Running head: Heterotrimeric G-proteins in Chara braunii

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Research Report

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Title: Characterization of heterotrimeric G-protein complex and its regulator from the green alga *Chara braunii* expands the evolutionarily breadth of plant G-protein signaling

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One-sentence summary: The presence of a complete repertoire of heterotrimeric G-protein complex components and its regulator in a green alga confirms that the origin of this signaling mechanism in plants is more ancient than previously thought and is not related to the land-plant evolution.
Footnotes:

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ABSTRACT

The lack of heterotrimeric G-protein homologs in the sequenced genomes of green algae has led to the hypothesis that in plants, this signaling mechanism co-evolved with the embryophytic life cycle and acquisition of terrestrial habitat. Given the large evolutionary gap that exists between the chlorophyte green algae and most basal land plants, the bryophytes, we evaluated the presence of this signaling complex in a charophyte green alga, Chara braunii, proposed to be the closest living relative of land plants. C. braunii genome encodes for the entire G-protein complex, the Gα, Gβ and Gγ subunits, and the regulator of G-protein signaling (RGS) protein. The biochemical properties of these proteins and their cross-species functionality show that they are functional homologs of canonical G-proteins. The subunit-specific interactions between CbGα and CbGβ, CbGβ and CbGγ, and CbGα and CbRGS are also conserved, establishing the existence of functional G-protein complex-based signaling mechanisms in green algae.
INTRODUCTION

Heterotrimeric G-proteins comprising of G\(\alpha\), G\(\beta\) and G\(\gamma\) (G-proteins, hereinafter) are key signaling intermediates in all eukaryotes. The catalytically active protein of the complex, G\(\alpha\), can bind GTP or GDP. GDP-bound G\(\alpha\) remains associated with G\(\beta\gamma\) (GDP\(\bullet\)G\(\alpha\)\(\beta\gamma\)) and represents the inactive signaling status. A signal-dependent exchange of GTP for GDP on G\(\alpha\) causes formation of G\(\alpha\)•GTP and releases the G\(\beta\gamma\) dimer. Both these entities can interact with a variety of effectors to transduce the signal. The intrinsic GTPase-activity of G\(\alpha\) causes hydrolysis of the bound GTP, and regenerates GDP•G\(\alpha\)\(\beta\gamma\), completing one signaling cycle (Cabrera-Vera et al., 2003; Offermanns, 2003; Urano et al., 2013). Regulators of G-protein Signaling (RGS) domain-containing proteins, which enhance the GTPase activity of G\(\alpha\), accelerate the rate of G-protein cycle (Siderovski and Willard, 2005).

The core G-protein components and their biochemical properties are largely conserved across the eukaryotic clade; however, important quantitative and qualitative differences emerge when comparing the well-characterized metazoan G-protein systems with those of plants (Anantharaman et al., 2011; Bradford et al., 2013; Urano et al., 2013). For example, there is a lineage-specific expansion of individual G-protein components in metazoans where each protein is represented by multiple members (e.g. 23 G\(\alpha\), 5 G\(\beta\), 12 G\(\gamma\) and 37 RGS proteins in humans) that have distinct biochemical properties (Anantharaman et al., 2011). In plants, analysis of G-protein functions in Arabidopsis, rice and soybean has emphasized the critical roles they play in plant growth, development and stress responses (Li et al., 2012; Zhang et al., 2012; Roy Choudhury and Pandey, 2013; Urano et al., 2013); however, the genome of most plants encodes for a single canonical G\(\alpha\) and G\(\beta\) protein and few G\(\gamma\) proteins (Urano et al., 2013) with the exception of plants where recent whole genome duplications have resulted in their higher numbers (e.g. 4 G\(\alpha\), 4 G\(\beta\), and 10 G\(\gamma\) proteins in soybean) (Bisht et al., 2011; Pandey, 2011; Roy Choudhury et al., 2011). Furthermore, at the biochemical level, the higher plant G\(\alpha\) proteins have significantly slower GTPase activity compared to the mammalian G\(\alpha\) proteins (Jones et al., 2011; Roy Choudhury et al., 2012; Urano et al., 2012).
Identification of the evolutionary origin of such critical differences is an active area of research. Available data points to the existence of rather complex and divergent regulatory mechanisms in plants compared to the established paradigms (Bradford et al., 2013; Urano et al., 2013). Specifically, many of the integral components of G-protein complex seem to be missing from the basal plant lineages. The liverwort *Marchantia polymorpha* contains the sequence homologs of Gα, Gβ and Gγ proteins, but not the regulatory protein, RGS. Interestingly, the Gα protein from *M. polymorpha* displays rapid GTP binding (similar to plants), but also rapid GDP-release (similar to metazoans) (Urano et al., 2012). Remarkably, the moss *Physcomitrella patens* encodes for the Gβ and Gγ proteins but no canonical Gα or RGS proteins. The fully sequenced genomes of chlorophyte green algae, *Volvox carteri*, *Chlamydomonas reinhardtii*, *Cocomyxa subellipsoidea* C-169, *Micromonas pusilla* CCMP1545, *Micromonas pusilla* RCC299 and *Osterococcus lucimarinus* do not contain G-proteins homologs (our analysis, www.phytozome.net). These observations have led to hypotheses that G-protein signaling in green plant lineage evolved with the evolution of land plants or alternatively with the evolution of a sporophytic lifestyle (Urano et al., 2012; Bradford et al., 2013; Urano et al., 2013). It has also been reasoned that G-protein signaling in plants is independent of a receptor-dependent regulation because sequence homologs of G-protein coupled receptor (GPCR)-like proteins from flowering plants are present in green algae, even though they do not encode for G-protein components themselves (Bradford et al., 2013; Urano et al., 2013).

To address the evolutionary origin of heterotrimeric G-protein signaling in plants and its relationship to the evolution of land plants and/or sporophytic lifestyle, we have analyzed the genome of *Chara braunii* (order: Charales; class: Charophyceae). We identified a complete repertoire of G-protein heterotrimer as well as a homolog of N-terminal transmembrane-containing RGS proteins in *C. braunii*. The proteins exhibit biochemical properties similar to their homologs in *Arabidopsis thaliana*. These findings challenge the established notions regarding the evolution of G-protein signaling in plants and confirm that the origin of this conserved signaling mechanism in plants is more ancient than previously proposed.

**RESULTS AND DISCUSSION**

The genome of a green alga, *Chara braunii*, encodes for a complete repertoire of heterotrimeric G-protein components
A large evolutionary gap exists between the chlorophyte green algae that do not seem to possess the G-protein components and the most ancient land plants, the bryophytes, which contain the Gβ and Gγ proteins. The charophyte green algae are especially interesting from an evolutionary perspective since they are the closest living relatives of the ancestors of the embryophytes (Becker and Marin, 2009; Niklas and Kutschera, 2010; Wodniok et al., 2011; Laurin-Lemay et al., 2012; Timme et al., 2012; Raven, 2013; Zhong et al., 2013). Superficially, these algae (e.g. Chara, Nitella) have morphologies similar to basal land plants with the presence of rhizoids, a main axis that is differentiated into nodes and giant cylindrical internodes, dimorphic branches and whorls of leaf-like giant cells. However, in contrast to embryophytes, the plant is a gametophyte with the diploid generation represented only by the zygote, similar to other algae.

To evaluate the extent to which the existence of plant G-proteins is correlated with the evolution of embryophytes, a search was performed in Chara braunii, a representative of the order Charales. RNA-Seq data generated from the C. braunii thalli at the vegetative growth phase were used for BLAST search with the A. thaliana Gα (AtGPA1), Gβ (AtAGB1), Gγ (AtAGG1, AtAGG2, AtAGG3) and AtRGS1 genes. This analysis identified a potential homolog for each of the genes, with the exception of AtAGG3, along with some hits with high similarity to Dictyostelium transcripts. To verify the accuracy of the assembled nucleotide sequences and their origin from the Chara genome, the corresponding transcripts were PCR-amplified from cDNA pools generated from independent C. braunii cultures. Full-length cDNAs were sequenced and analyzed for the characteristic features of G-protein subunits. C. braunii G-protein genes showed an overall nucleotide sequence similarity of 53.1%, 48.1% and 44.2/39.2% with the Arabidopsis GPA1, AGB1 and AGG1/2, respectively. No sequence homologs were identified for AtAGG3 genes in Chara, which is not surprising as sequence homologs of AtAGG3 are present only in higher plants (Chakravorty et al., 2011; Li et al., 2012; Trusov et al., 2012). Conceptually translated amino acid sequences of C. braunii Ga, Gβ and Gγ homologs (named CbGa, CbGβ and CbGγ, hereafter) exhibit 47.9%, 43.9%, 18.8/23.1% identity (66.1%, 58.4% and 28.6/41% similarity) with the corresponding Arabidopsis proteins.

Even though we have identified only a single homolog of each of the G-protein subunits and the RGS protein, the possibility that additional homologs of these genes exist in the Chara
genome cannot be ruled out at this stage. It should be noted however that that most plant species including moss, Selaginella, Adiantum, Pinus, Arabidopsis and rice possess only few, if not only one, homologs of G-protein genes and multiplicity is typically present with respect to the Gγ proteins (Urano et al., 2012). Moreover one type of Gγ proteins seems to be specific to higher plants. The only plants that have multiple homologs of each of the subunits are the ones that have undergone recent, whole genome duplication events e.g. soybean, that has 4 Gα, 4 Gβ and 10 Gγ proteins (Bisht et al., 2011; Roy Choudhury et al., 2011). A complete genome sequence assembly will be required to assess the existence of additional G-protein genes in the Chara genome. Nonetheless, these data clearly show that sequences encoding for each of the functional subunits of a complete heterotrimeric G-protein complex exist in green algae.

The signature motifs required for GTP-binding and GTPase activity, designated as G1, G2, G3, G4 and G5 domains in Gα proteins, are highly conserved in CbGα with few similar amino acids substitutions, compared to AtGPA1 (Fig. 1A). Additional critical motifs present in Gα proteins such as an ADP-ribosylation site and an invariant glutamine (corresponding to position 222 in AtGPA1) that affects its GTPase activity (Oki et al., 2005) are also conserved in CbGα. Homology modeling of the three-dimensional structure of CbGα, based on the published crystal structure of AtGPA1 (Jones et al., 2011) shows that the G1-G5 domains of CbGα attain a spatial orientation similar to that of AtGPA1, and predict the formation of a functional GTP-binding pocket.

The N-terminal region of CbGα shows some variation from other known plant Gα proteins. All known higher plant Gα proteins contain a conserved methionine which is a part of the signature sequences for palmitoylation (MGXXXS) and myristoylation (MGXXCS) modifications. These lipid modifications are required for the proper targeting of Gα proteins to the plasma membrane. This conserved methionine also represents the translational start codon for a number of Gα proteins (e.g. Arabidopsis). However, additional eight (e.g. soybean) or ten (most monocots) amino acids preceding this methionine are also common (Bisht et al., 2011). The significance of such modifications and whether it results in alternatively translated Gα proteins in planta remains unknown. Interestingly, CbGα contains a 26 amino acid overhang from the conserved methionine (Fig. 1A). However, the sequence motifs for palmitoylation and myristoylation are not present at the CbGα N-terminal region. A similar lack of these lipid-
modification motifs is also obvious in the Gα protein sequence from *M. polymorpha*, although it does not have any N-terminal overhangs (Fig. 1A). Whether these alterations affect the localization of basal Gα proteins or have any effect on their biochemical or signaling activity remains unknown at this time. The possibility that CbGα and MpGα remain plasma membrane-localized by additional modifications (prenylation of Gγ proteins) or protein-protein interactions (interaction with RGS protein or with the βγ dimer) can also be not ruled out.

Phylogenetic analysis using Gα sequences from species representing important taxa during plant evolution resulted in clear clusters of monocotyledons and eudicotyledons that cluster with gymnosperms, which is consistent with the evolution in land plants. Outside the seed plants, the liverwort *M. polymorpha*, the spikemoss *S. moellendorffii* and the *Chara braunii* Gα were nested without strong support (Fig. 1B).

CbGβ shows moderate (~44%) sequence identity with Gβ proteins of other plant species including the two *P. patens* Gβ proteins. The protein has 7 WD-repeat motifs, typical of all Gβ proteins. Sequences important for interaction with Gα and Gγ proteins are mostly conserved in CbGβ and AtAGB1 (Fig. S1).

The sequence identity between AtAGG1/2 and CbGγ is relatively modest (~19 and 23% with AGG1 and AGG2, respectively), which is typical of most Gγ proteins given the large variability observed between them (Roy Choudhury et al., 2011). However, signature motifs predicted to be essential for Gγ function, namely the coiled-coil domain at the N-terminus, the DPLL motif and the sequences predicted to be involved in its high affinity interaction with the Gβ proteins are mostly conserved in CbGγ. The protein shows relatively higher homology (27.9% identity) with *P. patens* Gγ1 protein (Fig. S2).

None of the basal plant lineages, including *P. patens* and *M. polymorpha* encode for a RGS protein homolog. Furthermore, many monocots do not possess a RGS protein homolog and it is predicted to have been lost multiple times during evolution (Urano et al., 2012). Remarkably, a sequence homolog of *AtRGS1* is present in the *C. braunii* transcriptome. The protein (1097 aa) is encoded by a 3.3 kb transcript and is almost twice the size of other known plant RGS proteins (Roy Choudhury et al., 2012). The N-terminal of the protein is predicted to possess nine transmembrane spanning helical domain (TM domain), in comparison to seven in Arabidopsis and
soybean, and a RGS domain at the C-terminal region (Fig. 1C). Comparison of the nucleotide sequences of the full-length \( CbRGS \) and \( AtRGS1 \) gene showed only 22.9% identity, while the corresponding aa sequences revealed 12.9/21.8% identity/similarity (Fig. S3). Relatively higher identity/similarity (~30/50%) was observed between the RGS domain of \( CbRGS \) and \( AtRGS1 \). Interestingly, \( CbG\alpha \) contains the conserved Thr residue (Thr194 of \( AtGPA1 \)), proposed to be critical for its regulation by RGS protein activity (Urano et al., 2012). Phylogenetic analysis of \( CbRGS \) with different plant RGS proteins (Fig. 1D) revealed clear monophyly of monocots, flowering plants, and seed plants. The branch length leading to \( C. braunii \) being slightly deeper than \( S. moellendorffii \) is consistent with orthologous relationships of these genes.

\textbf{C. braunii G-protein components are transcriptionally active}

To confirm the transcriptional activity of the sequence homologs of G-protein genes, additional expression analyses by real time quantitative PCR were performed, using cDNA generated from the internodes and dimorphic branches of \( C. braunii \). All G-protein genes and \( CbRGS \) exhibited a low, but unambiguously detectable expression, with no significant differences in expression levels between the internodes and dimorphic branches (Fig. 2). This is similar to the expression of higher plant G-protein genes, which show a low but ubiquitous expression (Bisht et al., 2011; Roy Choudhury et al., 2011). The relative expression levels of \( CbG\alpha, CbG\beta, CbG\gamma \) and \( CbRGS \) were between 2-15% of the expression of \( C. braunii \ EF-1\alpha \ gene for elongation factor 1-alpha (CbEF1) \) (GenBank: AF032728.1), which was used as internal control. In comparison, the level of 18S rRNA of \( C. braunii \) (GenBank: AB606030.1) were 500-1000 times more abundant than those of \( CbEF1 \). These results establish that each of the \( C. braunii \) G-protein genes are transcriptionally active and therefore possess protein-coding ability, a fundamental prerequisite for the existence of a functional G-protein signaling complex.

\textbf{CbG\alpha exhibits specific GTP-binding and GTPase activity which is regulated by \( CbRGS \) protein}

To establish \( CbG\alpha \) as an authentic G-protein, the recombinant, purified protein was subjected to GTP-binding and GTPase activity assay using fluorescent BODIPY-GTP (4,4-difluoro-4-bora-3\( \alpha,4\alpha\)-diaza-s-indacene-GTP). In this real-time assay, an increase in fluorescence over time corresponds to GTP-binding while a decrease in fluorescence due to the hydrolysis of bound GTP represents GTPase activity of an active G\( \alpha \) protein (Roy Choudhury et al., 2013).
CbGα is an active GTP-binding protein and displays GTPase activity as shown by a classic increase in fluorescence followed by a gradual decrease over time (Fig 3A). The CbGα nucleotide-binding is GTP-specific, as indicated by using non BODIPY-conjugated GTP, GDP, ATP or ADP as competitors to the labeled BODIPY-GTP FL in binding assays. Addition of GTP, but not of other nucleotides reduced the BODIPY-GTP FL-specific fluorescence signal.

To evaluate whether the functional interaction between CbGα and CbRGS proteins is conserved, the GTPase activity of CbGα was assayed in the presence of the purified, recombinant RGS domain of C. braunii. The CbGα GTPase activity can be significantly accelerated by the presence of CbRGS protein (Fig. 3B). Interestingly, despite the relative low sequence identity between the Arabidopsis and Chara RGS domains, their cross-species functionality is maintained. Purified recombinant AtRGS1 protein (RGS domain) accelerated the GTPase activity of CbGα to the similar extent as the CbRGS protein (Fig. 3B). Conversely, CbRGS protein was able to accelerate the GTPase activity of AtGPA1 (Fig. 3C). This suggests that the critical residues required for the activity of these proteins are conserved from the date of divergence between Chara and land plants.

**Interaction between different C. braunii G-protein subunits and its regulator is conserved**

A functional C. braunii G-protein complex necessitates the physical interaction between the individual subunits as well as between Gα with RGS proteins. To ascertain such interaction, targeted interaction analyses were performed between CbGα and CbGβ and CbGα with CbRGS, using the membrane-based split-ubiquitin assay, and between CbGβ and CbGγ using the classic yeast-two-hybrid assay. CbGβ and CbGγ subunits displayed strong and specific interaction, when used as fusion constructs with the DNA activation (AD) or DNA binding (DB) domains (Fig. 4A). Similarly, the interaction between the full-length CbGα and CbGβ proteins as well as the interaction of CbGα and the RGS domain of CbRGS could be verified in split ubiquitin assays (Fig. 4B). CbGα and the RGS proteins interacted in all of the tested combinations. However, CbGα and CbGβ proteins interacted only in specific combinations of different split-ubiquitin fusions. CbGα interacted with CbGβ only when the Nub fusion was made at its C-terminal (Gα::Nub + Gβ::Cub) or when the Gα protein was used as a Cub fusion protein (Gα::Cub + Nub::Gβ). This is expected as the N-terminal of Gα proteins is required for their interaction with the Gβ proteins, and fusion of the ubiquitin half at the N-terminal of the Gα.
protein might interfere with this interaction (Cabrera-Vera et al., 2003). When CbGβ was used as a Nub fusion protein, interaction was detected only in Nub-Gβ orientation (Nub::Gβ + Gα::Cub). It is well known that the Cub fusion constructs have a significantly higher affinity for the Nub-X constructs than for the X-Nub constructs. As a result, weaker interactions between two proteins may not be detected when the test protein is in the X-Nub orientation, as has also been shown for the interaction of KAT1 protein (Obrdlik et al., 2004). Nonetheless the interaction data suggest the existence of both, the trimeric and dimeric conformation of specific G-protein subunits in Chara, which is consistent with the classical G protein cycle mechanism.

Taken together these data firmly establish that the green algae of the Charales, which is the close relative to land plants, possessing a complete functional repertoire of G-proteins, reported to date. It is evident therefore, that G-protein signaling in the green lineage was established before embryophytes evolved and plants started occupying terrestrial habitats.

MATERIAL AND METHODS

Plant material and growth condition

Chara braunii cultures (kindly provided by Dr. Ilse Foissner, University of Salzburg, Austria) were cultivated in distilled water on a sand/soil/peat mixture at RT and 14/10 h light/dark cycle as described previously (Schmolzer et al., 2011).

G-protein genes identification

C. braunii G-protein genes were identified by BLAST analysis of a single lane of a transcriptome dataset with Arabidopsis G-protein sequences as queries. The RNA-Seq library was made using Chara braunii thalli at the vegetative growth phase. The transcriptome was sequenced using Illumina HiSEQ2000 platform and the sequence reads were assembled using ABaSS (http://www.bcgsc.ca/platform/bioinfo/software/abyss) (Abe et al., 2012). Full-length G-protein genes were amplified from C. braunii cDNA using gene-specific primers (Table S4). All G-protein genes were cloned into pCR™8/GW/TOPOR vector (Life Technologies, Carlsbad, USA) and confirmed by sequencing.

Phylogenetic analyses

Gα and RGS related genes were aligned using Clustal omega (Sievers et al., 2011). Phylogenetic analyses were conducted by the Maximum Likelihood method under the JTT
matrix-based model (Jones et al., 1992) using MEGA5 (Tamura et al., 2011). Initial tree(s) for
the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of
pairwise distances estimated using a JTT model. All positions containing gaps and missing data
were eliminated.

RNA isolation and quantitative expression analysis

Total RNA was isolated with Qiagen RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany)
according to the manufacture’s protocol using 100 mg of C. braunii internodal or dimorphic
branch tissue. Genomic DNA was removed from total RNA by using Turbo DNA-free™. One
µg of RNA was used for first strand synthesis using Superscript® III Reverse Transcriptase (Life
Technologies, Carlsbad, USA) along with 50 ng of random hexamer oligo nucleotides. Quantitative RT-PCR experiments were performed in 20 µL reactions (2 µL 1:10 diluted cDNA,
200 nM each primer, 0.4 µL ROX Reference Dye and 10 µL 2 × SYBR® Advantage® qPCR
Premix; Clontech, Mountain View, USA) with the StepOnePlus™ Real-Time PCR System. Amplicon integrity was verified by analysis of melting curves and agarose gel electrophoresis. Primer efficiency was determined by linear regression on the Log (fluorescence) of each PCR reaction using the LinRegPCR software (Ramakers et al., 2003) and transcript levels of each gene were normalized to C. braunii EF-1alpha gene for elongation factor 1-alpha (CbEF1)
(GenBank: AF032728.1).

Recombinant protein purification and G-protein activity assay

Full length CbGα and AtGPA1 as well as RGS domains of CbRGS (aa 401-606) and AtRGS
(aa 250-459) were cloned into the pET-28a vector (Novagen, Gibbstown, USA) and expressed in
Escherichia coli strain BL21 (DE3). Recombinant protein purification and real-time
fluorescence-based GTP-binding and GTP-hydrolysis assays were performed as described
previously (Roy Choudhury et al., 2013).

Protein-protein interaction assays

The interaction assay between CbGα and CbGβ and CbGα and CbRGS was performed using the
mating-based yeast split ubiquitin system (Obrdlik et al., 2004; Pandey and Assmann, 2004).
Protein-protein interactions were determined by growth of diploid yeast colonies on minimal
media lacking Leu, Trp, His and Ade, but containing 200 µM Met. Interactions were confirmed
by filter-lift assays using 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) as a substrate (Schneider et al., 1996). The interaction between CbGβ and CbGγ was determined using a GATEWAY-based yeast-two-hybrid assay (ProQuest Two Hybrid System, Life Technologies, Carlsbad, USA) as described previously (Roy Choudhury et al., 2011). Yeasts, co-transformed with the gene of interest and an empty vector were used as negative controls. Interactions between proteins were identified by growth on media lacking Leu, Trp, His and Ura as well as by filter-lift assays using X-GAL as a substrate.

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LITERATURE CITED


FIGURE LEGENDS

Figure 1. Ga and RGS proteins from Chara braunii. (A) Amino acid sequence alignment of plant Ga proteins representing major taxa was performed using Clustal W (www.clustal.org). Identical aa residues are highlighted in black and blocks of aa with similar biochemical features with grey. Black and grey fonts represent conserved and non-similar aa residues, respectively. Consensus sequences for GTP-binding and -hydrolysis are labeled with G1-G5. The conserved methionine and the invariant glutamine, corresponding to position 1 and 222 in AtGPA1, are labeled with asterisks. P/M represents the predicted sites for palmitoylation/myristoylation. (B) Phylogenetic relationship of plant Ga proteins was inferred using the Maximum Likelihood method based on the JTT matrix-based model. (C) Schematic structure of CbRGS protein possessing one central RGS domain and 9 putative transmembrane helices at its N-terminus (TM1 to TM9), as predicted by hydrophobicity plots generated with TMpred (Hofmann and Stoffel, 1993). (D) Phylogenetic relationship of plant RGS proteins was inferred using the Maximum Likelihood method based on the JTT matrix-based model. For (B) and (D) trees with the highest log likelihood (Ga: -5305.0502 and RGS: -2021.0637) are shown and percentage of trees in which the associated taxa clustered together is shown next to the branches. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Species names and branches are color encoded according their affiliation to eudicots, monocots, gymnosperms, lycophytes, liverworts, or charophyta.

Figure 2. Relative expression of G-proteins and RGS-protein encoding genes in Chara braunii. Relative expression levels were derived from quantitative PCR experiments. Expression levels were normalized to CbEF1 and average of 2^ΔCt values of three biological replicates are plotted with their standard deviation on logarithmic scaled y-axes. 18S rRNA expression levels were measured as internal control.

Figure 3. Activity assay of CbGa and CbRGS proteins. (A) GTP-binding and -hydrolysis of recombinant, purified CbGa were measured using BODIPY-FL in real-time fluorescence-based assay. Specificity of GTP binding was tested by adding nonfluorescent GTP, GDP, ATP or ADP in the reaction mix, where only nonfluorescent GTP was able to compete efficiently for fluorescent GTP binding.
(B) GTPase activity of CbGα is accelerated by recombinant, purified RGS domains of CbRGS or AtRGS1 proteins, in the BODIPY-FL based real-time fluorescence assay. (C) GTPase activity of AtGAP1 is accelerated by recombinant, purified RGS domains of AtRGS1 or CbRGS proteins. All data are one of three independent experiments, each with three replicates (mean ± SD).

**Figure 4. Physical interactions among Chara braunii G-protein components.** (A) Protein-protein interaction between CbGβ and CbGγ was demonstrated using yeast-two-hybrid-based yeast growth and colorimetric assay. Physical interaction between CbGβ and CbGγ could be demonstrated in either fusion construct combinations, as DNA activation (AD) or DNA binding domain (DBD) fusions. (B) Interaction between CbGα and CbGβ as well as CbGα and the RGS domain of CbRGS was tested in split ubiquitin-based system. CbGα and CbRGS interacted with each other in all orientations (N-terminal or C-terminal fusions with N-terminus of ubiquitin (Nub::X, X::Nub) as well as when fused with the C-terminus of ubiquitin (Y::Cub). CbGα interacted with CbGβ only when used as a Cub fusion protein, or as a CbGα::Nub fusion, whereas Nub::CbGβ but not CbGβ::Nub interacted with CbGα::Cub. Interactions were tested by X-Gal filter lift assays and Nub vector fusions with empty Cub vectors were used as negative controls.
The graph illustrates the relative expression of various genes and control RNA (18S rRNA) in two tissue types: internodal tissue and dimorphic branches. The y-axis represents the relative expression in the form of a log scale, ranging from $10^{-2}$ to $10^4$. The x-axis lists the genes: CbGα, CbGβ, CbGγ, CbRGS, and 18S rRNA. Each gene is compared between the two tissue types, showing a higher expression in dimorphic branches for CbGγ and CbRGS, and a lower expression for CbGα and CbGβ in the same comparison.