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Plant heterotrimeric G protein β subunit is associated with membranes via protein interactions involving coiled-coil formation

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Abstract Gβ subunits from animals are anchored to membranes via Gγ subunits. No Gγ has been identified in plants to date. Using differential centrifugation of *Arabidopsis* and broccoli extracts, Gβ was highly enriched in the microsomal pellet. Treatment of microsomes with detergents and salts indicates that plant Gβ is located at the membrane surface and attached to membranes by hydrophobic interactions. Analysis of transgenic plants expressing Gβ–GFP fusion proteins showed that mutations in the heptad repeat domain of Gβ severely diminished their membrane association. We propose that plant Gβ is anchored to membranes by an unknown protein similar to animal Gβ by Gγ, via coiled-coil formation.

Key words: Heterotrimeric G protein; Gβ subunit; Gγ subunit; Membrane attachment; Protein solubilization; *Arabidopsis*

1. Introduction

Heterotrimeric GTP-binding proteins (G proteins) consist of three subunits, α, β, and γ, and function as major signal transducers between G protein-coupled receptors (GPCRs) at membranes and intracellular effectors. The stimulation of a receptor at the extracellular face of the plasma membrane leads to an exchange of Gα-bound GDP for GTP and subsequent dissociation of the complex into Gα and the tightly associated Gβγ dimer [1,2]. Both Gα-GTP and Gβγ can then activate distinct signaling effectors [1–3].

G proteins composed of different α, β, and γ subunits have been shown to transduce various signals within the same cell [4–6]. Around 17 different Gα, five different Gβ, and at least 10 different Gγ subunits have been characterized in animals but only one gene encoding Gα and one gene encoding a Gβ homologue have been identified in *Arabidopsis thaliana* [6–8]. Genes that encode Gα and Gβ-like proteins have been isolated from several other diploid plant species [9]. Only in some tetraploid or hexaploid species like tobacco, soybean and oat two or three genes of Gβ or Gα subunits have been found [9]. Recently, one GPCR homologue has been identified in *Arabidopsis* [10,11]. However, no plant Gγ homologue has been isolated to date.

There is much indirect experimental evidence for G proteins regulating different signaling pathways such as light regulation, pathogen response, the activity of ion-channels and hormone signaling in higher plants [12–17]. Using patch clamp techniques, direct evidence has been obtained that plant Gα protein regulates Ca^{2+} channels in vitro [18]. Analysis of the recently identified *daikoku dwarf* mutants in rice indicates that Gα may be involved in gibberellin signaling and may play an important role in plant development [19,20]. In contrast, there are no data concerning possible functions of plant Gβ or its biochemical properties and localization.

Gα subunits are anchored to membranes through a myristoyl or/and palmitoyl moiety attached at the amino terminus whereas Gβ subunits are tethered to membranes by interaction with Gγ, which by itself is localized to the membrane via a prenyl group at the carboxyl terminus. These posttranslational modifications are required for proper localization and function of the G protein complex [21,22]. One characteristic feature of the Gβγ dimer structure is a two-stranded coiled-coil formed between the amino termini of β and γ [23,24]. The primary structures of Gβ proteins show a highly conserved heptad repeat domain at the amino terminus which enables the formation of the βγ coiled-coil [25].

In this report we characterize biochemically the interaction of the plant Gβ homologue with membranes and provide evidence for the existence of a Gγ-like protein in plants. Subcellular fractionation of *Arabidopsis* and broccoli extracts and solubilization studies using microsomes treated with different salts and detergents show that plant Gβ is a membrane-associated protein. Furthermore, these studies indicate that Gβ is associated with membranes by interaction with another, yet unknown, protein. Using transgenic tobacco plants that either express wild-type GβGFP or GβGFP fusion proteins which are mutated at the amino terminus we show that the heptad repeat domain of plant Gβ is crucial for its association with membranes.

2. Materials and methods

2.1. Production of antisera

The full-length *AtG*β cDNA encoding Gβ from *A. thaliana* [8] was cloned into the vector pQE9 and expressed as an amino terminal 6×His fusion protein in *Escherichia coli* M15 [pRep4] cells. The carboxy terminal region of *NtG*α cDNA (GenBank accession no. Y08154) spanning from amino acid residues 182 to 384 was cloned into the vector pQE11 and the resulting amino terminal 6×His fusion construct was expressed in *E. coli* BL21 cells. Both His-tagged proteins were purified from *E. coli* extracts under denaturing conditions using a Ni–NTA column (Qiagen) as described by the manufacturer. The purified proteins precipitated during dialysis against 10 mM Tris–HCl (pH 7.0), were collected, and resuspended in a buffer (0.2% SDS/0.1 M Na-Phosphate, pH 7.4). These AtGβ and NtGα antigen solu-

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Abbreviations: CMC, critical micellar concentration; GFP, green fluorescent protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; WT, wild-type

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tions were used to inject chinchilla bastard rabbits for raising anti-AtGβ and NtGα antibodies, respectively.

2.2. Affinity purification of the antibodies

The purification of anti-AtGβ antibodies was performed according to [26] with some modifications. Briefly, 60 μg of 6×His-Gβ antigen were transferred to PVDF membranes by Western blotting (10 μg per lane) and the corresponding protein band was cut out. This strip was blocked in blocking buffer (500 mM NaCl, 20 mM Tris–HCl, 4% skim milk powder, 0.05% NaN_3, pH 7.4), incubated for 8 h at room temperature with 300 μl of anti-AtGβ serum diluted 1:10 in blocking buffer and washed as described below. The antibodies were eluted in 3 ml 0.1 M glycine, pH 2.5, neutralized with 90 μl 2 M Tris (pH not adjusted) and stabilized with 3 ml 2×blocking buffer. For immunolabelling of Western blots the purified antibodies were used 1:10 diluted. The anti-NtGα antibodies were purified using the basic protocol described for anti-AtGβ except that the elution was performed with 1.5 ml 50 mM glycine, pH 2.8, and the eluate was neutralized with 32 μl 1 M Tris–HCl, pH 9.5. The specificity of the affinity-purified anti-NtGα antibodies was tested on a Western blot as described for anti-AtGβ antibodies (see also Fig. 1). In these tests anti-NtGα antibodies recognized one band at 45 kDa in *Arabidopsis* and broccoli extracts that corresponds to the size of the AtGα protein from *Arabidopsis* [27].

2.3. Isolation of microsomal membrane fraction

All steps of the homogenization and isolation procedure were performed on ice or at 4°C. Microsomal fractions were isolated from homogenized broccoli or *Arabidopsis* tissue by differential centrifugation as described by [28] in three centrifugation steps: at 5000×*g* (pellet P1) 15 000×*g* (pellet P2) and 100 000×*g* (microsomal pellet P3 and supernatant S3). The microsomal pellet (P3) was resuspended by mixing and sonication (Branson sonicator, 3×5 s at level I) in resuspension buffer (50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 10% glycerol, 5 mM 6-aminocaproic acid, 2 mM benzamidine) at 0.2 ml buffer/g fresh weight, aliquoted and stored at −20°C.

2.4. Solubilization of microsomal proteins

If not stated otherwise, 0.1 ml of broccoli microsomes was treated with different compounds or detergents (all from Sigma), incubated for 20 min on ice and centrifuged for 30 min at 100 000×*g*. The non-soluble pellets were resuspended in 0.1 ml resuspension buffer and, together with the soluble supernatants, directly applied for Western blot analysis. If necessary, detergents and salts were removed from samples by TCA and the precipitated protein pellet was dissolved in fresh resuspension buffer. The concentrations of detergents are given in fold critical micellar concentration (CMC) in aqueous solution at 25°C as described by the manufacturers. The given CMCs of the applied detergents are as follows: Triton X-100, 0.2 mM (Sigma/Aldrich); *n*-dodecylmaltoside, 0.18 mM (Roche Molecular Biochemicals); CHAPS, 4.1 mM (Sigma/Aldrich).

2.5. Generation of transgenic tobacco plants

The *WT-AtGβ* cDNA from *Arabidopsis* was amplified using amino terminal and carboxy terminal specific primers which carried *Bam*HI and *Sma*I restriction enzyme sites respectively. The point mutations resulting in *L20/24E-AtGβ* were generated from *AtGβ* cDNA by overlap-extension polymerase chain reaction (PCR) using two overlapping primers that introduced the mutations and the amino terminal and carboxy terminal primer mentioned above. The PCR products were cloned into the *Bam*HI and *Sma*I sites of the *pBIN 35S-mGFP4* binary vector [29] and the inserts were sequenced. *Agrobacterium tumefaciens* LBA 4004 was transformed with these *pBIN* constructs and the resulting lines were used for leaf disc transformation of tobacco SR1 plants [30]. The plants were propagated in sterile conditions on Murashige and Skoog medium with B5 vitamins (Duchefa), containing 3% sucrose and 100 μg/ml kanamycin.

2.6. Protein gels and Western blots

Total protein concentrations in fractions were measured after [31,32]. All chemicals were purchased from Sigma. Protein samples were separated by 10% SDS–PAGE and electroblotted onto a polyvinylidene fluoride (PVDF) membranes (Millipore). The protein bands were visualized by staining with Ponceau S (Sigma). Immunolabelling and detection were performed using the ECL system kit according to the manufacturer (Pharmacia/Amersham).

3. Results

3.1. Plant Gβ is a membrane-bound protein and highly enriched in broccoli flowers

The affinity-purified anti-AtGβ antibodies detected a single band at about 40 kDa in *A. thaliana* extracts on Western blots (Fig. 1B). A band of the same size was also detected in broccoli extracts (Fig. 1A). These antibodies were used in further experiments.

Homogenates from whole plants of *Arabidopsis* and from broccoli flowers were subjected to differential centrifugation, and the individual fractions were tested by Western blot analysis. Gβ was present in pellets P2 (15 000×*g*) and highly enriched in microsomal pellets P3 (100 000×*g*) from both broccoli and *Arabidopsis* extracts (Fig. 1). The microsomal pellets P3 correspond to the crude membrane cell fraction [28]. Some Gβ protein was also present in the P1 pellet from broccoli, but Gβ was not detectable in soluble cytosolic fractions (S3) from either *Arabidopsis* or broccoli flower extracts. Interestingly, Gβ protein is present in distinctly higher amounts in microsomes from broccoli flower tissue than in microsomes from the whole plant extracts of *Arabidopsis*. Therefore, we decided to use broccoli microsomes as the starting material for further biochemical characterization of plant Gβ protein.

3.2. Solubilization of Gβ with salts and urea

To study the biochemical properties of Gβ interaction with membranes the crude broccoli microsomes were treated with different chemicals, separated into soluble and non-soluble fractions and analyzed by Western blotting. Raising the ionic strength by increasing the KCl concentration in the resuspension buffer up to 0.5 M resulted in increased interaction of Gβ with membranes (Fig. 2A). In contrast, Gβ could be partially washed away from membranes with 2 M NaBr or with an alkaline 0.1 M Na_2CO_3 solution adjusted to pH 11.0 (Fig. 2C,D), treatments which are known to strip away peripheral membrane proteins without disrupting the lipid bilayer of membranes [33,34]. Gβ was also partially solubilized by urea

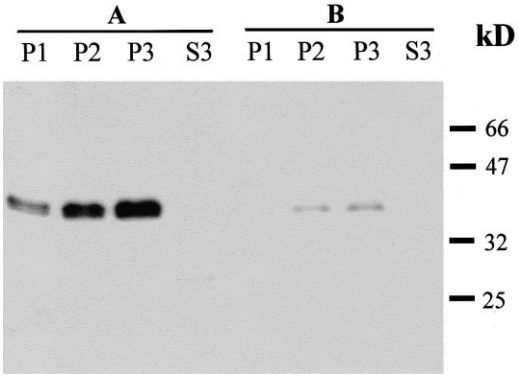

Fig. 1. Distribution of Gβ in subcellular fractions from broccoli (A) and *Arabidopsis* (B) after differential centrifugation. P1, P2 and P3 correspond to 5000×*g*, 15 000×*g* and 100 000×*g* pellets, S3 to 100 000×*g* supernatant, respectively. 30 μg of total protein per lane was separated by 10% SDS–PAGE, blotted onto a PVDF membrane and labelled with affinity-purified anti-AtGβ antibodies. Molecular mass sizes in kDa are given on the right.

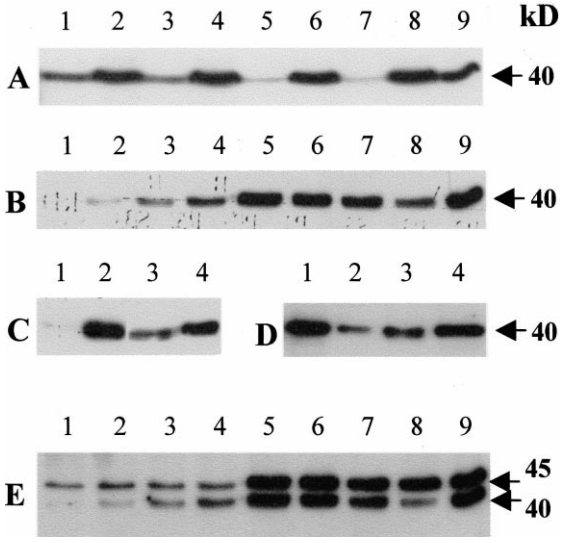

Fig. 2. Microsomal pellet fractions from broccoli flowers were treated with different compounds and re-centrifuged. The resulting supernatant and pellet fractions were analyzed on Western blots with anti-Gβ antibodies in (A–D), and with anti-Gα and anti-Gβ antibodies in (E) (see Section 2). Equal amounts (30 μg) of total protein per lane were loaded. A: Lanes 1, 3, 5, and 7 show the supernatant fractions after treatment with 0, 10, 100, and 500 mM KCl, lanes 2, 4, 6, and 8 show the corresponding pellet fractions. B: In lanes 1–4 soluble fractions after 0, 1, 2, and 4 M urea treatment are shown, lanes 5–8 represent the corresponding non-soluble pellets. Lanes A9 and B9 show controls before the treatments. C: Membranes were washed with 0.1 M Na_2CO_3 pH 11.0. Lanes 1 and 2 show the soluble and non-soluble Gβ in the absence of Na_2CO_3, lanes 3 and 4 the corresponding fractions after Na_2CO_3 treatment. D: Treatment of membranes with 2 M NaBr. Lane 1: non-treated membranes; 2: soluble fraction without 2 M NaBr; 3: soluble fraction after 20 min incubation in 2 M NaBr. E: The blot from B re-probed with anti-Gα antibodies.

(Fig. 2B,E). The 45 kDa band in Fig. 2E represents the Gα protein on a re-probed Western blot showing also Gβ to demonstrate that Gα is not dissociated from membranes by urea.

3.3. Solubilization of Gβ with different detergents

We also tested solubilization profiles of plant Gβ with detergents, which are commonly used for purification of native protein complexes. Concentrations are given in relation to the CMC of a detergent in distilled water at 25°C (Fig. 3). Triton X-100 solubilized a significant amount of Gβ only at concentrations 75 times higher than the specific CMC. With 50×CMC *n*-dodecylmaltoside the solubilization of Gβ was greater. Interestingly, Gβ was solubilized more efficiently with sub-CMC concentrations of the zwitterionic detergent CHAPS (Fig. 3).

3.4. Plant Gβ interacts with membranes via the amino terminal heptad repeat domain

In order to test if plant Gβ may be attached to membranes in a similar manner to animal Gβ subunits, we mutated AtGβ at two critical positions within its heptad repeat domain (Fig. 4A). Corresponding mutations in animals have been described to disrupt the interaction between Gβ and Gγ and lead to mislocalization of Gβ [23,35]. The *L20/24E-AtGβ* and *WT-AtGβ* were fused to *GFP* and transgenic SR1 tobacco plants were produced that expressed the fusion genes under control of a constitutive *35S* promoter. Whole plants of randomly selected transgenic lines were homogenized and fractionated by differential centrifugation; the resulting microsomal and soluble fractions were analyzed by Western blots. Fig. 4 shows that anti-AtGβ antibodies recognize in three out of four *WT-AtGβGFP* lines a band at 65 kDa, which correlates with the predicted size of the GβGFP fusion protein. In the corresponding soluble fractions there was either no or distinctly less wild-type GβGFP detectable. In contrast, the mutated GβGFP fusion protein of *L20/24E-AtGβGFP* lines was more abundant in soluble fractions than in microsomes. When all subcellular fractions from differential centrifugation where tested, WT-GβGFP was highly enriched in the P2 and P3 fractions whereas the L20/24E mutant protein was mainly present in the soluble S3 fraction (Fig. 4C,D).

4. Discussion

A single band at 40 kDa was detected with the affinity-purified anti-AtGβ antibodies in *A. thaliana* extracts. We conclude that the observed immunological reaction represents indeed the Gβ protein because *AtGβ* seems to be a single copy gene in *Arabidopsis* and the size of the band corresponds to the predicted size of AtGβ peptide [8]. The fact that the antibodies recognize a protein of the same size in broccoli, a species closely related to *Arabidopsis*, indicates that these antibodies are specific for plant Gβ proteins.

Subcellular fractionation showed that Gβ is a membrane-associated protein since it was highly enriched in microsomal pellet fractions of *Arabidopsis* and broccoli but was not detectable in soluble supernatant fractions (Fig. 1). The higher amounts of Gβ protein in broccoli flowers may originate from stronger expression of the *Gβ* gene in floral meristems as has been shown for plant Gα [36].

Treatments of microsomal membranes with 2 M NaBr and Na_2CO_3 (pH 11.0) show that Gβ is a peripheral membrane protein. Urea is a chaotropic agent which denatures proteins

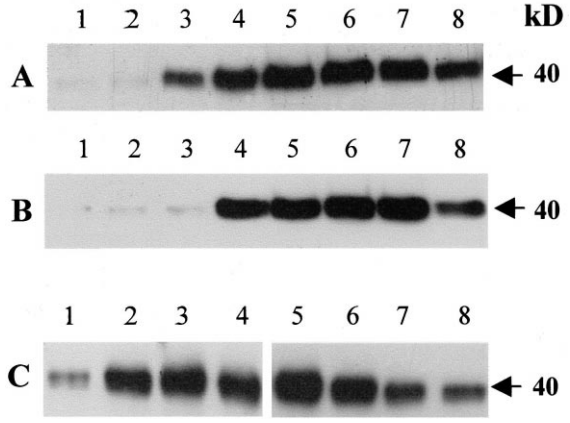

Fig. 3. Western blot analysis showing solubilization profiles of Gβ with different detergents. The solubilization tests were performed on ice. Lanes 1–4 represent soluble, lanes 5–8 the corresponding non-soluble fractions. The concentrations are given in fold CMC of the detergent (see Section 2). The same amount of protein was loaded in each lane. A: Lanes 1+5, 2+6, 3+7, and 4+8 show Gβ in supernatant and pellet fractions after treatment with 0, 0.25, 7.5, and 75×CMC of Triton X-100 respectively. B: Lanes 1+5, 2+6, 3+7, 4+8 correspond to supernatant and pellet fractions after solubilization of membranes with 0, 0.5, 5, and 50×CMC of *n*-dodecylmaltoside respectively. C: Lanes 1+5, 2+6, 3+7, 4+8 correspond to membranes treated with CHAPS at 0, 0.25, 10, and 50×CMC.

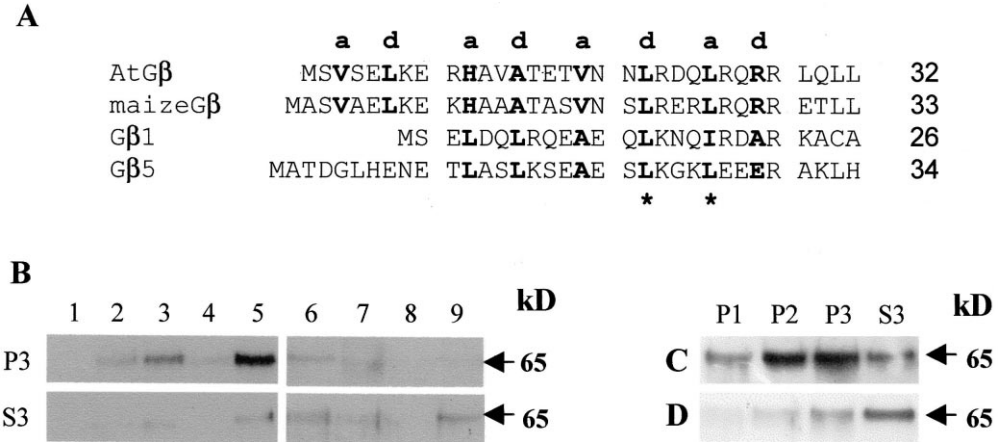

Fig. 4. Subcellular localization of wild-type and mutant Gβ fused to GFP in extracts from transgenic tobacco plants. A: Alignment of amino terminal heptad repeats from different Gβ subunits, adapted from [23] (*A. thaliana* AtGβ, U12232; maize Gβ, U12233; bovine Gβ1, M13236; mouse Gβ5 L34290). Positions 1 and 4 of each heptad repeat are designated with letters a and d, respectively. The numbers on the right give the position of the last amino acid residue in the corresponding peptide sequence. The asterisks show the positions where leucine residues at position 20 and 24 of AtGβ have been replaced by glutamate. B: Microsomes P3 and soluble fractions S3 from different tobacco lines. Lane 1: non-transformed tobacco SR1. Lanes 2–5: transgenic WT-GβGFP lines 2, 3, 4 and 6. Lanes 6–9: transgenic L20/24E-GβGFP lines 71, 75, 76 and 79. C and D: Subcellular distribution of WT-GβGFP transgenic line 6 and L20/24E-GβGFP transgenic line 71 after differential centrifugation (see also Fig. 1).

and, at high concentrations, can also destabilize the membrane bilayer. However, at lower concentrations urea may solubilize peripheral membrane proteins that are attached to membranes by protein interactions without disrupting the lipid bilayer. The solubility of Gβ in urea at concentrations lower than 4 M suggests the association of Gβ with membranes through another anchoring protein [37]. The fact that plant Gα, which presumably is anchored to membranes via the palmitoyl moiety at its amino terminus, was not solubilized at these urea concentrations indicates that the microsomal membranes were not disrupted (see Fig. 2E and Section 2). The results obtained with KCl suggest hydrophobic interactions between plant Gβ and membranes since raising the ionic strength with salts like KCl stabilizes hydrophobic protein interactions [38]. These findings correlate with the situation for G proteins in animals, where the membrane association of the Gβγ dimer is increased in high KCl concentrations [39].

According to the three-stage hypothesis of membrane solubilization with detergents, membrane components pass from non-solubilizing to solubilizing conditions through a transition stage II [40]. In this stage detergent-saturated membranes are thought to coexist with mixed lipid–detergent micelles at detergent concentrations close to the CMC. Therefore, in order to fully solubilize integral proteins or protein complexes from membrane vesicles, the detergent concentration should be distinctly higher than its CMC [41,42]. At concentrations below the CMC detergent may only permeabilize the vesicles or solubilize some peripheral proteins [43], for example those which interact with membranes via post-translational hydrophobic modifications. Interestingly, with the non-ionic detergents Triton X-100 and *n*-dodecylmaltoside, Gβ was only solubilized at concentrations far above the specific CMCs. Similar results were obtained when solubilization with Triton X-100 and *n*-dodecylmaltoside were performed at room temperature (data not shown), indicating that the low solubility of Gβ was not due to low temperatures. In contrast, Gβ was solubilized at concentrations below the CMC when treated with the zwitterionic detergent CHAPS. There are two possible interpretations for these results. Firstly, Gβ might be anchored to membranes via an integral protein and CHAPS would disrupt the interaction between Gβ and the anchor. The second explanation might be that plant Gβ and its anchor are localized in membrane domains which are poorly soluble with non-ionic detergents. We cannot exclude the first possibility because we do not know the interacting partner(s) of Gβ and it is unknown whether the membrane vesicles are still intact at concentrations of CHAPS below the given CMC. Nevertheless, we favor the hypothesis that the putative Gβ anchor is a peripheral protein since the solubilization profiles of plant Gα correlate with the results obtained for Gβ (data not shown). Additionally, the presence of several mammalian G proteins in membrane domains which are poorly solubilized by non-ionic detergents has been described [44,45].

The 65 kDa band that is detectable with the anti-AtGβ antibodies in extracts from SR1 tobacco plants transformed with *35S AtGβGFP* constructs clearly represents the GβGFP protein since the apparent size corresponds to the predicted size of the fusion protein and, in addition, no such band has been detected in extracts from non-transformed tobacco SR1 plants. Low amounts of GβGFP are also present in the soluble fraction S3 of WT-GβGFP lines 6 and 3. This may be explained by the fact that lines 6 and 3 express the fusion protein in excess and therefore some factors important for the membrane targeting of Gβ may become limiting. The interaction of the mutant L20/24E-GβGFP protein with membranes seems not to be completely destroyed but severely attenuated. The total amount of GβGFP in different extracts from *L20/24E-GβGFP* lines appeared to be lower than in the extracts from *WT-GβGFP* lines. This may be paralleled in animals, where Gβ protein is rapidly degraded if overexpressed alone or if its interaction with Gγ is prevented [23]. The differences in subcellular distribution of GβGFP between *WT*- and *L20/24E-GβGFP* lines indicate that plant Gβ interacts with its putative membrane anchor by formation of a coiled-coil.

In sum, the experiments described here clearly characterize

the plant Gβ homologue as a peripheral, membrane-associated protein. In addition, we provide evidence for the existence of a Gγ-like protein and thus for a heterotrimeric G protein complex in plants. However, no plant Gγ homologue has been identified to date. This may be partially due to the small size and the very low degree of conservation of Gγ subunits among species. Another possibility is that Gγ in plants may have a different structure. It is astonishing that despite the fact that more than 70% of the *Arabidopsis* genome has been sequenced only one Gα, one Gβ [7,8], and one putative GPCR homologue have been identified [10,11]. Thus, the variety of signal transduction pathways that are discussed to be regulated by trimeric G proteins in plants may be to a large extent determined by different Gγ subunits. Therefore, some additional structural motifs which are not present in Gγ subunits of animals and fungi may be present in plant Gγ.

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