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Evidence for an unusual transmembrane configuration of AGG3, a class C Gγ subunit of Arabidopsis

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SUMMARY

Heterotrimeric G proteins are crucial for the perception of external signals and subsequent signal transduction in animal and plant cells. In both model systems, the complex comprises one Gα, one Gβ, and one Gγ subunit. However, in addition to the canonical Gγ subunits (class A), plants also possess two unusual, plant-specific classes of Gγ subunits (classes B and C) that have not yet been found in animals. These include Gγ subunits lacking the C–terminal CaaX motif (class B), which is important for membrane anchoring of the protein; the presence of such subunits gives rise to a flexible sub-population of Gβ/γ heterodimers that are not necessarily restricted to the plasma membrane. Plants also contain class C Gγ subunits, which are twice the size of canonical Gγ subunits, with a predicted transmembrane domain and a large cysteine-rich extracellular C–terminus. However, neither the presence of the transmembrane domain nor the membrane topology have been unequivocally demonstrated. Here, we provide compelling evidence that AGG3, a class C Gγ subunit of Arabidopsis, contains a functional transmembrane domain, which is sufficient but not essential for plasma membrane localization, and that the cysteine-rich C–terminus is extracellular.

Keywords: Arabidopsis Gγ subunit 3, AGG3, membrane topology, heterotrimeric G protein, *Arabidopsis thaliana*, type II membrane protein.

INTRODUCTION

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are major components of the transmembrane signaling system in eukaryotes, and mediate various physiological responses (Urano *et al.*, 2013). The G protein complex is comprised of one Gα, one Gβ and one Gγ subunit. Gα binds and hydrolyzes guanosine triphosphate (GTP), thereby determining the active/inactive state of the heterotrimeric G protein complex, while the Gβ subunit possesses a seven-bladed propeller structure and forms a functional heterodimer with the Gγ subunit. Upon activation of the G protein, the GTP-bound Gα subunit and the Gβ/γ dimer dissociate from each other to subsequently modulate distinct downstream effectors (Cabrera-Vera *et al.*, 2003; Offermanns, 2003).

In contrast to the canonical mechanisms described in animals and fungi (Wess, 1997), activation of plant G protein signaling in Arabidopsis follows a different course of action, and involves internalization of the negative regulator AtRGS1, which functions as a seven-transmembrane, receptor-like GTPase-activating protein, maintaining Gα in its inactive, GDP-bound state (Chen *et al.*, 2003; Chen and Jones, 2004; Johnston *et al.*, 2007). Furthermore, the steady-state level of G protein subunits in plants is low and is probably rate-limiting for some aspects of G protein signaling (Fu *et al.*, 2014). Because cereals lack seven-transmembrane Regulator of G protein Signaling proteins, another mechanism for regulation of the active state of G protein signaling must exist. While the human genome,

for example, encodes 16 Gα, five Gβ and 12 Gγ subunits (Simon *et al.*, 1991), only one Gα (GPA1), one Gβ (AGB1) and three Gγ (AGG1–3) isoforms are present in *Arabidopsis thaliana* (Ma *et al.*, 1990; Weiss *et al.*, 1994; Mason and Botella, 2000, 2001; Chakravorty *et al.*, 2011). Thus, functional selectivity of the heterotrimer is determined by the Gγ subunits in Arabidopsis, rice, and probably all plants (Trusov *et al.*, 2007, 2008; Thung *et al.*, 2013).

The structure of the animal Gγ subunit is well understood (Gautam *et al.*, 1998; Robishaw and Berlot, 2004). The Gγ subunit forms a coiled-coil structure with its Gβ partner through the Gβ N–terminal γ domain (Pellegrino *et al.*, 1997; McCudden *et al.*, 2005), and the C–terminus contains a CaaX motif (where C = Cys; a = an aliphatic amino acid; X = any amino acid) that is prenylated, thus keeping the protein tethered to the cytoplasmic face of the plasma membrane (PM) (Simonds *et al.*, 1991; Chakravorty and Botella, 2007; Zeng *et al.*, 2007). All 12 human Gγ subunits are small membrane-associated proteins; however, no animal Gγ subunit is known to have a transmembrane or extracellular domain. In contrast, plants have at least three structurally distinct classes of Gγ subunits; those currently known are designated class A, B and C (Figure 1a) (Trusov *et al.*, 2012). Arabidopsis AGG1 and AGG2 belong to class A, and are structurally similar to the canonical Gγ subunits found in animal cells. Class B Gγ subunits possess the N–terminal γ domain but lack the CaaX motif. Therefore, the resulting sub-population of Gβ/γ dimers may not be localized to the PM. Representatives of this class are not found in Arabidopsis, but are present in most other flowering plants (Trusov *et al.*, 2012), as exemplified by RGG2 from rice (Kato *et al.*, 2004). AGG3 is a class C Gγ subunit; these possess special features compared to other Gγ subunits. With 251 amino acids, AGG3 is twice as large as AGG1 and AGG2 (Chakravorty *et al.*, 2011). AGG3 contains a typical N–terminal γ domain, but may also possess a transmembrane domain (TMD), and the cysteine-rich C–terminus may be extracellular (Botella, 2012; Li *et al.*, 2012). If confirmed, this unusual Gγ membrane topology is significant as it not only defines a new prototype of Gγ subunits, but also implies that class C Gγ subunits have an extracellular function. Extracellular functionality for a Gγ subunit is unprecedented. The importance of the cysteine-rich C–terminus for AGG3 function in plants was suggested in a previous study, which demonstrated that the phenotype of *agg3–3* knockout mutants is not rescued by complementation with a C–terminally truncated AGG3 protein (Chakravorty *et al.*, 2011). However, the previous study did not conclusively address the question of whether AGG3 possesses a TMD. Localization studies in stable Arabidopsis lines over-expressing translational GFP fusions of AGG3 suggested a PM localization for the protein, although fusion proteins were also detected in various other subcellular compartments, including the Golgi and the nucleus (Chakravorty *et al.*, 2011; Li *et al.*, 2012). While a function of the putative TMD in subcellular localization of AGG3 was postulated, the previous data did not fully support this because deletion of the transmembrane region did not entirely abolish the PM localization of the protein (Chakravorty *et al.*, 2011; Li *et al.*, 2012). Considering the uniqueness and physiological importance of a Gγ subunit with a transmembrane domain, it is critical to further assess the proposed membrane topology of the class C Gγ subunit, AGG3.

Using independent approaches, we provide strong evidence that AGG3 represents a membrane protein with an

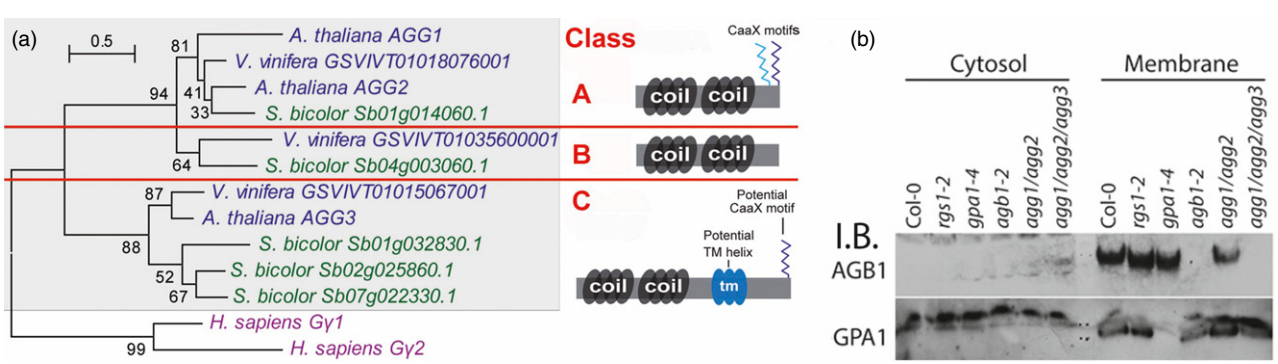

Figure 1. Three different Gγ subunits are found in flowering plants.
(a) Maximum-likelihood tree of representative Gγ subunits from *Arabidopsis thaliana Vitis vinifera*, *Sorhum bicolor* and *Homo sapiens*. The bootstrap support values are shown at each branch. A schematic overview of the domain structures of the three plant Gγ subunit classes is shown on the right.
(b) Immunoblot analyses of endogenous levels of Arabidopsis Gα and Gβ subunits in membrane and soluble fractions extracted from wild-type (Col–0) or G protein mutant leaves. GPA1 or AGB1 were detected using anti-GPA1 (GPA1) or anti-AGB1 (AGB1) antibodies. Soluble and membrane-associated samples were run on the same gel and blot for direct comparison. The signal intensities are directly comparable. Bands detected by the anti-GPA1 serum in the cytosol are non-specific, as indicated by the fact that they are also present in the *gpa1–4* null mutant sample. In the membrane fraction, the GPA1 protein runs as a split band under these conditions. Due to the low level of endogenous G protein subunits, sample overloading was necessary.

extracellular cysteine-rich C–terminus. A possible role for class C Gγ subunits in the perception of external signals and environmental cues is discussed.

RESULTS AND DISCUSSION

Three classes of Gγ subunits

The *agg1*/*agg2*/*agg3* triple mutant shares the known *agb1–2* mutant phenotypes (Thung *et al.*, 2012), and extensive homology searches failed to identify a class B subunit or additional class A and class C Gγ subunits in the Arabidopsis genome (Trusov *et al.*, 2012), suggesting that only three Gγ subunits are present in Arabidopsis. However, due to the limited homology and possible unexplored phenotypes of null mutations in the Gβ subunit, we sought biochemical evidence that Arabidopsis has only the three known Gγ subunits, and that no further subunits dimerize with Gβ. Based on studies of animal G proteins, it is well known that formation of a functional Gβ/γ dimer is crucial for the stability and localization of mammalian Gβ subunits (Dingus *et al.*, 2005; Mervine *et al.*, 2006). Based on this knowledge, we studied the stability of AGB1 proteins in the Gγ triple knockout mutant background (*agg1*/*agg2*/*agg3*). Proteins extracted from leaves of wild-type (WT) plants and various G protein mutants (*rgs1–2*, *gpa1–4*, *agb1–2*, *agg1*/*agg2* and *agg1*/*agg2*/*agg3*) were fractionated into membrane and soluble protein fractions, subjected to SDS–PAGE and detected using anti–AGB1 antibodies (Figure 1b). Because of the low abundance of the endogenous G protein subunits, detection by immunoblotting required sample overloading. AGB1 was detected in the membrane fraction from WT plants. The amount of Gβ protein was unchanged in *rgs1–2* and *gpa1–4* mutants, reduced in *agg1*/*agg2* double mutants, and severely decreased in *agg1*/*agg2*/*agg3* triple mutants. This result demonstrates that at least one of the three known Gγ subunits is needed to form a functional Gβγ dimer, and suggests that no further Gγ subunits are present in Arabidopsis, consistent with the previous conclusion (Thung *et al.*, 2012; Trusov *et al.*, 2012). In addition, these results indicate that loss of all three Gγ subunits destabilizes the Gβ subunit, as indicated by the reduced AGB1 signal in the soluble fraction. The Gα protein (AtGPA1), whose localization is independent of Gβ or Gγ, was used as a control. As expected, AtGPA1 was absent in *gpa1–4* mutants, but was detected in comparable amounts in membrane fractions from *rgs1–2*, *agb1–2*, *agg1*/*agg2* and *agg1*/*agg2*/*agg3* plants (Figure 1b).

Membrane topology of AGG3

Like other class C Gγ subunits (Fan *et al.*, 2006; Mao *et al.*, 2010), AGG3 contains a predicted TMD with a weak TM score (Figure S1). A comprehensive set of topology prediction algorithms returned either weak or strong TM scores for plant class C proteins (Figure S1). It was previously claimed that this postulated TMD is important for the subcellular localization of AGG3. However, deletion of the corresponding domain did not abolish PM localization (Li *et al.*, 2012). As the presence of a TMD in a Gγ subunit is unprecedented, it is critical to further investigate this topology.

We first assessed the existence of a potential extracellular domain in AGG3 using the split-ubiquitin membrane-based yeast two-hybrid system (Stagljar *et al.*, 1998). The N–terminal half of the ubiquitin I13G mutant (Nub_G) was fused to either the N- or C–terminus of AGG1, AGG2 or AGG3, while the C–terminal half (Cub) was fused to the C–terminus of AGB1, and vice versa with respect to the split ubiquitin tag. If the C–terminus of AGG3 is extracellular, a C–terminal fusion will place the ubiquitin fragment outside the cell, rendering it unable to complement growth. An N–terminal AGG3 fusion to the N–terminal half of WT ubiquitin (Nub_{WT}), which spontaneously interacts with Cub in yeast cells (Stagljar *et al.*, 1998), served as the positive control. Yeast strains co-expressing Nub_G–allene oxide cyclase 3 (AOC3), AOC3–Nub_G or free Nub_G (empty vector) were used as negative controls.

There was a clear difference in the growth of strains expressing AGG1–Cub and AGG2–Cub fusions compared to AGG3–Cub fusions. The Cub fragment contains the protein A-LexA-VP16 peptide (PLV) transcription factor, which is cleaved and released upon Nub–Cub reformation (Stagljar *et al.*, 1998). In some cases, soluble proteins that are capable of localizing to the nucleus show auto-activation independent of Nub–Cub reformation and PLV cleavage. In our experience, AGG1–Cub and AGG2–Cub cause auto-activation of the split-ubiquitin system, as exemplified by growth of strains expressing the Nub_G negative control, even on high-stringency SD medium containing 500 μM methionine (Figure S2e,k). Fusion of the Cub fragment to the C–terminus of AGG1 or AGG2 may cause disruption of the C–terminal isoprenylation motif, and therefore loss of membrane association, which may contribute to the observed auto-activation. In contrast, expression of AGG3–Cub combined with positive controls (Nub_{WT} combinations), or known interactors (Nub_G–AGB1 or AGB1-Nub_G) did not result in growth, even on low-stringency SD medium containing 0 μM methionine (Figure S2m–p,r). Therefore, there is an inherent difference between AGG1–Cub/AGG2–Cub and AGG3–Cub in the split-ubiquitin system, which renders AGG3–Cub non-functional, and this finding is consistent with an extracellular C–terminal topology but does not provide conclusive evidence.

When Nub–Gγ fusions were tested, the Nub_G–AGG1, Nub_G–AGG2, AGG1–Nub_G and AGG2–Nub_G fusions all resulted in yeast growth when combined with AGB1–Cub, as expected (Figure 2a, sub-panels a–h). Interactions between Nub_{WT}–AGG3/AGB1–Cub or Nub_G–AGG3/AGB1–Cub also complemented growth (Figure 2a, sub-panels i and j), as expected for an intracellular N–terminus of

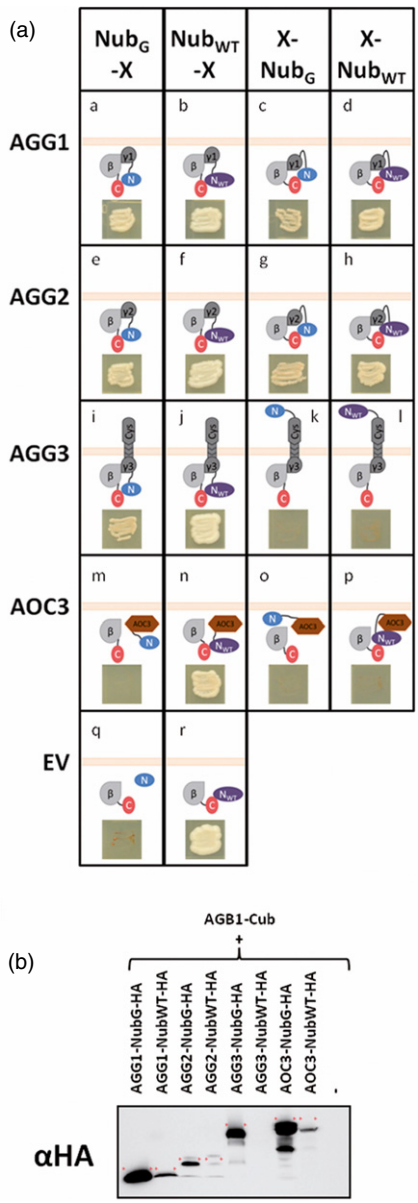

Figure 2. Membrane topology of AGG3.
(a) Split-ubiquitin assays between AGB1 and Gγ subunits, using yeast cells expressing AGB1 fused to the C–terminal half of ubiquitin (Cub), and AGG1, AGG2, AGG3 or AOC3 fused to the N–terminal half of the I13G mutant form of ubiquitin (Nub_G, showing weakened affinity for Cub) or wild-type (WT) ubiquitin (Nub_{WT}, showing high affinity for Cub). An interaction is indicated by growth of diploid cells on interaction-selective medium containing 50 μM methionine. The orientations of the Nub fusions are indicated above each column, where X is AGG1, AGG2 or AGG3, or the negative control AOC3 (as indicated on the left of each row). Schematic overviews of the Gγ and AGB1 fusions are shown above the yeast growth results.
(b) Immunoblot analysis of X–Nub_G and X–Nub_{WT} fusions in (a). Diploid cells were grown in SC liquid medium lacking Trp, Leu and Met, and gel loading was normalized by cell density. Nub fusion proteins were detected using an anti-HA (αHA) antibody that binds the HA epitope on the C–terminus of the fusion protein. All Nub fusion proteins (indicated by red asterisks) were detected except AGG3–Nub_{WT}, consistent with lack of interaction of that construct with AGB1–Cub, as seen in sub-panel l of (a). EV, empty vector.

AGG3. In contrast, no growth was detected when the ubiquitin fragments were attached to the C–terminus of AGG3 (AGG3–Nub_G/AGB1–Cub or AGG3–Nub_{WT}/AGB1–Cub). This result is consistent with the hypothesis that AGG3 contains a transmembrane domain, with the C–terminus being extracellular and thus unavailable for interaction in the split-ubiquitin assay (Figure 2a, sub-panels k and l).

To rule out the possibility that the negative results derive simply from a lack of expression of AGG3–Nub fusions, we performed a Western blot using an anti-HA antibody, targeting the HA epitope tag on the C–terminus of all Nub fusions. We observed that X–Nub_{WT} fusions (where x stands for the test protein) were expressed considerably more weakly than X–Nub_G fusions (Figure 2b). The weak expression of AOC3–Nub_{WT} and lack of expression of AGG3–Nub_{WT} (Figure 2b) probably explain why these 'positive control' fusions did not result in growth when combined with AGB1–Cub (Figure 2a, sub-panels l and p). However, when AGB1–Cub was combined with a strong interactor (e.g. AGG1–Nub_{WT} or AGG2–Nub_{WT}), yeast growth demonstrates that weak expression is sufficient to result in complementation in the case of a positive interaction (Figure 2a, sub-panels d and h). Furthermore, the expression of AGG1–Nub_G/AGG2–Nub_G was comparable to the expression level of AGG3–Nub_G, but expression of AGG1–Nub_G/AGG2–Nub_G resulted in growth whereas that of AGG3–Nub_G did not. Therefore, despite being a strong AGB1 interactor, and expressed at levels similar to AGG1–Nub_G and AGG2–Nub_G, AGG3–Nub_G displays some inherently different characteristics, consistent with an intracellular N–terminus and an extracellular C–terminus. Additionally, we performed a Western blot using anti-AGB1 antibody (Figure S3), and confirmed that the AGB1–Cub fusion was expressed in the relevant X–Nub_G and X–Nub_{WT} samples included in Figure 2(b).

As an independent method to determine the membrane topology of AGG3, we measured the relative fluorescence intensities of stably transformed Arabidopsis plants expressing GFP fused to either the N- or the C–terminus of AGG3. Our approach was based on the observation by Zheng *et al.* (2004) that fluorescence of apoplastic GFP is subject to quenching by low pH. Using stable expression, we show that, when GFP was fused to the C–terminus of AGG3, but not when it was fused to the N–terminus of AGG3, the GFP signal was subject to quenching by low pH (Figure 3), indicating that AGG3 is a membrane protein with an extracellular C–terminus.

Mechanism for membrane anchoring of AGG3

Conventional class A membrane-bound Gγ subunits (Figure 1a) contain C–terminal prenylation motifs (CaaX) that are essential for PM anchoring of the proteins. AGG3 contains a C–terminal CaaX motif (Chakravorty *et al.*, 2011),

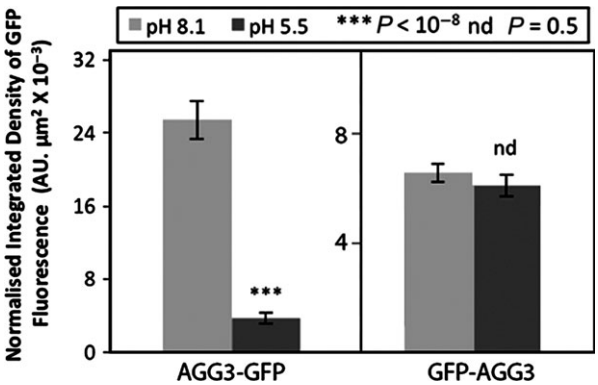

Figure 3. pH-dependent quenching of apoplastic GFP fluorescence. The integrated density of fluorescence of GFP–AGG3 and AGG3–GFP stably expressed in Arabidopsis root cells was normalized against autofluorescence observed in WT plants as discussed in Experimental Procedures. Values are means ± SEM of over 200 individual cell measurements.

although approximately half of the class C proteins listed in the databases lack a CaaX motif (Trusov *et al.*, 2012). Furthermore, the presence of a putative TMD leaves the function of the CaaX motif unclear. It was reported that deletion of the TMD of AGG3 was not sufficient to completely abolish the PM localization of AGG3 (Li *et al.*, 2012). Thus, it may be possible that prenylation of a cryptic CaaX motif substitutes for loss of the TMD to retain the AGG3ΔTMD mutant at the PM.

The classical experiment for assessment of protein topology involves expression of protein truncations and domain swaps in a heterologous system, and topology probing using extracellular proteolysis (Lorenz *et al.*, 2006; Wunder *et al.*, 2010). We attempted to express plant Gγ subunits in mammalian HEK293 and COS7 cells, but, even after extensive codon optimization, failed to reproducibly obtain sufficient protein levels for a robust conclusion. In our hands, only expression in plant protoplasts was successful.

Using transient expression in Arabidopsis mesophyll protoplasts, we tested the ability of distinct protein domains to localize AGG3 to the PM. A set of AGG3 mutant proteins was created (Figures 4a and S4), and the subcellular localization of the resulting proteins was analyzed using N–terminal GFP fusions. Additionally, co-localization studies with a PM-localized *myo*-inositol transporter (INT4) (Schneider *et al.*, 2006) were performed, to differentiate between soluble and membrane-bound fusion proteins (Figure 4b). Protoplasts transiently over-expressing *35S::GFP* were used as control for soluble GFP (Figure 4c; left). When soluble GFP was expressed, a ring of fluorescence with thicker and thinner sections was detected around the cell (Figure 4c; blue arrows), and GFP fluorescence was also detectable in the gaps between chloroplasts, indicating cytosolic localization. Upon co-localization with INT4–RFP, no overlap of the green fluorescence (yellow arrow) and red fluorescence (white arrow) was observed (Figure 4c; right). As a positive control for PM-localized GFP fusion proteins, protoplasts over-expressing *35S::RGS1-GFP* showed an even ring of fluorescence around the cell (Figure 4d; left). Protoplasts co-expressing *35S::RGS1-GFP* and *35S::INT4-RFP* showed complete overlap of the green and red fluorescence as indicated by the orange arrow (Figure 4d; right).

Unlike the results from previous reports (Chakravorty *et al.*, 2011; Li *et al.*, 2012), GFP–AGG3 was exclusively localized at the PM of the protoplasts, and no fluorescence was detected in the Golgi apparatus or the nucleus (Figure 4e). As expected for PM proteins (Bassham *et al.*, 2008), AGG3–GFP fusion proteins containing the TMD were often detected in these intermediary compartments in subcellular localization studies.

Amino acid substitutions leading to mutation of the C–terminal CaaX motif (AGG3ΔCaaX) did not affect the subcellular localization of AGG3, and GFP fluorescence was still observed solely at the PM (Figure 4f). Deletion of the AGG3 transmembrane region (AGG3ΔTMD) partially redistributed the GFP fusion protein, with some GFP fluorescence clearly detectable in the cytosol (Figure 4g; yellow arrow). However, the majority of GFP–AGG3ΔTMD was still localized at the PM (Figure 4g, orange arrow). This result is consistent with the previous observation by Li *et al.* (2012) that the TMD was not essential for membrane localization of AGG3. A possible explanation is that the cryptic CaaX motifs (Figure S4), which are normally not exposed to the farnesyl transferase complex in the cytosol, become farnesylated on an AGG3 molecule that lacks a transmembrane span, enabling PM association via this lipid modification. Similarly, AGG3 proteins lacking both the TMD and the C–terminal CaaX motif, but still containing the large C–terminal Cys-rich domain (AGG3ΔϖCaaXΔTMD), were partitioned to some extent to the PM (Figure 4h; orange arrow).

Consistent with the possibility that residues 108–125 form a TMD, removal of the entire C–terminus but retention of this putative TMD (AGG3ΔCT) did not influence the PM localization of AGG3 (Figure 4i). Only deletion of both the C–terminus and the putative TMD (AGG3ΔTMDΔCT) caused redistribution of the AGG3 mutant protein to the cytosol (Figure 4j).

AtGPA1 and AGB1 are not needed for correct localization of AGG3

Our localization studies in Arabidopsis protoplasts revealed that AGG3 is localized at the PM even when the putative TMD and the C–terminal CaaX motif are removed. Therefore, some other mechanism seems to be involved in PM association of this AGG3 mutant protein. Removal of the Cys-rich C–terminus in addition to the transmembrane region abolished the PM localization completely. As Cys-rich regions are often involved in protein–protein

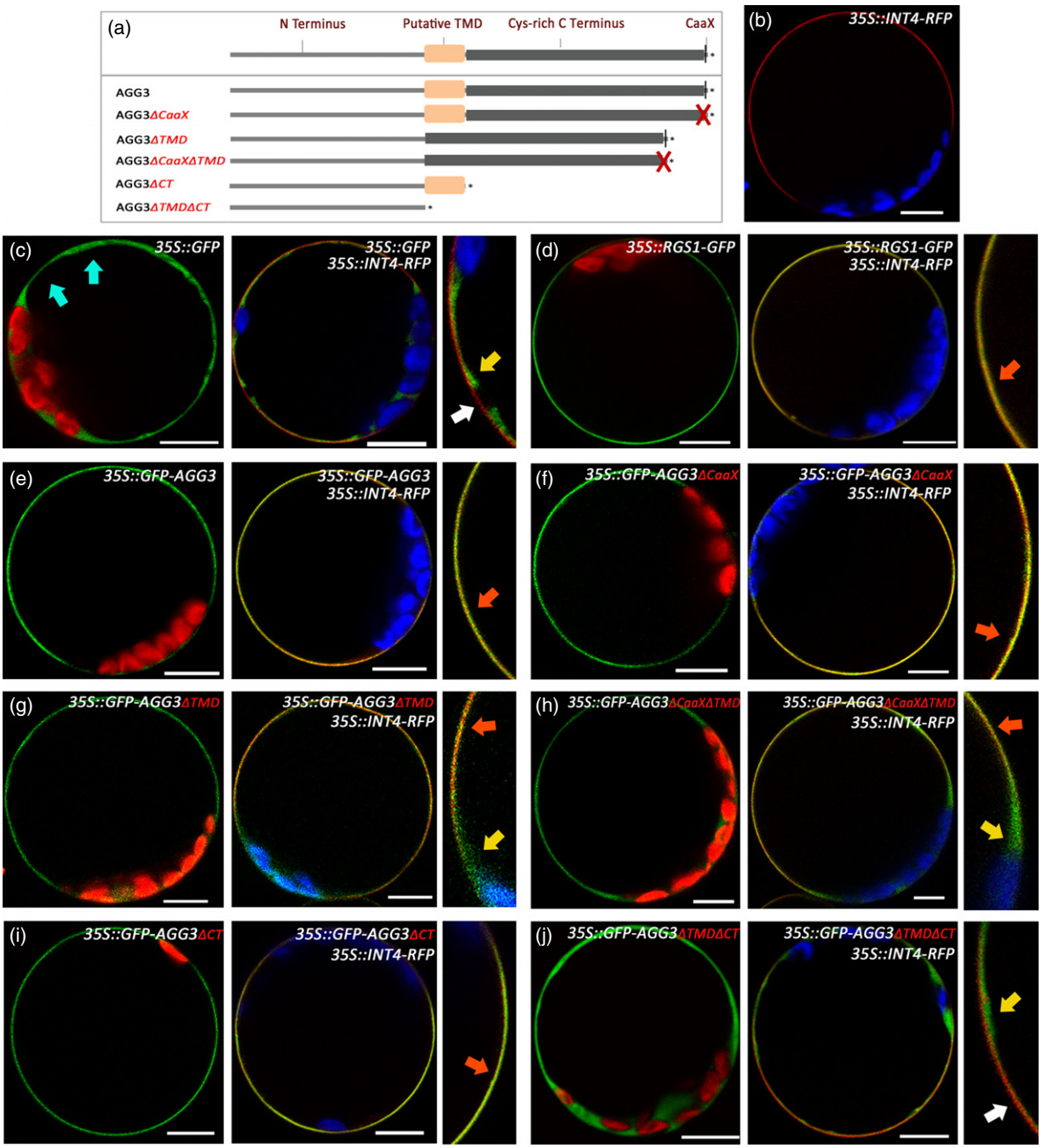

Figure 4. Subcellular localization of AGG3 mutants in Arabidopsis mesophyll protoplasts.
(a) Schematic overview of the various AGG3 mutants used for the localization studies.
(b) Subcellular localization of the PM marker INT4–RFP in mesophyll protoplasts.
(c) Protoplasts transiently over-expressing *35S::GFP* alone (left) or in combination with *35S::INT4-RFP* (middle); a magnified section is shown on the right.
(d) Protoplasts transiently over-expressing *35S::RGS1–GFP* alone (left) or in combination with *35S::INT4-RFP* (middle); a magnified section is shown on the right.
(e–j) Subcellular localization of AGG3 WT protein and AGG3 mutants in protoplasts transiently over-expressing the constructs indicated. Left: GFP signal for the various AGG3 GFP fusions; middle: co-localization studies with INT4–RFP; a magnified section of each picture in the middle is shown on the right. All images are optical sections. GFP fluorescence is shown in green, RFP fluorescence is shown in red; yellow signals indicate complete merging of green and red fluorescence. Depending on the experiment, the autofluorescence of the chloroplasts is either shown in red (sole localization of GFP fusions) or blue (co-localization studies with INT4–RFP). The blue arrows in (c) indicate the typical fluorescence pattern observed in protoplasts caused by soluble fusion proteins. The yellow arrows in (c) and (g–j) indicate soluble fractions of the respective GFP fusions. The white arrows in (c) and (j) indicate lack of co-localization between the GFP fusions tested and INT4–RFP. The orange arrows in (d–i) indicate co-localization between the GFP fusions tested and INT4–RFP. Scale bars = 10 μm.

interactions and complex formation (Voorberg *et al.*, 1991; Okada *et al.*, 1999; Labunskyy *et al.*, 2005), it is possible that other subunits of the heterotrimeric G protein complex are involved in the subcellular localization of AGG3. In accordance with this hypothesis, co-infiltration of *Nicotiana benthamiana* leaves with *35S::AGB1* and *35S::AGG3–GFP* significantly increased the total amount of measurable GFP fluorescence at the PM (Chakravorty *et al.*, 2011). To further investigate a putative role for AGB1 and GPA1 in the targeting of AGG3, we performed localization studies in protoplasts from *gpa1–4/agb1–2* double mutants.

Protoplasts expressing *35S::GFP* or *35S::RGS1-GFP* were used as controls for soluble or PM-localized fusion proteins, respectively (Figure 5a,b). As observed in WT protoplasts, AGG3 localized to the PM of *gpa1–4/agb1–2* mutant cells; this was confirmed via co-localization experiments with the PM marker INT4–RFP (Figure 5c). As Dense and Erect Panicle 1, a rice homolog of AGG3, directly or indirectly interacts with the rice Gα subunit (Sun *et al.*, 2014), it was necessary to determine whether localization of the Gγ subunits in Arabidopsis requires either AtGPA1 or AGB1. The localization of the various tested AGG3 mutants did not change in the Gα/β mutant background compared to their subcellular localization in WT protoplasts (Figure 5d–h). These results indicate that neither GPA1 nor AGB1 are involved in the membrane association of AGG3. Moreover, the localization of AGG3 or AGG3–CaaXΔTMD was also not affected in the *gpa1–4/agb1–2/agg1/agg2* quadruple mutant background (Figures S5 and S6).

AGG3 is a type II membrane Gγ subunit

In summary, despite weak indication from topology prediction algorithms, the results of our studies obtained using independent and complementary experimental approaches, are consistent with the hypothesis that AGG3 contains a TMD and a large extracellular Cys-rich C–terminus. Subcellular localization studies with various translational GFP fusions of AGG3 mutants revealed that both residues 108-125, encompassing the postulated TM region, and the C–terminal extracellular half are involved in PM anchoring of AGG3. However, neither the extracellular domain of AGG3 nor the CaaX motif were required to observe fluorescence at the PM.

To address the question of AGG3 membrane topology, split-ubiquitin yeast complementation assays and pH-sensitive fluorescence quenching assays were performed. Yeast growth facilitated by restoration of a functional ubiquitin molecule was only observed when the split ubiquitin tag was attached to the N–terminus but not the C–terminus of AGG3, and therefore (in agreement with the predicted membrane topology) was present on the cytosolic side. In addition, when a GFP tag was placed at the C–terminus of AGG3 but not at its N–terminus, pH sensitivity of fluorescence was observed in the quenching assay, indicating that the C–terminal domain is apoplastically located, and further supporting the presence of a single TMD.

Moreover, in contrast to intracellular proteins, extracellular proteins (or protein domains) contain a high percentage of cysteines and half-cystines that form disulfide bridges (Fahey *et al.*, 1977). The cysteine content of the hypothesized extracellular domain (residues 128–251) of AGG3 is approximately 34%, which strongly suggests that this domain is extracellular. This observation, in combination with the experimental evidence provided in this study, strongly supports the hypothesis that AGG3 has the membrane topology of a typical type II membrane protein.

AGG3 is the prototype of class C Gγ subunits

The presence of a large extracellular domain in a Gγ subunit raises the interesting and unprecedented possibility that additional extracellular signaling is mediated, at least in part, through the Gβ/γ dimer. Alternatively, or in addition, the extracellular Cys-rich region of AGG3 may play a structural or stabilizing role in the formation of protein complexes in the apoplast. Restricting Gβ/γ dimers, and thus G protein signaling as a whole, to microdomains of the PM raises another possibility for a G protein signaling control mechanism. As G proteins influence the sugar profile of cell walls (Klopffleisch *et al.*, 2011), we speculate that the sugar composition of the cell wall regulates G protein signaling in a feedback loop. If AGG3 is important for cell-wall composition, we speculate that the AGG3 extracellular domain may directly contact wall components as a mechanism to assess composition.

EXPERIMENTAL PROCEDURES

Plant material and reagents

The stably transformed Arabidopsis lines expressing GFP-tagged AGG3 have been described previously (Chakravorty *et al.*, 2011).

Phylogenetic analyses

Full-length protein sequences of Gγ subunits from *A. thaliana* (At3g63420.1, At3g22942.1 and At5g20635.1), *Vitis vinifera* (GSVIVT01018076001, GSVIVT01015067001 and GSVIVT0101 5067001), *Sorghum bicolor* (Sb01g014060.1, Sb04g003060.1, Sb01g032830.1, Sb02g025860.1 and Sb07g022330.1) and *Homo sapiens* were obtained from the National Center for Biotechnology Information or Joint Genome Institute proteome databases. The sequences were aligned using the CLUSTAL W algorithm (Chenna *et al.*, 2003) implemented in MEGA5.0, and regions containing 70% or more gaps were deleted from the aligned sequences. The maximum-likelihood tree was created using the Jones–Taylor–Thornton model (Jones *et al.*, 1992), with bootstrap analysis of 500 replicates.

Protein extraction and immunoblot analyses

Leaves from 7-week-old Arabidopsis WT plants or G protein null mutants were collected, frozen, and ground in liquid nitrogen. The ground leaves were suspended in extraction buffer (50 mM Tris/HCl, pH 8.0. 10% glycerol, 10 mM β–mercaptoethanol) containing

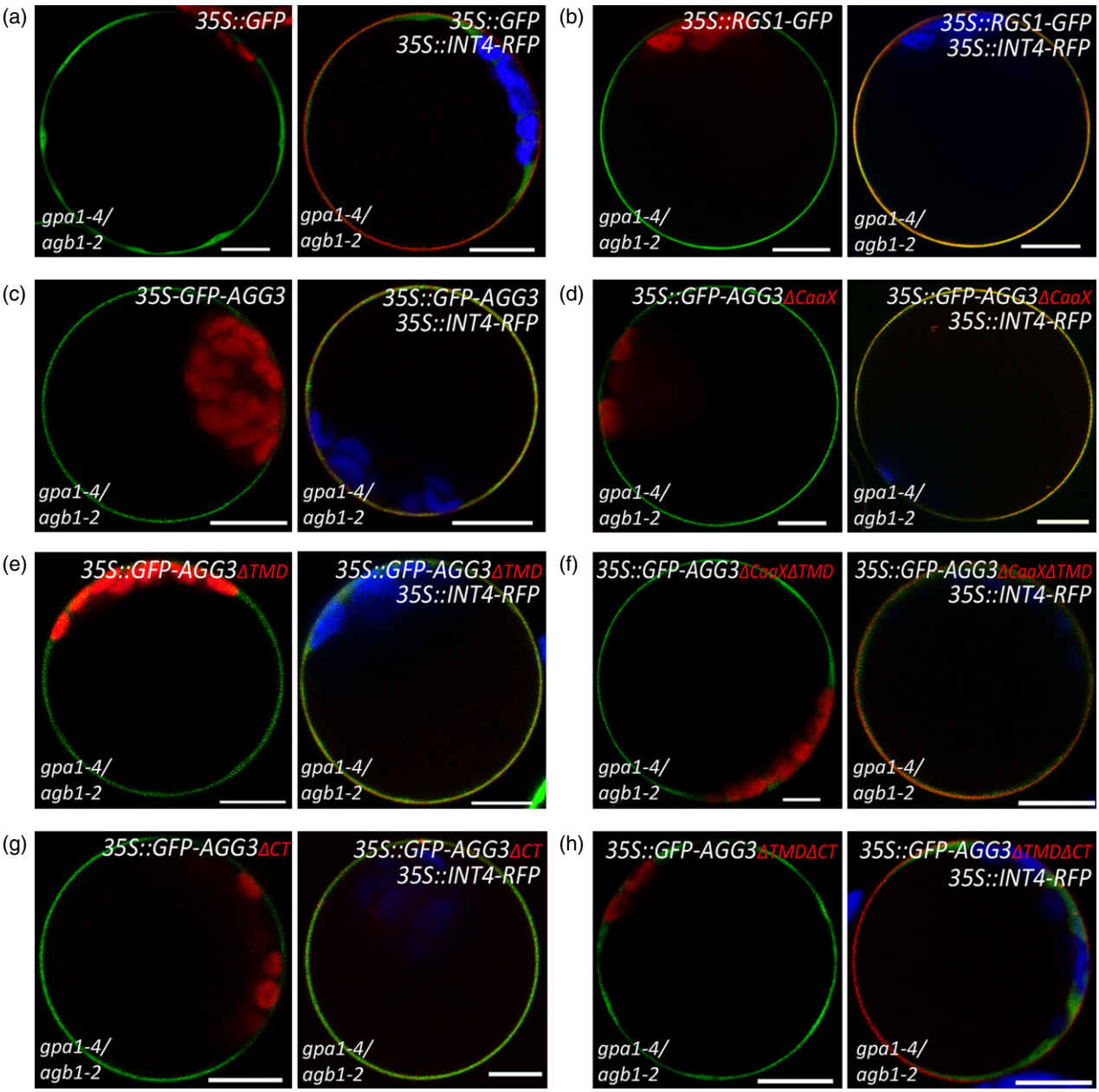

Figure 5. Subcellular localization of AGG3 mutants in *gpa1–4*/*agb1–2* mesophyll protoplasts.
(a) Protoplasts transiently over-expressing *35S::GFP* alone (left) or in combination with *35S::INT4-RFP* (right).
(b) Protoplasts transiently over-expressing *35S::RGS1–GFP* alone (left) or in combination with *35S::INT4-RFP* (right).
(c–h) Subcellular localization of AGG3 WT protein and various AGG3 mutants in protoplasts transiently over-expressing the constructs indicated. Left: GFP signal for the various AGG3 GFP fusions; right: co-localization studies with INT4–RFP. All images represent optical sections at the center of mesophyll protoplasts. GFP fluorescence is shown in green, RFP fluorescence is shown in red; yellow signals indicate complete merging of green and red fluorescence. Depending on the experiment, the autofluorescence of the chloroplasts is either shown in red (localization of GFP fusions) or blue (co-localization studies with INT4–RFP). Scale bars = 10 μm.

protease inhibitor cocktail (Sigma-Aldrich, http://www.sigmaaldrich.com/united-states.html), and centrifuged for 60 min at 50 000 ***g***, 2°C. The supernatants were collected and retained as cytosolic protein fractions, while the pellets were solubilized in extraction buffer containing 1% NP–40 or 2% SDS, and used as membrane fractions. Gα and Gβ proteins were detected by immunoblot analyses using anti-AtGPA1 or anti-AGB1 antibodies. The volumes of the samples were adjusted according to dilution during preparation in order that the levels of G protein subunit detected by the antiserum in the soluble and membrane fractions are directly comparable. Antiserum to GPA1 was prepared as described by Chen *et al.* (2003). Antiserum against AGB1 was prepared in rabbits by Open BioSystems (http://dharmacon.gelifesciences.com/) using the peptide TETVNNLRDQLRQRRLQLK as the antigen.

Split-ubiquitin membrane-based yeast assays

The mating-based split ubiquitin system was used to examine the interaction between AGB1 and Gγ subunits (AGG1, AGG2 and AGG3). The N–terminal half of the ubiquitin I13G mutant (Nub_G) was fused either to the N- or C–terminus of AGG1, AGG2 or AGG3. Nub and Cub constructs were generated by transferring sequence-verified clones from pCR®8/GW/TOPO® (Life Technologies, http://www.lifetechnologies.com) into the indicated yeast expression vector (Lalonde *et al.*, 2010) by Gateway cloning methods. Nub_{WT} fusion proteins were used as positive controls, and empty vector containing Nub_G only was used as a negative control. When expressed from an empty vector, unlike NubWT, NubG does not spontaneously bind to the Cub fragment, and therefore the PLV transcription factor is not released in X–Cub/Nub–EV fusion combinations (Obrdlik *et al.*, 2004). Each entire set of interaction assays was repeated twice, and combinations including AGG3 were repeated five times. Mating and growth conditions were as described previously (Obrdlik *et al.*, 2004). Expression of Nub constructs was verified by immunoblotting using a peroxidase-conjugated anti-HA antibody (clone 3F10; Roche Applied Science, www.roche-applied-science.com).

Generation of expression plasmids

All AGG3 sequences were amplified using primers that introduce flanking *Bsp*HI sites for subsequent cloning into protoplast expression vectors. The full-length coding sequence for the WT AGG3 protein was amplified using primers *AGG3–5-BspHI* (5′-TCAT GAGTGCTCCTTCTGGCGGTG-3′) and *AGG3–3-BspHI* (5′-TCAT GACGAAAGCTAAACAACAAGG-3′). To generate an AGG3 mutant with a deleted prenylation motif (ΔCaaX), the full-length coding sequence was amplified using primers *AGG3–5-BspHI* (5′-TCATGAGTGCTCCTTCTGGCGGTG-3′) and *AGG3–CaaX3-BspHI* (5′-TCATGACGAAAGCTAAAGAAGAAGG-3′), causing amino acid substitutions C247S and C248S in the resulting protein sequence. To generate the C–terminal truncation mutants AGG3ΔCT and AGG3ΔTMDΔCT, the forward primer *AGG3–5-BspHI* was combined with reverse primer *AGG3–dCT-BspHI–R* (5′-TCATGACTGC TTGGCAGCAACAGCAGAAACTC-3′) or *AGG3-dCT-dTM* (5′-TCATG ACTGCTCTTCGACTTTTTCGTTGTGCAG-3′), respectively. AGG3 and AGG3–CaaX mutants lacking the putative TMD (residues 108–125) were amplified using primers *AGG3–5-BspHI* and *AGG3–3-BspHI* or *AGG3–CaaX3-BspHI*, respectively, from plasmid DNA encoding $AGG3^{\Delta 108-125}$ (Li *et al.*, 2012). The full-length coding sequence of *RGS1* was amplified using primers *RGS1–5-NcoI* (5′-CCATGGCGAGTGGATGTGCTCTACATGGTGGTTG-3′) and *RGS1–3-NcoI* (5′-CCATGGCACCGGGACTACTGCATCTGGAACTCT TTGAC-3′). The resulting sequences were then cloned into protoplast expression vector pCS120 for C–terminal GFP fusions (Dotzauer *et al.*, 2010) or protoplast expression vector pSS87 for N–terminal GFP fusions (Schneider *et al.*, 2012). As a PM marker for co-localization studies, we used the *myo*-inositol transporter AtINT4 (At4g16480) C–terminally fused to RFP (Wolfenstetter *et al.*, 2012).

Mesophyll protoplast transformation

Protoplasts from Arabidopsis Col–0 WT plants and *gpa1–4/agb1–2* mutants were generated as described previously (Drechsel *et al.*, 2011) and transformed as described by Abel and Theologis (1994). Forty-eight hours after transformation, the subcellular localization of GFP and RFP fusion proteins was analyzed using a confocal laser-scanning microscope (Zeiss LSM 710 Duo, http://www.zeiss.com/). Only protoplasts with low or intermediate expression of fusion proteins were analyzed. Excitation of the fluorophores was performed using laser light of wavelength 488 nm (GFP) or 560 nm (RFP). Detection windows ranged from 493 to 531 nm for GFP and 573–641 nm for RFP and mCherry. Chloroplast autofluorescence was detected from 689 to 758 nm. All images were processed using ZEN 2009 confocal software (Carl Zeiss, http://www.zeiss.com/microscopy/en_us/downloads/zen.html).

GFP fluorescence quantification

For study of pH effects on GFP fluorescence, GFP fluorescence from root epidermal cells of 1-week-old Arabidopsis seedlings (grown on half-strength MS medium with pH adjusted to either 5.5 or 8.1 using MES or HEPES, respectively) was imaged and quantified as described previously (Sheahan *et al.*, 2004), but without optical sectioning. GFP fluorescence was imaged using an LSM 510 confocal laser-scanning microscope (Zeiss) equipped with a 40 x C–Apochromat water-immersion objective (NA 1.2), using a 488 nm argon laser and BP500–530IR filter. GFP fluorescence intensity was quantified from mid-plane cell sections of a minimum of 200 cells. Fluorescence values were normalized to account for the observed pH-dependent change in autofluorescence of Col–0 seedlings at the applied image acquisition settings. Normalization was performed by subtracting the ratio of fluorescence intensities in Col–0 plants at pH 8.1 and pH 5.5 from the same ratio in GFP–AGG3 or AGG3–GFP plants, and then multiplying this value by the fluorescence intensity at pH 5.5.

Accession numbers

The GenBank accession numbers for AGG3, INT4, RGS1, GPA1 and AGB1 are At5g20635.1, At4g16480.1, At3g26090.1, At2g26300 and At4g34460, respectively.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Transmembrane helix prediction for AGG3.

Figure S2. AGG1–Cub and AGG2–Cub fusions auto-activate, while AGG3–Cub fusions are non-functional.

Figure S3. Immunoblot analysis of AGB1–Cub + X–Nub_G and X–Nub_{WT} samples from fusions in Figure 2(b).

Figure S4. Arabidopsis AGG3 full-length coding sequence and translated protein sequence.

Figure S5. Subcellular localization of AGG3 in various G protein mutants.

Figure S6. Subcellular localization of AGG3–CaaXΔTMD in *gpa1–4/agb1–2/agg1/agg2* mutants.

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