Tyrosine phosphorylation switching of a G protein

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ABSTRACT

Heterotrimeric G protein complexes are molecular switches relaying extracellular signals sensed by G protein–coupled receptors (GPCRs) to downstream targets in the cytoplasm, which effect cellular responses. In the plant heterotrimeric GTPase cycle, GTP hydrolysis, rather than nucleotide exchange, is the rate-limiting reaction and is accelerated by a receptor-like regulator of G signaling (RGS) protein. We hypothesized that posttranslational modification of the Gα subunit in the G-protein complex regulates the RGS-dependent GTPase cycle. Our structural analyses identified an invariant phosphorylated tyrosine residue (Tyr-166 in the Arabidopsis Gα subunit AtGPA1) located in the intramolecular domain interface where nucleotide binding and hydrolysis occurs. We also identified a receptor-like kinase that phosphorylates AtGPA1 in a Tyr-166–dependent manner. Discrete molecular dynamics simulations predicted that phosphorylated Tyr-166 forms a salt bridge in this interface and potentially affects the RGS protein–accelerated GTPase cycle. Using a Tyr-166 phosphomimetic substitution, we found that the cognate RGS protein binds more tightly to the GDP-bound Gα substrate, consequently reducing its ability to accelerate GTPase activity. In conclusion, we propose that phosphorylation of Tyr-166 in AtGPA1 changes the binding pattern with AtRGS1 and thereby attenuates the steady-state rate of the GTPase cycle. We coin this newly identified mechanism “substrate phosphoswitching.”
The heterotrimeric G protein complex functions as a molecular switch that couples extracellular signals sensed by cell-surface G protein-coupled receptors (GPCR) to downstream targets in the cytoplasm that affect cellular behavior. The G protein complex contains Ga, Gβ, and Gγ subunits; the Ga subunit binds guanine nucleotides. When Ga binds GTP, the complex becomes active by separating into a conformationally-different Ga subunit and a released Gβγ dimer, each able to initiate downstream signaling through interaction with cellular targets called effectors. After the bound GTP is hydrolyzed to GDP by the intrinsic GTPase activity of Ga, the heterotrimeric complex reforms its inactive GDP-bound complex (resting state). This process is termed the GTPase cycle. The GTPase cycle is modulated by three known classes of proteins: GTPase-Accelerating Proteins (GAP, e.g. Regulator of G Signaling [RGS] proteins (1, 2)), Guanine-nucleotide Exchange Factors (GEF, e.g. GPCRs), and the Guanine nucleotide Dissociation Inhibitor (GDI). In animals, but not plants and protists, nucleotide exchange is the rate limiting step of the GTPase cycle (1). Unlike Ga homologs in metazoans and yeast, plant and protist Ga subunits spontaneously exchange guanine nucleotide, therefore they self-activate without the need of a GPCR or other GEF (3, 4). Consequently, the rate-limiting step is the GTP hydrolysis reaction. In the genetic model Arabidopsis thaliana, acceleration of GTP hydrolysis by the canonical Ga subunit, AtGPA1, is catalyzed by the prototype 7-transmembrane RGS protein, AtRGS1 (4–7). In the current model, AtRGS1 maintains G protein in the resting state by its GAP activity (8, 9). While sustained activation does occur through decoupling AtRGS1 from AtGPA1 by phosphorylation-dependent AtRGS1 endocytosis (10, 11), this cannot be the main mechanism because AtRGS1 endocytosis itself requires prior G protein activation (10).

Therefore, this leaves three possible mechanisms by which AtRGS1 keeps the GTPase cycle in the resting state: 1) Accelerated hydrolysis at a rate faster than guanine nucleotide exchange alone shifts AtGPA1GDP to the predominant state in the GTPase cycle 2) the GTPase cycle lingers or stops at the AtGPA1GDP state, 3) or both a combination of hydrolysis and cycling control. In animals, phosphorylation of the Ga subunit affects its activity. Tyrosine phosphorylation by pp60src or epidermal growth factor receptor increases Ga, GTPγS binding and GTPase activity (12–14). Serine phosphorylation by p21-activated protein kinase (PAK)-1 or PKC decreases the affinity of Ga to its Gβγ subunit, and confers Ga resistance to GAP proteins (15–17). While none of these reported phosphorylation sites are conserved in plant Ga subunits such as AtGPA1, reversible phosphorylation as a regulatory mechanism is not excluded. This prospect is explored here.

Based on acceptor specificity, plant kinases are classified into serine/threonine, and serine/tyrosine dual-specificity kinases. Despite the Arabidopsis genome lacking canonical tyrosine kinases (18), there are: 1) canonical phosphotyrosine phosphatases (19–21), 2) phosphotyrosine PTMs (22–24), and 3) Ser/Thr receptor kinases that have dual-specificity to tyrosine (25, 26). Specifically, some members of the Leucine-Rich-Repeat Receptor-Like Kinase (LRR RLKs) family phosphorylate tyrosine residues (27). The Arabidopsis genome encodes at least 223 LRR RLKs (28) and several of these LRR RLKs participate in the regulation of the same plant growth, defense, and stress pathways (29–34) as do the G protein components (35, 36). Among the LRR RLK family members, BR11 and BAK1 have dual-specificity, which initiate brassinosteroid signaling by auto- and trans-phosphorylation (37). BAK1 also phosphorylates AtRGS1 and regulates G protein signaling in...
response to stimulation by the Pathogen-Associated Molecular Pattern flagellin peptide 22 (flg22) (10).

AtGPA1 phosphorylation at Y_{166} is induced by several plant hormones (38). We show that the corresponding Y_{166} phosphorylation mimetic alters how AtRGS1 acts upon its substrate AtGPA1. Y_{166} phosphorylation significantly slows the rate of the GTPase cycle. We coined this newly-described substrate-enzyme relationship “substrate phosphoswitching”.

RESULTS

SAPH-ire predicts that AtGPA1 Y_{166} is an important phosphorylation site in one of three major clusters in the G\alpha protein family.

Structural analysis of PTM hotspots (SAPH-ire) is a quantitative informatics method to prioritize the function potential of co-aligning PTMs based on protein sequence and structural features. In brief, multiple sequence and structural features including PTM frequency across all eukaryotes, PTM residue conservation, protein interface residence, solvent accessible surface area, as well as nearest neighbor features, are integrated through a neural-network model trained on MAPs with known biological function. As a result, each MAP receives a probability score that can be used to rank-order the MAP from low to high function potential (low to high probability score, respectively). Previous results showed that SAPH-ire is highly effective for identification of functional PTM hotspots, and was validated both computationally as well as empirically (39, 40).

Nearly 160 PTMs, comprising 70 clusters designated Modified Alignment Positions; MAPs (MAPs), were experimentally observed in the protein family (Fig. S1) represented specifically by G\alpha subunits (IPR001019) – the vast majority of which are due to phosphorylation (39, 40). Y_{166} of AtGPA1 occurs within MAP IPR001019-1010, which exhibits several features consistent with functional PTM hotspots including multiple observations of phosphorylation, moderate solvent accessibility, a high degree of sequence identity between family members, and the presence of neighboring MAPs (39). Consequently, SAPH-ire ranks the function potential of IPR001019-1010 as the 22nd out of 70 MAPs (approx. 30th percentile) in the G\alpha protein family, above which ~60% of all MAPs with known regulatory function are ranked (Fig. 1A).

Surveying from N- to C-terminus the density of MAPs along the length of a protein family reveals regions of local structure that exceed the average density of modification for the family – a characteristic that is common for functional MAPs (39). Therefore, we compared the MAP cluster density for every MAP between the N- and C-terminus of the G\alpha family – reporting the number of total MAPs contained within +/- 2 residues of each modified position (Fig. 1B). We observed three distinct MAP clusters in the family: The base of the N-terminal \alpha-helix (\alphaN) that interfaces with receptors (AP_{682-AP_{685}}), the P-loop that is a well-conserved structure that is necessary for nucleotide/phosphate coordination (AP_{761-AP_{771}}), and the \alphaE helix that exhibits an uncharacterized phosphorylation cluster that includes AtGPA1 Y_{166} (AP_{1009-AP_{1019}}) (Fig. 1B). In addition to Y_{166}, the \alphaE helix is phosphorylated in several different positions across mouse and human G\alpha subunits, including mG\alphaS2 (pY_{177}), mG\alpha14 (pY_{156}), hG\alpha11 (pT_{162}), and mG\alpha0 (pS_{159}) (41–44).

The functional relevance of phosphorylation sites in the \alphaE helix has not yet been determined. MAP IPR001019-1010 exhibits 100% identity across all family
members, indicating that substitution at this site is evolutionarily constrained (Fig. 2A, middle panel). In addition to AtGPA1Y\textsubscript{166}, phosphorylation of mouse G\textalpha\,2\,Y\textsubscript{177} (equivalent to Arabidopsis Y\textsubscript{167}), was also observed experimentally in more than 10 independent high-throughput experiments across human, mouse, and rat (44). Despite having high sequence conservation, high PTM observation frequency or measured occurrence in diverse organisms, Y\textsubscript{166} (nor homologous sites in mammalian G\textalpha\, subunits) was not functionally characterized at the onset of this study. Given that Y\textsubscript{166} is remote from switch II and the G\textbeta\, subunit binding interface (Fig. 2B, left), we presumed it is unlikely that its phosphorylation directly affects AtGPA1 binding to the G\textbeta\gamma subunit. Rather, Y\textsubscript{166} is located in the intramolecular domain interface for GTP hydrolysis (Fig. 2B, right and Fig. 3A), suggesting that phosphorylation at this site may have an effect on GTPase activity, either intrinsic or accelerated by AtRGS1. Indeed, the corresponding residue of AtGPA1Y\textsubscript{166}, bovine G\textalpha\,t Y\textsubscript{150}, participates in guanine ring binding (45). Although such a structural important residue might belong to a rigid local structure, nonetheless phosphorylation occurs at or near this position in the \textalpha\,E helix in other G subunits (41–44) indicating that the \textalpha\,E helix is a prominent, yet uncharacterized, phosphorylation hotspot occurring in plants and animals. We hypothesized that phosphorylation of Y\textsubscript{166} within this region plays a regulatory role in the function of the G\textalpha\, subunit.

**DMD simulations predict pY\textsubscript{166} salt bridging at the intramolecular domain interface.**

Phosphorylation changes protein structure and consequently affects protein stability or catalytic activity. We hypothesized that phosphorylation of Y\textsubscript{166} changes AtGPA1 conformation. The local effect of phosphorylation is to change a neutral amino acid to a negatively charged amino acid. We expected that the major effects of phosphorylation would be evident in the formation of new salt bridges. To test our hypothesis *in silico*, we performed discrete molecular dynamics (DMD) simulations of phosphorylated and nonphosphorylated AtGPA1. We computed distance distributions between the phenolic oxygen of Y\textsubscript{166} and positively charged groups in nearby amino acids (Fig. 3A), and compared the results from simulations of phosphorylated and unphosphorylated Y\textsubscript{166}, using a distance of ≤ 6 Å as a metric for salt bridge formation. Positive charges at residue positions 56 and 288 (AtGPA1 numbering) are 100% conserved across eukaryotic G subunits; mostly conserved at position 173 and poorly conserved at position 294, although a lysine at position 295 is 100% conserved (Fig 2A). Predicted salt bridges formed between R\textsubscript{173} and pY\textsubscript{166} and R\textsubscript{173} and the phosphomimetic mutation Y\textsubscript{166}→E (Fig. 3G and 3K), but not unphosphorylated Y\textsubscript{166} or the Y\textsubscript{166}→D mutant (Fig. 3C and 3O). The small population of short distances between Y\textsubscript{166} and R\textsubscript{173} (Fig. 3C) may be the result of hydrogen bonding between Y\textsubscript{166} and R\textsubscript{173}, however the effect was noticeably less pronounced than for either pY\textsubscript{166} or Y\textsubscript{166}E. Both phosphorylated and unphosphorylated Y\textsubscript{166} interact with K\textsubscript{288} (Fig. 3D and 3H). However, while the Y\textsubscript{166}E mutation promoted interaction with K\textsubscript{288}, the Y\textsubscript{166}D mutation disrupted interaction with K\textsubscript{288} (Fig. 3L and 3P). Moreover, K\textsubscript{288} forms a hydrophobic interaction with the nucleotide and therefore its rotation away to form the salt bridge would be expected to alter the intrinsic exchange rate (46, 47). As will be shown later, a phosphomimetic did not alter the intrinsic exchange rate, therefore this potential pY\textsubscript{166}-K\textsubscript{288} salt bridge is given low probability. A salt bridge formed between K\textsubscript{294} and pY\textsubscript{166} or Y\textsubscript{166}E (Fig. 3I and 3M), but
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not unphosphorylated Y₁₆₆ or Y₁₆₆D (Fig. 3E and 3Q). Finally, a salt bridge formed between K₅₆ and pY₁₆₆ (Fig. 3F) but not between K₅₆ and unphosphorylated Y₁₆₆, Y₁₆₆E or the Y₁₆₆D mutant (Fig. 3B, 3J, and 3N). On the basis of these simulation results, we predict that AtGPA1 Y₁₆₆E mimics pY₁₆₆, while the Y₁₆₆D substitution is insufficient to form salt bridges. All implicated residues except K₂₉₄ are conserved (Fig. 2A), emphasizing the importance of the potential function elicited by formation of these salt bridges. This predicted distinction between the two mimetic forms is tested below.

Amino acid residues that potentially form salt bridges with pY₁₆₆ face toward the interface between the helical and Ras domains (Fig. 3A) prompting the hypothesis that phosphorylation changes the local but not the global conformation of AtGPA1. To test this hypothesis, circular dichroism (CD) spectroscopy was employed to measure the secondary structure formation (Fig. 4A). The two minimum peaks appeared near 208 nm and 222 nm indicated that all of the mutant proteins contained the characteristic wild type helical structure. The mutations also showed comparable thermal stability with wild type AtGPA1, excluding aggregation caused by mutagenesis (Fig. 4B). Taken together, we conclude that the Y₁₆₆E mutation conserved the global structure of AtGPA1. GTP binding and hydrolysis rates that are at wild type levels which are described later, are further support that the point mutation does not affect global structure.

Identification of 18 AtGPA1 kinases, one requires Y₁₆₆ for AtGPA1 phosphorylation

To identify kinase(s) that phosphorylate a tyrosine residue on AtGPA1, we purified 70 recombinant LRR RLKs, containing the intracellular juxtamembrane, the catalytic kinase and the C-terminal domains (48). We biochemically screened these kinases for AtGPA1 substrate specificity (Fig. 5A and Fig. S2). No signal was found in [γ-³²P] ATP containing buffer (lane #1) or the “GST only” reaction (lane #2) indicating no contaminating kinases were present. Not all of the LRR RLKs showed autophosphorylation suggesting that: 1) some plant RLKs do not autophosphorylate, 2) the condition for the in vitro kinase assay was not optimal for every kinase to autophosphorylate, or 3) some LRR RLKs lost phosphorylation activity during purification. Among the 70 kinase domains, 18 transphosphorylated AtGPA1 under the conditions tested and not all of these kinases displayed autophosphorylation (Table S1). As shown in Fig. 5B, these candidates were predicted to be involved in many biological processes and functional categorization (49), including protein metabolism, developmental processes, response to stress, signal transduction, and abiotic or biotic stimulus, cell organization and biogenesis. The DAVID bioinformatics resource (50, 51) was used to analyze pathways shared by these LRR RLKs. Among them, LRR RLK family protein (PSY1R, AT1G72300, lane #19), LRR RLK family protein (AT5G37450, lane #66), LLR transmembrane protein kinase (GSO1, AT4G20140, lane #31), and PEP1 receptor 1 (PEPR1, AT1G73080, lane #20) are involved in protein tyrosine kinase signaling pathways, however, none of the four were previously reported to have tyrosine-protein kinase activity either in vivo or in vitro; BRI1-associated receptor kinase 1 (BAK1, AT4G33430, lane #12), LRR RLK family protein (BRI1, AT4G39400, lane #35), and somatic embryogenesis receptor-like kinase 1 (SERK1, AT1G71830, lane #18) are involved in the brassinosteroid mediated signaling pathway and are known dual-specificity kinases (27, 52, 53).

We chose brassinosteroid-associated kinase 1 (BAK1, AT4G33430, lane #12) to characterize not only because it is a known dual-specificity kinase (27, 54) but also
because it operates in G-protein coupled signaling (10, 55, 56). The stoichiometry of BAK1 phosphorylation of AtGPA1 (i.e. serine, threonine and tyrosine) was 5.5 moles P_i to 1.0 mole AtGPA1 as described in Experimental Procedures, indicating multiple phosphorylation sites on AtGPA1. The phosphotyrosine specificity of BAK1 on AtGPA1 was further confirmed using a phosphotyrosine antibody. BAK1 phosphorylated GST-tagged AtGPA1 (Fig. 5C, open triangle). There was no band at the corresponding position of the lanes with either GST-GPA1 only or BAK1 only, despite detectable BAK1 autophosphorylation (Fig. 5C, open circle) indicating an active BAK1 kinase in the reaction. The total amount of BAK1 was determined immunochemically using an antibody to the BAK1 poly-histidine tag (Fig. 5C, solid circle). GST and GST-AtGPA1 were detected by anti-GST tag antibody (Fig. 5C, solid square and solid triangle).

A requirement for Y_{166} for BAK1-mediated phosphorylation was confirmed by site-directed mutagenesis in combination with immune-detection using a phosphotyrosine-specific antibody. As shown in Fig. 5D, Y_{166} \rightarrow D/E mutagenesis reduced phosphorylation two-fold, confirming that Y_{166} is required for BAK1 phosphorylation of AtGPA1. SAPH-ire indicated that phosphorylation at Y_{166} has the largest functional potential. Nonetheless, we did not detect a pY_{166} tryptic peptide by tandem MS. This indicates that either: 1) Y_{166} is not directly phosphorylated by BAK1 or 2) that the pY_{166} tryptic peptide is not detected by tandem mass spectrometry. Because TiO_{2}-enriched tryptic peptides containing pY_{166} have been repeatedly detected (38), the latter is less plausible leaving the possibility that BAK1 does not directly phosphorylate Y_{166}. Because we have shown that Y_{166} is necessary for some BAK1 phosphorylation of AtGPA1 (Fig. 5D), we do not rule out the possibility that pY_{166} is required for other BAK1 phosphorylation sites.

The AtGPA1 phosphorylation mimetic mutation affects the RGS-dependent GTPase cycle.

Based on the predicted importance of pY_{166}, Y_{166} \rightarrow E mutagenesis was used as a phosphorylation mimetic protein in further experiments, while Y_{166} \rightarrow D mutagenesis was used as a negative control for reasons described above (Fig. 3). Nonetheless, we do not exclude the possibility that other phosphorylated AtGPA1 residues play functional roles, although the lack of conservation lowers the priority for investigation at this time.

The intrinsic single turnover and steady-state rates of GTP hydrolysis were unaffected by the Y_{166} \rightarrow D/E mutations (Fig. 6A and 6C, respectively). GTP hydrolysis accelerated by AtRGS1 was slightly reduced by the Y_{166} \rightarrow E mutation (Fig. 6B). At low concentration of AtRGS1 (~1:1 ratio AtRGS1:AtGPA1), GTP hydrolysis was about half for the phosphomimetic mutant compared to wild type. At a high concentration of AtRGS1, the reaction approached saturation.

Most informative is the effect of AtRGS1 on GTP hydrolysis at steady-state. The AtRGS1-accelerated steady-state GTP hydrolysis was reduced in the Y_{166} \rightarrow E mutant (Fig. 6D). As predicted (see Fig 3), the Y_{166} \rightarrow D mutant behaved like wild type AtGPA1 (Fig. 6D).

To understand the basis for this difference in steady-state cycling, we examined the nucleotide exchange rate using a GTP_{\gamma}S binding assay. In the absence of AtRGS1, GTP_{\gamma}S binding by the mutants were statistically indistinguishable from wild type (Fig. 6E). AtRGS1 (1 \mu M) slightly reduced the GTP_{\gamma}S binding rate nearly equally by both the wild type and mutant (Fig.
Thus, the strong reduction in AtRGS1-accelerated GTPase cycle by the phosphomimetic mutation was not caused an altered nucleotide exchange rate.

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As a modulator, AtRGS1 acts as a GAP to accelerate GTP hydrolysis by AtGPA1, therefore it reduces the free energy of the transition state for the GTPase reaction (4, 6). RGS proteins selectively bind Ga subunits in its transition state conformation which is mimicked by Ga·GDP aluminum tetrafluoride (Ga-GDP AlF₄⁻) (57). As previously shown (e.g. 6, 58), AtRGS1 preferentially binds the transition state of the wild type AtGPA1 (Fig. 7A, lane 3 vs lanes 1 and 2). However, AtRGS1 strongly bound both the transition state and GDP-bound form of the phosphomimetic Y₁₆₆E mutant AtGPA1 (Fig. 7A, lanes 4 and 6, Fig. 7B).

To confirm and quantitate these different binding affinities by an independent method, we used surface plasmon resonance with AtRGS1 immobilized on the chip and the wild type and the Y₁₆₆E mutant AtGPA1 as the analytes (Fig. 7C to 7K and Table 2). There was no specific binding between AtRGS1 and GDP- or GTPγS-bound AtGPA1 (Fig. 7C and 7D), while AtRGS1 bound to wild type AtGPA1 in its transition state with a binding affinity of 2.51 nM from kinetic analysis (Fig. 7E) and 4.83 nM from equilibrium analysis (Fig. 7J). Despite that there was no specific binding with the AtGPA1 Y₁₆₆E mutant in its GTPγS-bound state (Fig. 7G), AtRGS1 bound to the GDP-bound state with a binding affinity of 5.99 nM from kinetic analysis (Fig. 7F) and 23.4 nM from equilibrium analysis (Fig. 7J). This was similar to its binding affinity in the transition state which was 5.39 nM from kinetic analysis (Fig. 7H) and, 11.1 nM from equilibrium analysis (Fig. 7K). The affinities calculated from kinetic and equilibrium analyses were comparable and the Chi² values were small compared to the Rₘₐₓ, indicating these affinity constants were reliable. These result were perfectly consistent with the binding pattern shown by co-immunoprecipitation (Fig. 7A and B).

To test the effect of Y₁₆₆ phosphorylation on AtGPA1-AtRGS1 interaction in vivo, FRET experiments were conducted using a donor AtGPA1 with and without the Y₁₆₆ phosphomimetic mutation tagged with a C-terminal CFP and coexpressed with acceptor AtRGS1 protein (wild type, phosphorylation and GTPase mutants, 3SA and E320K respectively) with C-terminal YFP tag in N. benthamiana. As shown in Figure 5A (left side), there was no difference in FRET Efficiency % between wild type AtGPA1 donor and the wild type AtRGS1 vs. phosphorylation-dead mutant (3SA) FRET acceptors. As expected, mutations that disrupt the AtRGS1-AtGPA1 interactions brought FRET Efficiency % to its baseline value (~3.3-fold lower, P<0.039).

As shown in Figure 5A (right), as compared to wildtype AtGPA1, the Y₁₆₆ phosphomimetic mutation had a lower FRET Efficiency % indicating either a greater distance between the FRET pairs or a different orientation between AtGPA1 and wild type AtRGS1 (Fig. 8A, ~ 1.5 fold, P<0.047). The 3SA AtRGS1 mutation abrogated this decrease indicating that the phosphorylation state of AtRGS1 is also important for its interaction orientation with the AtGPA1 isoforms.

AtRGS1 phosphorylation by BAK1 is important for AtRGS1 endocytosis, G protein dynamics, and G protein activation in response to flg22 (59). AtGPA1 Y₁₆₆ phosphorylation shown here fits into this mechanism whereby increasing the AtRGS1-AtGPA1 transient complex consequently increases freed Gβγ dimer to recruit kinases that phosphorylate AtRGS1, necessary for its endocytosis and thus sustained activation.
In response to flg22, the AtGPA1-AtRGS1 alters orientation within 3 min (Fig. 8, right) (P < 0.032), however the donor AtGPA1 Y<sub>166</sub>E-AtRGS1 complex, appeared to be already in this altered orientation in the absence of flg22.

**SUMMARY**

We propose that phosphorylation on Y<sub>166</sub> of the substrate AtGPA1 changes the binding pattern with its enzyme, AtRGS1. As a consequence, the steady-state rate of the GTPase cycle attenuates. Phosphorylation of Y<sub>166</sub> increases the binding affinity of the GDP state of AtGPA1 to AtRGS1. Typically, RGS1 proteins only bind the GDP+Pi transition state of their cognate Gα subunit. This unprecedented change in AtRGS1-AtGPA1 binding pattern prompts an obvious question as to whether or not AtRGS1 theoretically behaves as a GDI to the phosphorylated Gα subunit.

This unprecedented change in AtRGS1-AtGPA1 binding pattern prompts an obvious question as to whether or not AtRGS1 theoretically behaves as a GDI to the phosphorylated Gα subunit. Typically, GDIs impair the dissociation rate of GDP from the Gα subunit thus preventing guanine nucleotide exchange and consequently attenuating the GTPase cycle (1). A statistical difference between wild type and the AtGPA1 Y<sub>166</sub> mutant was not observed here (Fig. 6E) as determined by GTPγS binding.

In animals, RGS proteins typically bind the switch region on their corresponding Gα proteins (60–64). The reported GAP resistant phosphorylation sites on Gα<sub>z</sub> were all at N terminus nearby the switch region(15–17). To our surprise, Y<sub>166</sub> is located in the intramolecular domain interface for GTP hydrolysis, spatially remote from the switch region(3). Thus, this leaves two possible mechanisms by which phosphomimetic mutagenesis on Y<sub>166</sub> affects the interaction: 1) AtRGS1 binds to different region on AtGPA1 where can be affected by the residue of Y<sub>166</sub>, 2) Y<sub>166</sub>→E mutant changes the conformation of switch region. Although we cannot exclude a novel RGS binding region exists on Arabidopsis Gα protein, given that both AtGPA1 and the RGS domain of AtRGS1 are conserved among the respective eukaryotic homologs (3, 6), the first hypothesis is less likely. Therefore, according to the molecular dynamics prediction (Fig 3), we propose that phosphorylation at Y<sub>166</sub> forms a salt bridge with positive residues near the guanine nucleotide binding pocket to serve as a sensor of the state of nucleotide binding and consequently affects AtRGS1 binding.

Definitive tests involve reconstitution of a full-length 7 transmembrane AtRGS1 protein with the other G protein complex subunits and await a method produce the full-length AtRGS1 protein. While, we developed a method by which the full-length protein can be expressed and purified from a cell-free system (65), it does not yet produce sufficient quantity to move forward at this time.

**EXPERIMENTAL PROCEDURES**

**Protein purification**

Briefly, His-tagged LRR RLKs-cDNA encoding the complete cytoplasmic domain was transformed into BL21 (DE3) pLysS cells(48) as previously described (10).

AtGPA1 mutants were made by QuikChange Lightning Site-Directed Mutagenesis™ Kit (Agilent Technologies, Cat. #210518) and cloned into the pDEST15 or pDEST17 for GST-tagged or His-tagged recombinant proteins. Wild type and mutant proteins were expressed and purified as described previously (10, 66). The purification procedure for GST-tagged AtGPA1 was similar to His-tagged AtGPA1 purification with minor modifications. To determine the specific activity of the protein, the concentration of purified AtGPA1 wild type and its
mutants were compared to total [γ-35S]GTPγS binding.

His-tagged and GST-tagged RGS+Ct (AtRGS1, residues from 284 to 459) were cloned into pDEST17 or pDEST15 destination vector as previously described (11). The procedure for expression and purification was similar to His-tagged and GST-tagged AtGPA1 purification except without GDP. Detailed information is provided in the SI Text.

**SAPH-ire analyses**

SAPH-ire is described in detail (39, 40) and was utilized here with only minor adjustments. Briefly, neural network models for function potential probability scores were generated using maximum observed solvent accessible surface area for each hotspot. PTM hotspot cluster analysis was accomplished by determining the count of neighboring hotspots within +/- 2 residues and including the hotspot in question. All PTM and structural data used for SAPH-ire corresponds to experimental values and strictly exclude predicted or putative information.

**DMD simulations**

The starting structure for all simulations was the previously published crystal structure for AtGPA1 (PDB code: 2XTZ). Simulations were performed using the Medusa force field(67). Phosphorylated tyrosine is not a natural amino acid included within the Medusa force field. For consistency, side chains for both phosphorylated and unphosphorylated Y166 in AtGPA1 were modeled as ligands. The Y166A mutant was modeled using the protein design platform Eris(68, 69). Eris was used to perform 100 independent Monte Carlo side chain optimizations for residues within 10 Å of the mutation site.

Note that we did not consider the formation of a cation-π interaction. The reason is two-fold. First the distance, while not unreasonable for cation-π interaction, is more consistent with H-bonding. The peak in question is centered at ~4 Å. The expected distance between the measured groups for H-bonding is also ~ 4 Å, while for a cation-π interaction the distance is ~ 5 Å. The second reason is that cation-π and aromatic stacking interactions are not typically well-captured by MD simulations.

For each system, energy minimization was performed according to the protocol implemented in Chiron (71). Systems were then equilibrated by linearly increasing the simulation temperature from 0.1 to 0.45 kcal mol⁻¹k_B⁻¹ over 100,000 steps of discrete molecular dynamics (DMD)(72, 73). Five replicate DMD simulations were performed for each system at a constant temperature of 0.45 kcal mol⁻¹k_B⁻¹. Each simulation was performed for 2 million steps (~100 ns).

**Circular dichroism (CD) spectroscopy**

The CD spectra of purified AtGPA1 and its mutations were collected with a Chirascan plus CD spectrometer at 20 °C from 185 nm to 260 nm in 0.5 nm scan steps in CD spectroscopy buffer (100 nM in 4.3 mM
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Na$_2$HPO$_4$, 1.4 mM NaH$_2$PO$_4$ and 50 mM Na$_2$SO$_4$, 50 µM GDP). The results were analyzed with Chirascan software.

**Protein thermostability**

Thermostability of AtGPA1 was analyzed by the method of Isom and coworkers (74). Briefly, 100 ng/µL AtGPA1 wild type or the mutants were incubated with 1 mM 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD; TCI America; A5597) in Tris buffer (20 mM Tris-HCl pH 7.6, 50 mM NaCl, 1 mM MgCl$_2$, 1 mM PMSF, 50 µM GDP). 20 µL aliquots were distributed into thin-walled 0.5 mL microfuge tubes, then placed in a gradient thermocycler (Biometra TProfessional Thermocycler), incubated for 3 minutes at the indicated temperatures. Then, the samples were aliquoted into a 384-well plate, and the fluorescence values were measured by BMG Labtech PHERAstar plate reader with excitation and emission bandpass filters of 400 and 500 nm. Per requirements of JBC, the quantitative results were expressed as individual curves.

**In vitro kinase assays**

To screen the potential kinase for AtGPA1, purified LRR RLKs was mixed with 1.25 µg RGS+Ct and 2.5 µg GST-AtGPA1 in 25 µL kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl$_2$, 10 mM MnCl$_2$, 1 mM DTT, 1 µg/ml leupeptin, 0.1 µM calycin A and 1 µM [γ-$^{32}$P] ATP (Perkin Elmer, NEG004Z250UC)) at ambient temperature. Previously, a set of 70 LRR RLK was screened for AtRGS1 phosphorylation (59). As described therein, we used purified His-tagged RGS + Ct as the positive control for the activity of each kinase. The reactions were quenched by 4 × Laemmli buffer and separated by SDS-PAGE. The phosphorylated proteins were detected by autoradiography.

To further confirm the transphosphorylation by BAK1, GST-AtGPA1 (8 µg) or GST were incubated with 1 µg His-BAK1 in 30 µL kinase buffer (50 mM Tris-BAK1 pH 7.5, 1 mM MgCl$_2$, 50 µM ATP, and 1 mM DTT) for 2 h at 25°C. The reactions were quenched by 4 × Laemmli buffer and separated by SDS-PAGE. The transphosphorylation and autophosphorylation on AtGPA1 and BAK1 were detected by anti-phosphotyrosine (Santa Cruz, PY99, sc-7020). GST-tagged or His-tagged protein were detected by polyclonal rabbit anti-GST antibody (Life Technologies, A5800) or mouse anti-His tag monoclonal antibody (Roche, clone BMG-His-1, Ref 11 922 416 001).

**AtGPA1 GTP hydrolysis rate**

For measuring the single-turnover GTP hydrolysis rate, purified His-AtGPA1 (wildtype or mutant) was preloaded with [γ-$^{32}$P] GTP (Perkin Elmer, NEG004Z250UC) in single-turnover GTPase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 1 mM DTT, and 0.05% Thesit) for 5 minutes on ice. The hydrolysis reaction was then started by adding an equal volume of GTP$\gamma$S containing buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 1 mM DTT, 0.05% Thesit and 400 µM GTP$\gamma$S) with or without His-tagged RGS cytoplasmic domain and move to a 20 °C water bath. At the indicated times, duplicate samples were denatured by mixing with 1 mL quench buffer (5% (w/v) activated charcoal, 50 mM phosphoric acid pH 2.0) to remove non-hydrolyzed [γ-$^{32}$P] GTP and proteins. The charcoal-treated samples were centrifuged and the amount of [γ-$^{32}$P] in solution was measured by scintillation counting.

The steady-state hydrolysis rate of AtGPA1 was measured as previously described with some modification (4, 8). Briefly, purified His-AtGPA1 (wild type or mutant) was preloaded with [γ-$^{32}$P] GTP...
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(Perkin Elmer, NEG004Z250UC) in steady-state GTPase buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, and 0.05% Thesit) at 20 ºC for 5 min. Then, the reaction was started by adding an equal volume of 20 mM MgCl₂ containing buffer with or without His-tagged RGS + Ct. At the indicated time points, 1 mL ice-cold quench buffer (5% (w/v) activated charcoal, 50 mM phosphoric acid pH 2.0) was added to remove non-hydrolyzed [γ-³²P] GTP and proteins. The charcoal-treated samples were centrifuged and the amount of [γ-³²P] in solution was measured by scintillation counting.

[γ-³⁵S] GTPγS binding

The rate of GTPγS binding was measured by [γ-³⁵S] GTP (Perkin Elmer, NEG030H250UC) (9). The entire procedure was performed on ice. Purified His-AtGPA1 (wild type or mutant) was mixed with GTPγS binding buffer (50 mM Tris-HCl pH 7.0, 5 mM MgCl₂, 1 mM EDTA, and 5 µM GTPγS (~ 5,000 cpm/pmol)) with or without His-tagged RGS + Ct. The reaction was quenched at the indicated time points by quench buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 25 mM MgCl₂, and 50 µM GTP) and vacuum-filtered onto 0.45 µm nitrocellulose membrane (BIO-RAD, 1620115) by 1225 Sampling Manifold (Millipore, XX2702550). The filter was washed 3 times with 3 mL quench buffer and dried thoroughly. The [γ-³⁵S] bound to the filter was quantified by scintillation counting.

In vitro pull-down assays

3 µg His-tagged AtGPA1 (wild type or Y₁₆₆E mutant) was incubated with 3 µg of GST-tagged RGS + Ct in 500 µL pull down buffer (25 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM DTT, 10 mM MgCl₂), in the presence of either 50 µM GDP, 50 µM GTPγS or AlF₄⁻ (50 µM GDP, 10 mM NaF and 100 µM AlCl₃). GST-tagged RGS + Ct were precipitated by glutathione sepharose 4B (GE Healthcare Life Sciences, Product code: 17075601) and washed 3 times with pull down buffer. The precipitated proteins were eluted by Laemmli sample buffer and separated by SDS-PAGE gel and detected by polyclonal rabbit anti-GST antibody (Life Technologies, A5800) or mouse anti-His tag monoclonal antibody (Roche, clone BMG-His-1, Ref 11 922 416 001).

Surface Plasmon Resonance (SPR)

Binding affinity constants were measured by the ProteOn XPR36 protein interaction array system and a ProteOn™ GLC Sensor Chip (BIO-RAD #1765011). GST-tagged RGS+Ct (AtRGS1, residues from 284 to 459) was immobilized as ligand. His-tagged AtGPA1 wild type or Y₁₆₆E mutant was dosage diluted by running buffer (10 mM HEPES pH 8.0, 150 mM NaCl, 10 mM MgCl₂, 3 mM EDTA, 10 µM GDP and 0.01% Tween-20) in the absence or presence of 10 µM GTPγS or AlF₄⁻ (50 µM GDP, 10 mM NaF and 100 µM AlCl₃) as analyte. Both association and dissociation were performed for 300s at a flow rate of 50 µl/min at 25 ºC. Kinetic analysis was performed by fitting the original curves with a 1:1 Langmuir binding model. The affinity constants were also calculated by equilibrium analysis.

Förster Resonance Energy Transfer (FRET) analyses

Transient expression in N. benthamiana for FRET was performed as described by Tunc-Ozdemir and Jones (75). Briefly, Agrobacterium carrying a binary plasmid encoding either AtRGS1-YFP, AtRGS13SA-YFP, AtRGS1E320K AtGPA1-CFP, AtGPA1Y₁₆₆E-CFP and RNA silencing suppressor P19 were infiltrated into N. benthamiana leaves’ abaxial side with a needleless syringe. Here, the AtGPA1-CFP coding cDNA was carried by pEG100. The coding region of the CFP was inserted between amino acids 97 and 98 of AtGPA1.
For acceptor photobleaching FRET, 514-nm and 458-nm argon lasers were used to excite YFP (acceptor) and CFP (donor) respectively. Acceptor and donor channels’ emissions were detected within the range of 516–596, 460–517 nm respectively. Region of interests were scanned 5 times (each for 50 iterations) using a 514-nm argon laser line at 100% intensity with a pinhole diameter set to 1.00 airy units. Acceptor was photobleached until it reached ~20–30% of its initial value. FRET efficiency was then estimated via Zen Software (http://www.zeiss.com/microscopy/en_de/downloads/zen.html). Regions of interests with similar intensity levels to start with were selected and samples with decreased donor fluorescence intensity after bleaching were excluded in the calculations:

\[
\text{FRET eff\%} = \frac{\left( (F_{Dpost} - F_{\text{bckgrnd post}}) - (F_{Dpre} - F_{\text{bckgrnd pre}}) \right)}{F_{Dpost} - F_{\text{bckgrnd post}}} \times 100
\]

\text{eq 1}

where:

- \( F_{Dpost} \): Fluorescence intensity of donor after photobleaching
- \( F_{\text{bckgrnd}} \): Fluorescence intensity of a non-fluorescence background area
- \( F_{Dpre} \): Fluorescence intensity of donor before photobleaching

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**Conflict of interest.** The authors declare that there is no competing interest.

**Author contributions**

BL, DU, and AMJ conceived the study. AMJ managed the project. Primarily, BL and AMJ wrote the paper, but all authors contributed. MTO designed, performed and analyzed the experiments shown in Figure 8. EGW and JH designed and performed experiments phosphomapping AtGPA1 proteins. MPT designed, performed and analyzed the experiments shown in Figures 1 and 2. DDM designed, performed and analyzed the experiments shown in Figure 3. BL designed, performed and analyzed the experiments shown in Figures 4 to 7. DU and NVD provided technical assistance and contributed to the preparation of the figures. Funding to AMJ, MPT, LMH and NVD enabled these experiments. All authors approved the final version of the manuscript.
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**Abbreviations**

The abbreviations used are: GPCR, G protein-coupled receptor; GAP, GTPase-Accelerating/Activating Protein; GEF, Guanine-nucleotide Exchange Factors; GDI, Guanine nucleotide Dissociation Inhibitor; PAK-1, p21-Activated protein Kinase; PTM, Post-Translational Modification; LRR RLK, Leucine-Rich-Repeat Receptor-Like Kinase; SAPH-ire, Structural Analysis of PTM Hotspots; MAPs, Modified Alignment Positions; DMD, Discrete Molecular Dynamics; CD, Circular Dichroism; BAK1, BRI1-Associated receptor Kinase 1; ABD, 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; SPR, Surface Plasmon Resonance.
Table 1. The reaction rate for steady-state GTPase assay in the presence of AtRGS1.

| AtRGS1 (nM) | AtGPA1 wild type (min⁻¹) | AtGPA1 Y166D (min⁻¹) | AtGPA1 Y166E (min⁻¹) |
|-------------|--------------------------|----------------------|----------------------|
| 0           | 0.034 ±0.004             | 0.041 ± 0.009        | 0.027 ± 0.004        |
| 30          | 0.046 ± 0.005            | 0.051 ± 0.009        | 0.041 ± 0.008        |
| 100         | 0.069 ± 0.007            | 0.101 ± 0.012        | 0.035 ± 0.005        |
| 300         | 0.086 ± 0.01             | 0.146 ± 0.017        | 0.051 ± 0.006        |
| 1000        | 0.19 ± 0.02              | 0.222 ± 0.02         | 0.078 ± 0.01         |
| 3000        | 0.36 ± 0.007             | 0.396 ± 0.05         | 0.149 ± 0.02         |

The reaction rates for steady-state GTPase assay in the presence of AtRGS1 were calculated based on the result in Fig. 6D. The quantitative results were fitted to linear regression using GraphPad Prism version 5.0.

Table 2. Summary of affinity constants between AtRGS1 and Gα subunits.

|                    | Kinetic Analysis | Equilibrium Analysis |
|--------------------|------------------|----------------------|
|                    | kₐ (1/Ms)        | kₜ (1/s)  | K_D (nM) | R_max (RU) | Chi² (RU) | K_D (nM) | R_max (RU) | Chi² (RU) |
| AtGPA1 wild type   | N.A.             | N.A.      | N.A.     | N.A.       | N.A.      | N.A.     | N.A.       | N.A.      |
| GDP                | 1.24E+06         | 3.11E-03  | 2.51     | 27.12      | 2.81      | 4.83     | 30.01      | 0.148     |
| GDP-AlF₄           | 1.22E+05         | 7.29E-04  | 5.99     | 32.81      | 8.25      | 23.4     | 33.92      | 1.36      |
| AtGPA1 Y166E       | 6.56E+05         | 3.54E-03  | 5.39     | 15.19      | 1.90      | 11.1     | 17.48      | 0.121     |

Binding affinity constants were measured using a ProteOn XPR36 surface plasmon resonance instrument. For the kinetic analysis, the original curves were fit with a 1:1 Langmuir binding model. kₐ, association rate constant; kₜ, dissociation rate constant; K_D, calculated by kₐ/kₜ; R_max, maximum response; Chi², the average of squared residuals. For the equilibrium analysis, K_D was calculated by RU at steady state. N.A., no specific binding was detected with the GDP-bound state of wild type AtGPA1.
Figure Legends

Figure 1. SAPH-ire analyses of AtGPA1 Y\textsubscript{166}. (A) Log plot of experimentally observed PTM hotspots in the \(G\alpha\) protein family IPR001019. PTM hotspots generated by SAPH-ire were plotted in rank order with respect to calculated function potential probability score (SAPH-ire NN Score). Hotspots known to be involved in regulating protein function were color and size-coded by the number of literature sources (known function source count, KFSC) that provide evidence of the biological function. Hotspot IPR001019-1010, containing AtGPA1 pY\textsubscript{166} was colored green for ease of viewing. (B) PTM hotspot cluster density from N- to C-terminus of the \(G\alpha\) protein family (Alignment Position, AP). The native residue position of AtGPA1 (a member of the \(G\alpha\) family) is shown within the nested x-axis for clarity (Native Position, NP). Circle size is proportional to the number of PTMs found within the +/- 2 residue cluster centered on each hotspot. Circle color indicates whether the hotspot has a known function (red), is known by proximity to a hotspot with known function (yellow), or is uncharacterized (grey).

Figure 2. Conservation analysis of AtGPA1 Y\textsubscript{166}. (A) The conservation of \(\alpha1\) helix (upper panel, amino acid residues: 51-62), \(\alpha E\) helix (middle panel, amino acid residues: 163-175), and \(\beta5\) brand + \(\alpha G\) helix (lower amino acid residues: 281-298) were aligned and calculated by the online Web-Logo program (76). The x-axis represented the corresponding position on AtGPA1, and the y-axis represented the bit-score. Empty triangles denoted the amino acid residue of K\textsubscript{56}, Y\textsubscript{166}, K\textsubscript{173}, K\textsubscript{288}, and K\textsubscript{294} respectively. (B) Crystal structure of AtGPA1 (gray, PDB: 2XTZ (3)) aligned with a heterotrimeric G protein (tan for \textit{Bos} hybrid \(G\alpha\) subunit, yellow for \(G\beta\), and orange for \(G\gamma\). PDB: 1GOT (77)). Arrows point to Y\textsubscript{166} on AtGPA1 and its corresponding residue Y\textsubscript{150} on \textit{Bos} hybrid \(G\alpha\) subunit.

Figure 3. Discrete molecular dynamics (DMD) simulations for unphosphorylated, phosphorylated, and phosphomimetic AtGPA1. (A) Positions of K\textsubscript{56}, Y\textsubscript{166}, R\textsubscript{173}, K\textsubscript{288}, and K\textsubscript{294} around the intramolecular domain interface were shown. (B-Q) Distance histograms between the phenolic oxygen of unphosphorylated (B-E), phosphorylated Y\textsubscript{166} (F-I), phosphomimetic Y\textsubscript{166}E (J-M) or phosphomimetic Y\textsubscript{166}D (N-Q) and the amino nitrogen or guanidinium carbon of nearby positive charge residues (K\textsubscript{56}, R\textsubscript{173}, K\textsubscript{288}, and K\textsubscript{294}). Peaks in the histograms in the range indicated salt bridge formation (< 6 Å) are highlighted (*).

Figure 4. AtGPA1 mutants retain global wild type structure. (A) CD spectra of His-tagged AtGPA1 wild type (red) and its mutations Y\textsubscript{166}D (blue), and Y\textsubscript{166}E (green) on far UV spectra (185 nm to 260 nm) in 0.5 nm scan steps at 20 °C. The protein solutions were all present at 100 nM and the cell path length was 0.5 cm. The spectra results were analyzed with Chirascan software. (B) Fast quantitative cysteine reactivity (fQCR) unfolding curves for His-tagged AtGPA1 wild type (red circle) and its mutations Y\textsubscript{166}D (blue square), and Y\textsubscript{166}E (green triangle).
Figure 5. Phosphorylation of AtGPA1 by Leucine-Rich Repeat Receptor-like kinases. (A) *In vitro* kinase assays were performed to screen kinases for AtGPA1. Naive reactions ([γ-^32^P] ATP containing buffer only (lane #1) and GST (lane #2) were performed as negative controls. Seventy purified LRR RLKs (48) (the migration range on SDS-PAGE was denoted by the vertical line, lane #3~72) were incubated with GST-AtGPA1 (~70 kDa, empty triangle) in kinase assay buffer containing [γ-^32^P] ATP as described in the Materials and Methods. Purified RGS + Ct (~28 kDa, solid triangle) was employed as positive control for the kinase activity of LRR RLKs. Apparent molecular masses were indicated on the left in kDa. The TAIR locus number of each LRR RLK was indicated at the top of the corresponding lane and the lane number at the bottom. Autophosphorylation and transphosphorylation were detected by autoradiography. Supporting material in Table S1. (B) Functional categorization by annotation for AtGPA1 kinase candidates based on gene ontology (GO) biological process. The number indicates the percentage of annotations to terms in each GO slim category to the total annotations to terms in ontology. (C) Detection of phosphotyrosine residues on wild type AtGPA1. Triangles denote the position of GST-AtGPA1, circles denote the position of His-BAK1, and squares denote the position of GST. Apparent molecular masses (kDa) are indicated on the right. Tyrosine phosphorylation on AtGPA1 or BAK1 was detected by a phosphotyrosine specific antibody. Polyhistidine tagged-BAK1 was detected by His-tag antibody. Total GST-GPA1 and GST were detected by GST-tag antibody. (D) The phosphorylation of AtGPA1 wild type, Y₁₆₆D, and Y₁₆₆E were detected by phosphotyrosine specific antibody. Total GST-GPA1 were detected by GST-tag antibody. The intensity of each band was quantified with Image J. The ratio of phosphorylated/total AtGPA1 were calibrated to wild type. The quantitative results were expressed as the means ± SD of three experiments. Statistical significance was determined by an analysis of variance (ANOVA). ‘***’ represented differences with P values <0.001.

Figure 6. AtGPA1 Y₁₆₆E impairs AtRGS1 accelerated GTPase cycle. The GTPase hydrolysis rate was measured by single-turnover GTP hydrolysis assay in the absence (A) or presence (B) of AtRGS1. Wildtype AtGPA1 or equivalent mutants was incubated with [γ-^32^P] GTP at 20 ºC in the presence of GTPγS. The reactions were quenched at the indicated time points (A) or 10 min (B), and the amount of ^32^PO₄ in solution was measured by scintillation counting. The GTPase cycle was measured by steady-state GTP hydrolysis assay in the absence (C) or presence of AtRGS1 (D). Wild type AtGPA1 or equivalent mutants were incubated with [γ-^32^P] GTP at 20 ºC. The reactions were quenched at the indicated time points (C) or 10 min (D) and the amount of ^32^PO₄ in solution was measured by scintillation counting. (E) The GTPγS binding ability were measured by GTPγS binding assay in the absence or presence of 1 µM AtRGS1. Wild type AtGPA1 or equivalent mutants were incubated with [γ-^35^S] GTPγS on ice. The reactions were stopped at the indicated time points. The amount of AtGPA1 bound [γ-^35^S] GTPγS was filtered on nitrocellulose membrane and measured by scintillation counting. The quantitative results were expressed as the means ± SD of at least three experiments, and fitted to exponential one-phase association functions (A and E), linear regression (C) or log (agonist) vs. response (D) using GraphPad Prism version 5.0. Statistical significance was determined by an analysis of variance (ANOVA). ‘***’ represented differences with P values <0.001. Supporting material for panel D is shown in Table 1.
Figure 7. AtGPA1 Y166E changes its binding specificity with AtRGS1. (A) Direct interaction between GST-tagged AtRGS1 (RGS + Ct) and His-tagged AtGPA1 wild type or Y166E mutant in the presence of GDP, GTPγS or GDP-AlF₄⁻ were detected by in vitro pull down assay. Protein complexes were purified by glutathione Sepharose, separated by SDS-PAGE, and detected by anti-GST or anti-His antibodies. (B) The intensities of His-AtGPA1 wild type and Y166E mutant were quantitated with Image J and normalized as relative values to each interaction in the presence of GDP-AlF₄⁻. The quantitative results were expressed as the means ± SD of four experiments. Statistical significance was determined by an analysis of variance (ANOVA). '*' represented differences with P values of <0.05. '***' represented differences with P values of <0.01. The binding affinity constants of AtRGS1 and AtGPA1 wild type or Y166E mutant were measured by surface plasmon resonance with a ProteOn XPR36 instrument. The GST-tagged AtRGS1 (RGS + Ct) was immobilized on the surface of GLC sensor chip as ligand, and the His-tagged AtGPA1 wild type or Y166E in GDP-bound, GTPγS-bound or the GDP-AlF₄⁻-bound state were diluted into dosage concentrations as analyte. The affinity constants were calculated by kinetic analysis (C-H) or equilibrium analysis (I-K) and shown in Table 2.

Figure 8. Y166 modulates AtGPA1 interaction with AtRGS1 in vivo. (A) FRET Efficiency between transiently expressed C-terminal YFP-tagged AtRGS1 wild type, phosphorylation mutant (3SA), or GAP mutant (E320K), and C-terminal CFP-tagged AtGPA1 wild type or Y166E mutant in N. benthamiana. Values based on eq 1 in Experimental Procedures. (B) FRET changes in response to 3 min 1 µM flg22 treatment between AtRGS1 and AtGPA1 wild type or Y166E mutant in N. benthamiana. The quantitative results were expressed as the means ± SD of ROIs (n = 6 to 37). Statistical significance was determined by t-test. '*' represented differences with P values of <0.05.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A

Substrate phosphoswitching

B

Cell organization and biogenesis, 4.348
Response to abiotic or biotic stimulus, 5.435
Transport, 1.087
Other biological processes, 6.522
Signal transduction, 8.996
Response to stress, 9.783
Developmental processes, 11.957
Protein metabolism, 13.043
Other cellular processes, 26.087
Other metabolic processes, 13.043

C

GST
GST-AIGPA1
IB: α-pY-AIGPA1
IB: α-pY-BAK1
IB: α-His-BAK1
IB: α-GST-AIGPA1

D

Ratio to wt phosphorylation

wt Y186D Y186E GST-AIGPA1

IB: α-pY-AIGPA1
IB: α-GST-AIGPA1
Figure 6
Substrate phosphoswitching

Figure 7
Figure 8
Tyrosine phosphorylation switching of a G protein substrate
Bo Li, Meral Tunc-Ozdemir, Daisuke Urano, Haiyan Jia, Emily G Werth, David D Mowrey, Leslie M Hicks, Nikolay V Dokholyan, Matthew P Torres and Alan M. Jones

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