

Plant G protein heterotrimers require dual lipidation motifs of G α and G γ and do not dissociate upon activation

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Summary

In plants one bona fide G α subunit has been identified, as well as a single G β and two G γ 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* G α subunit, GP α 1, contains two lipidation motifs that were essential for plasma membrane localization. The *Arabidopsis* G β subunit, AG β 1, and the G γ subunit, AGG1, were dependent upon each other for tethering to the plasma membrane. The second G γ subunit, AGG2, did not require AG β 1 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 AG β 1 and AGG1 or AG β 1 and AGG2 can form heterodimers independent of lipidation. In addition, FLIM-FRET revealed the existence of GP α 1-AG β 1-AGG1 heterotrimers at the plasma membrane. Importantly, rendering GP α 1 constitutively active did not cause a FRET decrease in the heterotrimer, suggesting no dissociation upon GP α 1 activation.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/119/24/5087/DC1>

Key words: heterotrimeric G protein, *Arabidopsis thaliana*, GFP, lipid modification, FRET, FRAP

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 G α subunit to release the bound GDP and exchange it for GTP. This exchange promotes the dissociation of the G α subunit from the G $\beta\gamma$ 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 G α 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 G α subunit (GP α 1, 45 kDa), which is most identical (36%) to G α_{i1} , a member of the mammalian G $_i$ class of G α subunits (Ma, 1994; Ma et al., 1990). GP α 1 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 GP α 1, only G-5 is slightly different from the consensus sequence (Kaydamov et al., 2000). GP α 1 binds GTP γ S with a dissociation constant in the nanomolar range (Wise et al., 1997) and displays GTPase activity that can be stimulated by both phospholipase D α (PLD α) and the recently cloned *Arabidopsis* regulator of G protein signaling 1 (RGS1) protein (Chen et al., 2003; Zhao and Wang, 2004). The plant G α 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 GP α 1 is a bona fide candidate for a plant heterotrimeric G α subunit since it only shows minor differences in structure when compared to the crystal structures of mammalian G α

subunits. Cell fractionation and immunofluorescence studies demonstrate that GP α 1 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 GP α 1 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 G β subunit, AG β 1 (41 kDa), which has 44% sequence identity to mammalian G β ₂ (Weiss et al., 1994). Similar to mammalian G β subunits, AG β 1 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 G β in the crude membrane fractions of broccoli and *Arabidopsis*. Plant G γ subunits have only recently been described because of the lack of amino acid similarity among the known G γ subunits. The two G γ subunits that are expressed in *Arabidopsis*, AGG1 and AGG2, have been found by means of a yeast two-hybrid screen using tobacco G β 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 G β . This type of interaction causes mammalian G β and G γ subunits to function as an obligate heterodimer (Clapham and Neer, 1997). The model by Ullah et al. (Ullah et al., 2003) of GP α 1 also provides putative structures for AG β 1 and AGG1. When comparing these structures with the mammalian heterotrimer they showed that the GP α 1 residues interacting with AG β 1, as well as the AG β 1 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 G α subunits are either myristoylated, palmitoylated or decorated by both lipid tails. G γ subunits also depend on lipidation for plasma membrane localization and signaling (Manahan et al., 2000). Mammalian G γ 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* G α 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* G γ 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			
<i>HsG$\alpha_{i/o/z}$</i>	<i>N</i> -myristoylation	MG CxxS	(Galbiati et al., 1994; Grassie et al., 1994; Parenti et al., 1993; Wilson and Bourne, 1995)
	<i>S</i> -palmitoylation	where x=TL/TV in α_i x=TL in α_o x=RQ in α_z	
<i>HsGα_s</i>	<i>N</i> -palmitoylation	MG CLGNS	(Kleuss and Krause, 2003)
	<i>S</i> -palmitoylation		
<i>HsGα_q</i>	<i>S</i> -palmitoylation	MTLESIMACC	(Wedegaertner et al., 1993)
<i>HsGα_t</i>	<i>N</i> -myristoylation	MGAGAS	(Yang and Wensel, 1992)
<i>HsGα_{12}</i>	<i>S</i> -palmitoylation	MSGVVRTL SRC	(Ponimaskin et al., 1998)
<i>HsGα_{13}</i>	<i>S</i> -palmitoylation	MAD-¹⁴CFPQC^{18,37}CLS	(Ponimaskin et al., 2000)
<i>ScGpa1</i>	<i>N</i> -myristoylation	MGCTVS	(Song and Dohlman, 1996; Song et al., 1996)
	<i>S</i> -palmitoylation		
<i>AtGPα1</i>		MGLLCS	
Gγ subunits			
<i>HsGγ_1</i>	Farnesylation	KELKGGCVIS	(Sanford et al., 1991)
<i>HsGγ_2</i>	Geranylgeranylation	REKKFFCAIL	(Sanford et al., 1991)
<i>ScSTE18</i>	<i>S</i> -palmitoylation	NSNSVCCTLM	(Manahan et al., 2000; Whiteway and Thomas, 1994)
	Farnesylation		
<i>AtAGG1</i>		GGEGCRCLIL	
<i>AtAGG2</i>		EAKRCGC SIL	

*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 $G\alpha$ and the $G\gamma$ subunit AGG2, contrary to what has been described in mammals.

Results

Subcellular location and lipidation motifs of the *Arabidopsis* $G\alpha$ subunit, GP α 1

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 $G\alpha$ subunits were found to be non-functional (Yu and Rasenick, 2002), yellow fluorescent protein (YFP) (Tsien, 1998) was inserted into the *Arabidopsis* $G\alpha$ subunit (GP α 1). The insertion site was based on the paper by Chen et al. (Chen et al., 2003), in which they describe a GP α 1-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 GP α 1 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 α AB-loop of the α -helical domain to obtain a functional mammalian GFP-tagged $G\alpha_i$. Fig. 1A shows that GP α 1-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 GP α 1 contains the classical myristoylation consensus sequence: MGxxxS (Utsumi et al., 2004) that is conserved among $G\alpha$ 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, MGLLCS, 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 GP α 1(G2A)-YFP and GP α 1(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 GP α 1. Interestingly, the location of fluorescence was comparable to that of unfused YFP and the double mutant GP α 1(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, GP α 1(G2A)-YFP, GP α 1(C5S)-YFP and GP α 1(G2A/C5S)-YFP, we observed a very fast fluorescence recovery, which is typical of fast diffusing cytoplasmic proteins. However, GP α 1-YFP diffused significantly slower (Fig. 1F). The diffusion data thus confirm the localization data, showing that only GP α 1-YFP was able to bind to the plasma membrane.

Subcellular location of the *Arabidopsis* $G\beta$ and $G\gamma$ subunits, AG β 1, AGG1 and AGG2

The *Arabidopsis* $G\beta$ subunit (AG β 1) and the *Arabidopsis* $G\gamma$ subunits (AGG1 and AGG2) were fused to the C terminus of spectral variants of GFP. The two subunits, AG β 1 and AGG1, both showed a diffuse distribution in the cytoplasm (Fig. 2A and B, respectively). The other $G\gamma$ subunit, AGG2, was predominantly located at the plasma membrane (Fig. 2C). Occasionally, Golgi-like structures were observed in the cells expressing AG β 1 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 colocalization of YFP-AGG1 and ST-RFP.

Next, the effect of expression of YFP-AGG1 on the distribution of CFP-AG β 1 was studied. Co-expression of CFP-AG β 1 and YFP-AGG1 showed

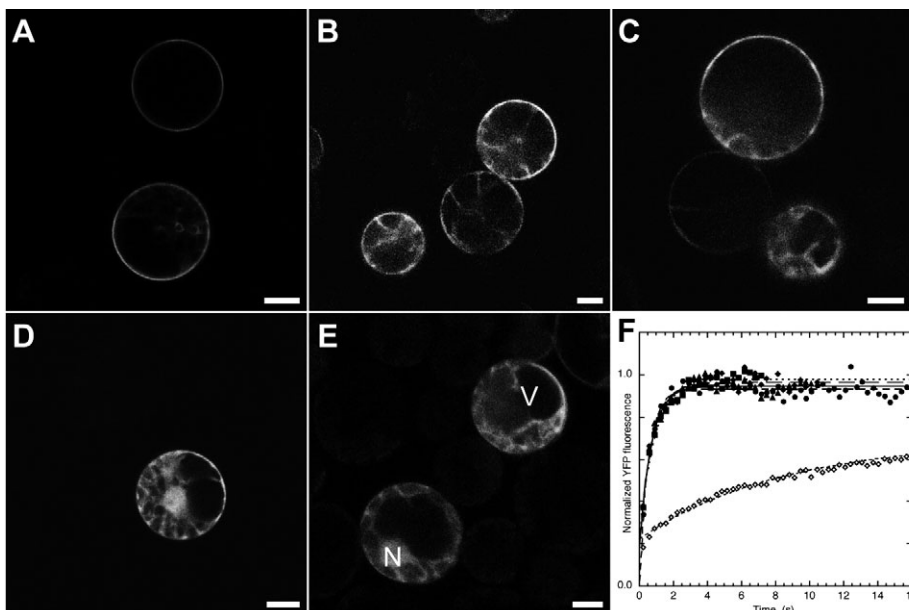
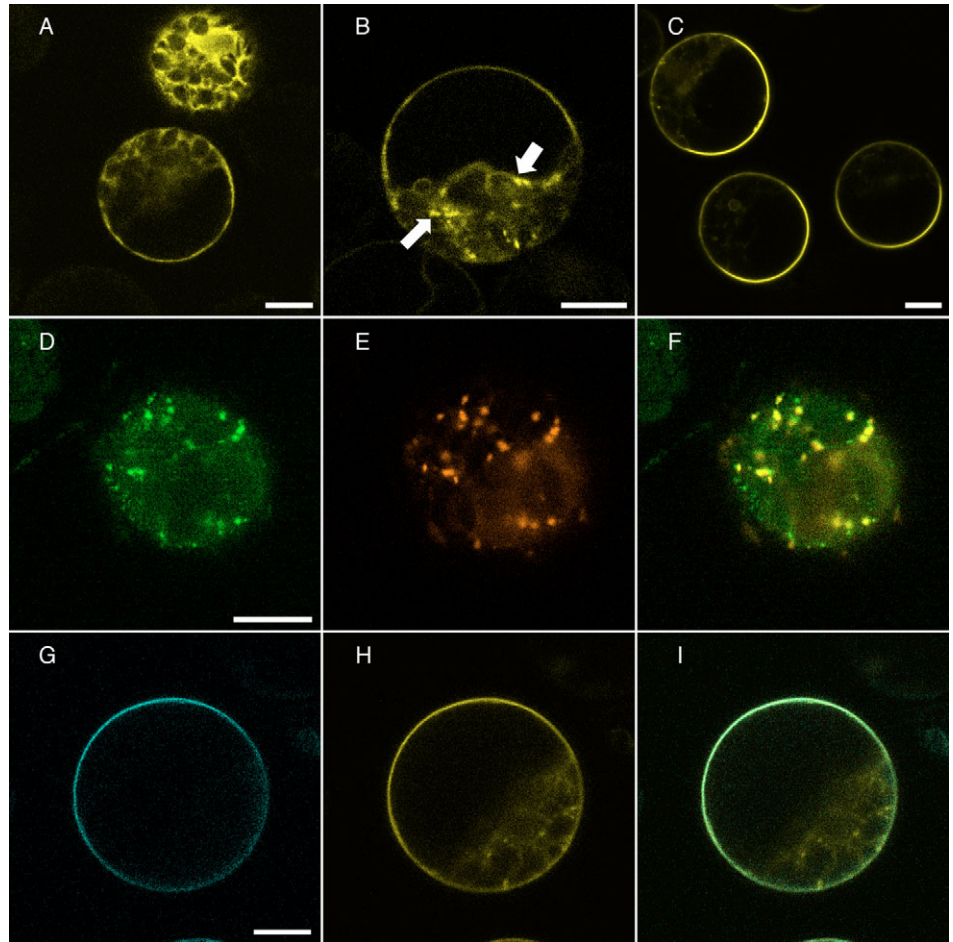


Fig. 1. Subcellular location and dynamics of GP α 1-YFP and its lipid motif mutants in cowpea protoplasts. Protoplasts expressing (A) GP α 1-YFP, (B) GP α 1(G2A)-YFP, (C) GP α 1(C5S)-YFP, (D) GP α 1(G2A/C5S)-YFP, (E) unfused YFP. N, nucleus; V, vacuole. (F) FRAP curves of YFP (triangles); GP α 1-YFP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

Fig. 2. Subcellular location of plant G β and G γ subunits in cowpea protoplasts. (A-C) Protoplasts expressing (A) YFP-AG β 1, (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-AG β 1 and (H) YFP-AGG1; (I) the overlaid images of G and H. Arrows indicate Golgi-like structures. Bars, 10 μ m.



plasma membrane targeting of both proteins (Fig. 2G,H). AGG2 was equally effective in targeting AG β 1 to the plasma membrane (Fig. S1 in supplementary material). During co-expression studies of AG β 1 and AGG1 we observed improved expression of AG β 1. Therefore, the number of YFP-AG β 1-expressing cells was quantified in the presence and absence of AGG1. We observed an approximate twofold increase in the number of cells expressing YFP-AG β 1 upon co-expression of AGG1, as compared to cells expressing only AG β 1 (Fig. 3; Table 2).

Lipidation motifs of AGG2 and heterodimerization of AG β 1 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 AG β 1 (Fig. 4C,D and Fig. S2 in supplementary material, respectively). These observations implicate a direct interaction between AG β 1 and AGG2.

Therefore, we analyzed interactions between AG β 1 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 $<100\text{\AA}$ and is characterized by a decrease in the fluorescence intensity and fluorescence lifetime of the donor

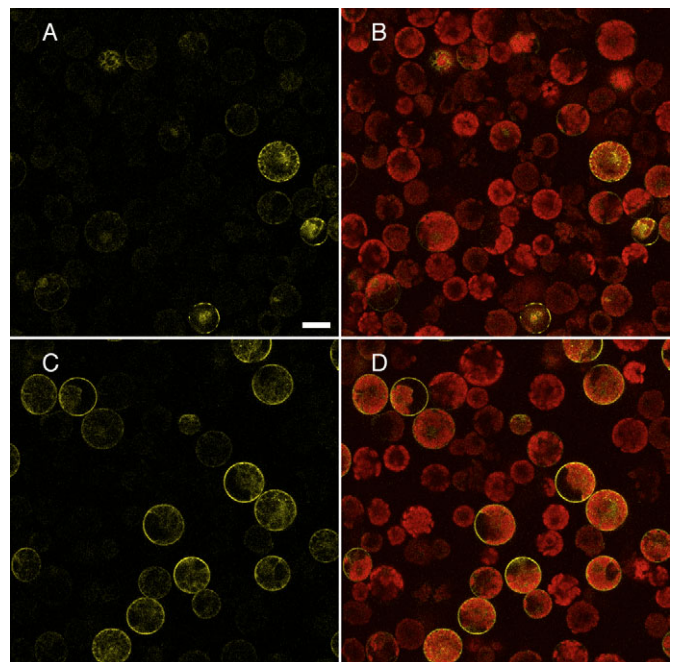


Fig. 3. The influence of AGG1 expression on the amount of YFP-AG β 1 expressors. (A,B) Protoplasts transfected with YFP-AG β 1. (C,D) Protoplasts transfected with both YFP-AG β 1 and AGG1. A and C show yellow fluorescence of protoplasts expressing YFP-AG β 1, B and D show yellow fluorescence of YFP-AG β 1-expressing protoplasts combined with the red chlorophyll autofluorescence of all protoplasts. Bar, 20 μ m.

Table 2. Number of protoplasts expressing YFP-AG β 1

Constructs	# cells	# YFP-AG β 1 cells	% YFP-AG β 1 cells
YFP-AG β 1	368	82	22
YFP-AG β 1+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 G β and G γ 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-AG β 1, 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-AG β 1 and again a decrease in lifetime was observed, indicating that AG β 1 interacts with AGG2mut. This suggests that in plants G $\beta\gamma$ dimerization is independent of the lipidation motif of the plant G γ subunit AGG2.

Formation of the heterotrimer

We studied heterotrimer formation in living plant cells with FLIM-FRET using GP α 1-YFP and RFP-AG β 1 in cowpea protoplasts, expressed together with unfused AGG1 in order to assure plasma membrane distribution of the G β subunit. The lifetime of GP α 1-YFP decreased upon expression with RFP-AG β 1, suggestive of the formation of a heterotrimer (Table 3).

Mutation of the glutamine that is present in the switch II of mammalian G α 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

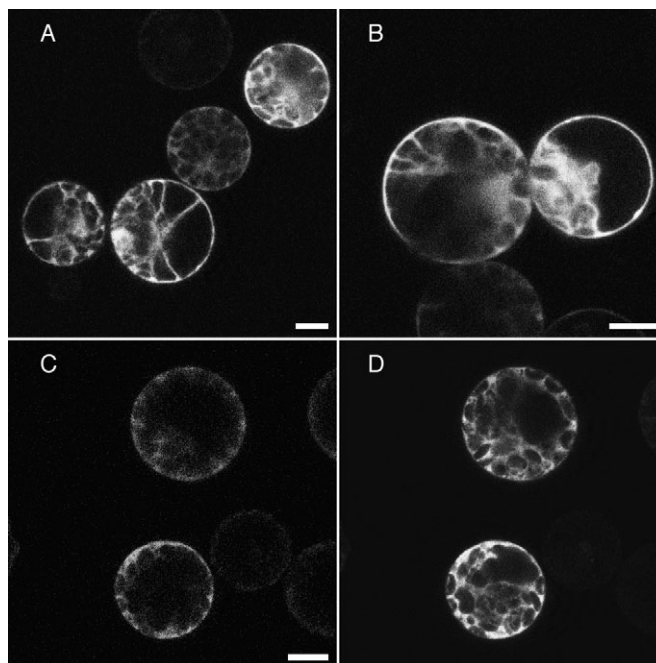


Fig. 4. Subcellular location of YFP-AGG2 lipidation mutants and the effect on localization of AG β 1. Protoplasts expressing (A) the prenylation-deficient construct YFP-AGG2mut and (B) YFP-AGG2(C95S). (C,D) Protoplasts co-expressing (C) CFP-AG β 1 and (D) YFP-AGG2mut. Bars, 10 μ m.

of this mutation in GP α 1, Q222L, did not change the subcellular location of GP α 1(Q222L)-YFP as compared to GP α 1-YFP, and FRAP analysis reported similar mobility characteristics (Fig. S3 in supplementary material), whereas GP α 1(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-AG β 1, since the lifetime of GP α 1(Q222L)-YFP upon expression with RFP-AG β 1 was efficiently quenched (Table 3). These above observations suggest that G α subunits containing this mutation still reside in the membrane as a heterotrimer with G $\beta\gamma$. Fig. S4 in supplementary material shows the distribution of the phase

Table 3. Fluorescence lifetime data

Donor (YFP)	Acceptor (RFP)	Unlabelled	<i>n</i>	YFP phase lifetime (nanoseconds) \pm s.d.	YFP modulation lifetime (nanoseconds) \pm s.d.
AGG1		AG β 1	8	2.75 \pm 0.11 ^a	2.85 \pm 0.09 ^{aa}
AGG1	AG β 1		24	2.52 \pm 0.12 ^a	2.67 \pm 0.09 ^{aa}
AGG2		AG β 1	24	2.70 \pm 0.10 ^b	2.68 \pm 0.06
AGG2	AG β 1		26	2.58 \pm 0.12 ^b	2.63 \pm 0.07
AGG2mut		AG β 1	8	2.65 \pm 0.05 ^c	2.80 \pm 0.04 ^{cc}
AGG2mut	AG β 1		19	2.35 \pm 0.17 ^c	2.57 \pm 0.07 ^{cc}
GP α 1		AG β 1+AGG1	33	2.64 \pm 0.08 ^d	2.79 \pm 0.05 ^{dd}
GP α 1	AG β 1	AGG1	30	2.43 \pm 0.09 ^{d,e}	2.62 \pm 0.06 ^{dd,ee}
GP α 1(Q222L)		AG β 1+AGG1	34	2.64 \pm 0.09 ^f	2.78 \pm 0.02 ^{ff}
GP α 1(Q222L)	AG β 1	AGG1	43	2.41 \pm 0.08 ^{f,g}	2.61 \pm 0.06 ^{ff,gg}
GP α 1	AG β 1	AG β 1+AGG1	26	2.57 \pm 0.06 ^e	2.78 \pm 0.07 ^{ee}
GP α 1(Q222L)	AG β 1	AG β 1+AGG1	35	2.53 \pm 0.07 ^g	2.76 \pm 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.

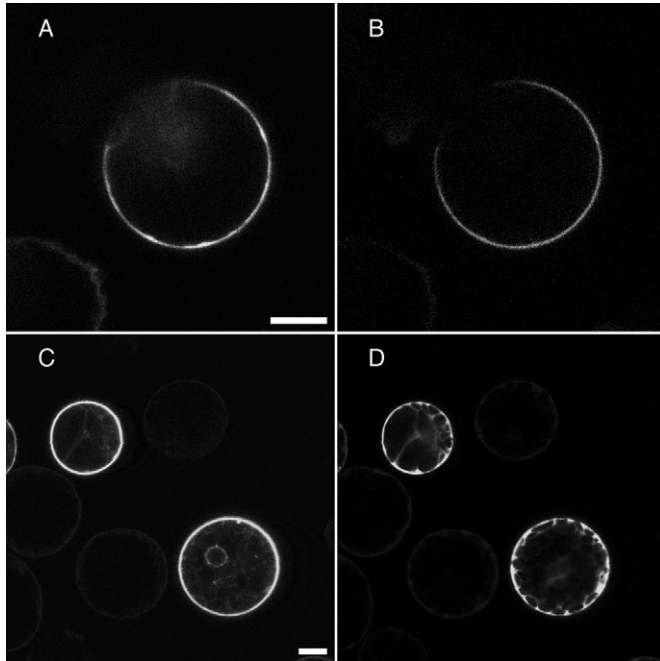


Fig. 5. The effect of co-expression on the localization of lipidation mutants. (A,B) Protoplast co-expressing (A) GP α 1(C5S)-YFP and non-fluorescent AGG1 and (B) CFP-AG β 1 and non-fluorescent AGG1. (C,D) Protoplasts co-expressing (C) GP α 1-YFP and non-fluorescent AG β 1 and (D) CFP-AGG2mut and non-fluorescent AG β 1. Bars, 10 μ m.

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 AG β 1 on the FRET-efficiency between G α and G β . If the interactions that were described here are specific, the unfused AG β 1 subunit should be able to compete with the RFP-AG β 1 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 AG β 1 increased the lifetime values of both GP α 1-YFP and GP α 1(Q222L)-YFP. This demonstrates that FRET between GP α 1-YFP and RFP-AG β 1 is specific and not due to surface concentration. Because co-expression of unfused AG β 1 increased the lifetime of GP α 1-YFP and GP α 1(Q222L)-YFP with an equal efficiency, this confirms the notion that also GP α 1(Q222L)-YFP is present as a heterotrimer with G β γ in plants. Additionally, we found that the lifetimes measured for GP α 1-YFP did not correlate negatively with the intensity of RFP-AG β 1, 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 AG β 1 and AGG1 together with the lipidation motif mutants of GP α 1. Surprisingly, plasma membrane distribution of GP α 1(C5S)-YFP could not be

restored (Fig. 5A,B), as was also observed for GP α 1(G2A)-YFP (Fig. S5 in supplementary material). Similarly, GP α 1 was not able to rescue plasma membrane localization of the AG β 1-AGG2mut heterodimer (Fig. 5C,D). Since the wild-type heterodimer and the GP α 1 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* G α subunit, GP α 1

The N terminus of GP α 1 contains the classical myristoylation motif (Table 1), which suggests that GP α 1 is a genuine target of NMT *in vivo*. Indeed, the N-terminal peptide of GP α 1 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 GP α 1 is myristoylated at position 2 and acylated at position 5 and that both lipid modifications are required for plasma membrane localization of GP α 1.

Subcellular location of the *Arabidopsis* G β and G γ subunits, AG β 1, AGG1 and AGG2

In order to visualize their location and interaction in cells, we fused the *Arabidopsis* AG β 1 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 AG β 1 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 G γ 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 AG β 1 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 AG β 1 must have membrane affinity. Interestingly, in

mammalian G β_i an electrostatic region close to the farnesyl group of G γ 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 AG β 1 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 AG β 1-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 AG β 1 (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 AG β 1, 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 G γ subunits of both monocots and dicots. Similar observations have been made for Ras proteins in mammals and G γ in yeast (Manahan et al., 2000; Willumsen et al., 1996). Interestingly, double-lipid-modified G γ subunits have not been described in mammals. However, engineering a palmitoylation site into mammalian G γ rescued plasma membrane localization of the G $\beta\gamma$ dimer in the absence of co-expressed G α (Takida and Wedegaertner, 2003).

Heterodimerization of AG β 1 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 G α dissociate from G $\beta\gamma$ upon activation, whereas others show a mere conformational change (Bunemann et al., 2003; Frank et al., 2005).

Our FRET studies demonstrate that the plant G β and G γ 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-AG β 1,

indicating a direct interaction, independent of lipidation. In mammals, lipidation has been shown to be dispensable for G $\beta\gamma$ 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 G β and G γ subunits occurs through the formation of a coiled-coil structure by the N termini of both proteins (Clapham and Neer, 1997). When AG β 1 was expressed without its partner, a clear decrease in the number of cells expressing AG β 1 was observed. Similar results were previously reported for an AG β 1 N-terminal mutant (Obrdlik et al., 2000). A decrease in the amount of G β subunits upon removal of G γ 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 GP α 1 subunit interacts with the AG β 1 subunit. Rendering the GP α 1 subunit constitutively active by mutating Q222 to L222 did not cause a loss of FRET, indicating that GP α 1(Q222L) and AG β 1 still interact. Hence, the Q222L mutation in GP α 1 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 G α and G β 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 GP α 1(Q222L)-YFP interacts with AG β 1 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 α_i subunit and the yeast G α 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 AG β 1 mutants displaying a similar phenotype as GP α 1 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 GP α 1. However, opposite effects have

also been described for GP α 1 and AG β 1 mutants. Presumably, GP α 1 or AG β 1 signals independently of the other in these cases.

Wild-type GP α 1 might be constitutively active, since its intrinsic GTPase activity is low and spontaneous nucleotide exchange is very fast, compared with most mammalian G α subunits (Willard and Siderovski, 2004). This could suggest that wild-type GP α 1 and the Q222L mutant do not differ significantly. Notably, the G5 region in GP α 1 diverges from the conserved sequence in mammalian G α 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 G α subunits and G β and G γ 1/2 subunits in membrane fractions of rice plants, as assayed by gel filtration. Activation of the G α subunit caused these large complexes to fall apart, which was interpreted to suggest that the G α subunit in monocots dissociates from the G $\beta\gamma$ dimer. Our results are markedly different: first, activation of GP α 1 (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.

Kato et al. (Kato et al., 2004) also proposed that lipidation of rice G α may be necessary for G β interaction, since G α subunits produced in *E. coli* did not interact with G β . Our observation that AG β 1 and AGG1 co-expression could not rescue the lipidation motif mutants of GP α 1 indicates that in plants the G α subunit and G $\beta\gamma$ 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 G $\beta\gamma$ dimer (Manahan et al., 2000). Also, GP α 1 was not able to recruit AG β 1-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 of the signaling by dynamic de/repalmitoylation of the GP α 1 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 G α subunits (for review, see Qanbar and Bouvier, 2003). Also, methylation of the prenylated cysteine in G γ 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 GP α 1 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.

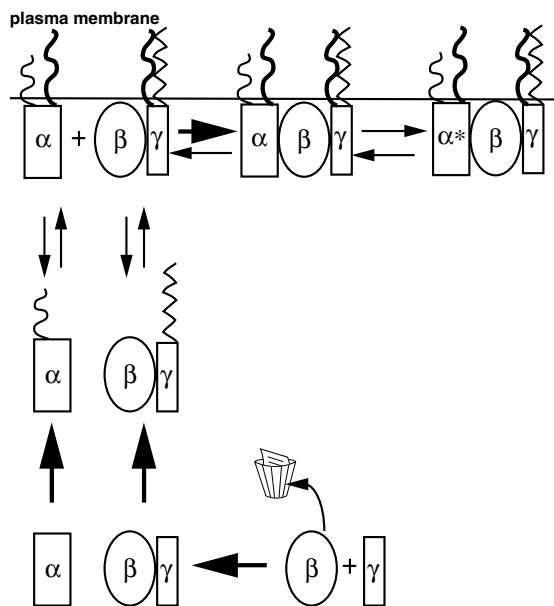


Fig. 6. Heterotrimer formation at the plant plasma membrane requires dual lipidation of G α and G γ . The model shows the formation of G $\beta\gamma$ dimers in the cytosol. The waste-basket symbolizes the breakdown of G β that occurs in the absence of G γ . Dimerization does not require lipidation.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

Materials and Methods

Constructs

The pGEM7Zf(+) vector containing the cDNA encoding GP α 1 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'-GCTCTAGAcaccatgggctactctgcagtagaa-3' and 5'-GGAATTCtataaaaggccagcctccagta-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,

- Huang, J., Taylor, J. P., Chen, J. G., Uhrig, J. F., Schnell, D. J., Nakagawa, T., Korth, K. L. and Jones, A. M. (2006). The plastid protein THYLAKOID FORMATION1 and the plasma membrane G-protein GPA1 interact in a novel sugar-signaling mechanism in Arabidopsis. *Plant Cell* **18**, 1226-1238.
- Iiri, T., Herzmark, P., Nakamoto, J. M., van Dop, C. and Bourne, H. R. (1994). Rapid GDP release from Gs alpha in patients with gain and loss of endocrine function. *Nature* **371**, 164-168.
- Jones, A. M. and Assmann, S. M. (2004). Plants: the latest model system for G-protein research. *EMBO Rep.* **5**, 572-578.
- Jones, A. M., Ecker, J. R. and Chen, J. G. (2003). A reevaluation of the role of the heterotrimeric G protein in coupling light responses in Arabidopsis. *Plant Physiol.* **131**, 1623-1627.
- Katada, T., Northup, J. K., Bokoch, G. M., Ui, M. and Gilman, A. G. (1984). The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. Subunit dissociation and guanine nucleotide-dependent hormonal inhibition. *J. Biol. Chem.* **259**, 3578-3585.
- Kato, C., Mizutani, T., Tamaki, H., Kumagai, H., Kamiya, T., Hirobe, A., Fujisawa, Y., Kato, H. and Iwasaki, Y. (2004). Characterization of heterotrimeric G protein complexes in rice plasma membrane. *Plant J.* **38**, 320-331.
- Kaydamov, C., Tewes, A., Adler, K. and Manteuffel, R. (2000). Molecular characterization of cDNAs encoding G protein alpha and beta subunits and study of their temporal and spatial expression patterns in Nicotiana glauca. *Biochim. Biophys. Acta* **1491**, 143-160.
- Klein, S., Reuveni, H. and Levitzki, A. (2000). Signal transduction by a nondissociable heterotrimeric yeast G protein. *Proc. Natl. Acad. Sci. USA* **97**, 3219-3223.
- Kluss, C. and Gilman, A. G. (1997). Galpha contains an unidentified covalent modification that increases its affinity for adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **94**, 6116-6120.
- Kluss, C. and Krause, E. (2003). Galpha(s) is palmitoylated at the N-terminal glycine. *EMBO J.* **22**, 826-832.
- Koroleva, O. A., Tomlinson, M. L., Leader, D., Shaw, P. and Doonan, J. H. (2005). High-throughput protein localization in Arabidopsis using Agrobacterium-mediated transient expression of GFP-ORF fusions. *Plant J.* **41**, 162-174.
- Kremers, G. J., Goedhart, J., van Munster, E. B. and Gadella, T. W., Jr (2006). Cyan and yellow super fluorescent proteins with improved brightness, protein folding, and FRET Förster radius. *Biochemistry* **45**, 6570-6580.
- Lippincott-Schwartz, J. and Patterson, G. H. (2003). Development and use of fluorescent protein markers in living cells. *Science* **300**, 87-91.
- Ma, H. (1994). GTP-binding proteins in plants: new members of an old family. *Plant Mol. Biol.* **26**, 1611-1636.
- Ma, H., Yanofsky, M. F. and Meyerowitz, E. M. (1990). Molecular cloning and characterization of GPA1, a G protein alpha subunit gene from Arabidopsis thaliana. *Proc. Natl. Acad. Sci. USA* **87**, 3821-3825.
- Manahan, C. L., Patnana, M., Blumer, K. J. and Linder, M. E. (2000). Dual lipid modification motifs in G(alpha) and G(gamma) subunits are required for full activity of the pheromone response pathway in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **11**, 957-968.
- Mason, M. G. and Botella, J. R. (2000). Completing the heterotrimer: isolation and characterization of an Arabidopsis thaliana G protein gamma-subunit cDNA. *Proc. Natl. Acad. Sci. USA* **97**, 14784-14788.
- Mason, M. G. and Botella, J. R. (2001). Isolation of a novel G-protein gamma-subunit from Arabidopsis thaliana and its interaction with Gbeta. *Biochim. Biophys. Acta* **1520**, 147-153.
- Maurer-Stroh, S., Eisenhaber, B. and Eisenhaber, F. (2002). N-terminal N-myristoylation of proteins: refinement of the sequence motif and its taxon-specific differences. *J. Mol. Biol.* **317**, 523-540.
- Michaelson, D., Ahearn, I., Bergo, M., Young, S. and Philips, M. (2002). Membrane trafficking of heterotrimeric G proteins via the endoplasmic reticulum and Golgi. *Mol. Biol. Cell* **13**, 3294-3302.
- Muntz, K. H., Sternweis, P. C., Gilman, A. G. and Mumby, S. M. (1992). Influence of gamma subunit prenylation on association of guanine nucleotide-binding regulatory proteins with membranes. *Mol. Biol. Cell* **3**, 49-61.
- Murray, D., McLaughlin, S. and Honig, B. (2001). The role of electrostatic interactions in the regulation of the membrane association of G protein beta gamma heterodimers. *J. Biol. Chem.* **276**, 45153-45159.
- Orbdlík, P., Neuhaus, G. and Merkle, T. (2000). Plant heterotrimeric G protein beta subunit is associated with membranes via protein interactions involving coiled-coil formation. *FEBS Lett.* **476**, 208-212.
- Offermanns, S. (2003). G-proteins as transducers in transmembrane signalling. *Prog. Biophys. Mol. Biol.* **83**, 101-130.
- Parenti, M., Viganò, M. A., Newman, C. M., Milligan, G. and Magee, A. I. (1993). A novel N-terminal motif for palmitoylation of G-protein alpha subunits. *Biochem. J.* **291**, 349-353.
- Parish, C. A., Smrcka, A. V. and Rando, R. R. (1995). Functional significance of beta gamma-subunit carboxymethylation for the activation of phospholipase C and phosphoinositide 3-kinase. *Biochemistry* **34**, 7722-7727.
- Pellegrino, S., Zhang, S., Garritsen, A. and Simonds, W. F. (1997). The coiled-coil region of the G protein beta subunit. Mutational analysis of Ggamma and effector interactions. *J. Biol. Chem.* **272**, 25360-25366.
- Perfus-Barbeoch, L., Jones, A. M. and Assmann, S. M. (2004). Plant heterotrimeric G protein function: insights from Arabidopsis and rice mutants. *Curr. Opin. Plant Biol.* **7**, 719-731.
- Ponimaskin, E., Harteneck, C., Schultz, G. and Schmidt, M. F. (1998). A cysteine-11 to serine mutant of G alpha12 impairs activation through the thrombin receptor. *FEBS Lett.* **429**, 370-374.
- Ponimaskin, E., Behn, H., Adarichev, V., Voyno-Yasenetskaya, T. A., Offermanns, S. and Schmidt, M. F. (2000). Acylation of Galpha(13) is important for its interaction with thrombin receptor, transforming activity and actin stress fiber formation. *FEBS Lett.* **478**, 173-177.
- Qanbar, R. and Bouvier, M. (2003). Role of palmitoylation/depalmitoylation reactions in G-protein-coupled receptor function. *Pharmacol. Ther.* **97**, 1-33.
- Rocks, O., Peyker, A., Kahms, M., Vermeer, P. J., Koerner, C., Lumbierres, M., Kuhlmann, J., Waldmann, H., Wittinghofer, A. and Bastiaens, P. I. (2005). An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. *Science* **307**, 1746-1752.
- Rodriguez-Concepcion, M., Toledo-Ortiz, G., Yalovsky, S., Caldelari, D. and Gruissem, W. (2000). Carboxyl-methylation of prenylated calmodulin CaM53 is required for efficient plasma membrane targeting of the protein. *Plant J.* **24**, 775-784.
- Rolland, N., Ferro, M., Ephritikhine, G., Marmagne, A., Ramus, C., Brugiere, S., Salvi, D., Seigneurin-Berny, D., Bourguignon, J., Barbier-Brygoo, H. et al. (2006). A versatile method for deciphering plant membrane proteomes. *J. Exp. Bot.* **57**, 1579-1589.
- Ruiz-Velasco, V. and Ikeda, S. R. (2001). Functional expression and FRET analysis of green fluorescent proteins fused to G-protein subunits in rat sympathetic neurons. *J. Physiol.* **537**, 679-692.
- Sanford, J., Codina, J. and Birnbaumer, L. (1991). Gamma-subunits of G proteins, but not their alpha- or beta-subunits, are polyisoprenylated. Studies on post-translational modifications using in vitro translation with rabbit reticulocyte lysates. *J. Biol. Chem.* **266**, 9570-9579.
- Schroeder, H., Leventis, R., Shahinian, S., Walton, P. A. and Silvius, J. R. (1996). Lipid-modified, cysteinyl-containing peptides of diverse structures are efficiently S-acylated at the plasma membrane of mammalian cells. *J. Cell Biol.* **134**, 647-660.
- Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E. and Tsien, R. Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat. Biotechnol.* **22**, 1567-1572.
- Simon, M. I., Strathmann, M. P. and Gautam, N. (1991). Diversity of G proteins in signal transduction. *Science* **252**, 802-808.
- Smotrys, J. E. and Linder, M. E. (2004). Palmitoylation of intracellular signaling proteins: regulation and function. *Annu. Rev. Biochem.* **73**, 559-587.
- Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E. and Sigler, P. B. (1996). Crystal structure of a G-protein beta gamma dimer at 2.1 Å resolution. *Nature* **379**, 369-374.
- Song, J. and Dohlman, H. G. (1996). Partial constitutive activation of pheromone responses by a palmitoylation-site mutant of a G protein alpha subunit in yeast. *Biochemistry* **35**, 14806-14817.
- Song, J., Hirschman, J., Gunn, K. and Dohlman, H. G. (1996). Regulation of membrane and subunit interactions by N-myristoylation of a G protein alpha subunit in yeast. *J. Biol. Chem.* **271**, 20273-20283.
- Sprang, S. R. (1997). G protein mechanisms: insights from structural analysis. *Annu. Rev. Biochem.* **66**, 639-678.
- Svoboda, P. and Novotny, J. (2002). Hormone-induced subcellular redistribution of trimeric G proteins. *Cell. Mol. Life Sci.* **59**, 501-512.
- Swarthout, J. T., Lobo, S., Farh, L., Croke, M. R., Greentree, W. K., Deschenes, R. J. and Linder, M. E. (2005). DHHC9 and GCP16 constitute a human protein fatty acyltransferase with specificity for H- and N-Ras. *J. Biol. Chem.* **280**, 31141-31148.
- Takida, S. and Wedegaertner, P. B. (2003). Heterotrimer formation, together with isoprenylation, is required for plasma membrane targeting of Gbetagamma. *J. Biol. Chem.* **278**, 17284-17290.
- Tsien, R. Y. (1998). The green fluorescent protein. *Annu. Rev. Biochem.* **67**, 509-544.
- Ullah, H., Chen, J. G., Temple, B., Boyes, D. C., Alonso, J. M., Davis, K. R., Ecker, J. R. and Jones, A. M. (2003). The beta-subunit of the Arabidopsis G protein negatively regulates auxin-induced cell division and affects multiple developmental processes. *Plant Cell* **15**, 393-409.
- Utsumi, T., Nakano, K., Funakoshi, T., Kayano, Y., Nakao, S., Sakurai, N., Iwata, H. and Ishisaka, R. (2004). Vertical-scanning mutagenesis of amino acids in a model N-myristoylation motif reveals the major amino-terminal sequence requirements for protein N-myristoylation. *Eur. J. Biochem.* **271**, 863-874.
- van Bokhoven, H., Verver, J., Wellink, J. and van Kammen, A. (1993). Protoplasts transiently expressing the 200K coding sequence of cowpea mosaic virus B-RNA support replication of M-RNA. *J. Gen. Virol.* **74**, 2233-2241.
- Van Munster, E. B. and Gadella, T. W., Jr (2004a). phiFLIM: a new method to avoid aliasing in frequency-domain fluorescence lifetime imaging microscopy. *J. Microsc.* **213**, 29-38.
- van Munster, E. B. and Gadella, T. W., Jr (2004b). Suppression of photobleaching-induced artifacts in frequency-domain FLIM by permutation of the recording order. *Cytometry A* **58**, 185-194.
- Vermeer, J. E., Van Munster, E. B., Vischer, N. O. and Gadella, T. W., Jr (2004). Probing plasma membrane microdomains in cowpea protoplasts using lipidated GFP-fusion proteins and multimode FRET microscopy. *J. Microsc.* **214**, 190-200.
- Wedegaertner, P. B., Chu, D. H., Wilson, P. T., Levis, M. J. and Bourne, H. R. (1993). Palmitoylation is required for signaling functions and membrane attachment of Gq alpha and Gs alpha. *J. Biol. Chem.* **268**, 25001-25008.
- Weiss, C. A., Huang, H. and Ma, H. (1993). Immunolocalization of the G protein alpha subunit encoded by the GPA1 gene in Arabidopsis. *Plant Cell* **5**, 1513-1528.

- Weiss, C. A., Garnaat, C. W., Mukai, K., Hu, Y. and Ma, H. (1994). Isolation of cDNAs encoding guanine nucleotide-binding protein beta-subunit homologues from maize (ZGB1) and Arabidopsis (AGB1). *Proc. Natl. Acad. Sci. USA* **91**, 9554-9558.
- Weiss, C. A., White, E., Huang, H. and Ma, H. (1997). The G protein alpha subunit (GP alpha1) is associated with the ER and the plasma membrane in meristematic cells of Arabidopsis and cauliflower. *FEBS Lett.* **407**, 361-367.
- Whiteway, M. S. and Thomas, D. Y. (1994). Site-directed mutations altering the CAAX box of Ste18, the yeast pheromone-response pathway G gamma subunit. *Genetics* **137**, 967-976.
- Willard, F. S. and Siderovski, D. P. (2004). Purification and in vitro functional analysis of the Arabidopsis thaliana regulator of G-protein signaling-1. *Methods Enzymol* **389**, 320-338.
- Willumsen, B. M., Cox, A. D., Solski, P. A., Der, C. J. and Buss, J. E. (1996). Novel determinants of H-Ras plasma membrane localization and transformation. *Oncogene* **13**, 1901-1909.
- Wilson, P. T. and Bourne, H. R. (1995). Fatty acylation of alpha z. Effects of palmitoylation and myristoylation on alpha z signaling. *J. Biol. Chem.* **270**, 9667-9675.
- Wise, A., Thomas, P. G., Carr, T. H., Murphy, G. A. and Millner, P. A. (1997). Expression of the Arabidopsis G-protein GP alpha1: purification and characterisation of the recombinant protein. *Plant Mol. Biol.* **33**, 723-728.
- Wu, P. and Brand, L. (1994). Resonance energy transfer: methods and applications. *Anal. Biochem.* **218**, 1-13.
- Yalovsky, S., Rodr Guez-Concepcion, M. and Gruissem, W. (1999). Lipid modifications of proteins – slipping in and out of membranes. *Trends Plant Sci.* **4**, 439-445.
- Yang, Z. and Wensel, T. G. (1992). N-myristoylation of the rod outer segment G protein, transducin, in cultured retinas. *J. Biol. Chem.* **267**, 23197-23201.
- Yu, J. Z. and Rasenick, M. M. (2002). Real-time visualization of a fluorescent G(alpha)(s): dissociation of the activated G protein from plasma membrane. *Mol. Pharmacol.* **61**, 352-359.
- Zhang, F. L. and Casey, P. J. (1996). Protein prenylation: molecular mechanisms and functional consequences. *Annu. Rev. Biochem.* **65**, 241-269.
- Zhao, J. and Wang, X. (2004). Arabidopsis phospholipase Dalphal1 interacts with the heterotrimeric G-protein alpha-subunit through a motif analogous to the DRY motif in G-protein-coupled receptors. *J. Biol. Chem.* **279**, 1794-1800.