Plant Physiology and Biochemistry 68 (2013) 32–36

Contents lists available at SciVerse ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Short communication

Abscisic acid binds to recombinant *Arabidopsis thaliana* G-protein coupled receptor-type G-protein 1 in *Sacaromycese cerevisiae* and *in vitro*

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ARTICLE INFO

Article history:
Received 27 February 2013
Accepted 29 March 2013
Available online 12 April 2013

Keywords:
G-protein couple receptor-type G-Protein
Abscisic acid
Recombinant expression
S. cerevisiae
Protein–ligand interaction

ABSTRACT

The G-protein coupled receptor-type G-proteins (GTG) 1 and 2 from *Arabidopsis thaliana* have been proposed to function in the modulation of abscisic acid (ABA) mediated responses to stress and development. In particular it has been suggested that they function as ABA receptors based on *in planta* and *in vitro* analyses. However a recent independent report was inconsistent with this, suggesting that there is no link between the GTGs and ABA *in planta*. Here we provide an independent assessment of the ability of ABA to bind to recombinant GTG1 *in vitro* and *in vivo* in *Sacaromycese cerevisiae*. Radio-labelled binding assays on enriched lipid-reconstituted recombinant GTG1, demonstrated specific concentration dependent binding of [^{3}H]-ABA with a dissociation constant (K_D) of 80 nM, corroborating previous reports. Assessment of the binding of [^{3}H]-ABA to intact GTG1 expressing yeast, showed GTG1-dependent binding *in vivo*, yielding a physiologically relevant K_D of 0.6 μM. Together these results provide independent evidence of a binding-interaction between ABA and GTG1 *in vitro* and *in vivo*, in support of the previously proposed possibility of a biologically relevant interaction between GTG1 and ABA.

1. Introduction

Abscisic acid (ABA) is a phytohormone known to modulate plant development and stress responses [1]. The recent past has seen a variety of reports highlighting plant molecular receptors for ABA, currently including the pyrabactin resistant receptor-1 (PYR1)/PYR1-like(PYL)/regulatory components of ABA receptors (RCAR) family, the G-protein coupled receptor (GPCR)-Type G-proteins (GTGs) and the magnesium chelatase subunit H [2–5]. While the case for the PYR1/PYL/RCAR as ABA receptors is well supported, inconsistencies in the literature for the GTGs and Mg Chelatase leaves their role as ABA receptors in question, and gaps in our understanding of the mechanism of ABA action [4–7].

With respect to the GTGs, in 2009, the original characterization of the two homologs appeared to provide compelling evidence that these ubiquitously expressed, cell membrane localized proteins could bind ABA *in vitro* and mediate ABA responses *in planta* [4]. This early report was further substantiated by a recent, quantitative proteomics-based analysis performed on *Arabidopsis* roots of the original *gtg1gtg2* line, demonstrating that expression of the majority of ABA-responsive proteins in roots is dependent on the presence of GTG1 and GTG2 [8]. In particular, identified links between the GTG proteins and well-established effectors of the ABA signalling pathways, as well as mediators of phytohormone cross-talk, lend further support to the original proposal that GTG's mediate ABA signalling.

However, a homolog of the plant GTGs, GPR89, was shown to function as a voltage-gated anion channel in the endoplasmic reticulum of mammalian systems [9], suggesting this family of proteins may not actually function as GPCR-type G-proteins at all. Indeed a more recent report hypothesizes that none of the currently annotated GPCRs in *Arabidopsis* actually function as GPCRs [10]. While, such a role (i.e. ion channel) for the GTG proteins would be more consistent with the nine, rather than seven, transmembrane segments detected in the protein's structure, neither role (channel versus GPCR-type G-protein) precludes the possibility of a biologically relevant signalling interaction with ABA. But, in 2012 an independent report was published which contradicted the original 2009 report, providing equally compelling evidence that GTG1 and GTG2 are expressed in the endoplasmic reticulum of

Abbreviations: ABA, abscisic acid; [^{3}H]-ABA, DL-*cis*-[G-^{3}H]-ABA; K_D, Dissociation constant; GTG, G-protein coupled receptor-type G-protein; Ni-NTA, nickel-nitrilotriacetic acid; OG, octyl β-D-glucopyranoside; *S. cerevisiae*, *Sacaromycese cerevisiae*.

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0981-9428/$ – see front matter © 2013 Published by Elsevier Masson SAS.
http://dx.doi.org/10.1016/j.plaphy.2013.03.025

plants and are not involved in mediating ABA responses *in planta* [6]. Unfortunately this latter report did not provide an independent assessment of the ability of GTG1 or GTG2 to bind ABA directly. One possible explanation for the diametrically opposed results could lie with the details of the conditions under which the genetically modified plants were propagated and tested in the two independent studies.

In this context, we report here an independent investigation of the ability of GTG1 to bind ABA. Our findings demonstrate that ABA can interact with recombinant (produced in *Sacaromycese cerevisiae*) GTG1 with physiologically relevant binding constants, both *in vitro* after enrichment and after reconstitution into proteoliposomes, as well as when applied *in vivo* to intact GTG1 expressing yeast cells. While this investigation does not directly address the biological relevance of the relationship of ABA and the GTGs *in planta*, it provides an independent corroboration of the original report that GTGs can interact with ABA.

2. Results and discussion

2.1. Recombinant expression of GTG1 in S. cerevisiae

The coding sequence for GTG1 was subcloned into the pYES.DEST52 (Invitrogen) vector as described in the Methods section, placing it immediately downstream of the GAL4 promoter and including a both a V5 epitope and His tags at the 3′ end. Expression of fusion GTG1 protein from this construct in *S. cerevisea* was initially assessed over a time course of galactose induction. Induced cells were harvested and lysed at the indicated time intervals, and analyzed by Western blot using anti-V5-HRP antibody (Fig. 1A). A single band in the induced cultures cross-reacted with the antibody at a Mw of ~50 kDa, a bit lower than the expected Mw of 57 kDa for the fusion protein, suggesting the possibility of some proteolytic degradation of the protein product. Maximum intensity was observed after 8 h of galactose induction. No bands were observed in the corresponding galactose induced empty vector expressing cell samples. Localization of the recombinant integral GTG1 membrane fusion protein was subsequently assessed. Yeast cells were induced and soluble and membrane fractions obtained as described in the methods. Western blot analysis confirmed the presence of GTG1 in the membrane fraction and not the soluble fraction (Fig. 1B). The observation of two bands here, one at the expected 57 kDa and at the lower ~50 kDa further suggests the possibility of proteolytic or spontaneous degradation of the protein, possibly arising during storage of the harvested cell pellet at −20 °C, or during the preparation of cell samples. While this analysis does not discriminate between microsomal fractions, i.e. we cannot say cell membrane versus endoplasmic reticulum, it does show an association to the membrane fraction in general, suggesting properly folded and membrane integrated GTG1. No bands were detected in the corresponding fractions of the empty vector control expression sample.

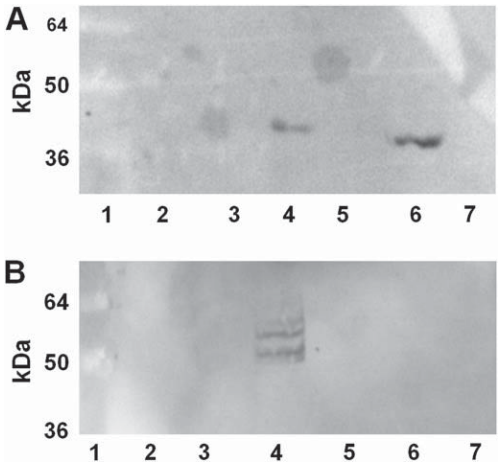

Fig. 1. GTG1 recombinant expression in *S. cerevisiae*. (A) Time course of GTG1 expression. The relative expression of recombinant fusion GTG1-His-V5 from pYESDEST52 in *S. cerevisiae* total cell extracts was assessed, at various time points after induction with galactose, by Western blot. lane 1, Mw markers; lanes 2, 4 and 6 pYES.DEST52-GTG1-His-V5 expressing *S. cerevisiae* after 0, 4, and 8 h of galactose induction respectively; lanes 3, 5, 7 and 9, empty pYES.DEST52 vector in *S. cerevisiae* after 0, 4 and 8 h of galactose induction. (B) Localization of GTG1. Western blot analysis of soluble and membrane fractions of galactose induced GTG1 and empty vector expressing *S. cerevisiae* cultures. lane 1: Mw markers; lanes 2–4, pYES.DEST52-GTG-His-V5 expressing *S. cerevisiae* induced for 8 h; lanes 5–7, pYESDest52 empty vector expressing *S. cerevisiae* induced for 8 h lanes 2 and 5, total cell extract; lane 3 and 6, soluble fraction; lanes 4 and 7, membrane fraction; anti-V5-HRP antibody was used for the Western analyses.

2.2. Ni-NTA enrichment and in vitro assay of ABA binding to GTG1

The ability of the recombinant GTG1 to bind to ABA was subsequently tested *in vitro*. GTG1 fusion protein was extracted from cell lysates by detergent solubilisation with Octyl β-D-glucopyranoside (OG) and enriched by Ni-NTA affinity chromatography (Fig. 2A). Similar results were obtained using 0.25% Triton X-100 (data not shown). The eluted protein was reconstituted into proteoliposomes and the binding of racemic [^{3}H]-ABA assessed. In particular the concentration dependence of the binding of [^{3}H]-ABA to enriched GTG1 was evaluated. For each concentration of [^{3}H]-ABA tested, the amount of binding observed to a heat inactivated enriched GTG1 sample was subtracted from the total

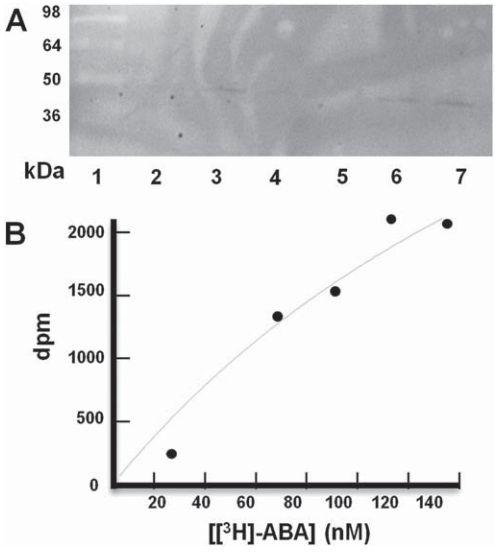

Fig. 2. Enrichment and binding of [^{3}H]-ABA to recombinant GTG1 *in vitro*. (A) Ni-NTA affinity purification of recombinant GTG1. Western blot of the GTG1 purification performed with modifications: The protein solubilization was achieved using 1% OG. Anti-V5-HRP antibody was used as a detection system. The expression of GTG1 in PYES.DEST52 was induced by galactose for 8 h. Lane 1, Mw markers; lane 2, total cell extract; lane 3, flow-through, lanes 4–6 washes, lane 7, elution. (B) Concentration dependence of [^{3}H]-ABA binding to GTG1. Ni-NTA purified and reconstituted GTG1 was incubated with increasing concentrations of [^{3}H]-ABA and the amount of bound ABA remaining detected after washing. Values for [^{3}H]-ABA binding to heat inactivated GTG1 were subtracted from the experimental values and the difference plotted. All experiments were $n = 3$. Michaelis–Menten analysis of the difference data using Enzfitter software (Biosoft) yielded a K_m of 80 nM.

binding observed to an intact enriched GTG1 sample to account for any background binding. A plot of the differences obtained highlights GTG1 specific binding of ABA showing a trend toward saturation at around 120 nM [^{3}H]-ABA (Fig. 2B). Michaelis–Menten analysis of this data yielded a K_D of 80 nM for the binding of [^{3}H]-ABA to recombinant GTG1 *in vitro*. This is comparable to the value previously reported for the *in vitro* binding of ABA to Escherichia coli expressed and enriched recombinant GTG1 at 36 nM [4].

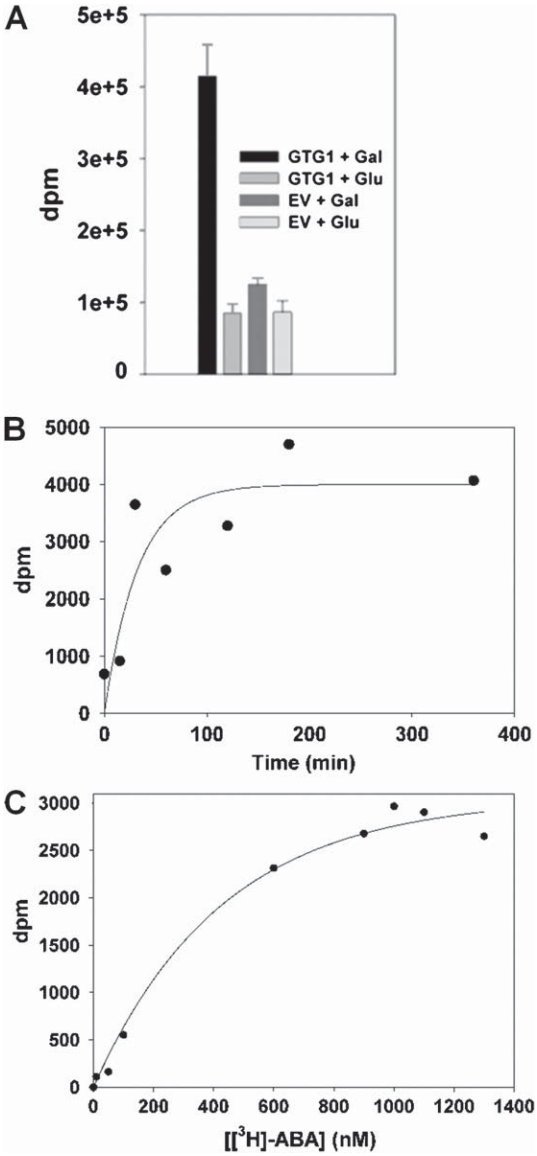

Fig. 3. GTG1-dependent binding of [^{3}H]-ABA to *S. cerevisiae*. (A) ABA binds to intact *S. cerevisiae* in a GTG1-dependent manner. [^{3}H]-ABA was applied to either galactose or glucose-induced intact *S. cerevisiae* cells containing either the pYESDEST52-GTG1 expression construct or an empty vector (EV) pYESDEST52 expression construct ($n = 3$). (B) Time course of [^{3}H]-ABA binding to intact *S. cerevisiae* cells overexpressing GTG1. Values ($n = 3$) obtained for binding of [^{3}H]-ABA to *S. cerevisiae* containing the GTG1 expression construct from glucose-induced negative control samples were subtracted from the galactose-induced samples. The obtained differences were plotted versus time. (C) Concentration dependence of [^{3}H]-ABA binding to GTG1 in intact *S. cerevisiae*. Values ($n = 3$) obtained for binding of [^{3}H]-ABA to *S. cerevisiae* containing the GTG1 expression construct from glucose-induced negative control samples were subtracted from the galactose-induced samples. The mean values of the obtained differences were plotted versus concentration of [^{3}H]-ABA.

2.3. Binding of ABA to GTG1 expressing yeast

Finally, the binding of [^{3}H]-ABA to intact GTG1 expressing *S. cerevisiae* cells was assessed. Galactose induced GTG1 expressing cells were treated with 100 nM [^{3}H]-ABA, harvested at the indicated time points and assessed for bound [^{3}H]-ABA. To account for background binding, the relative background in three different negative controls were assessed (Fig. 3A). These included a non-galactose induced (glucose-induced) control containing the GTG1 expression construct, and both galactose-induced and glucose-induced controls transformed with the pYESDEST52 vector without the GTG1 coding sequence. These results show a comparable amount of binding of [^{3}H]-ABA to all three negative controls with a 5-fold higher level of binding to the GTG1 expressing strain. This demonstrates the GTG1-dependent binding of ABA to intact *S. cerevisiae*. Expanding on this finding, a time course analysis of ABA binding to GTG1 expressing *S. cerevisiae* was carried out. Values obtained for binding of [^{3}H]-ABA to *S. cerevisiae* containing the GTG1 expression construct from glucose-induced negative control samples were subtracted from the galactose-induced samples. A plot of obtained differences against time shows saturable GTG1-dependent binding of [^{3}H]-ABA to the yeast cells (Fig. 3B). Under these conditions saturation occurred at about 200 min. A concentration-dependence analysis, carried out as described above (using glucose-induced GTG1 expression construct containing cells as a negative control), but over a range of [^{3}H]-ABA concentrations, further confirmed the GTG1-dependent binding of [^{3}H]-ABA to the yeast cells (Fig. 3C). A Michaelis–Menten analysis of the obtained concentration-dependence difference plot yielded a K_D of 0.6 μM. While this is approximately 100-fold higher than the K_D value obtained in the *in vitro* studies, it is as expected based on other *in vitro–in vivo* comparisons for interactions of ABA to recombinant membrane proteins [11,12].

3. Conclusion

The experiments reported here do not address the role or mechanism of action, be it as a receptor or anion channel or other, of GTG1 in modulation of ABA signalling. However these findings do clearly demonstrate that ABA can interact with GTG1 both *in vitro* and in intact yeast cells, with physiologically relevant binding constants. In light of the recent discrepancies in the literature regarding the relationship between GTG1 and ABA, we present these results as independent evidence in support of the possibility of a relevant binding-interaction between ABA and GTG1.

4. Materials and methods

4.1. Materials

All materials were purchased from Sigma–Aldrich unless otherwise noted. ECL (enhanced chemiluminescence) biotinylated protein markers, ECLplus Western blotting detection reagents and [^{3}H]-ABA (DL-*cis*-[G-^{3}H]-ABA) were all obtained from Amersham Biosciences. (+)-ABA was prepared as described previously [13].

4.2. Cloning and recombinant expression of GTG1 fusion protein

Arabidopsis thaliana Col-0 plant material was grown in liquid Germination Media (0.431% MS Salts, 1% Sucrose, 0.05% MES and Gamborg's Vitamins) for 16 days under continuous light. Tissue was macerated under liquid nitrogen and RNA extracted as per the QIAGEN RNAeasy kit protocol. *C. therm* polymerase one-step RT-PCR (Roche) kit was used to produce cDNA from the RNA according to the manufacturers specifications. This cDNA (NCBI Accession HM776216) was used with the following primers for PCR

amplification of the GTG1 encoding cDNA: forward 5′-CACCATGAGTTACGGATGGGCGATATACGAAGGC C-3′ and reverse 5′-GTCTATTGGGTGCTTATCAATTTGACGAGATGTG-3′ to remove the stop codon. The product was cloned into pENTR.D Topo as per Invitrogen's protocol. The obtained GTG1 coding sequence was confirmed by sequencing and subsequently recombined into the pYES.DEST52 (Invitrogen) yeast expression vector, producing a pYES.DEST52-GTG1-V5-HIS fusion expression construct, which was again confirmed by sequencing. Yeast transformation was performed according to the Invitrogen protocol, with either pYES.DEST52-GTG1-V5-HIS or empty pYES.DEST52 empty vector into the INVSc1 yeast strain (Invitrogen). Positive transformants were selected on SC-URA selective plates. For the GTG1 expression time course, a single colony for each construct was inoculated into SC-URA minimal medium overnight at 30 °C with shaking and then induced with 2% galactose in 50 ml SC-URA minimal medium. Aliquots of cells were harvested at 0, 4, 8 and 24 h. Cells were lysed by sonication with 30 s pulses followed by 30 s rest intervals for up to 5 min and samples analyzed by Western blot using anti-V5 epitope antibody at 1:5000 ratio. For detection of protein in membrane fractions, protein expression was induced with 2% galactose for 8 h. The cell pellet was collected and resuspended in the 50 mM Tris buffer, pH 7.9, containing 250 mM KCl, 0.1% Tween20, 10% glycerol and lysed as described above. The cell lysate was centrifuged at 2000 × *g* for 15 min. The cleared lysate was further centrifuged at 15,000 × *g* for 5 h yielding the soluble and membrane fractions, which were further analyzed by Western blot as described above.

4.3. Affinity purification and proteoliposome reconstitution of GTG1 fusion protein

Galactose induced cell pellets was collected and resuspended in buffer 1 (50 mM Tris buffer, pH = 7.5, 250 mM NaCl, 0.1% Tween20, 10% glycerol, with 1% Octyl β-D-glucopyranoside (OG) or 0.25% Triton x-100 as indicated) containing 5 mM β-mercaptoethanol and phenylmethanesulfonyl fluoride protease inhibitor. Cells lysis was, followed by a 1 h solubilization on ice and then centrifugation at 10,000 × *g* for 15 min. The obtained cleared supernatant was applied to a Ni-NTA column, and washed twice with three column volumes of buffer 1 containing 15 mM imidazole, and then twice with the three column volumes of buffer 1 with 30 mM imidazol. The protein was eluted with 4 × 1 ml buffer 1 containing 300 mM imidazol and 100 ng/ml phosphatidyl choline [UPC, Avanti Polar Lipids]. The detergent was subsequently removed, and protein reconstituted in proteopliposomes by reiterative concentration/dilution with buffer 1 using Amicon (Millipore) concentration devises (10,000 MW cut-off) or using the Extracti-Gel D detergent removing gel column with buffer 1 (Pierce).

4.4. [^{3}H]-ABA binding to GTG1 fusion protein in vitro

Purified GTG1 reconstituted into phosphatidyl choline proteoliposomes as described above, was incubated with 100 nM [^{3}H]-ABA in the absence or presence of excess unlabelled (+)-ABA for 1 h on ice. The samples were filtered through SPIN-X columns and washed extensively with buffer 1. The filters were separated from the columns and transferred into scintillation vials containing 4 mL scintillation fluid. Bound radioactivity was measured using a Beckman Coulter multi-purpose scintillation counter. For the concentration-dependence experiments, a range of concentrations of [^{3}H]-ABA was added to the reaction mixtures containing either GTG1 prepared as described above or heat treated GTG1 (as a negative control for non-specific binding). Binding of [^{3}H]-ABA was measured as described above. Values obtained for heat treated GTG1 were subtracted from values obtained for untreated experimental samples. The differences obtained were plotted and kinetic values were obtained using Enzfitter software (Biosoft). The plotted values represent the mean of three experiments.

4.5. [^{3}H]-ABA binding to intact yeast cells

Evaluation of the binding of [^{3}H]-ABA binding to intact yeast cells was performed essentially as described in Ref. [14] with the following modifications. INVSc1 cells either untransformed or transformed with pYES.DEST52-GTG1-V5-HIS or empty pYES.DEST52 were grown overnight at 30 °C. The cells were collected by centrifugation, washed once, and split into two equal volumes supplemented with either 2% glucose or 2% galactose (this latter yielding an 'un-induced' sample) and grown again overnight. For the initial experiment [^{3}H]-ABA was added to the yeast cultures at 100 nM concentration and binding measured at 30 min thereafter by removal of 100 μL aliquots of cells. The cell aliquots were immediately filtered through SPIN-X column, washed on the filter with 50 mM potassium phosphate buffer, pH 6.5, 2% SDS and then heated to 80 °C for 5 min. Bound [^{3}H]-ABA was detected as described above. Each data point represents the average of at least three replicates. For the time course experiment, working only with the glucose and galactose induced pYES.DEST52-GTG1-V5-HIS transformed cells, experiments were as described above except aliquots were taken at a variety of time points after the addition of [^{3}H]-ABA. Similarly, the concentration-dependence of the interaction was analyzed as described above, but with the addition of a range of concentrations of [^{3}H]-ABA, again working only with the glucose and galactose induced pYES.DEST52-GTG1-V5-HIS transformed cells. For both the time course and concentration dependent experiments values obtained for the glucose supplemented samples were subtracted from the galactose induced sample values to account for background binding. Differences were plotted (mean values of at least three experiments) and kinetic values obtained by Michaelis–Menten analysis using Enzfitter software (Biosoft).

Acknowledgements

We are grateful to K. Nelson and S. Abrams for supplying (+)-ABA, and J. Boyd for initial cloning of the cDNA. This work was funded by the National Research Council of Canada, Genomics and Health Initiative as well as a Natural Sciences and Engineering Research Council grant to M.C.L.

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