylglycerol and inositol trisphosphate [17], and $G_{12/13}$ subunits signal to small G protein-dependent pathways. Some mammalian effectors, such as adenylyl cyclase, couple with $G\alpha$, some are targeted by both $G\alpha$ and the $G\beta\gamma$ dimer, and some are specific to $G\beta\gamma$ [17–19]. Additional examples of effectors are phosphodiesterases that degrade cyclic nucleotides, phospholipase D (PLD) which produces the signaling metabolite phosphatidic acid, and Ca^{2+} , Na^+ , K^+ , and Cl^- ion channels that alter cytosolic ion concentrations, modulate membrane potential (for example, in nerve impulse transmission) or, particularly in the case of Ca^{2+} , initiate signaling cascades. Another important set of signaling components downstream of G proteins are protein kinases, particularly the serine/threonine (S/T) protein kinase Cs (PKCs), with diverse roles including regulation of smooth muscle contraction, secretion, opioid perception, neuronal excitation and memory, and the cAMP-dependent Ser/Thr protein kinase As (PKAs), with major roles in carbohydrate and lipid metabolism.

Following activation, GPCR desensitization to persistent stimuli in mammals is achieved by the binding of β -arrestin proteins to GPCRs that have been phosphorylated by G protein-coupled receptor kinases (GRKs) [20]. β -Arrestin binding both prevents G protein association with the GPCR and promotes receptor internalization by endocytosis [20,21]. The extent to which internalized GPCRs continue to signal is an active area of current research [22].

The heterotrimeric G protein system in budding yeast

As a single cell eukaryotic model organism that is genetically malleable and easily assayed, yeast has proved to be a rich source of information regarding signal transduction mechanisms. Heterotrimeric G protein signaling in the yeast *Saccharomyces cerevisiae* [23] largely parallels the mammalian paradigm, albeit with a greatly reduced set of components (Table 1). G protein signaling in yeast is integral to the pheromone-stimulated mating response [24]. Because the ligands of the two prototypical GPCRs of yeast are the cell-type specific mating factors α and α , impairment of G protein signaling results in inability of the two haploid cell types to conjugate. Thus, G protein pathway genes have been uncovered by genetic screens for sterility and have received the designation ‘*STE*’ (sterile) genes. Haploid mating-type α cells (*MAT α*) possess the GPCR Ste2, which recognizes the α -mating factor. Reciprocally, mating-type α cells (*MAT α*) possess the GPCR Ste3, which recognizes the α -mating factor. Upon binding of the mating factor from the opposite cell type, Ste2 and Ste3 act as GEFs that activate the single yeast G protein heterotrimeric complex, comprising the $G\alpha$ Gpa1 and the $G\beta\gamma$ dimer Ste4–Ste18. Freed $G\alpha$ interacts with the mitogen-activated protein kinase (MAPK) Fus3 and an RNA-binding protein Sc160, which promote cellular changes such as chemotropic shmoo formation that are important for mating [23]. However, primacy in signaling is via the freed $G\beta\gamma$, which activates a MAPK kinase cascade that results in activation of the MAPK, Fus3. Active Fus3 phosphorylates downstream targets that include the formin Bni1 which promotes actin polymerization and shmoo formation, the transcription factor Ste12 which results in transcription of mating-essential genes, and the cyclin Far1. Far1 phosphorylation leads to cell cycle arrest and thus requisite cell cycle synchronization in the G1 phase prior to mating and formation of a diploid zygote [24]. Although there is no evidence in yeast for orthologs of mammalian-like GRKs, phosphorylation by casein kinases (CKs) is implicated in internalization of Ste2 (and presumably Ste3) pheromone receptors [25,26] (see Table 2 for the conservation of selected kinase families between humans, Arabidopsis, and yeast). Furthermore, while there are no true β -arrestins in yeast [27], termination of plasma membrane-localized signaling in yeast also occurs via GPCR internalization, facilitated by arrestin-related trafficking adaptor proteins (ARTs/ α -arrestins) [27,28]. As in mammals, heterotrimer reassociation is accelerated by an RGS protein with GAP activity. Indeed, the sole yeast RGS, Supersensitive 2 (Sst2), was the first RGS protein to be identified in any organism. Yeast also contains a second $G\alpha$ -like protein, Gpa2, which couples with a nutrient-sensing GPCR, GPR1. However, Gpa2 does not interact with the only known $G\beta\gamma$ dimer in yeast, Ste4–Ste18 [29,30], and is not discussed further in this review.

The heterotrimeric G protein system in plants

With regard to G protein signaling in plants, Arabidopsis and other model plant species have dozens of predicted 7TM domain proteins, some of which have been shown to physically interact with $G\alpha$ [31]. Among these, the most intriguing is GCR1, which harbors structural homology to classical GPCRs [32] and sequence homology to the *Dictyostelium* cAMP receptor, CAR1 [32–34]. However, unequivocal evidence for the ligand-induced GEF activity that typifies mammalian-type 7TM GPCRs is still lacking for any plant candidate GPCR [35], and plants lack sequence orthologs of arrestins or GRKs [36]. Cytosolic GEFs

and GDIs also have not been identified in plants to date, although such functionalities can be conferred by a diversity of motifs, such that the absence of orthologous proteins [37] does not suffice to conclude that these regulatory mechanisms are absent from plants. Arabidopsis has one RGS protein, RGS1, with documented GAP activity [38,39]. RGS1 also harbors a predicted 7TM N-terminal extension, analogous to the 7TM structure of mammalian GPCRs, and has been implicated in sugar sensing [40,41]. However, not all plant genomes encode RGS homologs; notably, some monocots, including the major grain crops rice and maize, lack *RGS* genes [42]. The Arabidopsis phospholipase D, PLD α 1, has been observed to weakly stimulate the GTPase activity of the G α subunit [43], similar to the GAP activity observed for mammalian phospholipase Cs [44]. Therefore, it is possible that soluble proteins, such as phospholipases, confer feedback GAP activity to plant G proteins in addition to, or instead of, RGS proteins. Below, we discuss the possibility that the ~600 member receptor-like kinase (RLK) family of plants [45,46] might serve as plant GPCRs [47]. Although yeast lacks RLK homologs [48], RLKs are evolutionarily monophyletic with the IRAK (interleukin-1 receptor-associated kinase)/Pelle kinases of metazoans [49,50] (Table 2). BLAST analysis with RLKs identifies both the mammalian IRAK kinases and invertebrate Pelle kinases, and the reverse is true for BLAST analysis with metazoan IRAK and Pelle kinases.

The G protein heterotrimer of plants (Table 1) has both conserved and unique elements [36,51]. Plant genomes encode all three subunit types, but diploid plant genomes contain many fewer G protein subunit genes than are found in mammalian genomes [36,51,52]. Arabidopsis has one canonical G α subunit, GPA1, and three extra-large G α s [52,53] (XLG1, XLG2, and XLG3), which are plant-specific [they are not homologous to metazoan extra-large variant G α s (XL α s)]. The plant XLGs consist of an N-terminal half that is unique to this family of proteins and a C-terminal half that comprises the G α region. The XLGs have been shown to physically couple with Arabidopsis G $\beta\gamma$ dimers and to mediate a subset of G protein-related phenotypes [54]. Arabidopsis has one canonical G β subunit, AGB1, and three G γ subunits, AGG1, AGG2, AGG3, that share the characteristic G γ -specifying motifs, although AGG3 additionally has a C-terminal likely single-membrane-pass extension [55]. Rice, the best-characterized monocot system, has orthologs of all subunit types, including both canonical and non-canonical G α s [56]. Interestingly, several useful agronomic characteristics of specific rice cultivars related to yield, grain shape, drought tolerance, and nutrient use efficiency arise from naturally occurring mutations in G protein subunits [57–60].

Despite relatively few subunits, plant G proteins, like those of metazoans, regulate a multitude of processes including the development of all major organs [59,61,62], signaling via the majority of plant hormones [63–69], infectious disease susceptibility [47,67,70–72], and environmental sensing, including responses to desiccation [60], salinity [73], and light [74]. The fact that plants, like yeast, have relatively few G protein subunits but, like mammals, exhibit a multiplicity of G protein-regulated responses suggests that the few plant G protein subunits interact with large numbers of partners, and raises the possibility that specificity of effector interaction is conferred by post-translational modifications (PTMs) of the G protein subunits. It is well known that G protein subunits are subject to lipid modifications, including myristoylation, palmitoylation (G α) and isoprenylation (G γ),

which are crucial for membrane anchoring [75,76]. Proteomic approaches are categorizing new types of PTM of proteins [77], including acetylation, nitrosylation, glutathionylation, and cysteine oxidation, and thus, there may be other covalent modifications of G proteins that are yet to be described. However, available information currently implicates phosphorylation as a prime candidate for G protein signal biasing. Although plant G protein interactomes based on yeast two-hybrid screening have been published [78,79], few interactors have been functionally validated. Among the confirmed GPA1 effectors are PLD α 1 [80], implicated in signaling downstream of the plant stress hormone abscisic acid (ABA), and THF1 [81], which plays roles in chloroplast development [82]. Validated AGB1 effectors include NDL1 (N-MUC DOWNREGULATED-LIKE-1), which modulates transport of the hormone auxin [83], and the acireductone dioxygenase ARD1 [84]. Few XLG interactors have been validated, other than XLG2 interaction with the cytosolic kinase, BIK1 (BOTRYTIS-INDUCED KINASE1) [85], which is involved in hormone and defense signaling.

G α subunit phosphorylation in mammalian systems

The above summary illustrates that regulation of GPCRs by kinases and kinases as signaling elements that function downstream of G protein subunits are both well-established paradigms in G protein signaling. In contrast, phosphorylation of the G protein subunits *themselves* as a regulatory mechanism has received relatively little attention. Studies on subunit phosphorylation in the early 1990s largely focused on specific kinases known to be downstream of G protein-mediated signaling and utilized biochemical assays. Commonly investigated downstream kinases included the cAMP-dependent Ser/Thr protein kinase, PKA, and the Ser/Thr PKC. In this section, we summarize the early evidence for PKA and PKC phosphorylation of G α subunits, as well as studies demonstrating that G α subunits are targeted by tyrosine kinases. The signaling or physiological impacts of a given phosphosite, where known, are summarized in Table 3.

Early studies showed PKA-mediated phosphorylation of two G α subunits, G α_{i2} and G α_{i3} . Phosphorylation of G α_{i2} at sites that were not definitively mapped was promoted in hepatocytes by a membrane-permeant analog of cAMP [108], which activates PKA. G α_{i3} was shown to be phosphorylated by PKA *in vitro* and loss of phosphorylation in a T203A mutant implicated this specific residue, contained within both the G2 motif and the Switch I region (features indicated in Figure 2A–C), as the phosphosite [97]. The T203A phosphonull mutant of G α_{i3} showed reduced interaction with G $\beta\gamma$ in pulldown experiments [97], which might suggest prolongation of the active state of the G α . PKA regulation at this particular residue may be specific to the G $\alpha_{i2/3}$ G α s as, though the position is strictly conserved, T203 resides within a PKA consensus sequence that is absent from G α_i , G α_s , G α_q , and G α_z . However, most G α subunits contain predicted [109] PKA phosphorylation sites at other locations, as summarized in Table 4.

Results from early studies with PKC also provided important clues supporting regulatory impacts of G α phosphorylation on G protein signaling intensity and duration. Phosphorylation of G α_{i2} was promoted in hepatocytes by phorbol esters, which activate PKC, suggesting that this G α subunit is targeted by both PKA and PKC [108]. Insulin

treatment attenuated both basal and phorbol ester-stimulated $G\alpha_{i2}$ phosphorylation, and a consequent increase in non-phosphorylated (active) $G\alpha_{i2}$ was proposed as a possible mechanism underlying the observed inhibition of adenylate cyclase activity by insulin [110]. Consistent with this hypothesis, basal and phorbol ester-stimulated $G\alpha_{i2}$ phosphorylation was promoted in mice by the chemotherapy agent, streptozotocin, which induces type I (insulin-deficient) diabetes [111].

In the 1990s, PKC was also shown to phosphorylate $G\alpha_z$, $G\alpha_{12}$, and members of the $G\alpha_q$ family. $G\alpha_z$ was shown to be a PKC target both *in vitro* and in permeabilized platelets upon PKC activation by phorbol esters. Phosphorylation occurred at S16 and at $G\alpha_z$ -specific S27 and was impeded, likely sterically, by $G\beta\gamma$ [112–115].

While phosphonull mutation did not affect heterotrimer dissociation [116], phosphorylation impeded $G\beta\gamma$ binding and RGS binding [87,88]. These results indicate that PKC-mediated phosphorylation may prolong the active GTP-bound state [75], although resultant impacts on effector activity remain elusive [116]. $G\alpha_{12}$ (but not $G\alpha_{13}$) was also shown *in vitro* and in cultured cells to be a PKC substrate, with phosphorylation and $G\beta\gamma$ binding again showing reciprocal inhibition [117]. $G\alpha_z$ was also shown to be phosphorylated by p21-activated protein kinase (PAK) at S16, with similar impacts on $G\beta\gamma$ interaction [87]. PAK is also an effector of $G\beta\gamma$ and in mammalian cells, interaction with $G\beta\gamma$ inhibits PAK phosphorylation of substrates, including $G\alpha_z$. PAK phosphorylation of $G\alpha_{i1}$, $G\alpha_{i2}$, G_o , G_s , and G_q was not observed, suggesting subunit specificity [87]. These impacts of PKC and PAK are likely examples of phosphorylation-based effector/ $G\beta\gamma$ reciprocal inhibition, as diagrammed in Figure 3A.

PKC has also been shown to phosphorylate $G\alpha_q$ family members. For example, phorbol ester treatment stimulates phosphorylation of $G\alpha_{15}$ (then known as $G\alpha_{16}$; see the footnote in Table 1), with direct phosphorylation by PKC confirmed in *in vitro* assays [96]. Interestingly, phosphorylation at S336 occurs within a PKC consensus sequence present in a $G\alpha_{15}$ -specific C-terminal domain, as opposed to phosphorylation at the N-terminal $G\beta$ interaction surface, as observed for $G\alpha_z$. This $G\alpha_{15}$ domain has been implicated as a region of receptor interaction, and phosphorylation may affect this interaction. $G\alpha_{15}$ S336A mutants failed to transduce ligand activation of the M2R or β_2 AR receptors into PLC activation; however, when this mutation was coupled with another, constitutively activating mutation, PLC activation was restored. These results implicate the C-terminal S336 phosphosite as a determinant of receptor, rather than effector, coupling. Similar results were also observed for the murine ortholog of $G\alpha_{15}$ [96].

Tyrosine phosphorylation has also been implicated as a regulatory mechanism for mammalian $G\alpha$ signaling. For example [95], carbachol stimulation of the M1 mAChR receptor evoked phosphorylation of $G\alpha_q$ and $G\alpha_{11}$. Y356 was implicated as the $G\alpha_{11}$ phosphosite based on loss of phosphorylation in an Y356F mutant. Y356 phosphorylation in $G\alpha_{11}$, like serine phosphorylation in $G\alpha_{15}$, occurred following agonist exposure. $G\alpha_{11}$ activation of IP_3 production was impaired by tyrosine kinase inhibitors, implicating tyrosine phosphorylation in transduction of the activated state of the GPCR [95]. Tyrosine phosphorylation of $G\alpha_{11}$ also increased its direct stimulatory effect on PLC activity *in vitro*

[118], suggesting a role of tyrosine phosphorylation not only in $G\alpha$ signal reception from the GPCR but also in modulating the efficacy of $G\alpha_{11}$ — effector coupling (cf. Figure 3B).

Specific tyrosine kinases that have been implicated as targeting mammalian $G\alpha$ subunits include the protooncogene pp60^{c-src} tyrosine kinase, which was shown to phosphorylate $G\alpha_s$ at Y37 and Y391 (originally designated as Y377) [89]. Tyrosine phosphorylation of $G\alpha_s$ by pp60^{c-src} was shown to stimulate GTP γ S binding and receptor-stimulated GTP hydrolysis [90]. $G\alpha_s^{Y391}$ aligns with $G\alpha_{11}^{Y356}$, indicating that it too may play a role in receptor coupling. src also phosphorylates Y142 in the helical domain of transducin, and phosphorylation was confirmed *in vivo* [90,119]. In addition to soluble tyrosine kinases, receptor tyrosine kinases (RTKs) are also implicated in mammalian G protein signaling, as many RTK-mediated pathways have been shown to require $G\alpha_s$. RTKs are single span transmembrane proteins that are activated upon binding of growth factors and neuropeptides. In some cases, direct RTK– $G\alpha$ interaction/regulation is known or supported, while in others $G\alpha$ -interacting vesicle-associated protein- (GIV-) mediated transactivation is demonstrated [120]. The insulin receptor kinase (IRK) is a well-studied example of a tyrosine kinase. Although current paradigms emphasize transactivation following IRK phosphorylation of GIV–GEF [121], early studies showing insulin-dependent phosphorylation of $G\alpha_o$ and $G\alpha_i$ reconstituted with the insulin receptor in phospholipid vesicles are consistent with direct phosphorylation by this receptor [122]. Similarly, another RTK, the epidermal growth factor receptor, was shown to directly phosphorylate tyrosine residues of $G\alpha_s$, which in turn led to greater $G\alpha_s$ -mediated stimulation of adenylyl cyclase [123], a possible example of phosphorylation-stimulated effector coupling (Figure 3B). Further studies will be required to fully elucidate the respective contributions to G protein signaling of GIV-mediated transactivation vs. direct regulation by RTKs.

G $\beta\gamma$ subunit phosphorylation in mammalian systems

Relatively less attention was paid in early studies to phosphorylation of mammalian $G\beta$ and $G\gamma$ subunits. Chronic morphine exposure was shown to promote phosphorylation of $G\beta$ in muscle and neuronal tissue. Phosphorylation was mediated by PKC and PKA and phosphorylated $G\beta\gamma$ was found to increase the activity of adenylyl cyclase II [124–126]. $G\gamma_{12}$ was shown to be a PKC substrate *in vivo*, and phosphorylation at the first Ser conversely increased $G\beta_1\gamma_{12}$ affinity for $G\alpha_o$, i.e. promoted the heterotrimeric configuration [98,99]. These results demonstrate that $G\beta\gamma$ phosphorylation, like $G\alpha$ phosphorylation, can feed back to influence both effector activation and heterotrimer assembly. With regard to effector interactions, PKC-mediated phosphorylation of $G\gamma$ did not affect $G\beta_1\gamma_{12}$ activation of the phospholipase PLC- β , but decreased the ability of $G\beta_1\gamma_{12}$ to activate adenylyl cyclase II [99]. This result importantly illustrates a subunit- and effector-specific role of G protein phosphorylation on effector interaction/activity, i.e. phosphorylation-dependent biased signaling, a mechanism illustrated in Figure 3C.

Phosphoproteomics studies in mammalian systems

It is now possible to identify G protein phosphosites from the datasets of modern phosphoproteomic studies that have no deliberate focus on G proteins. In

phosphoproteomics, phosphopeptides are typically first enriched based on charge, and phosphosites are then identified by the characteristic increase in mass conferred by phosphorylation. We codified the phosphorylation data from over 50 high-throughput metazoan studies available at PhosphoSitePlus (<http://www.phosphosite.org/homeAction.action>) [127], and found several intriguing examples of G protein phosphorylation. We mapped these phosphosites onto BioEdit alignments as features with annotated details and include this information in Supplementary Files S1–S3.

The G1–G5 GTP-binding loops of $G\alpha_s$ (Figure 2A,B) [12] are crucial for function. The invariant (first) serine in the GAGESGKS/T G1 motif is in close proximity to the guanine nucleotide, as illustrated by the red highlighted S49 residue of the AtGPA1 structure in Figure 2D, and was initially implicated as a phosphosite of $G\alpha_{i2}$ in a targeted study of morphine-induced μ -opioid receptor desensitization [86]. Several high-throughput studies subsequently identified this G1 position as a phosphosite of $G\alpha$ subunits; however, due to the high degree of conservation of the G1 motif, the identified peptides cannot unequivocally be attributed to a specific $G\alpha$ subunit. For example, phosphorylation at this position was identified in HeLa cells [128], a non-small cell lung cancer tumor [129], mouse hippocampal slices [130], and mouse brain [131], and in all cases, the phosphopeptide was consistent with $G\alpha_{i1}^{S44}$, $G\alpha_{i2}^{S44}$, $G\alpha_{i3}^{S44}$, $G\alpha_o^{S44}$, $G\alpha_{t1}^{S40}$, $G\alpha_{t2}^{S44}$, $G\alpha_{t3}^{S44}$, $G\alpha_s^{S51}$, or $G\alpha_{olf}^{S55}$. Similarly, in HeLa cells arrested at various stages of the cell cycle [132] as well as in human embryonic stem cells [133], observed phosphosites could be either $G\alpha_{i2}^{S68}$ or $G\alpha_{i3}^{S59}$. A screenshot from Bioedit of the portion a $G\alpha$ alignment around the G1 motif is shown in Figure 4A, with phosphosites indicated; the full alignment is provided in Supplementary File S1. Aside from the G1 motif, another proteomics study on nine mouse organs found $G\alpha_o$ phosphorylated at S62 [134], with phosphorylation subsequently identified at the equivalent position in human $G\alpha_o$, $G\alpha_{i3}$ [135], $G\alpha_q$, $G\alpha_{i1}$, and $G\alpha_{i5}$ [136]. Proteomics studies have likewise reported tyrosine (Y) phosphorylation at sites including, but not limited to, the non-conserved Y44 of $G\alpha_{i3}$ [132], the partially conserved Y61 and Y69 of $G\alpha_{i2}$ [137,138] and $G\alpha_{i3}$ [139], the non-conserved Y91 of $G\alpha_{t1}$ [140], and at Y167 of $G\alpha_{i3}$ [128], which is broadly conserved. The S352 of $G\alpha_s$ is the most highly represented $G\alpha$ phosphosite in high-throughput datasets, as to date it has been identified in eight human, 11 mouse, and one rat phosphoproteomic studies (Supplementary File S1). This position is not conserved in other $G\alpha$ subunits, though as a Thr it is a potential phosphosite in the closely related $G\alpha_{olf}$. Much like $G\alpha_{i5}$ S336 discussed previously, $G\alpha_s$ S352 phosphorylation and perhaps other phosphorylation events in the C-terminal region are likely to play a role in receptor coupling and specificity.

The phosphosite information that we compiled (Supplementary Files S1–S3) from high-throughput studies reveals many phosphosites in human $G\beta_1$ – $G\beta_4$ and in $G\gamma_s$, with no investigation to date of their functional relevance. Phosphorylation of S2 appears common for $G\beta$, with position S2 identified in 10 high-throughput metazoan phosphoproteomic studies for $G\beta_1$ [131,135,141–148] and 16 studies for $G\beta_2$ or $G\beta_4$ [131,132,135, 141–153] (subunit identity could not be differentiated based on the peptide sequence). Similarly, phosphorylation of $G\beta$ at S72 (in $G\beta_1$, $G\beta_2$, $G\beta_3$, or $G\beta_4$) and S74 (in $G\beta_3$ or $G\beta_4$) occurred but could not be differentiated among the $G\beta$ subunits due to strong conservation. These

positions are noteworthy in that they are conserved between humans, yeast, and Arabidopsis (see Supplementary File S2 for the alignment with annotation).

Regarding G γ phosphorylation, G γ ₁₂ has commonly been found to be phosphorylated, with 10 phosphosites identified across the span of the protein, six of which are clustered in the first 10 residues. Indeed, the N-terminus of metazoan G γ subunits appears to correspond to a phosphorylation hotspot (Figure 4C), with the majority of sites observed to date falling within the first 14 residues, and one or more sites identified in eight of the 12 G γ s, namely G γ ₁, G γ ₂, G γ ₃, G γ ₄, G γ ₅, G γ ₇, G γ ₁₀, and G γ ₁₂ (Supplementary File S3). Given poor positional conservation of the N-termini of G γ subunits, these phosphosites do not readily align (Figure 4C); however, as G γ ₁₂ N-terminal phosphorylation has been implicated as a determinant of heterotrimer formation and effector specificity for G β ₁ γ ₁₂ (discussed above), it is plausible that N-terminal G γ phosphorylation is a general mechanism for biasing G $\beta\gamma$ dimer interactions with G α and effectors (Figure 3C).

The *in vivo* relevance of G α phosphorylation in human genetic and infectious disease and drug response

Many of the early studies on G protein subunit phosphorylation had the limitation that they were conducted *in vitro* or in cell lines, but had the advantage of revealing roles of specific kinases and their impact on heterotrimer assembly or association with specific effectors. Conversely, recent phosphoproteomics studies are tantalizing in that they show G protein subunit phosphorylation in intact cells or tissues, but do not address the identity of the phosphorylating kinase, nor the significance of the phosphorylation event. However, several recent studies have more directly demonstrated the importance of G α phosphorylation in human health and disease. As described above, phosphoproteomics studies have cataloged phosphorylation of S44 (using the G α _{i1} numbering) in the G1 motif (Figure 4A). In a study suggestive of pharmacological relevance, a triple phosphonull (S44A/S144A/S302A/C352L) of G α _{i2} was unable to be phosphorylated by PKC ϵ and, unlike the wild-type protein, the mutant protein did not allow morphine-induced desensitization of the μ -opioid GPCR [86].

The second serine in the G1 GXXXSGKS/T motif (Figure 4A) is also conserved (as S or T) and is critical for guanine nucleotide binding and Mg²⁺ co-ordination in both small G proteins and G α s. Early studies showed that mutation at this site interferes with GTP-binding and G protein signaling. In 2016, a mutation at this residue, G α _{i3}^{S47R}, was shown to result in a dominant-negative G α _{i3} that outcompetes G α _q for its effectors, and is ultimately causative of auriculo-condylar syndrome, which causes cranio-facial deformation [154]. While this study did not demonstrate the importance of S47 phosphorylation *per se*, we now know that the corresponding residue in G α _q, S53, (though notated by the authors as S47 perhaps due to a splice variant) is the primary site of G α phosphorylation by the YpkA (Yersinia protein kinase A) virulence factor, produced by pathogens of the *Yersinia* genus, including *Yersinia pestis*, the causative agent of bubonic plague. Phosphorylation by YpkA at this site impedes GTP-binding and downstream signaling events [91]. These two reports suggest that we are only beginning to uncover the impact of subunit phosphorylation on human genetic and infectious disease states.

G protein subunit phosphorylation in yeast

The relatively small genome of yeast has fostered functional identification of the majority of genes and construction of a comprehensive set of knockout lines. Dohlman and colleagues utilized these resources to screen 109 yeast kinase deletion mutants for resultant loss of phosphorylation of the yeast Gα, Gpa1. The screen relied on the fact that post-translationally modified proteins often show retarded mobility during gel electrophoresis, and loss of the mobility shift upon *in vitro* phosphatase treatment is indicative of prior phosphorylation. Yeast Gα, Gpa1, demonstrates this diagnostic shift, indicating that it is phosphorylated *in vivo*. Dohlman and colleagues thereby identified the kinase Elongated morphology 1 (Elm1) as necessary and sufficient for Gpa1 phosphorylation, at least under the nutrient-rich conditions that they employed for yeast growth [101]. A mutated Gpa1^{S200A} subunit displayed a modest reduction in Elm1 phosphorylation *in vitro*, demonstrating that, among other sites, Elm1 phosphorylates the S200 residue, located within a yeast-specific region of the helical domain. Ubiquitination of Gpa1 was reduced in *elm1* cells, suggesting that phosphorylation of Gpa1 is required for efficient ubiquitination and protein turnover. Elm1 is integrally involved in the regulation of bud emergence, mitosis, and cytokinesis and is most highly expressed during S and G2 phases; phases in which maximal Gpa1 phosphorylation was also observed. Conversely, reduced Gpa1 phosphorylation as assessed by gel shift assays was observed in cells treated with α mating factor and thus arrested in G₁ phase, as occurs prior to mating [101]. The S200 position is the most commonly identified Gpa1 phosphosite in high-throughput datasets, appearing in three studies (Supplementary File S1), including one conducted on yeast under α mating factor arrest [155], and another in which reduced phosphorylation at S200 was observed in two kinase mutants: *ribosome biogenesis and tRNA synthetase-associated kinase 1 (rtk1)* and *pkb-activating kinase homolog 1 (pkh1)* [156]. These results implicate these two additional kinases as acting directly or indirectly in the phosphorylation of Gpa1 S200. Other phosphosites identified in high-throughput datasets include S175 [157], T189 [158,159], S199 [160], and S429 [157] (Supplementary File S1).

Under conditions of reduced glucose availability, not only Elm1 but also Snf1 activating kinase 1 (Sak1) and Target of Sbf 3 (Tos3) kinases mediate rapid phosphorylation of Gpa1, with the major role played by Sak1 [161]. These three kinases also phosphorylate Sucrose nonfermenting 1 (Snf1) [the yeast homolog of mammalian AMP-activated protein kinase (AMPK)]; consequent Snf1 activation helps to maintain energy status under nutrient-limiting conditions. Antagonistically, the PP1 phosphatase containing the regulatory subunit Resistance to glucose repression 1 (Reg1), which dephosphorylates Snf1, also appears to act on Gpa1. *reg1* cells display enhanced phosphorylation of Gpa1 under high glucose conditions and an extended duration of phosphorylation under low glucose conditions [161].

Gα phosphorylation thus appears as a point of cross-talk between pheromone sensing and perception of glucose depletion in yeast [161]. Consistent with repression of the mating pathway by Gpa1 phosphorylation, reduced mating efficiency is observed under the glucose-limiting conditions that promote Gpa1 phosphorylation, suggesting a tradeoff between energy homeostasis and sexual reproduction under stress conditions. Consistent with this scenario, the reduction in pheromone-stimulated Gpa1 phosphorylation that is observed in

triple *elm1 sak1 tos3* kinase mutants is associated with increased activity of the pheromone response pathway. Clement et al. transformed kinase and phosphatase mutants with a *FUS1::LacZ* reporter and quantified β -galactosidase activity as a measure of activity of the pheromone pathway. They observed increases in both basal and pheromone-responsive levels of β -galactosidase activity in the *elm1 sak1 tos3* kinase mutant. Moreover, reduced β -galactosidase reporter activity was observed upon pheromone treatment of cells lacking the Reg1 phosphatase subunit [161]. Comparison of the exact phosphosites of Gpa1 following glucose depletion vs. pheromone treatments would help to further elucidate this cross-talk. Such experiments would also allow ascertainment of specific phosphosite conservation and thus potential relevance of yeast phosphosites to mammalian and plant G protein phosphorylation.

With regard to the yeast G β subunit, Ste4, pheromone-induced phosphorylation has been observed in a yeast-specific region encompassing amino acids 310–346 [102]. S335, T320, and T322 were implicated as the phosphorylated residues by assessment of radiolabeled G β phosphorylation intensity in various G β mutants [103]. Additional phosphosites were subsequently identified in this region from high-throughput studies (Supplementary File S2) [158,162]. Fus3 kinase is directly or indirectly involved, based on decreased G β phosphorylation levels in a *fus3* mutant [103]. It was hypothesized that Ste5, a scaffold protein for Fus3, preferentially binds the phosphorylated form of G β *in vivo*, based on identification of phosphorylated G β in Ste5 immunoprecipitates. However, Ste5 binding is also required for G β phosphorylation [163]. Therefore, it is possible that Ste5 binding simply promotes phosphorylation of G β by the complexed Fus3, resulting in an enrichment of phosphorylated Ste4 in Ste5 immunoprecipitates. Phosphorylation of G β is predicted to create a casein kinase substrate motif, resulting in additional phosphorylation of G β , and indeed the Ste4^{T320A/S335A} phosphonull mutant displays reduced affinity for casein kinases compared with the wild-type G β [164].

Surprisingly, despite pheromone-induced phosphorylation of G β , two G β phosphonull double mutants, T320A/S335A and T322A/S335A, each exhibit no impairment in pheromone sensing, adaptation, or response [103], demonstrating that G β phosphorylation is not central to mating. Nevertheless, G β phosphorylation may still play a role in a default pathway of chemotropism. When yeast cells detect diffuse, non-directional pheromone, they form a 'default mating-type projection' at a site that is otherwise marked as a site for budding (asexual reproduction). This site is marked by the small GTPase BUD1. *bud1* null mutants that additionally are G β phosphonulls as a result of T320A/S335A mutations exhibit shmooing abnormalities, with multiple projections that often exhibit early termination [104]. These mutants also display impaired unidirectional growth in the presence of a directional pheromone signal, implying that G β phosphorylation helps to maintain a stable axis of cell polarity [104]. Consistent with this hypothesis, G β phosphonull proteins visualized as GFP fusions show less stable localization to the incipient outgrowth site. Interestingly, Far1, a known G β -binding protein that is involved in establishing cell polarity [165], has a decreased affinity for the phosphorylated form of G β vs. a non-phosphorylated form in pulldown assays. The decreased affinity for the phosphorylated G β is hypothesized to promote recycling of G $\beta\gamma$ into the heterotrimer and thus allow greater frequency in temporal sampling of pheromone localization [104]. However, no generic

mechanisms can be extrapolated from these yeast data because this phosphorylated region of Ste4 is not widely conserved in G β subunits; it is not found in either human or Arabidopsis G β s (Supplementary File S2).

Regarding the yeast G γ subunit, Ste18, a large-scale analysis of 12 PTM proteomics databases identified three potential phosphosites in the G γ N-terminus. Position S7 was confidently shown to be phosphorylated, while it was ambiguous which of positions T2 or S3 was phosphorylated [105,166,167]. As for G β , pheromone stimulation results in rapid phosphorylation of G γ , and pheromone-stimulated phosphorylation correlated with an increase in abundance of the G γ . Perhaps surprisingly, G γ triple phosphonull mutants (T2A/S3A/S7A) exhibited a higher basal G γ protein level than the wild-type protein, but abundance of the G γ phosphonull was insensitive to regulation by pheromone treatment [105]. The downstream kinase, Fus3, was subsequently implicated in the phosphorylation of the G γ subunit, Ste18, as it has been for the G β subunit, Ste4 (discussed above). Fus3-regulated phosphorylation of G γ is suggestive of a negative feedback mechanism, because when compared with WT or a phosphomimic, cells expressing the Ste18^{T2A/S3A/S7A} phosphonull display an increase in speed and amplitude of pheromone-stimulated Fus3 phosphorylation and therefore activation of G $\beta\gamma$ -mediated MAPK signaling [106]. Because of the low cross-species conservation of G γ sequences, Ste18 sites do not readily align with mammalian or plant G γ sequences, and thus generalizable regulatory mechanisms cannot confidently be inferred. However, as can be seen in Figure 4C and Supplementary File S3, phosphorylation of G γ subunits appears universally enriched in the N-terminal region.

G protein subunit phosphorylation in plant systems

G α phosphorylation in plants

To date, targeted experiments of the type that were conducted in the 1990s with mammalian G protein subunits and the downstream kinases PKA and PKC are lacking in plants. However, incontrovertible evidence for subunit phosphorylation, particularly for the model plant species, Arabidopsis, is present in phosphoproteomics databases. All Arabidopsis G α subunits (except XLG2) conserve the S and S/T of the G1 motif of mammalian G α s (Figure 4A). Phosphorylation of the canonical Arabidopsis G α , GPA1, within the G1 motif at S49, highlighted in red on the GPA1 structure (Figure 2D), is confirmed in Arabidopsis experimental phosphoproteomic data compiled at P3DB and PhosPhAt4.0 [168–170] (Figure 4A and Supplementary File S1). Four other GPA1 phosphosites are also present in these databases: T12, T15, T19, and Y166 [171–174]. Of these, phosphorylation in the N-terminal regions (albeit not at these exact sites) is frequent in human G α s, as discussed above and shown in Supplementary File S1. Y166 is at a strictly conserved position within the helical domain of human, yeast, and Arabidopsis G α subunits, and phosphorylation at this position has been observed in G α_{i2} , G α_z , and G α_s (Supplementary File S1). Based on simulations, Li et al. [107] hypothesized that phosphorylation of Y166 (highlighted in white in Figure 2D) may result in the formation of several intramolecular salt bridges leading to a subtle alteration of G α conformation, particularly locally around the GDP-/GTP-binding pocket. In support of this hypothesis, it was shown [107] that, unlike wild-type GPA1, a GPA1^{Y166E} phosphomimic bound RGS1 while in the GDP-bound state. The wild-type

GPA1, as is typical, only bound RGS1 while in the transition state, as mimicked by the addition of GDP and AlF_4^- .

To date, none of the phosphosites of XLGs observed in large-scale datasets are at positions that are conserved in canonical G α s. Interestingly, the Arabidopsis XLGs all contain a 13–22 C-terminal extension rich in Ser/Thr/Tyr residues (Figure 4B), suggestive of a phosphorylation hotspot, and indeed, the only experimentally defined phosphosites for XLG1, all identified from seedlings treated with the cellulose inhibitor isoxaben, fall within this region (Y876, Y879, and S887) [170]. On the other hand, the majority of XLG2 phosphosites identified to date are within the N-terminal XLG-specific domain; specifically, S13 [174,175], S23 [174], S71 [176], S75 [174,177], S151, S154 [174], and S169 [174,176–179] have been identified in high-throughput studies. A targeted study [85] of XLG2 in protoplasts treated with the pathogen elicitor peptide, flg22, confirmed several of these sites (S13, S23, S154, and S169), while also identifying additional sites that could be unambiguously attributed: S69, S141, S148, S150, S156, S191, S194, and S489 (incorrectly listed as S488 in the original publication). As Liang et al. observed a cluster of four phosphosites, S141, S148, S150, and S151, which represented nearly half of the sites they identified, they complemented *xlg2* null mutants with an XLG2^{S141A/S148A/S150A/S151A} phosphonull and an XLG2^{S141E/S148E/S150E/S151E} phosphomimic. The phosphomimic fully complemented the flg22-induced reactive oxygen species burst mutant phenotype, although phosphomimicry at these sites did not suffice to cause constitutive pathogen resistance. The phosphonull resulted in only partial complementation, and resistance to the bacterial pathogen *Pseudomonas syringae* was also impaired in the phosphonull. Therefore, phosphorylation of the plant-specific N-terminal domain of XLG2 is required for full pathogen resistance. XLG2 S489 is the only known XLG phosphosite in a region that readily aligns with canonical G α s, but most G α s encode a glycine at this position. The only other Arabidopsis XLG phosphosites currently known from high-throughput studies, namely S530 [180] of XLG2 and S506 [181,182] of XLG3, reside within an XLG-specific insertion of 17–31 residues located within the G α helical domain. However, in the absence of comprehensive studies on XLG phosphorylation, it would be premature to conclude that phosphosites conserved with canonical G α subunits do not exist.

Though high-throughput phosphoproteomic studies of plant species other than Arabidopsis are to date uncommon, available studies have found evidence for phosphorylation of the canonical rice G α , RGA1, at S10 [183] and S110 [173,184–186]. Multiple phosphosites were also identified for an XLG in rice, encoded by the gene Os06t0111400. These rice XLG phosphosites span the protein from the N-terminal domain (S68 [183], S183 [173,183,184], and Y186 [173]) to the G α -like domain (T669 [184], which is proximal to the G3 motif) to the C-terminal extension that we suggested above to be a potential phosphorylation hotspot (S859/S860/S861 — the exact site could not be conclusively attributed within these three consecutive serines [173]).

G $\beta\gamma$ phosphorylation in plants

The only plant G β phosphosite derived from large-scale phosphoproteomics is the AGB1 S40 equivalent in *Brassica napus* (canola), a crop species closely related to Arabidopsis

[187]. We immunoprecipitated the Arabidopsis G β , AGB1, from plasma membranes of Arabidopsis *agb1* null lines complemented with an epitope-tagged AGB1 [188] and analyzed the immunoprecipitate (IP) for G protein subunit phosphorylation. We found evidence for two AGB1 phosphosites: S267 and T271. S267 is conserved in human and yeast G β subunits, and T271 is not conserved in yeast but is conserved in human G β s (except as an S in G β 3, which still conserves it as a potential phosphosite; Supplementary File S2). It is informative that the identified AGB1 phosphosites at S267 and T271 correspond to human G β ₁ S245 and T249, which are adjacent to the G α contact residue at D246 [189]. Unlike G α , G $\beta\gamma$ does not undergo a conformational change during activation. Instead, G α dissociation from G $\beta\gamma$ relieves steric inhibition of the G α -effector-binding surface. Therefore, the S267 and T271 AGB1 phosphosites reside proximal to the interaction interface and are prime candidates for biasing G $\beta\gamma$ interactions (Figure 3C), likely by altering the local charge environment.

Regarding G γ subunits, current plant phosphoproteomics databases show no phosphosites for AGG1, and one phosphosite each for AGG2 (S8) [190] and AGG3 (S37) [174]. S37 is in a highly conserved block of seven amino acids in plant AGG3-type G γ subunits, with the motif PXP(K/R)SPP. In our own AGB1 immunoprecipitates, G γ s were also present, as expected, and we found evidence consistent with phosphorylation of T5 in AGG1 and of a site(s) among S23/S24/S28/S29 in AGG3 [188] (the phosphosite could not conclusively be attributed within the peptide). Similarly, the rice G γ subunits RGG2 and DEP1 have been found to be phosphorylated at S38 [184] and S15 [183], respectively. These G γ phosphosites are reminiscent of the N-terminal sites identified in metazoan G γ s (Figure 4B).

RLKs as candidates for GPCRs that phosphorylate plant G protein subunits

Despite the prevalence of 7TM GPCRs in animals, metazoans also have non-7TM proteins, such as the RTKs, that couple with heterotrimeric G proteins [120], establishing a general precedent for novel receptor types functioning as GPCRs. Newly emerging genetic and protein–protein interaction evidence suggests that in plants, a family of single-pass transmembrane kinases, the RLKs, play such a role. This would make RLKs prime candidates for kinases mediating subunit phosphorylation. RLKs are a large superfamily (Table 2) in Arabidopsis [45,46], and are implicated in both biotic and abiotic stress response [45,191], yet the majority remain orphan receptors with surprisingly few ligands identified to date. Table 5 summarizes the RLKs discussed in this section, with known ligands and roles indicated.

A first hint that RLKs and G proteins might function together was provided by the observation in 2001 that null mutation of *AGB1* (G β) resulted in certain developmental phenotypes that were shared with null mutants of the RLK *ER* (*ERECTA*); double mutant analysis implicated AGB1 in a subset of organ development phenotypes dependent on ER [227]. Additional clues of functional relationships arose from protein–protein interaction studies that described physical association of RLKs with G protein subunits. For example, AGB1 was found to physically interact with the RLK ZAR1, which regulates zygote development in Arabidopsis [207]. In maize, the RLK FASCIATED EAR2 (FEA2) and the canonical G α named COMPACT PLANTS2 (CT2) were found in the same

immunoprecipitation complex, and knockout of each was shown to increase the size of apical meristems [228]. (Meristems are regions of stem cells that are preserved in the adult plant and are the source of the indeterminate growth that characterizes the plant body form.) FEA2 interaction with co-receptors is hypothesized to enable distinct FEA2–receptor complexes to sense different peptide ligands. Recent genetic analyses [197] suggest that one of these ligands, ZmFCP1, signals through FEA2 via a downstream pseudokinase, CRN, while another peptide ligand, ZmCLE7, appears to signal through FEA2 via the Gα CT2. Enlarged shoot apical meristems were also observed in Arabidopsis CLAVATA receptor mutants, *clv1*, *clv2*, and *rpk2* (*RECEPTOR-LIKE PROTEIN KINASE2*), and this phenotype was enhanced by knockout of the Gβ subunit AGB1 [229]. AGB1 was confirmed to interact with RPK2 in bimolecular fluorescence complementation (BiFC) and co-IP assays [229].

In Arabidopsis, our immunoprecipitation of the Gβ subunit from purified plasma membrane allowed us to perform mass spectrometric identification of Gβ-associated proteins. Five RLKs (Table 5) were present in the immunoprecipitates and absent from control IPs: FER (FERONIA), MIK2, AT5G39000, AT1G48480 (RKL1), and AT5G63410. This subset of RLKs has a range of functions in signaling by hormones [68,230,231] and perception of the microbe-associated molecular patterns (MAMPs/elicitors) that trigger innate immune responses [213,220,232,233]; pathways in which plant G proteins are also implicated.

We performed a targeted study of the G protein dependency of one of the RLKs in our Gβ immunoprecipitate, FER. FER is one of the 17 members of the *Catharantus roseus* RLK1-like (CrRLK1L) subfamily of RLKs in Arabidopsis [234]. FER is activated by endogenous polypeptide ligands of the RALF (Rapid Alkalinization Factor) family, encoded by 34 genes [215]. RALF1 exposure induces extracellular alkalization, and changes in the phosphostatus of FER and other proteins [215]. FER, like G proteins, is implicated in diverse plant responses, including salinity sensing [210,211], responses to the plant hormones ABA, ethylene, and brassinosteroids [212,214,235], root growth [236] and mechano-sensing [237], pollen tube rupture during fertilization [209,238,239], and plant–pathogen responses [213,240], in which FER was found to promote association of the MAMP receptors FLS2/EFR and their co-receptor BAK1 following exposure to elicitors [213].

The discovery of FER in our AGB1 immunoprecipitate comprised the first evidence for a role of G proteins in signaling via this RLK. We confirmed FER and AGB1 physical interaction by BiFC [241]. We next discovered a new functional role of FER in stomatal regulation. Stomata are the microscopic pores in leaf surfaces through which plants take up CO₂ and release O₂ and H₂O to the atmosphere. Stomatal apertures are regulated by osmotic swelling and shrinking of a pair of guard cells that surround and delineate each pore. We found that the known FER ligand RALF1 [215,242] promotes stomatal closure and inhibits stomatal opening in wild-type guard cells [188]. We then demonstrated that AGB1 and FER interact genetically in this response: stomata of *agb1* mutants are essentially insensitive to RALF1, with epistasis verified by identical RALF1 insensitivity phenotypes of the *fer* single mutant and the *fer agb1* double mutant. As expected for a response mediated by the Gβγ dimer, we observed a similar lack of sensitivity to RALF1 in stomata of the triple Gγ mutant [188]. Interestingly, null mutants of the canonical Gα, GPA1, exhibited wild-type RALF1 responses, but the *xlg* triple mutant showed insensitivity to RALF1 in stomatal movements,

implicating XLGs rather than GPA1 as the G α s in the RALF1-regulated heterotrimer. These data demonstrate a functional role of G proteins in signal transduction from an RLK ligand and therefore implicate FER as a plant GPCR. Given an emerging role of FER in pathogen defense [213,240], this result might be linked to defense against infectious disease: bacteria invade plants through open stomata and guard cell sensing of MAMPs inhibits stomatal opening and provokes stomatal closure [243,244]. Interestingly, and analogous to observations with the maize RLK, FEA2, FER also couples to parallel G protein-independent pathways in some responses, as indicated by synergistic impacts of *fer* and *agb1* mutation in RALF1 modulation of salinity response and inhibition of root growth by the plant hormone ABA [73,188,210].

One group of plant RLKs that is important in pathogen defense responses consists of plant analogs of human Toll receptors in innate (MAMP-triggered) immunity [245]. Early work implicated the plant G α , GPA1, in signaling from the archetypal member of this RLK family, FLS2, showing that inhibition of stomatal opening and inward K⁺ channel activity by the FLS2 ligand, flg22, is impaired in *gpa1* mutants [244]. Null mutation of the G β subunit, AGB1, also impairs downstream defensive responses to flg22 as well as to the elicitor EF-Tu [70]; *gpa1* and *agb1* mutants show more bacterial colonization than wild type, consistent with their impaired stomatal closure [71].

Liang et al. [85] showed that the non-canonical G α , XLG2, is also involved in FLS2-mediated responses to flg22. XLG2, in a complex with AGB1 and AGG1/2, physically interacts with non-liganded FLS2, as well as with a downstream kinase, BIK1 (RLK BOTRYTIS-INDUCED KINASE 1). XLG2 and AGB1 binding of BIK1 in the absence of elicitor opposes proteasome-mediated degradation of BIK1, leaving the system poised for activation. Activation of FLS2 by the flg22 peptide elicitor causes heterotrimer dissociation and phosphorylation of XLG2 by BIK1; phosphorylated XLG2 then interacts with the NADPH oxidase RbohD and promotes production of reactive oxygen species as an integral component of the defense response.

In other genetic evidence of G protein involvement in RLK-mediated defense responses, null mutants of another defense-associated RLK, *BIR1* (*BAK1-INTERACTING RECEPTOR-LIKE KINASE1*), exhibit spontaneous cell death and seedling lethal phenotypes and these phenotypes are suppressed when *AGB1* [47,246] or *XLG2* [246] is mutated. In contrast with the previous examples where RLKs and G proteins show similar phenotypes, suggestive of receptor-mediated G protein stimulation, BIR1 and XLG2/AGB1 are antagonistic within the cell death pathway. Therefore, we hypothesize that RLK-mediated phosphorylation can either stimulate or inhibit G protein signaling, depending on the positions phosphorylated. The G α , GPA1, and G γ s, AGG1, and AGG2 also physically interact [72] with BIR1, as well as with the BRI1 co-receptor BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE), and with CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1) which perceives oligosaccharide elicitors. BAK1 acts as a co-receptor for a variety of RLKs including FLS2, BRI1, and BIR1 (reviewed by Kim et al. [247]) and has recently been found to play a role in RALF signaling [248] and to complex with FER [213]. BAK1 therefore plays a role in multiple diverse processes that overlap with known G protein functions (e.g. pathogen recognition, hormone signaling, and RALF signaling). Indeed, BAK1 and BRI1 were shown to phosphorylate

GPA1 [107], RGS1 [249], AGB1, and the AGGs [250] *in vitro*, consistent with diverse physiological and developmental roles of G proteins identified largely from genetic studies. One caveat is that the results on AGB1 and the AGGs [250] should be treated with caution, since these proteins were not recombinantly expressed and purified in the functional dimeric (G $\beta\gamma$) form but rather as individual subunits, which could result in subunit misfolding and artifactual kinase susceptibility. It should also be noted that Li et al. [107] recombinantly expressed the complete cytoplasmic domains of 70 Arabidopsis RLKs and found that 18 were capable of phosphorylating the G α subunit, GPA1, *in vitro*, further demonstrating direct phosphorylation of G proteins by RLKs.

In summary, the evidence that G protein subunits are substrates for RLK kinases is strong, but the field is still in its infancy. Demonstration of direct phosphorylation of plant G protein subunits by RLKs, coupled with *in vivo* genetic and phosphoproteomic studies, is likely to become an exciting frontier in G protein research.

Future directions

As is evident from this review, the impact of subunit phosphorylation in control of G protein signaling has not yet been the subject of comprehensive study in any of the model organisms discussed herein. However, given that biochemical and phosphoproteomic investigations now unequivocally confirm widespread and dynamic *in vivo* phosphorylation of G protein subunits, the functional roles of this PTM are ripe for elucidation. Based on clues from early work with mammalian kinases such PKA and PKC, it seems likely that ligand-induced phosphorylation of G protein subunits will be found to control the G protein cycle and provide specificity to subunit–effector interactions. Such proposed mechanisms are testable with a combination of phosphoproteomics to identify kinase target sites, site-specific mutations to create phosphomimic and phosphonull versions of G protein subunits whose biochemical activity can be interrogated, *in vivo* protein–protein interaction assays to assess resultant impacts on heterotrimer assembly and effector binding, and *in vivo* assays of ensuing effector activity and downstream phenotypic consequences. Anticipated tissue specificity of kinase, G protein, and effector expression, as well as tissue-specific ligand availability will likely add additional layers of complexity.

Studies in yeast, plants, and other non-mammalian model species [251,252] both provide new insights into conserved regulatory systems and highlight novel points of attack that can be incorporated into the design of new strategies to manipulate human G proteins for the benefit of human health. The conservation of G α phosphosites across the vast evolutionary distance between plants and animals raises the hypothesis that in metazoans, the Pelle and IRAK kinases, which are related to RLKs [49,50] but lack a transmembrane domain, may serve a transactivation role, coupling non-7TM ligand receptors to G protein subunits in a phosphorylation-dependent manner. Phosphoregulation of G protein subunits is also likely to be relevant to informing the mode of action of RTKs [120]. With regard to new clinical interventions, biased signaling from phosphorylated subunits, as first elucidated in model systems, could form the basis for medical approaches designed to deliberately impose such biased signaling in repair of genetic dysfunction.

Investigation of the mechanisms by which kinases phosphorylate G proteins will also be of interest. While some G protein phosphosites are readily accessible (e.g. $G\alpha_{15}$ S336, GPA1 Y166, N-terminal $G\beta$, and $G\gamma$ sites), and others are close to the protein surface and presumably could be phosphorylated with a moderate amount of backbone flexibility (e.g. $G\alpha_{11}$ S154 and AGB1 T271), some sites such as $G\alpha_{11}$ S44/GPA1 S49, $G\beta_{1-4}$ S72, and AGB1 S267 are buried internally. Mechanisms such as co-translational phosphorylation or protein unfolding (potentially itself a result of phosphorylation events) may be required to achieve phosphorylation of these sites.

Finally, it will be of interest to assess phosphorylation as a plausible mechanism that regulates other players which affect the G protein cycle. Given the numerous cytosolic GDIs, GEFs, and arrestins that control G protein signaling duration and intensity in mammals, it seems likely that these accessory proteins are also targets of regulatory kinases. Clues may again be provided from plant systems; for example, several recent reports have implicated RLK phosphoregulation of plant RGS [249,253]. In conclusion, this review of the literature on G protein subunit phosphorylation from the 1990s to the present exemplifies the adage 'everything old is new again.' We can anticipate many new insights and scientific surprises from the revitalization of this field.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

7TM	seven-transmembrane domain
ABA	abscisic acid
BAK1	<i>BRI1-ASSOCIATED RECEPTOR KINASE</i>
BiFC	bimolecular fluorescence complementation
BIK1	BOTRYTIS-INDUCED KINASE1
BIK1	RLK BOTRYTIS-INDUCED KINASE 1
BIR1	BAK1-INTERACTING RECEPTOR-LIKE KINASE1
CKs	casein kinases

Elm1	Elongated morphology 1
FER	FERONIA
GAP	GTPase accelerating
GDI s	guanine nucleotide dissociation inhibitors
GEF s	guanine nucleotide exchange factors
GPCR s	G protein-coupled receptors
GRK s	G protein-coupled receptor kinases
IP	immunoprecipitate
IRK	insulin receptor kinase
MAMP s	microbe-associated molecular patterns
MAPK	mitogen-activated protein kinase
MATα	mating-type α cells
MATa	mating-type a cells
PAK	p21-activated protein kinase
PKA s	protein kinase A
PKC s	protein kinase Cs
PLC	phospholipase C
PLD	phospholipase D
PTM s	post-translational modifications
RALF	Rapid Alkalinization Factor
Reg1	Resistance to glucose repression 1
RGS	regulator of G protein signaling
RLK s	receptor-like kinases
RPK2	<i>RECEPTOR-LIKE PROTEIN KINASE2</i>
RTK s	receptor tyrosine kinases
S/T	serine/threonine
Snf1	Sucrose nonfermenting 1
STE	sterile
XLG s	extra-large Gas

YpkA*Yersinia* protein kinase A**References**

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Figure 1. The classical G protein signaling paradigm, elucidated in yeast and metazoans. The inactive heterotrimer, consisting of $G\alpha$ -GDP and $G\beta\gamma$, associates at a plasma membrane spanning receptor. Upon ligand-mediated activation, the GEF activity of the receptor stimulates the exchange of GDP for GTP by the $G\alpha$ subunit, dissociation of $G\alpha$ from $G\beta\gamma$, and thus active signal transduction to effectors (E1 and E2) by $G\alpha$ -GTP and $G\beta\gamma$. Signaling termination is achieved by GTP hydrolysis to GDP by the intrinsic GTPase activity of the $G\alpha$ subunit, a process that can be expedited by the activity of an RGS protein, and the complex returns to the inactive conformation.

Figure 2. Features and key conserved phosphosites of $G\alpha$ subunits mapped onto the structure of the Arabidopsis $G\alpha$ subunit, GPA1.

(A) G1–5 binding motifs, switches I–III (SI–III), and the helical domain indicated on a schematic of the GPA1 protein. (B–D) The empirically derived structure of GPA1 bound to $GTP\gamma S$ (PDB: 2XTZ) [13] color-coded by feature. Blue corresponds to the Ras domain, purple to the helical domain, and yellow indicates the bound $GTP\gamma S$ molecule. (B) The G1–5-binding motifs are highlighted (G1 = orange, G2 = green, G3 = red, G4 = aqua, and G5 = pink). In (C), the switch regions I–III are highlighted (switch I = orange, switch II = green, and switch III = red) and GPA1 is rotated 180° around (x -axis) and tilted 20° back (y -axis) to show the reverse side, compared with the view presented in (B) and (D). (D) Key conserved and experimentally verified phosphosites between humans and Arabidopsis, namely GPA1 S49 in the Ras domain/G1 motif (red) and Y166 in the helical domain (white) are highlighted.

Figure 3. Implicated mechanisms of phosphorylation-mediated regulation of G protein signaling. (A) Effector kinase-mediated feedback; phosphorylation of the $G\alpha$ subunit by a downstream kinase (E-K) impairs heterotrimer formation, while $G\beta\gamma$ -binding inhibits, likely sterically, $G\alpha$ phosphorylation by E-K. (B) Phosphorylation as a determinant of effector coupling efficiency; phosphorylation of the G protein subunit, in this example $G\alpha$, increases the affinity for an effector. (C) Phosphorylation-mediated biased signaling; the unphosphorylated G protein subunit, in this example $G\beta\gamma$, strongly activates effector 1 (E1), but only weakly activates effector 2 (E2); upon phosphorylation, signaling dynamics are altered such that E2 becomes the primary activated effector.

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Table 1

Summary of the genes encoding heterotrimeric G protein subunits in the model organisms focused on in this review: human, Arabidopsis, and yeast

Subunit	Genes	
Human		
G α	G α families	Family members
	G α_i	<i>HsGNAI1</i> (G α_{i1}), <i>HsGNAI2</i> (G α_{i2}), <i>HsGNAI3</i> (G α_{i3}), <i>HsGNAO</i> (G α_o), <i>HsGNAZ</i> (G α_z), <i>HsGNAT1</i> (G α_{t1}), <i>HsGNAT2</i> (G α_{t2}), <i>HsGNAT3</i> (G α_{t3})
	G α_s	<i>HsGNAS</i> (G α_s), <i>HsGNAL</i> (G α_{olf})
	G α_q	<i>HsGNAQ</i> (G α_q), <i>HsGNA11</i> (G α_{11}), <i>HsGNA14</i> (G α_{14}), <i>HsGNA15</i> [*] (G α_{15})
	G $\alpha_{12/13}$	<i>HsGNA12</i> (G α_{12}), <i>HsGNA13</i> (G α_{13})
G β	<i>HsGNB1</i> (G β_1), <i>HsGNB2</i> (G β_2), <i>HsGNB3</i> (G β_3), <i>HsGNB4</i> (G β_4), <i>HsGNB5</i> (G β_5)	
G γ	<i>HsGNG1</i> (G γ_1), <i>HsGNG2</i> (G γ_2), <i>HsGNG3</i> (G γ_3), <i>HsGNG4</i> (G γ_4), <i>HsGNG5</i> (G γ_5), <i>HsGNG7</i> (G γ_7), <i>HsGNG8</i> (G γ_8), <i>HsGNG9</i> (G γ_9), <i>HsGNG10</i> (G γ_{10}), <i>HsGNG11</i> (G γ_{11}), <i>HsGNG12</i> (G γ_{12}), <i>HsGNG13</i> (G γ_{13})	
Arabidopsis		
G α	Canonical G α	<i>AtGPA1</i>
	Extra-large G α s	<i>AtXLG1</i> , <i>AtXLG2</i> , <i>AtXLG3</i>
G β	<i>AtAGB1</i>	
G γ	<i>AtAGG1</i> , <i>AtAGG2</i> , <i>AtAGG3</i>	
Yeast		
G α	<i>ScGPA1</i>	
G β	<i>ScSTE4</i>	
G γ	<i>ScSTE18</i>	

* Originally named GNA16, before it was determined to be the ortholog of the mouse GNA15 and renamed.

Table 2

G protein-related kinase families within the proteomes of humans, Arabidopsis, and yeast. In the cases of PKC and IRAK/RLKS, where there is a multi-fold proliferation of a family within one of the organisms, numbers of known proteins are indicated in parentheses

Kinase family	Humans	Arabidopsis	Yeast
Protein kinase C	PKCs (15)	Not found	PKC1 (1)
Protein kinase A	PKAs *	Not found	PKAs *
Interleukin-1 receptor-associated kinase/receptor-like kinase	IRAKs ** (4)	RLKs ** (600+)	Not found
Casein kinase 1	CK1s	CKs	YCKs
Casein kinase 2	CK2s ***	CK2s ***	CK2s ***
G protein-coupled receptor kinase	GRKs	Not found	Not found
Mitogen-activated protein kinase	MAPKs	MAPKs	MAPKs

* Tetramers of two catalytic and two regulatory subunits.

** Kinase domains of IRAKs and RLKs are identified in reciprocal BLAST searches. IRAKs uniquely contain an N-terminal death domain (DD) and RLKs uniquely contain N-terminal extracellular and transmembrane domains.

*** Tetramers of two α subunits (CKAs) and two β subunits (CKBs).

Table 3

G protein subunit phosphosites with known signaling impacts

Organism	Subunit	Phosphosite	Method ¹	Kinase (s)	Reported signaling implications of phosphorylation
Human	Gα ₁₂	S44/S144/S302	Phosphonull	PKC	Promotes morphine-induced μ-opioid receptor desensitization [86]
	Gα _z	S16	Phosphonull	PKA	Inhibits Gβγ-binding [87]
		S16/S27	Phosphonull	PKC	Inhibits Gβγ- and RGS-binding [87,88]
	Gα _s	Y37/Y391	Mapped to peptides	SRC	Promotes receptor-stimulated GTPγS-binding and GTP hydrolysis [89,90]
	Gα _q	S53	Mass spectrometry	YpkA	Impairs GTP-binding/Gα activation [91]
		Y356	Phosphonull	PMT	Potentiates agonist-stimulated activation of Gα _q and therefore PLC-β activation [92]
	Gα ₁₁	S154	Phosphonull	PKC?	Reduces agonist-stimulated
				CaMK? ²	PLC-β activation (agonist-induced desensitization mechanism) [93,94]
		Y356	Phosphonull	Tyr kinases	Reduces receptor (M1) interaction [95]
	Gα ₁₅	S336	Mapped to peptide and phosphonull	PKC	Promotes receptor (M2 and β ₂ AR) coupling [96]
Yeast	GPA1	T203	Phosphonull	PKA	Reduces Gα ₁₃ -mediated Rho activation [97]
		S2	Site-specific antibody	PKC	Increases Gα _o affinity and reduces stimulation of adenylyl cyclase II by Gβγ (no change to PLC-β stimulation) [98–100]
	GPA1	S200	Phosphonull	Elm1	Required for efficient ubiquitination of GPA1 [101]
	STE4	T320/S335	Phosphonull	Fus3? ³	Required for chemotropic growth towards pheromone sources (shmooing) [102–104]
	STE18	S7 (T2/S3?) ⁴	Mass spectrometry	Fus3? ³	Reduced Gβγ-mediated activation of Fus3 (negative feedback) [105,106]
	GPA1	Y166	Mass spectrometry	γ ⁵	Promotes RGS1 binding even when GPA1 is in the inactive GDP-bound form [107]
	XLG2	S141/S148/S150/S151	Mass spectrometry	BIK1 (FLS2?) ⁶	Required to promote flg22-stimulated ROS burst (occurs via RbohD) and pathogen resistance [85]

¹The method used to determine phosphorylation at the indicated site. Most often a phosphonull mutation was made at a predicted/suspected site and a decrease/abolition of phosphorylation was observed in the mutated subunit.

²Gα₁₁^{S154} was predicted to reside within PKC and CaMK consensus sites [93].

³Phosphorylation at these sites was abrogated in *fus3* mutants, indicating, but not conclusively proving, Fus3 as the responsible kinase [104,106].

⁴Phosphorylation of STE18 was identified at either T2 or S3 in addition to S7, so studies subsequently utilized STE18^{T2A/S3A/S7A} phosphonulls and STE18^{T2E/S3E/S7E} phosphomimics [105,106].

⁵BAK1 phosphorylates the wild-type GPA1, whereas reduced BAK1-mediated phosphorylation was observed for GPA1 mutants of the Y166 site. However, following a GPA1–BAK1 *in vitro* kinase reaction, no phosphorylation of Y166 was identified by tandem mass spectrometry. Therefore, it is unclear if BAK1 directly phosphorylates GPA1 at Y166, or if prior phosphorylation of Y166 potentiates BAK1 phosphorylation of GPA1.

⁶BIK1 was shown to directly phosphorylate XLG2 *in vitro*; however, XLG2 also couples to FLS2, an RLK. As XLG2 phosphorylation was observed *in vivo* in response to the FLS2 ligand, flg22, direct phosphorylation of XLG2 by FLS2 is also plausible.

Table 4

Summary of PKA phosphorylation sites of human heterotrimeric G protein sites as predicted by pkaPS (<http://mendel.imp.ac.at/sat/pkaPS/>)

Predicted sites	
Gα subunit	
HsGNAI1 (Gα _{i1})	S134
HsGNAI2 (Gα _{i2})	T317
HsGNAT2 (Gα _{t2})	S16 T181
HsGNAT3 (Gα _{t3})	S16
HsGNAZ (Gα _z)	S6 S16 ¹ S27 ¹ T48 S145
HsGNAS (Gα _s)	S84 S286 T335 ⁴
HsGNAL (Gα _{olf})	S256 ⁴ S273
HsGNAQ (Gα _q)	S65
HsGNA14 (Gα ₁₄)	S18 S61
HsGNA15 (Gα ₁₅)	S114 T189 S254 S271 S341 S362
HsGNA12 (Gα ₁₂)	S2 S9 S27 S40 ^{4,5} S161 T208 S309 S343
HsGNA13 (Gα ₁₃)	T87 S124 T203 ¹
Gβ subunit	
HsGNB1 (Gβ ₁)	T50 S98 S316
HsGNB2 (Gβ ₂)	S2 ^{4,6} T50 S98 S316
HsGNB3 (Gβ ₃)	T50 S98 S140 S316
HsGNB4 (Gβ ₄)	S2 ^{4,6} T50 S98 S316
HsGNB5 (Gβ ₅)	S30 T100 S190 S361 S371 S376
Gγ subunit	
HsGNG3 (Gγ ₃)	S35
HsGNG5 (Gγ ₅)	S2 ^{4,5} S29
HsGNG7 (Gγ ₇)	S2 ⁵ S29
HsGNG9 (Gγ ₉)	T25
HsGNG10 (Gγ ₁₀)	S2 ⁴ S29
HsGNG11 (Gγ ₁₁)	S69
HsGNG12 (Gγ ₁₂)	S2 ^{2,5} S7 ^{4,5} T17 S33 S36

G protein subunits lacking predicted PKA sites are not included.

¹Experimentally validated phosphosite in humans (targeted study/studies).

²Experimentally validated phosphosite in mice (targeted study/studies).

³Experimentally validated phosphosite in rats (targeted study/studies).

⁴Experimentally validated phosphosite in humans (high-throughput study/studies).

⁵Experimentally validated phosphosite in mice (high-throughput study/studies).

⁶Experimentally validated phosphosite in rats (high-throughput study/studies).

Experimentally validated phosphosites are mapped in Supplementary Files S1 (G α), S2 (G β), and S3 (G γ).

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Table 5

Summary of RLKs discussed in this review, with known roles and ligands indicated

Name	AGI #	Roles	Ligands
ER (ERECTA)	AT2G26330	Organ [192,193] and stomatal [194] development	EPF peptides [195]
Fasciated Ear 2 (FEA2)	N/A — Maize CLV2	Meristem and organ development [196]	CLE peptides [197]
CORYNE (CRN)	AT5G13290	Meristem and organ development — pseudokinase that lacks an extracellular ligand-binding domain [198] but localizes CLV2 to the plasma membrane [199]	CLV2 performs ligand perception [199]
CLAVATA 1 (CLV1)	AT1G75820	Meristem and organ development [200]	CLE peptides [201]
CLAVATA 2 (CLV2)	AT1G65380	Meristem and organ development [202] — contains an extracellular LRR domain, but lacks a kinase domain [203]	CLE peptides [204]
Receptor-like Protein Kinase 2 (RPK2)	AT3G02130	Involved in CLE peptide signaling, possible member of CLV1 receptor complex [205]	CLE peptides? [206], Co-receptor for CLE peptides?
Zygote Arrest 1 (ZAR1)	AT2G01210	Asymmetric division of the zygote [207]	Unknown
PEP1 Receptor 1 (PEPR1)	AT1G73080	Amplification of innate immunity responses [208]	AtPep1 [208]
FERONIA (FER)	AT3G51550	Fertilization [209], salinity tolerance [210–212], pathogen resistance [213], stomatal movement [188,214]	RALF peptides [215]
MDISI-Interacting Receptor-Like Kinase 2 (MIK2)	AT4G08850	Directional growth of the pollen tube towards the ovule [216], salt tolerance [217], cell wall integrity sensing [218]	LURE peptides [216]
AT5G39000	AT5G39000	CrRLK family	Unknown
RKL1	AT1G48480	Unknown	Unknown
AT5G63410	AT5G63410	Unknown	Unknown
Flagellin-Sensitive 2 (FLS2)	AT5G46330	Pathogen-associated molecular pattern (PAMP) perception [219]	flg22 [219]
EF-Tu Receptor (EFR)	AT5G20480	PAMP perception [220]	EF-Tu [220]
BRI1-Associated Receptor Kinase 1 (BAK1)	AT4G33430	Co-receptor * [221]	N/A
BAK1-Interacting Receptor-Like Kinase (BIR1)	AT5G48380	Repression of cell death pathways [222]	Unknown
Brassinosteroid Insensitive 1 (BRI1)	AT4G39400	Brassinosteroid perception [223]	Brassinosteroids [224]
Chitin Elicitor Receptor Kinase 1 (CERK1)	AT3G21630	PAMP perception [225]	Chitin [226]

Abbreviations: EPF, epidermal patterning factor; CLE, CLAVATA3 (CLV3)/EMBRYO-SURROUNDING REGION (ESR); RALF, Rapid Alkalization Factor; flg22, flagellin22; EF-Tu, Elongation Factor-Thermo unstable; LRR, leucine-rich repeat.

* BAK1 is a co-receptor for several RLKs (including FLS2, BIR1, FER, and BRI1) and therefore has been implicated in several diverse responses, as is discussed in the text.