Plant G protein heterotrimers require dual lipidation motifs of Gα and Gγ and do not dissociate upon activation

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Summary

In plants one bona fide Gα subunit has been identified, as well as a single Gβ and two Gγ subunits. To study the roles of lipidation motifs in the regulation of subcellular location and heterotrimer formation in living plant cells, GFP-tagged versions of the *Arabidopsis thaliana* heterotrimeric G protein subunits were constructed. Mutational analysis showed that the *Arabidopsis* Gα subunit, GPα1, contains two lipidation motifs that were essential for plasma membrane localization. The *Arabidopsis* Gβ subunit, AGβ1, and the Gγ subunit, AGG1, were dependent upon each other for tethering to the plasma membrane. The second Gγ subunit, AGG2, did not require AGβ1 for localization to the plasma membrane. Like AGG1, AGG2 contains two putative lipidation motifs, both of which were necessary for membrane localization. Interaction between the subunits was studied using fluorescence resonance energy transfer (FRET) imaging by means of fluorescence lifetime imaging microscopy (FLIM). The results suggest that AGβ1 and AGG1 or AGβ1 and AGG2 can form heterodimers independent of lipidation. In addition, FLIM-FRET revealed the existence of GPα1-AGβ1-AGG1 heterotrimers at the plasma membrane. Importantly, rendering GPα1 constitutively active did not cause a FRET decrease in the heterotrimer, suggesting no dissociation upon GPα1 activation.

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Introduction

Mammalian heterotrimeric G proteins are important signal transducers composed of α, β and γ subunits that couple to a plethora of receptors (G protein coupled receptors; GPCRs). Activated receptors, residing in the plasma membrane, function as guanine nucleotide exchange factors (GEFs) causing the Gα subunit to release the bound GDP and exchange it for GTP. This exchange promotes the dissociation of the Gα subunit from the Gβγ dimer and both units can subsequently activate various effectors (Cabrera-Vera et al., 2003; Offermanns, 2003). Heterotrimeric G protein signaling in plants differs from that in animals. Only one canonical Gα subunit gene has been identified in dicots and monocots, as compared to 17 in mammalian species (Jones and Assmann, 2004). Also, the quest for the identification of a classical GPCR is ongoing. The failure in finding a feasible candidate could also indicate that plant heterotrimeric G proteins are not activated by classical GPCRs. The plant heterotrimeric G protein has been implicated in auxin, gibberellin and abscisic acid signaling, light responses, cell division and ion-channel regulation (for reviews, see Assmann, 2002; Perfus-Barbeoch et al., 2004). Evidence for this involvement comes mostly from pharmacological assays. However, such assays might not always be specific enough (Fujisawa et al., 2001). Indeed, using different methods, Jones et al. (Jones et al., 2003) re-evaluated the alleged direct role that G proteins play in red and far-red light signal transduction, but failed to find one.

The structure of the plant heterotrimeric G protein does share important characteristics with its putative mammalian counterparts. The *Arabidopsis thaliana* gene *GPA1* encodes a Gα subunit (GPα1, 45 kDa), which is most identical (36%) to $G\alpha_{i1}$, a member of the mammalian G_i class of Gα subunits (Ma, 1994; Ma et al., 1990). GPα1 contains a Ras-like GTPase domain, which is conserved among all members of the G protein superfamily. This domain consists of several conserved regions (G-1 to G-5) that are critical for GDP/GTP binding and exchange (Bourne et al., 1991). In GPα1, only G-5 is slightly different from the consensus sequence (Kaydamov et al., 2000). GPα1 binds GTPγS with a dissociation constant in the nanomolar range (Wise et al., 1997) and displays GTPase activity that can be stimulated by both phospholipase Dα (PLDα) and the recently cloned *Arabidopsis* regulator of G protein signaling 1 (RGS1) protein (Chen et al., 2003; Zhao and Wang, 2004). The plant Gα subunit also has an α-helical domain, indicating that it belongs to the family of heterotrimeric G proteins (Sprang, 1997). The 3D-model of Ullah et al. (Ullah et al., 2003) supports the notion that GPα1 is a bona fide candidate for a plant heterotrimeric Gα subunit since it only shows minor differences in structure when compared to the crystal structures of mammalian Gα

subunits. Cell fractionation and immunofluorescence studies demonstrate that GPα1 localizes to the ER and plasma membrane in meristematic cells of *Arabidopsis* and cauliflower (Weiss et al., 1997). In tobacco embryos a similar distribution is observed (Kaydamov et al., 2000). The amount of GPα1 is especially high in meristems, as compared to cells of mature organs. This indicates that expression is developmentally regulated (Weiss et al., 1993).

The *Arabidopsis* genome also encodes one putative heterotrimeric Gβ subunit, AGβ1 (41 kDa), which has 44% sequence identity to mammalian $G\beta_2$ (Weiss et al., 1994). Similar to mammalian Gβ subunits, AGβ1 consists of a WD-40 motif with seven tandem repeats and an N-terminal heptad repeat. Obrdlik et al. (Obrdlik et al., 2000) described an enrichment of plant Gβ in the crude membrane fractions of broccoli and *Arabidopsis*. Plant Gγ subunits have only recently been described because of the lack of amino acid similarity among the known Gγ subunits. The two Gγ subunits that are expressed in *Arabidopsis*, AGG1 and AGG2, have been found by means of a yeast two-hybrid screen using tobacco Gβ as bait (Mason and Botella, 2000; Mason and Botella, 2001). Both AGG1 and AGG2 are small (10.8 kDa and 11.1 kDa, respectively) and their N termini are predicted to form a coiled-coil with the N terminus of Gβ. This type of interaction causes mammalian Gβ and Gγ subunits to function as an obligate heterodimer (Clapham and Neer, 1997). The model by Ullah et al. (Ullah et al., 2003) of GPα1 also provides putative structures for AGβ1 and AGG1. When comparing these structures with the mammalian heterotrimer they showed that the GPα1 residues interacting with AGβ1, as well as the AGβ1 residues interacting with AGG1, are conserved. Although the existence of a heterotrimer in *Arabidopsis* has not yet been experimentally verified, the formation of heterotrimeric G protein complexes was recently reported in rice plasma membranes (Kato et al., 2004).

Lipid modifications play an important role in the correct functioning of mammalian and yeast heterotrimeric G proteins. The different Gα subunits are either myristoylated, palmitoylated or decorated by both lipid tails. Gγ subunits also depend on lipidation for plasma membrane localization and signaling (Manahan et al., 2000). Mammalian Gγ subunits are either farnesylated or geranylgeranylated. Table 1 (and references therein) gives an overview of lipid modifications found in G proteins and the amino acid motifs that dictate the type of modification. The *Arabidopsis* Gα subunit is thought to be myristoylated and palmitoylated by *N*-myristoyl transferase (NMT) and palmitoyl transferase (PAT), respectively. NMT has been cloned from *Arabidopsis* (Maurer-Stroh et al., 2002). A PAT has not yet been found, although a superfamily of putative PATs is currently being investigated (Swarthout et al., 2005). *Arabidopsis* Gγ subunits are predicted to be geranylgeranylated since they are equipped with CaaL box motifs. Enzymatic activity of geranylgeranyltransferase-I (GGTase-I) has been detected in plants (Yalovsky et al., 1999) and its gene has been cloned (Crowell, 2000). Here we describe a study of the hitherto poorly defined role of lipidation motifs in subcellular targeting of plant heterotrimeric G proteins. We opted for an experimental approach based on live cell analysis in which we studied lipidation and oligomerization in unperturbed intact plasma membranes, since it is well known that for plant cells with a high turgor pressure membrane fractionation causes rupture of the vacuole, leading to proteolysis (Rolland et al., 2006). Moreover, upon disruption of cells, the labile palmitoyl groups are easily lost from G protein subunits (Kleuss and Gilman, 1997). In addition, intact plasma membranes preserve transient inhomogeneities in the distribution of membrane lipids and proteins (also known as microdomains or rafts), which may be crucial for G protein signaling (Svoboda and Novotny, 2002; Vermeer et al., 2004). Therefore, localization was studied by using GFP-tagged versions of the subunits. Heterodimer and heterotrimer formation were studied using FLIM-FRET techniques in living

Table 1. Lipidation motifs in heterotrimeric G proteins

G protein	Lipid modification	Motif*	References
Gα subunits			
*Hs*Gα$_{i/o/z}$	*N*-myristoylation	M**G**CxxS	(Galbiati et al., 1994; Grassie et al., 1994; Parenti et al., 1993; Wilson and Bourne, 1995)
	S-palmitoylation	where x=TL/TV in α_i x=TL in α_o x=RQ in α_z	
*Hs*Gα$_s$	*N*-palmitoylation *S*-palmitoylation	M**G**CLGNS	(Kleuss and Krause, 2003)
*Hs*Gα$_q$	*S*-palmitoylation	MTLESIMA**CC**	(Wedegaertner et al., 1993)
*Hs*Gα$_t$	*N*-myristoylation	M**G**AGAS	(Yang and Wensel, 1992)
*Hs*Gα$_{12}$	*S*-palmitoylation	MSGVVRTLSR**C**	(Ponimaskin et al., 1998)
*Hs*Gα$_{13}$	*S*-palmitoylation	MAD-^{14}**C**FPQ**C**^{18}-^{37}**C**LS	(Ponimaskin et al., 2000)
*Sc*Gpa1	*N*-myristoylation *S*-palmitoylation	M**G**CTVS	(Song and Dohlman, 1996; Song et al., 1996)
*At*GPα1		MGLLCS	
Gγ subunits			
*Hs*Gγ$_1$	Farnesylation	KELKGG**C**VIS	(Sanford et al., 1991)
*Hs*Gγ$_2$	Geranylgeranylation	REKKFF**C**AIL	(Sanford et al., 1991)
*Sc*STE18	*S*-palmitoylation Farnesylation	NSNSV**CC**TLM	(Manahan et al., 2000; Whiteway and Thomas, 1994)
*At*AGG1		GGEGCRCLIL	
*At*AGG2		EAKRCGCSIL	

*Amino acids are indicated by their single letter code. Lipidated amino acids are in bold. Underlined amino acids are subject of this study.

plant cells. Our findings support a model in which heterodimer and heterotrimer formation at the plasma membrane requires dual lipidation of Gα and the Gγ subunit AGG2, contrary to what has been described in mammals.

Results

Subcellular location and lipidation motifs of the *Arabidopsis* Gα subunit, GPα1

In order to determine the subcellular location of plant heterotrimeric G proteins fluorescent protein-tagged versions of these proteins were prepared. Since N- and C-terminal fusions of mammalian Gα subunits were found to be non-functional (Yu and Rasenick, 2002), yellow fluorescent protein (YFP) (Tsien, 1998) was inserted into the *Arabidopsis* Gα subunit (GPα1). The insertion site was based on the paper by Chen et al. (Chen et al., 2003), in which they describe a GPα1-GFP fusion protein that accumulates at the nascent cell plate in dividing *Arabidopsis* cells. Recently, this fusion protein was used to study the interaction of GPα1 with the plastid protein thylakoid formation 1 in *Arabidopsis* roots (Huang et al., 2006). Bunemann et al. (Bunemann et al., 2003) used a similar insertion site in the αAB-loop of the α-helical domain to obtain a functional mammalian GFP-tagged $G\alpha_i$. Fig. 1A shows that GPα1-YFP was enriched in the plasma membrane of living cowpea (*Vigna unguiculata* L.) mesophyll protoplasts. By contrast, unfused YFP was found in the nucleus (N) and in the cytosol, which appears as strands crossing the cell and surrounding the large vacuole (V; Fig. 1E).

The N terminus of GPα1 contains the classical myristoylation consensus sequence: M**G**xxxS (Utsumi et al., 2004) that is conserved among Gα subunits in dicots and monocots (Kaydamov et al., 2000) and can be recognized by NMT. This enzyme catalyzes the transfer of myristoyl ($C_{14:0}$) from CoA to the primary amino group of glycine-2. Myristoylation most often occurs co-translationally after removal of the initiating methionine by methionine aminopeptidase (MAP) (Yalovsky et al., 1999). A closer look at the amino acid sequence, MGLL**C**S, hints at a second possible lipid modification: the cysteine at position 5 might be a target for palmitoylation, which involves the attachment of palmitic acid ($C_{16:0}$) (Smotrys and Linder, 2004).

In order to investigate possible lipidation of G2 and C5, the mutants GPα1(G2A)-YFP and GPα1(C5S)-YFP were analyzed. Fig. 1B,C shows the distribution of the two mutants in cowpea protoplasts, which was strikingly different from the distribution of wild-type GPα1. Interestingly, the location of fluorescence was comparable to that of unfused YFP and the double mutant GPα1(G2A/C5S)-YFP (Fig. 1E and D, respectively), indicating cytoplasmic localization.

Next, the difference in diffusion between the wild-type protein, the two single-point mutated proteins and the double mutant was investigated by means of fluorescence recovery after photobleaching (FRAP) (Lippincott-Schwartz and Patterson, 2003). Upon photobleaching of unfused YFP, GPα1(G2A)-YFP, GPα1(C5S)-YFP and GPα1(G2A/C5S)-YFP, we observed a very fast fluorescence recovery, which is typical of fast diffusing cytoplasmic proteins. However, GPα1-YFP diffused significantly slower (Fig. 1F). The diffusion data thus confirm the localization data, showing that only GPα1-YFP was able to bind to the plasma membrane.

Subcellular location of the *Arabidopsis* Gβ and Gγ subunits, AGβ1, AGG1 and AGG2

The *Arabidopsis* Gβ subunit (AGβ1) and the *Arabidopsis* Gγ subunits (AGG1 and AGG2) were fused to the C terminus of spectral variants of GFP. The two subunits, AGβ1 and AGG1, both showed a diffuse distribution in the cytoplasm (Fig. 2A and B, respectively). The other Gγ subunit, AGG2, was predominantly located at the plasma membrane (Fig. 2C). Occasionally, Golgi-like structures were observed in the cells expressing AGβ1 and AGG2 (data not shown). In cells expressing AGG1, these structures were more abundant (arrows, Fig. 2B). In order to examine the nature of these structures, YFP-AGG1 was co-transfected with the Golgi-marker sialyltransferase fused to red fluorescent protein (RFP) (Boevink et al., 1998). When the YFP signal (depicted in green) was merged with the RFP signal, the resultant image showed several bright yellow spots (Fig. 2D-F), indicative of a significant co-localization of YFP-AGG1 and ST-RFP.

Next, the effect of expression of YFP-AGG1 on the distribution of CFP-AGβ1 was studied. Co-expression of CFP-AGβ1 and YFP-AGG1 showed

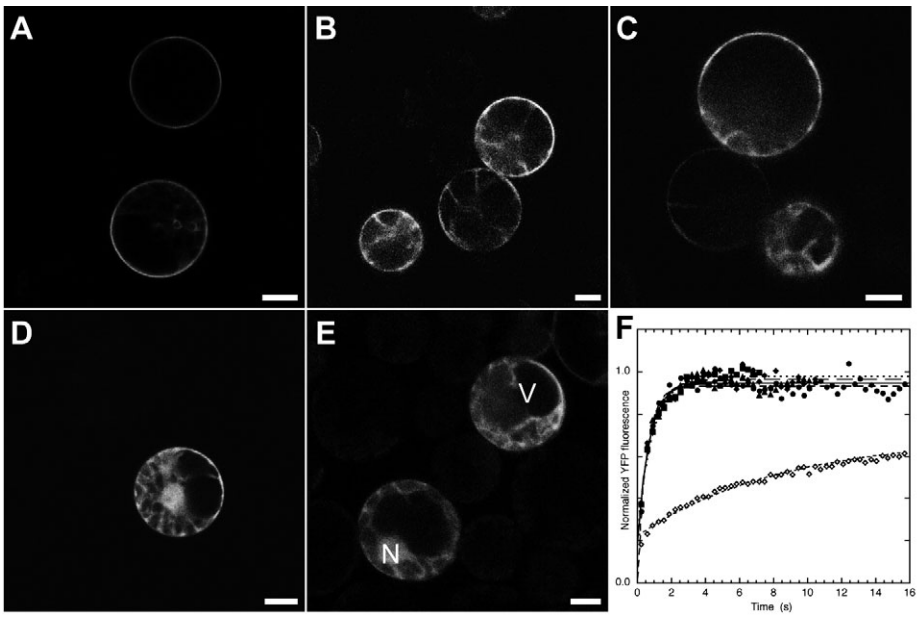

Fig. 1. Subcellular location and dynamics of GPα1-YFP and its lipid motif mutants in cowpea protoplasts. Protoplasts expressing (A) GPα1-YFP, (B) GPα1(G2A)-YFP, (C) GPα1(C5S)-YFP, (D) GPα1(G2A/C5S)-YFP, (E) unfused YFP. N, nucleus; V, vacuole. (F) FRAP curves of YFP (triangles); GPα1-YFP (open diamonds); GPα1(G2A)-YFP (circles), GPα1(C5S)-YFP (squares) and GPα1(G2A/C5S)-YFP (filled diamonds). Bars, 10 μm.

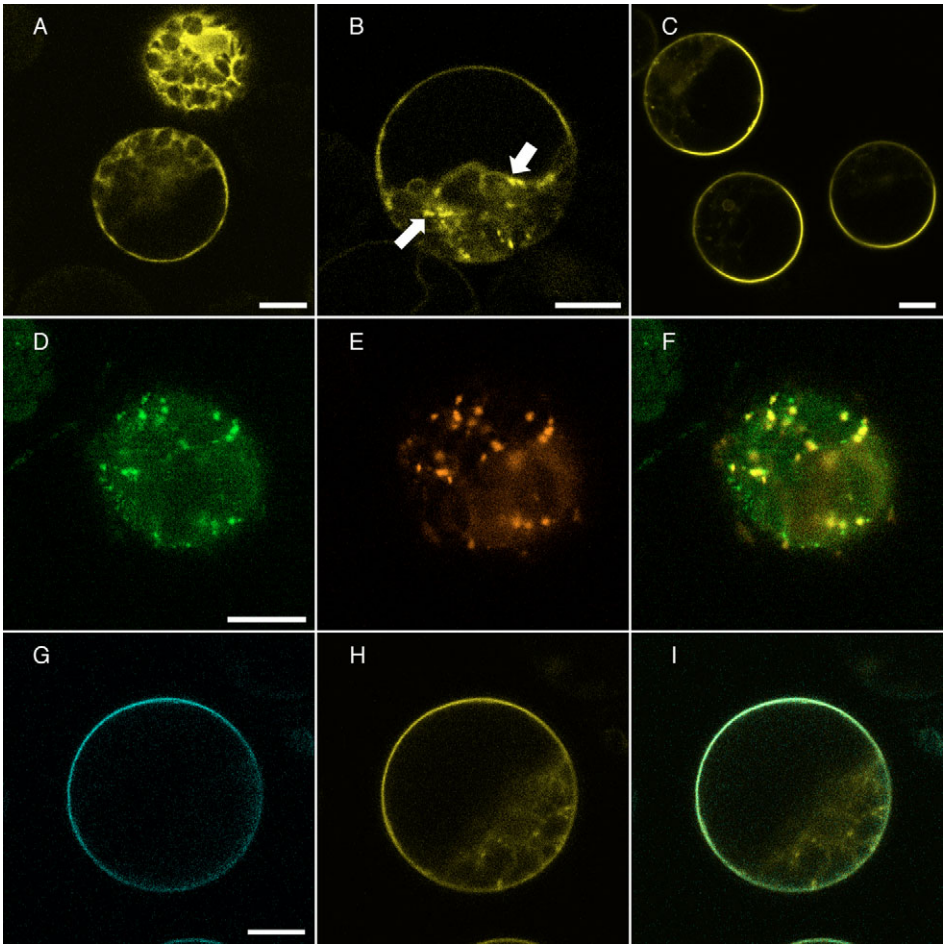

Fig. 2. Subcellular location of plant Gβ and Gγ subunits in cowpea protoplasts. (A-C) Protoplasts expressing (A) YFP-AGβ1, (B) YFP-AGG1 and (C) YFP-AGG2. (D-F) Protoplast co-expressing (D) YFP-AGG1 and (E) ST-RFP, as seen in F which is the overlaid images of D and E. (G-I) Protoplast co-expressing (G) CFP-AGβ1 and (H) YFP-AGG1; (I) the overlaid images of G and H. Arrows indicate Golgi-like structures. Bars, 10 μm.

plasma membrane targeting of both proteins (Fig. 2G,H). AGG2 was equally effective in targeting AGβ1 to the plasma membrane (Fig. S1 in supplementary material). During co-expression studies of AGβ1 and AGG1 we observed improved expression of AGβ1. Therefore, the number of YFP-AGβ1-expressing cells was quantified in the presence and absence of AGG1. We observed an approximate twofold increase in the number of cells expressing YFP-AGβ1 upon co-expression of AGG1, as compared to cells expressing only AGβ1 (Fig. 3; Table 2).

Lipidation motifs of AGG2 and heterodimerization of AGβ1 and AGG1/2

In order to investigate the role that C-terminal sequences play in plasma membrane targeting, the AGG2 subunit was mutated by introducing five additional amino acid residues behind its CaaX box, thereby rendering the protein unrecognizable for prenylation enzymes. This mutation, AGG2mut, caused the AGG2 protein to become cytosolic (Fig. 4A). We also mutated the putative palmitoylation site in AGG2 at residue C95 (Table 1), and observed a loss in plasma membrane affinity (Fig. 4B). The prenylation as well as the palmitoylation motif of YFP-AGG2 appeared to be necessary for plasma membrane targeting of the heterodimer, as YFP-AGG2mut and YFP-AGG2(C95S) were found in the cytosol together with AGβ1 (Fig. 4C,D and Fig. S2 in supplementary material, respectively). These observations implicate a direct interaction between AGβ1 and AGG2.

Therefore, we analyzed interactions between AGβ1 and AGG1/2 with fluorescence resonance energy transfer (FRET) microscopy. FRET is a quantum-mechanical phenomenon that can be used to measure distances or changes in distance between two fluorescently tagged molecules. FRET can occur at distances <100Å and is characterized by a decrease in the fluorescence intensity and fluorescence lifetime of the donor

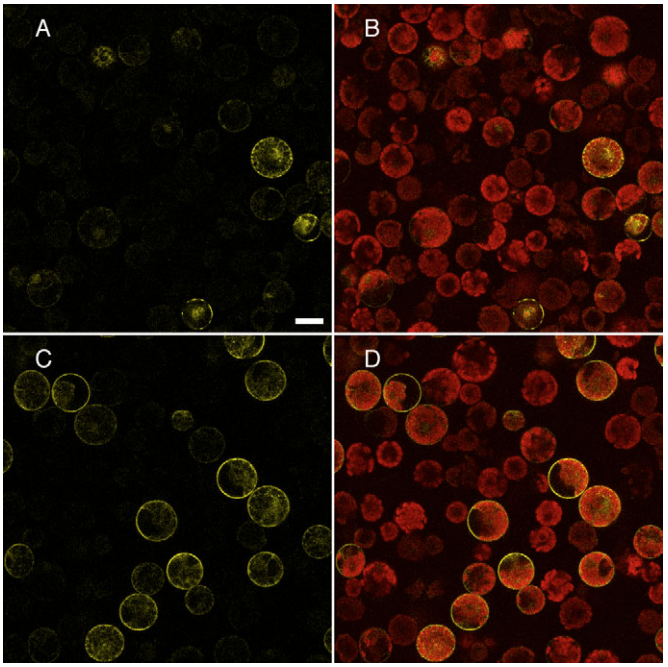

Fig. 3. The influence of AGG1 expression on the amount of YFP-AGβ1 expressors. (A,B) Protoplasts transfected with YFP-AGβ1. (C,D) Protoplasts transfected with both YFP-AGβ1 and AGG1. A and C show yellow fluorescence of protoplasts expressing YFP-AGβ1, B and D show yellow fluorescence of YFP-AGβ1-expressing protoplasts combined with the red chlorophyll autofluorescence of all protoplasts. Bar, 20 μm.

Table 2. Number of protoplasts expressing YFP-AGβ1

Constructs	# cells	# YFP-AGβ1 cells	% YFP-AGβ1 cells
YFP-AGβ1	368	82	22
YFP-AGβ1+AGG1	361	163	45

fluorophore (Gadella et al., 1999). Several techniques are available to measure FRET. Initial experiments using acceptor photobleaching (Bastiaens and Squire, 1999) showed an increase in donor fluorescence upon bleaching of the acceptor, suggestive of FRET (data not shown). Since FLIM (fluorescence lifetime imaging microscopy) is more suitable for live cell experiments (Bastiaens and Squire, 1999) this technique was used to study in detail the existence of a direct interaction between plant Gβ and Gγ subunits. In frequency-domain FLIM, the sample is excited with sinusoidally modulated light. As a consequence, the emitted fluorescence is also sinusoidally modulated at the same frequency, but displays a shift in phase and a reduction in modulation depth, from which a phase lifetime and a modulation lifetime can be measured, respectively. A reduction in lifetime of both YFP-AGG1 and YFP-AGG2 was observed upon co-expression of RFP-AGβ1, which demonstrates the occurrence of FRET and is indicative of the formation of a heterodimer (Table 3). Lifetime data were also obtained for YFP-AGG2mut expressed together with RFP-AGβ1 and again a decrease in lifetime was observed, indicating that AGβ1 interacts with AGG2mut. This suggests that in plants Gβγ dimerization is independent of the lipidation motif of the plant Gγ subunit AGG2.

Formation of the heterotrimer

We studied heterotrimer formation in living plant cells with FLIM-FRET using GPα1-YFP and RFP-AGβ1 in cowpea protoplasts, expressed together with unfused AGG1 in order to assure plasma membrane distribution of the Gβ subunit. The lifetime of GPα1-YFP decreased upon expression with RFP-AGβ1, suggestive of the formation of a heterotrimer (Table 3).

Mutation of the glutamine that is present in the switch II of mammalian Gα subunits and small G proteins renders them constitutively active, since they are no longer able to hydrolyze bound GTP molecules and hence are present in the 'active' GTP-bound state (Simon et al., 1991). Remarkably, application of this mutation in GPα1, Q222L, did not change the subcellular location of GPα1(Q222L)-YFP as compared to GPα1-YFP, and FRAP analysis reported similar mobility characteristics (Fig. S3 in supplementary material), whereas GPα1(Q222L) has been shown to change the phenotype in whole plants (Perfus-Barbeoch et al., 2004). It is even more remarkable that incorporation of the mutation Q222L did not abolish FRET to RFP-AGβ1, since the lifetime of GPα1(Q222L)-YFP upon expression with RFP-AGβ1 was efficiently quenched (Table 3). These above observations suggest that Gα subunits containing this mutation still reside in the membrane as a heterotrimer with Gβγ. Fig. S4 in supplementary material shows the distribution of the phase

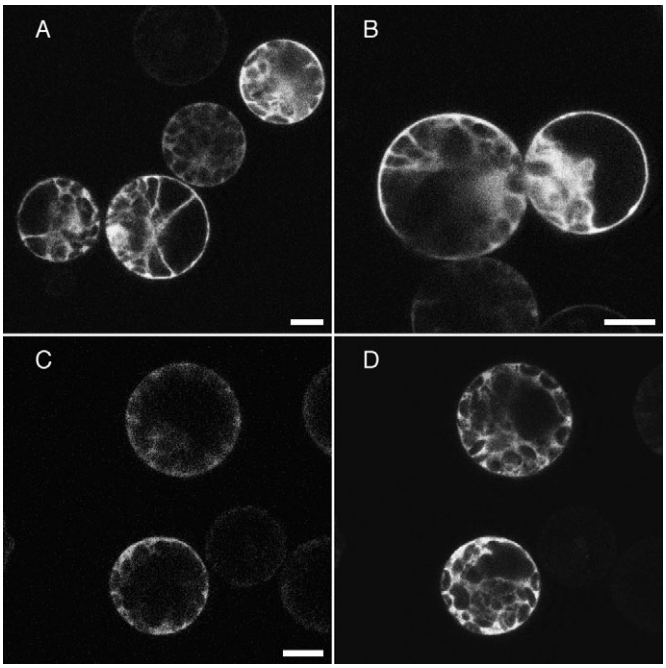

Fig. 4. Subcellular location of YFP-AGG2 lipidation mutants and the effect on localization of AGβ1. Protoplasts expressing (A) the prenylation-deficient construct YFP-AGG2mut and (B) YFP-AGG2(C95S). (C,D) Protoplasts co-expressing (C) CFP-AGβ1 and (D) YFP-AGG2mut. Bars, 10 μm.

Table 3. Fluorescence lifetime data

Donor (YFP)	Acceptor (RFP)	Unlabelled	*n*	YFP phase lifetime (nanoseconds) ± s.d.	YFP modulation lifetime (nanoseconds) ± s.d.
AGG1		AGβ1	8	2.75±0.11[a]	2.85±0.09[aa]
AGG1	AGβ1		24	2.52±0.12[a]	2.67±0.09[aa]
AGG2		AGβ1	24	2.70±0.10[b]	2.68±0.06
AGG2	AGβ1		26	2.58±0.12[b]	2.63±0.07
AGG2mut		AGβ1	8	2.65±0.05[c]	2.80±0.04[cc]
AGG2mut	AGβ1		19	2.35±0.17[c]	2.57±0.07[cc]
GPα1		AGβ1+AGG1	33	2.64±0.08[d]	2.79±0.05[dd]
GPα1	AGβ1	AGG1	30	2.43±0.09[d,e]	2.62±0.06[dd,ee]
GPα1(Q222L)		AGβ1+AGG1	34	2.64±0.09[f]	2.78±0.02[ff]
GPα1(Q222L)	AGβ1	AGG1	43	2.41±0.08[f,g]	2.61±0.06[ff,gg]
GPα1	AGβ1	AGβ1+AGG1	26	2.57±0.06[e]	2.78±0.07[ee]
GPα1(Q222L)	AGβ1	AGβ1+AGG1	35	2.53±0.07[g]	2.76±0.06[gg]

Each of the comparisons a, b, c, d, e, f, g, aa, cc, dd, ee, ff, gg indicates a significant difference between fluorescence lifetimes, i.e. confidence level of 95%, according to Student's *t*-test.

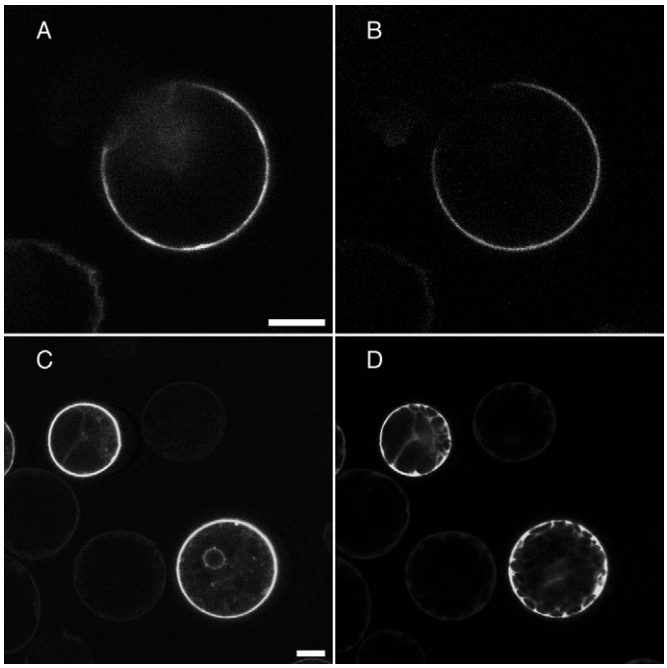

Fig. 5. The effect of co-expression on the localization of lipidation mutants. (A,B) Protoplast co-expressing (A) GPα1(C5S)-YFP and non-fluorescent AGG1 and (B) CFP-AGβ1 and non-fluorescent AGG1. (C,D) Protoplasts co-expressing (C) GPα1-YFP and non-fluorescent AGβ1 and (D) CFP-AGG2mut and non-fluorescent AGβ1. Bars, 10 μm.

lifetimes plotted against the modulation lifetimes obtained for the various combinations. A clear reduction of both phase lifetime and modulation lifetime is observed as compared to the control situation. This results in two significantly different distributions, which is indicative of FRET.

To ensure that the FRET observed in the heterotrimer was not due to surface concentration effects, we performed a competition assay. For this we studied the effect of co-expression of unfused AGβ1 on the FRET-efficiency between Gα and Gβ. If the interactions that were described here are specific, the unfused AGβ1 subunit should be able to compete with the RFP-AGβ1 version (Ruiz-Velasco and Ikeda, 2001), thereby reducing FRET and increasing the lifetime of YFP towards the values observed for the controls. Indeed, Table 3 shows that unfused AGβ1 increased the lifetime values of both GPα1-YFP and GPα1(Q222L)-YFP. This demonstrates that FRET between GPα1-YFP and RFP-AGβ1 is specific and not due to surface concentration. Because co-expression of unfused AGβ1 increased the lifetime of GPα1-YFP and GPα1(Q222L)-YFP with an equal efficiency, this confirms the notion that also GPα1(Q222L)-YFP is present as a heterotrimer with Gβγ in plants. Additionally, we found that the lifetimes measured for GPα1-YFP did not correlate negatively with the intensity of RFP-AGβ1, which again indicates that the FRET observed is not due to surface concentration effects (data not shown).

In order to gain insight into the location of heterotrimer formation, we co-expressed AGβ1 and AGG1 together with the lipidation motif mutants of GPα1. Surprisingly, plasma membrane distribution of GPα1(C5S)-YFP could not be restored (Fig. 5A,B), as was also observed for GPα1(G2A)-YFP (Fig. S5 in supplementary material). Similarly, GPα1 was not able to rescue plasma membrane localization of the AGβ1-AGG2mut heterodimer (Fig. 5C,D). Since the wild-type heterodimer and the GPα1 subunit were able to tether to the plasma membrane in the presence of their nonlipidated interacting partners these results indicate that the heterotrimer is assembled at the plasma membrane.

Discussion

Subcellular location and lipidation motifs of the *Arabidopsis* Gα subunit, GPα1

The N terminus of GPα1 contains the classical myristoylation motif (Table 1), which suggests that GPα1 is a genuine target of NMT in vivo. Indeed, the N-terminal peptide of GPα1 was demonstrated to be myristoylated by the *Arabidopsis* NMT1 (Boisson et al., 2003), a homologue of mammalian and yeast NMTs (Maurer-Stroh et al., 2002). Mutation of the glycine residue at position 2 caused cytosolic localization of the protein, as verified by means of FRAP. Exchanging the cysteine residue at position 5 for a serine had a similar effect. Cysteines in close proximity to myristoylated glycines are often palmitoylated or *S*-acylated by other fatty acids such as stearate or oleoyl (Schroeder et al., 1996). Hence, our results suggest that GPα1 is myristoylated at position 2 and acylated at position 5 and that both lipid modifications are required for plasma membrane localization of GPα1.

Subcellular location of the *Arabidopsis* Gβ and Gγ subunits, AGβ1, AGG1 and AGG2

In order to visualize their location and interaction in cells, we fused the *Arabidopsis* AGβ1 and AGG1/2 subunits to the C terminus of GFP, since this strategy was successful in obtaining functional proteins in mammalian cells (Ruiz-Velasco and Ikeda, 2001). Single transfection of either AGβ1 or AGG1 showed that the subunits are not able to bind to the plasma membrane. Contrary to AGG1, AGG2 alone did localize at the plasma membrane. We speculate that the higher plasma membrane affinity of AGG2 is caused by its overall lower negative charge, a higher amount of positively charged amino acids proximal to the CaaX box and/or a palmitoylated cysteine residue next to the CaaX box. Interestingly, AGG2 is expressed at its highest levels in 4-week-old roots, while AGG1 expression levels are low at that stage (Mason and Botella, 2001). Possibly, the difference in plasma membrane affinity between AGG1 and AGG2 described here might play a role in root development.

Importantly, Golgi-like structures were observed in cells expressing AGG1 or AGG2. This can be explained by processing of lipidated Gγ subunits on the ER and Golgi, since the enzymes that remove the three C-terminal amino acids and methylate the modified cysteine are found to be integral parts of these membranes (Bracha et al., 2002; Cadinanos et al., 2003; Rodriguez-Concepcion et al., 2000). In addition, the localization on the Golgi, makes it less likely that the fusion proteins are incorrectly folded.

Co-transfections of AGβ1 and AGG1 showed that the two subunits together attained enough affinity for the plasma membrane. Since monomeric AGG1 does not localize to the plasma membrane, we believe that an, as yet, unidentified region in AGβ1 must have membrane affinity. Interestingly, in

mammalian $G\beta_t$ an electrostatic region close to the farnesyl group of Gγ in crystal structures (Sondek et al., 1996) was implicated to increase membrane partitioning of the heterodimer (Murray et al., 2001). Possibly, a similar region in AGβ1 may serve as a membrane-targeting signal that synergistically interacts with the putative lipid group(s) attached to the AGG1 subunit.

The prenylation motif of AGG2 was necessary for plasma membrane localization of the heterodimer, as shown using AGG2mut. We therefore speculate that a similar domain in AGG1 is needed for plasma membrane targeting of the AGβ1-AGG1 dimer. Apparently, lipidation of AGG1 is insufficient to accomplish plasma membrane localization of AGG1 alone, but is sufficient to act in synergy with the low membrane affinity of AGβ1 (discussed above) to target the heterodimer to the plasma membrane. Alternatively, the fluorescent protein fused to AGG1 somehow shields its plasma membrane interaction site, but upon interaction with AGβ1, the orientation of the fluorescent protein on AGG1 is changed, no longer interfering with the membrane-interaction site. However, YFP-AGG1 alone is localized on the Golgi, demonstrating its ability to interact with membranes, which argues against this alternative. The possible interference of the GFP tag with membrane affinity of AGG1 can be tested when antibodies against AGG1 and AGG2 become available.

Our results indicate that the cysteine proximal to the CaaX box of AGG2 is involved in membrane tethering as well. This residue is conserved in Gγ subunits of both monocots and dicots. Similar observations have been made for Ras proteins in mammals and Gγ in yeast (Manahan et al., 2000; Willumsen et al., 1996). Interestingly, double-lipid-modified Gγ subunits have not been described in mammals. However, engineering a palmitoylation site into mammalian Gγ rescued plasma membrane localization of the Gβγ dimer in the absence of co-expressed Gα (Takida and Wedegaertner, 2003).

Heterodimerization of AGβ1 and AGG1/2

FLIM was used to examine direct interaction between plant G protein subunits in protoplasts overexpressing GFP fusions of these subunits. Sometimes the use of native promoters to regulate the expression of the proteins studied is preferred. However, we chose the 35S promoter and this transient expression system, since we obtain a clearly detectable fluorescent signal and a variation in expression levels between different protoplasts. The variation in (relative) expression levels allows for the evaluation of how critical the expression level is for localization and interaction (Koroleva et al., 2005). In general, the lifetimes we observed showed a small variability, irrespective of the relative concentration of the interacting proteins. In support, studies with mammalian G proteins have shown that using a strong promoter does not interfere with location or formation of the heterotrimer. The use of overexpressed G proteins and FRET microscopy in mammalian systems demonstrated that certain types of Gα dissociate from Gβγ upon activation, whereas others show a mere conformational change (Bunemann et al., 2003; Frank et al., 2005).

Our FRET studies demonstrate that the plant Gβ and Gγ subunits interact. Remarkably, the lipid motif mutant AGG2mut that remained in the cytosol also showed a clear decrease in the YFP lifetime in the presence of RFP-AGβ1, indicating a direct interaction, independent of lipidation. In mammals, lipidation has been shown to be dispensable for Gβγ formation (Muntz et al., 1992), exemplified by the fact that dimerization occurs before prenylation (Zhang and Casey, 1996). Hence, our results point to the existence of a similar mechanism in plants. In mammals, direct interaction between Gβ and Gγ subunits occurs through the formation of a coiled-coil structure by the N termini of both proteins (Clapham and Neer, 1997). When AGβ1 was expressed without its partner, a clear decrease in the number of cells expressing AGβ1 was observed. Similar results were previously reported for an AGβ1 N-terminal mutant (Obrdlik et al., 2000). A decrease in the amount of Gβ subunits upon removal of Gγ or impairment of their interaction has also been observed in both mammals and yeast, and therefore seems to be a general phenomenon among eukaryotes (Hirschman et al., 1997; Pellegrino et al., 1997).

Formation of the heterotrimer

In dicots the formation of the heterotrimer has not been reported yet. The FRET data in this study suggest that the GPα1 subunit interacts with the AGβ1 subunit. Rendering the GPα1 subunit constitutively active by mutating Q222 to L222 did not cause a loss of FRET, indicating that GPα1(Q222L) and AGβ1 still interact. Hence, the Q222L mutation in GPα1 does not appear to induce dissociation of the heterotrimer. However, it is important to consider the Förster equation for FRET, implying that an unchanged FRET efficiency between two states of a complex can indicate: (i) no change in orientation and distance between the chromophores, (ii) no change in distance but a change in orientation between the chromophores, with the restriction that the κ^2 orientation factor is the same for both states; and (iii) a change in distance, which is compensated by a change in orientation between the transition moments of the chromophores (Clegg, 1996; Wu and Brand, 1994). Strictly spoken, condition (iii) could imply that there is a slight increase in distance between Gα and Gβ upon activation (somewhere within a 0-1 nm distance scale) with restrictions on orientation, but no loss of the interaction. Since we strictly used monomeric GFPs and did controls for surface concentration effects, we exclude artifacts related to fluorescent protein dimerization or overexpression.

Clearly, the observation that GPα1(Q222L)-YFP interacts with AGβ1 in the presence of AGG1 is not in agreement with the dissociation dogma (Katada et al., 1984). Recently, similar observations have been reported for the mammalian $G\alpha_i$ subunit and the yeast Gα subunit (Bunemann et al., 2003; Klein et al., 2000). Interestingly, a signaling role for the intact heterotrimer has been described in mammalian cells (Clancy et al., 2005) as well as in plants, where the trimer was required to negatively regulate cell proliferation in the root apical meristem (Chen et al., 2006). Moreover, several observations have been made of AGβ1 mutants displaying a similar phenotype as GPα1 mutants (Ullah et al., 2003), which is in line with a signaling function of the heterotrimer. Hence, we propose, based on our results and those of others (Ullah et al., 2003; Chen et al., 2006), that the plant G protein operates as a non-dissociating heterotrimer at the plasma membrane and toggles between two conformations, representing the GDP- and GTP-bound state of GPα1. However, opposite effects have

also been described for GPα1 and AGβ1 mutants. Presumably, GPα1 or AGβ1 signals independently of the other in these cases.

Wild-type GPα1 might be constitutively active, since its intrinsic GTPase activity is low and spontaneous nucleotide exchange is very fast, compared with most mammalian Gα subunits (Willard and Siderovski, 2004). This could suggest that wild-type GPα1 and the Q222L mutant do not differ significantly. Notably, the G5 region in GPα1 diverges from the conserved sequence in mammalian Gα subunits and has been implicated in receptor-independent spontaneous GDP release (Iiri et al., 1994).

Kato et al. (Kato et al., 2004) demonstrated the formation of large complexes composed of Gα subunits and Gβ and Gγ1/2 subunits in membrane fractions of rice plants, as assayed by gel filtration. Activation of the Gα subunit caused these large complexes to fall apart, which was interpreted to suggest that the Gα subunit in monocots dissociates from the Gβγ dimer. Our results are markedly different: first, activation of GPα1 (mimicked by Q222L) does not seem to induce dissociation; and second, our FRET competition experiments showed no surface concentration effects or extensive higher complex formation between the subunits. Yet, our FRET data cannot resolve heterodimeric and small higher order complexes. The discrepancy could imply a difference between monocots and dicots. Alternatively, the disruption of the complex might be interpreted as other proteins dissociating from it, leaving the integrity of the heterotrimer intact.

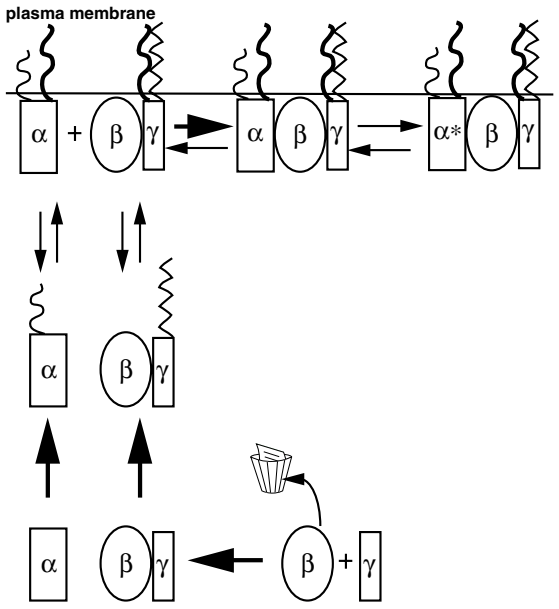

Fig. 6. Heterotrimer formation at the plant plasma membrane requires dual lipidation of Gα and Gγ. The model shows the formation of Gβγ dimers in the cytosol. The waste-basket symbolizes the breakdown of Gβ that occurs in the absence of Gγ. Dimerization does not require lipidation. Dual lipidation of Gγ targets the Gβγ dimer to the plasma membrane where it can form a heterotrimer with Gα that has been myristoylated in the cytosol and palmitoylated, probably at the plasma membrane. The heterotrimer does not fall apart upon activation of Gα (α*). The arrows indicate the putative direction(s) of the reactions. Heterodimer and heterotrimer formation might be regulated by de/repalmitoylation cycles.

Kato et al. (Kato et al., 2004) also proposed that lipidation of rice Gα may be necessary for Gβ interaction, since Gα subunits produced in *E. coli* did not interact with Gβ. Our observation that AGβ1 and AGG1 co-expression could not rescue the lipidation motif mutants of GPα1 indicates that in plants the Gα subunit and Gβγ dimer reach the plasma membrane independently of each other. In mammals, heterotrimer formation seems to occur in the cytosol or at Golgi membranes and is thought to be necessary to attain a high enough affinity for the plasma membrane (Evanko et al., 2001; Fishburn et al., 1999; Michaelson et al., 2002; Takida and Wedegaertner, 2003). In yeast, as in plants, loss of myristoylation or palmitoylation of Gpa1p resulted in mislocalization that no longer could be compensated by overexpression of the Gβγ dimer (Manahan et al., 2000). Also, GPα1 was not able to recruit AGβ1-AGG2mut to the membrane, again indicating that trimer formation occurs at the plasma membrane. It therefore seems that lateral association in the two-dimensional space of the plasma membrane is the only way in which the heterotrimer is being formed in plants (Fig. 6).

Importantly, the mechanism of heterotrimer assembly by means of lipidation enables the cell to regulate trimer formation in addition to regulation of GDP-GTP exchange and GTP hydrolysis. Since myristoylation and prenylation are stable types of protein modification that cannot be removed during the lifetime of the protein, regulation could occur at different developmental stages by changing the production of these lipid tails. However, regulation at the level of signaling by dynamic de/repalmitoylation of the GPα1 and/or the AGG1/2 subunits could also play a role. Such a phenomenon has recently been described for Ras signaling, for which Bastiaens and coworkers found a constitutive de/reacylation cycle to be indispensable for localization and activity of Ras isoforms (Rocks et al., 2005). Isoform-specific kinetics of this cycle resulted in downstream diversification of signaling. De/repalmitoylation cycles have also been described for several different mammalian Gα subunits (for review, see Qanbar and Bouvier, 2003). Also, methylation of the prenylated cysteine in Gγ subunits has been shown to be a dynamic modification, which has regulatory functions (Parish et al., 1995).

In conclusion, the data presented in this paper suggest that dual lipid modification of GPα1 and AGG2 is required for plasma membrane localization and heterodimer and heterotrimer formation, contrary to what has been described for mammals, providing the plant cell with novel ways of regulating G protein signaling.

Materials and Methods

Constructs

The pGEM7Zf(+) vector containing the cDNA encoding GPα1 of *Arabidopsis thaliana* ecotype Landsberg *erecta* (acc. no. M32887) was a kind gift from H. Ma (Penn State University, University Park, PA, USA). The cDNA was cloned into pMON999, making use of PCR with the primers: 5′-GCTCTAGAcaccatg-ggcttactctgcagtagaa-3′ and 5′-GGAATTCtcataaaaggccagcctccagta-3′, thereby introducing *Xba*I and *Eco*RI sites. Plasmid pMON999 is a plant expression vector that contains the enhanced cauliflower mosaic virus (CaMV) 35S promoter and the NOS terminator (van Bokhoven et al., 1993). A spectral variant of GFP, mVenus (Kremers et al., 2006), was inserted in the *Nhe*I site created by means of site-directed mutagenesis (Stratagene, La Jolla, CA, USA) in between residue A97 and Q98,

thereby changing Q98 to S98 (primer: 5′-caaaggagtttgctAGCaatgaaacagattc-3′). The linker present at the N terminus of YFP is GS, and at the C terminus the linker connecting YFP to GPα1 is GAS. Lipidation mutants were made using PCR with the following primers: 5′-GCTCTAGACACCatgGCTttactctgcagtagaagt-3′ for GPα1(G2A)-YFP and 5′-GCTCTAGACACCatgggcttactcTCTagtagaagtcgacatcat-3′ for GPα1(C5S)-YFP as forward and 5′-GGAATTCtcataaaaggccagcctccagta-3′ as reverse primer.

The cDNA encoding AGβ1 was obtained by PCR on an *Arabidopsis thaliana* cDNA library in the vector pGAD10 (Clontech, Mountain View, CA, USA). This library was constructed using 3-week-old green vegetative tissue of ecotype Columbia and contains 3 million independent cDNAs. The primers used (forward: 5′-AGATCTTCGatgtctgtctccgagctca-3′; reverse: 5′-GAATTCCGtcaaatcactctcct-gtg-3′) introduced the sites *Bgl*II and *Eco*RI. The PCR product was cloned into pECFP(A206K)-C1 (Kremers et al., 2006), pmVenus-C1 or pmStrawberry-C1 (Shaner et al., 2004). mStrawberry, a variant of RFP, was a kind gift from R. Y. Tsien (Howard Hughes Medical Institute, CA, USA). The cassette consisting of FP and AGβ1 (*Nhe*I/*Eco*RI) was cloned into pMON999 by means of *Xba*I/*Eco*RI.

The cDNA encoding AGG1 was kindly donated by J.,R. Botella (University of Queensland, Brisbane, Australia) and cloned into the pmVenus-C1 vector with *Bgl*II and *Eco*RI, using the following primers: 5′-AGATCTTCGatgcgagaggaaactgtgg-3′ as forward and 5′-GAATTCCGtcaaagtattaagcatctgcagcc-3′ as reverse primer. The cassette consisting of FP and AGG1 was cloned into pMON999 as described for AGβ1.

Arabidopsis AGG2 was obtained by means of PCR on the cDNA library described above (primers: 5′-AGATCTTCGatggaagcgggtagctcc-3′ and 5′-GAAT-TCCGtcaaagaatggagcagcc-3′) and cloned similarly as AGG1. One of the PCR products contained a frameshift in the STOP codon causing a larger protein; CSILTEFNHStop instead of CSILStop. This construct was used to investigate the role of prenylation and was named: AGG2mut. mVenus-AGG2(C95S) was made by means of PCR with the following primers: forward 5′-AGATCTTCGatgg-aagcgggtagctcc-3′ and reverse 5′-GAATTCCGtcaaagaatggagcagccaGatcgttttgc-ttctttagg-3′. The resulting PCR product was cloned as described above for AGG1.

The vector ST-RFP was a kind gift of Joop Vermeer (University of Amsterdam, Amsterdam, The Netherlands).

All constructs were verified by means of sequencing (BaseClear, Leiden, The Netherlands).

Preparation and transient transfection of cowpea protoplasts

Cowpea (*Vigna unguiculata* L.) mesophyll protoplasts were prepared and transfected as described by van Bokhoven et al. (van Bokhoven et al., 1993). After overnight incubation under continuous light, the samples were mounted in eight-well Lab-Tek chambered coverglasses (Nalge Nunc International, Rochester, NY, USA).

Confocal microscopy

Plant cells were imaged using a Zeiss LSM 510 confocal laser scanning microscope (Carl-Zeiss GmbH, Germany). The objectives used were either a Zeiss C-A 40×/1.2 W or a Zeiss Plan-A 63×/1.4 oil. Samples were excited with 458, 488, 514 nm argon and/or 568 nm argon/krypton laser lines controlled by an acousto-optical tunable filter. For CFP/YFP/chlorophyll the following settings were used: as primary dichroic mirror, HFT 458/514; as secondary dichroic mirror, NFT 635, thereby splitting the chlorophyll fluorescence from the CFP/YFP fluorescence. The third dichroic mirror, NFT 515, was used to discriminate between CFP and YFP fluorescence. To yield the chlorophyll image the LP 650 filter was used, for CFP the BP 470-500 nm filter was used and for YFP the BP 530-600 nm filter. In order to abolish crosstalk, the images were acquired in the multi-tracking mode, where the 514 nm laser line was coupled to the YFP detection channel and the 458 nm laser line to the CFP/chlorophyll detection channel. For YFP/RFP/chlorophyll the primary, secondary, tertiary A and tertiary B dichroic mirrors were HFT 488/568, NFT 570, NFT 490 and NFT 635, respectively. YFP fluorescence was induced at 488 nm, transmitted by the primary, reflected by the secondary and tertiary A dichroic mirrors and filtered using the BP 505-550 nm filter. RFP fluorescence was induced at 568 nm, transmitted through the primary and secondary dichroic mirrors and reflected by the tertiary B dichroic mirror and additionally filtered through the LP 585-615 filter. Chlorophyll fluorescence was induced at 488 nm, transmitted through the primary, secondary and tertiary B dichroic mirrors and detected after filtering through the LP 650 filter. Again, the images were taken in the multi-tracking mode.

Fluorescence recovery after photobleaching

The fluorescence recovery after photobleaching (FRAP) experiments were done using the two laser lines 488 and 514 nm set at maximum output (Acousto Optical Tunable Filter, AOTF, at 100%) to bleach a region of interest at the plasma membrane (with a duration of 400 mseconds), after which the recovery of fluorescence was measured every 250 mseconds with a very low laser output (AOTF at 3%, for which bleaching did not exceed 5-10%) and similar settings as described above.

Fluorescence lifetime imaging microscopy

Frequency-domain FLIM measurements were performed using the set-up described by van Munster and Gadella (van Munster and Gadella, 2004a). The objective used was a Zeiss plan Neofluar 40× 1.3 NA, oil-immersion objective. Samples were excited by means of a 514 nm Argon laser modulated at 75.1 MHz and a BP 530-560 nm emission filter was used to detect YFP fluorescence. FLIM stacks of 12 phase images permutated in recording order (van Munster and Gadella, 2004b) were acquired with an exposure time of 0.4-1.5 seconds each.

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