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D-Glucose sensing by a plasma membrane regulator of G signaling protein, *At*RGS1

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Abstract **Plants use sugars as signaling molecules and possess mechanisms to detect and respond to changes in sugar availability, ranging from the level of secondary signaling molecules to altered gene transcription. G-protein-coupled pathways are involved in sugar signaling in plants. The *Arabidopsis thaliana* regulator of G-protein signaling protein 1 (*At*RGS1) combines a receptor-like seven transmembrane domain with an RGS domain, interacts with the *Arabidopsis* Gα subunit (*At*GPA1) in a D-glucose-regulated manner, and stimulates *At*GPA1 GTPase activity. We determined that *At*RGS1 interacts with additional components, genetically defined here, to serve as a plasma membrane sensor for D-glucose. This interaction between AtRGS1 and *At*GPA1 involves, in part, the seven-transmembrane domain of *At*RGS1.**

Structured summary:
MINT-6743118:
RGS1 (uniprotkb:Q8H1F2) and GPA1 (uniprotkb:P18064) *physically interact* (MI:0218) by *bimolecular fluorescence complementation* (MI:0809)

Keywords: *Arabidopsis thaliana* regulator of G-protein signaling protein 1; Glucose sensing; Heterotrimeric G protein complex; Regulator of G signaling protein 1; *Arabidopsis*

1. Introduction

Signal transduction pathways mediated by sugars play roles in virtually all aspects of life and development for most organisms [1]. For example, in *Saccharomyces cerevisiae*, a complex network of sugar signaling pathways has been characterized, involving at least four receptors for glucose. These include both intracellular receptors, such as hexokinase-1, and cell surface receptors, including the hexose transporter-like proteins Snf3 and Rgt2 and the G protein-coupled receptor Gpr1 [2]. Despite the wealth of knowledge on sugar signaling pathways in *S. cerevisiae*, relatively little is known about the apical signaling elements or downstream pathways involved in sugar signaling in multicellular organisms. Animals use G-protein signaling for taste perception of sugars; in humans, this is accomplished via the gustducin-coupled T1R2–T1R3 heterodimer, and recent evidence indicates that this G protein signaling network is also expressed in the gut, where it regulates expression of the Na^+-dependent glucose co-transporter protein SGLT1 [3–5].

Like *S. cerevisiae*, *Arabidopsis thaliana* has a simple repertoire of G protein signaling elements, one canonical G_α subunit (*At*GPA1), one Gβ subunit (AGB1), at least two Gγ subunits and one regulator of G signaling protein (RGS1), *At*RGS1 [6]. The *Arabidopsis* heterotrimeric G protein complex has been implicated in an array, potentially a mélange, of plant physiologies [7] such as abscisic acid signaling [8–15], biotic and abiotic stress [16–22], germination and early development [23–25], and glucose signaling. The involvement of G-protein signaling pathways in the response of plants to changes in glucose availability has previously been suggested by the phenotypes of heterotrimeric G-protein signaling mutants in response to chronic treatment with high sugar concentrations, which inhibit seed germination and arrest growth of wild-type seedlings [26–34]. For example, *A. thaliana* Gα subunit (*At*GPA1)-null mutants are hypersensitive to glucose during germination and seedling development [30–33]. *At*RGS1, comprised of a C-terminal RGS domain coupled to an N-terminal domain with a predicted seven transmembrane (7TM) topology [27], interacts with the *At*GPA1 at the plasma membrane and functions as a GTPase-activating protein (GAP) for *At*GPA1 [27,35,36]. Several lines of evidence also indicate the involvement of *At*RGS1 in sugar-mediated regulatory pathways in *Arabidopsis*. In *Atrgs1-null* mutants, seed germination and seedling development are insensitive to D-glucose [28,29], while overexpression of *At*RGS1 results in hypersensitivity to glucose during seedling growth [27,37]. Treatment with D-glucose also alters the interaction of *At*RGS1 and *At*GPA1 [36]. However, G protein signaling is poorly characterized in *Arabidopsis* and other plants relative to what is known from yeast and animals systems, and relatively little is known about processes lying downstream of *At*GPA1.

Here, we conclude that *At*RGS1, a putative extracellular receptor for D-glucose, together with the heterotrimeric G protein complex mediates the steady-state level of transcripts from a small set of sugar-regulated genes in a G protein-coupled signaling network.

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2. Materials and methods

2.1. Arabidopsis

All experiments were conducted using *A. thaliana* of the Columbia ecotype. The generation and characterization of the majority of Arabidopsis lines containing T-DNA insertions and transgenic alleles used in these studies are described in the literature [27,36,38–40]. Gene accession numbers are: *AGB1*, At4g34460; *At*GPA1, At2g26300; *At*RGS1, At3g26090; *THF1*, At220890.

2.2. Cultivation of Arabidopsis seedlings for gene chip and real-time PCR analysis

Arabidopsis seeds were sterilized by sequential treatments with 70% ethanol + 0.05% Triton-X (15 min), 95% ethanol + 0.05% Triton-X (5 min) and 95% ethanol (5 min) followed by air-drying in a sterile hood. Roughly 200 seeds per sample were then transferred to 250-mL flasks containing 50 mL 1/2 Murashige and Skoog (MS) medium (pH 5.7) + 50 mM D-glucose. The flasks were incubated in the dark for 2 days at 4 °C and were then transferred to a shaker at 22 °C under constant low light conditions and incubated for 7 days. To sugar starve seedlings, the media was replaced with 1/2 MS medium containing no D-glucose and the seedlings were grown on a shaker in the dark for 2 days. Following sugar starvation, seedlings were removed from the dark and incubated under constant low light on a shaker with 1/2 MS media containing the indicated concentrations of D-glucose (0–300 mM) for the indicated time periods. Following this incubation, the seedlings were snap-frozen in liquid nitrogen. Cyclohexamide treatments were performed as described by Scherer et al. [41], except seedlings were grown as described above. Briefly, seedlings were treated with cycloheximide for 1 h before sugar treatment, and then throughout the sugar treatment. Concentrations of cyclohexamide used were 1 μg/μl and 10 μg/μl. These concentrations provided virtually identical results; therefore, the results were pooled for the final analysis. Experiments using 1 μg/μl cyclohexamide were performed twice, and experiments using 10 μg/μl were performed three times.

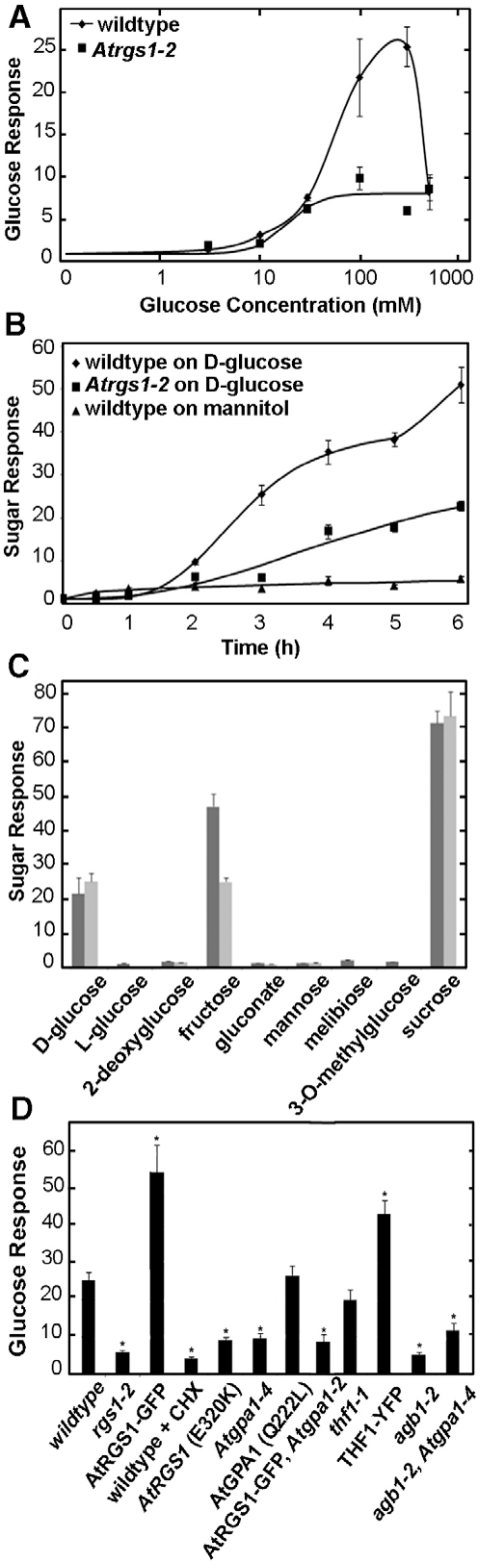

Fig. 1. Induction of *At4g01080* steady-state transcript level (glucose/sugar response). (A) D-glucose dose-dependence of *At4g01080* transcript increase by D-glucose. Wild-type and *Atrgs1*-27 day old seedlings were sugar-starved for 2 days and then treated with various concentrations of D-glucose for 3 h. Wild-type is indicated here as Col-O and *Atrgs1* null mutants indicated as *rgs1-2*. (B) Time dependency of *At4g01080* transcript levels in response to treatment with D-glucose or mannitol. Wild-type and *Atrgs1*-27 day old seedlings were sugar-starved for 2 days and then treated with 300 mM D-glucose for varying time periods. (C) *At4g01080* transcript level increase in response to a range of sugars and sugar analogues. *At4g01080* transcript increase in response to treatment with various sugars and sugar analogues for 3 h. Dark grey, 100 mM D-glucose; light grey, 300 mM D-glucose. (D) Regulation of *At4g01080* transcript level in various genetic backgrounds in response to treatment with D-glucose. Treatment was 300 mM D-glucose for 3 h. *rgs1-2*: seedlings null for *At*RGS1; RGS1-GFP: seedlings null for *At*RGS1 and overexpressing an *At*RGS1–GFP chimera; WT + CHX: wild-type seedlings treated with cycloheximide (1 or 10 μg/ml) for 1 h before and during D-glucose treatment; RGS1–E320K: seedlings null for *At*RGS1 over-expressing an *At*RGS1 mutant containing mutation (E320K) that disrupts the interaction between *At*RGS1 and *At*GPA1; *gpa1-4*: seedlings null for *At*GPA1; RGS–GFP + *gpa1-4*: seedlings null for *At*RGS1 and *At*GPA1 and over-expressing an *At*RGS1–GFP chimera; GPA1 (Q222L): seedlings null for *At*GPA1 overexpressing an *At*GPA1 mutant containing mutation (Q222L), which results in a constitutively active form of the protein; *thf1-1*: seedlings null for THF1; THF1–YFP: seedlings over-expressing an THF1–YFP chimera; *agb1-2*: seedlings null for AGB1; *agb1-2*/*gpa1-4*: seedlings null for AGB1 and *At*GPA1. * Indicates values that are significantly different from wild-type plants ($p < 0.01$). (A–D) Bars or points represent means +/− S.E. After the indicated treatment, RNA was isolated from whole seedlings and used to generate cDNA using oligo dT primers as described in Section 2. These cDNA samples were then used for real-time PCR reactions with primers specific for the *At4g01080* sequence and the *TUB4* sequence (the reference) to determine the level of increase of the *At4g01080* transcript level. Each mean is from at least three biological replicates with 3 internal replicates for each to assure precision. For the means presented in panel D, the number of replicates are: Col-O, eleven times1; *rgs1-2*, eight times; RGS1–GFP, six times; *gpa1-4*, three times; *agb1-2*, two times; *agb1-2*/*gpa1-4*, three times; GPA1(QL), two times; RGS1(E320K), six times; *thf1-1*, two times; cyclohexamide, five times.

2.3. Bimolecular fluorescence complementation (BiFC)

The coding sequences of *At*RGS1 and the mutant version *At*RGS1(E320K) were cloned into BiFC vector pBatL-sYFP-N to generate RGS1-sYFP-N and RGS1(E320K)-sYFP-N vectors. The coding sequences of GPA1, PIP2A and p31 (AT3G01290) were cloned into BiFC vector pBatL-sYFP-C to generate GPA1-sYFP-C, PIP2A-sYFP-C and p31-sYFP-C vectors. All vectors were transformed into *Agrobacteria* stain GV3101 (pMP90). Overnight-grown *Agrobacteria* were resuspend in infiltration solution (10 mM MES, pH 5.7, 10 mM MgCl, 150 μM acetosyringone) to OD = 1.5 and incubated at room temperature for 4 h. The indicated pair of *Agrobacteria* and *Agrobacteria* harboring p19 to suppress gene silencing [42] were mixed and used to infiltrate the leaves of 4–5 week-old *Nicotiana benthamiana*. Four days after infiltration, leaves were detached from plants and observed under an Olympus IX 81epi-fluorescence microscope. Images were captured by a cooled charge-coupled device camera (Photometrics Cascade digital camera, Roper Scientific).

2.4. Expression arrays

Wild-type and *Atrgs1-1* seedlings were grown to analyze expression profiles as in [43,44] except that after 7 days the seedlings were transferred to a fresh medium that contained no sugar, rather than nitrogen, and after an additional 2 days 15 or 100 mM glucose was added to the starved seedlings. Quality controls, RNA preparation, dye swaps, and replications are as described by Scheible et al. [43]. Measurements of carbohydrates showed that the seedlings were carbon depleted (data not shown). Plant material was harvested, RNA prepared and used for hybridization of Affymetrix ATH1 arrays, and the raw Affymetrix data (CEL files) processed using the RMA (log scale Robust Multi-array Analysis) software as in [43]. RMA is based on the Quantile normalization method and has better precision than MicroArray Suite 5.0 (Affymetrix, Santa Clara, CA) and dCHIP (http://www.dchip.org/), especially for low expression values [45,46]. In addition, all signals called 'not present' by the Affymetrix MASC software were excluded from the data (and are marked as 'A' in the table in Supplementary material). The data were also visualized and figures produced using MapMan software [47]. A downloadable version for local application and a servlet version are available at http://gabi.rzpd.de/projects/MapMan/.

2.5. RNA extraction and cDNA synthesis

RNA was extracted from *Arabidopsis* seedlings by use of the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was generated using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 3 μg total RNA with dNTPs (Invitrogen, 0.5 mM final concentration) and Oligo(dT)$_{20}$ oligomers (Invitrogen, 2.5 μM final concentration) was incubated at 65 °C for 5 min. First-strand cDNA synthesis was then performed by adding SuperScript III Reverse Transcriptase (Invitrogen, 200 U), DTT (Invitrogen, 5 mM final concentration), RNAseOut (Invitrogen, 2 U) and RNAse-free water to a final volume of 20 μl and incubating the samples at 50 °C for 45 min. The reactions were terminated by incubation at 70 °C for 15 min. Following first-strand cDNA synthesis, 1 μl RNAse H was added to the reactions, and the samples were incubated for 30 min at 37 °C and stored at −80 °C.

2.6. Real-time PCR technique and analysis

A 69-bp fragment of the *At4g01080* gene (GenBank accession number NM_116338) was amplified to quantitate transcript levels in seedlings exposed to different treatments. Real-time PCR reactions were assembled in a total volume of 50 μl using 25 μl of SYBR GREEN PCR Master Mix (Applied Biosystems, Foster City, CA), 2 μl of cDNA from the 20 μl first-strand cDNA synthesis reactions and primers specific for *At4g01080* or the reference gene tubulin beta-4 chain (*TUB4*; At5g44340) at final concentrations of 0.2 pmol/μl. Reactions were performed in triplicate. Primer sequences for *At4g01080* were 5′-GAA GAA CAA ATG GTG GGC TT-3′ and 5′-ATG CAG ATG AGA GAC TGG ACA-3′; primer sequences for tubulin beta-4 chain were 5′-AGA GGT TGA CGA GCA GAT GA-3′ and 5′-ACC AAT GAA AGT AGA CGC CA-3′.

Real-time PCR was performed in a DNA Engine Opticon 2 System (Bio-Rad, Hercules, CA) using Opticon Monitor 3.1 software with the following thermocycler program: 2 min of preincubation at 94 °C followed by 40–45 cycles of 15 s at 94 °C, 15 s at 55 °C, and 15 s at 72 °C. SYBR Green dye fluorescence was monitored at the end of the annealing phase. A melting curve from 65 °C to 95 °C was used to confirm the presence of single products. All amplification curves were baseline-adjusted by subtracting the lowest fluorescence signal measured in each well over all cycles and the average of the blank wells (global minimum baseline adjustment in the Opticon Monitor 3.1 software). The threshold was set manually to a position at which signal intensities were low but had significantly surpassed levels and begun to increase exponentially, and the number of cycles required to reach this value, CT, was determined for each sample.

2.7. Quantification of relative gene expression from real-time PCR data

A general mathematical model was used to determine the ratio of the expression of a gene following two different treatments by real-time PCR and was applied to expression of *At4g01080* in different *Arabidopsis* genetic lines following different glucose treatments. For these calculations, tubulin beta-4 chain was used as a reference gene. The basic equation describing the ratio calculation based on real-time PCR amplification data is

$$\text{Ratio} = \frac{(E_{\text{target}})\Delta C_{\text{t}}\ \text{target}(\text{treatment1} - \text{treatment2})}{(E_{\text{ref}})\Delta C_{\text{t}}\ \text{ref}(\text{treatment1} - \text{treatment2})}$$

where C_t is the threshold cycle number, ΔC_t target (treatment1 − treatment2) is the difference in C_t values for the target gene (*At4g01080*) between the two treatments being compared, ΔC_t ref (treatment1-treatment2) is the difference in C_t values for the reference gene (tubulin beta-4 chain) between the same two treatments, E_{target} is the PCR efficiency for the target gene ($E = 1$ corresponds to 100% efficiency) and E_{ref} is the PCR efficiency for the reference gene. E is assumed to be independent of N in the particular amplification range and was calculated by the Opticon Monitor 3.1 software from the slope of a plot of C_t vs. log N_0:

$$E = 10^{-(\text{slope})^{-1}} - 1$$

2.8. Statistical analysis

The statistical significance of changes in mRNA induction between groups was assessed using an unpaired Student's *t*-test. *P*-values <0.05 were considered to be significant.

3. Results and discussion

3.1. AtRGS1 mediates D-glucose regulation of expression of a limited set of genes

To investigate genetic and structural requisites of G protein- and *At*RGS1-mediated sugar signaling in *Arabidopsis*, we first compared the D-glucose-induced gene expression profiles of wild-type and *Atrgs1-2* null seedlings. Glucose-starved seedlings were treated with 100 mM mannitol or two concentrations of D-glucose for 3 h. The arrays were normalized using the Robust Multi-array Analysis software [45,46] and all signals called absent by the MASC software were excluded from the analysis. The raw data sets for the various treatments are provided in Supplementary data (S1) and deposited at http://www.ncbi.nlm.nih.gov.geo/ with the series number [upon notice of acceptance]. As expected, the profiles in *Atrgs1*-null seedlings were similar to wild-type plants, with regression coefficients of 0.999 and 0.992 in control comparisons of sugar-starved seedlings and of seedlings that received 100 mM mannitol as an osmotic control. A small number of genes showed strong responses to 100 mM mannitol in both genotypes, indicating they respond to mild water deficits, and were excluded from subsequent analyses. The regression decreased slightly

in the presence of 15 and 100 mM glucose (0.987 and 0.986, respectively).

Addition of glucose leads to dramatic changes in the steady-state level of transcripts from many genes involved in central metabolism in wild-type and *Atrgs1*-null seedlings. Direct comparison of the expression profiles for this set of ca. 2000 genes in 100 mM glucose revealed small differences between the two genotypes (Supplementary material S2). Ten genes were identified that showed a marked attenuation of the response to glucose in *Atrgs1*-null seedlings (highlighted in Supplementary data (S1)). The specific transcript levels often of these are shown in Supplementary material S3. These include five that encode putative myrosinase-binding proteins (At1g52000, At1g52040, At1g54020, At2g39330, and At5g48850), a predicted receptor kinase (At1g35710), a MYB transcription factor (*At1g56650*), a trehalose 6-P phosphatase (*At1g78090*) and two proteins of unknown function (At4g01080 and At5g48850).

Of the transcripts identified to be differentially regulated in wild-type and *Atrgs1*-null seedlings, *At4g01080* showed the strongest increase in wild-type plants in response to 100 mM D-glucose treatment, little response in *Atrgs1*-null plants and no response in either line to treatment with mannitol (Fig. S3), and was thus selected on the basis of these characteristics as a candidate for a marker of AtRGS1-mediated sugar sensing. The *At4g01080* gene encodes a 442 amino acid protein of unknown function with a predicted molecular weight of 50687.3. The *At4g01080* gene product is predicted by TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0), DAS-TMfilter (http://www.enzim.hu/DAS/DAS.html) and SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/) to contain a transmembrane domain (residues 51–73) with a cytoplasmic N-terminus, and is predicted by Plant-PLoc (http://chou.med.harvard.edu/bioinf/plant/) and PSORT (http://psort.hgc.jp/) to localize to the plasma membrane or extracellularly [48–55]. The At4g01080 protein contains an InterPro DUF231 domain (residues 256–430), as well as domains similar to those found in leaf senescence-related proteins from *Arabidopsis* and rice [56,57].

3.2. D-glucose induction of At4g1080 is time, dose, and AtRGS1 dependent

At4g01080 displayed a robust differential response to D-glucose between wild-type and *Atrgs1*-null plants (Fig. 1A and B), with little or no response to treatment with mannitol (Fig. 1B), confirming findings from the gene chip analysis. Above 10 mM glucose, the *At4g01080* transcript level increased dramatically in wild-type plants, with the greatest increase over baseline levels observed in plants treated with 300 mM D-glucose (25.4-fold increase vs. control plants). Much smaller increases in *At4g01080* transcript levels were observed in *Atrgs1*-null plants, where a roughly 6-fold increase was observed relative to untreated plants following incubation with 300 mM D-glucose. Three hundred millimolar glucose is commonly used in experiments linking altered sugar sensitivity to genotypes [28,31,32,40,58,59].

At4g01080 transcript levels in wild-type plants increased substantially with incubations of 3 h or longer. In *Atrgs1*-null plants, a 3-h incubation with 300 mM D-glucose resulted in little change in *At4g01080* mRNA levels above baseline; however, after 4 h *At4g01080* gene expression increased over the baseline levels in these plants (Fig. 1B). The *At*RGS1-independent increase in *At4g01080* mRNA levels seen in *Atrgs1*-null plants at later time points is D-glucose-mediated, because treatment of wild-type plants with 300 mM mannitol as an osmotic control resulted in little increase in transcript level (Fig. 1B).

At4g01080 is not a primary response gene because sugar induction of *At4g01080* has a 2–3 h lag period (Fig. 1B) and because treatment with cycloheximide blocked D-glucose-mediated *At4g01080* transcript level increase (Fig. 1D). This is consistent with findings from Price et al. [60] demonstrating that gene induction by glucose requires protein translation on a global scale, while glucose gene repression is largely translation-independent.

3.3. AtRGS 1-mediated At4g01080 transcription is sugar selective

Wild-type and *Atrgs1*-null seedlings displayed differential growth sensitivities to high concentrations of various sugars and sugar analogues that is dependent in part upon whether these molecules are able to be transported into plant cells, phosphorylated by hexokinases (HXKs) or metabolized [28]. In wild-type plants, monosaccharides (D-glucose, D-fructose) or a disaccharide (sucrose), which are transportable, metabolizable and phosphorylatable by HXKs, induced At4g01080 transcription (Fig. 1C).

3.4. AtRGS1 regulates At4g01080 transcription in a dose-dependent manner

To better understand the signaling pathway involved in the *At*RGS1-mediated transcriptional response to glucose, real-time PCR experiments were used to investigate the D-glucose-induced increase in *At4g01080* transcript level in a number of G protein- and sugar-signaling-specific mutant genetic backgrounds. In agreement with the results from our gene chip analysis and from dose-response real-time-PCR experiments using wild-type and *Atrgs1-2* plants, the induction of *At4g01080* transcript level was significantly decreased in *Atrgs1-2* seedlings relative to wild-type (Fig. 1D; 6.0-fold induction for *Atrgs1-2* vs. 25.4-fold induction for wild-type, $P < 0.0001$). In *Atrgs1-2* plants over-expressing an AtRGS1-GFP construct (driven by a 35S cauliflower mosaic virus promoter and previously shown to rescue the *Atrgs1-2* phenotype), *At4g01080* transcript level was significantly increased relative to wild-type (54.9-fold induction, $P = 0.0005$), suggesting a dose–response effect for *At4g01080* transcriptional or post-transcriptional regulation that is dependent upon the level of *At*RGS1 protein expression.

3.5. At4g01080 transcript level regulation requires AtRGS1–AtGPA1 interaction, but does not require intrinsic GTPase activity

Since the best-described role for *At*RGS1 is its function as a GAP for *At*GPA1, we determined *At4g01080* transcriptional levels following glucose treatment in *Atrgs1-2* plants over-expressing an *At*RGS1–GFP or *At*RGS1 construct in which the *At*RGS1 protein contains a mutation known to eliminate *At*RGS1 GAP activity [36] and to eliminate interaction between *At*RGS1 and *At*GPA1 (Fig. 1D, *At*RGS1–E320K). The D-glucose-induced increase in At4g01080 transcript level was significantly decreased in these plants relative to wild-type (9.2-fold induction, $P = 0.0002$), suggesting a requirement for

*At*RGS1 GAP activity and/or an interaction of *At*RGS1 with the Arabidopsis Gα subunit. In *Atgpa1*-null *Arabidopsis* seedlings (*At*gpa1-4), the increase in At4g01080 transcript level was significantly less than in wild-type (9.7-fold induction, $P = 0.0057$); over-expression of *At*RGS1 was unable to rescue this decrease in *At4g01080* induction in *At*gpa1-2 seedlings (9.6-fold induction, $P = 0.0065$). In *At*gpa1-4 plants over-expressing a constitutively active form of *At*GPA1 (*At*GPA1–Q222L), At4g01080 transcript level was significantly increased over the levels observed in *At*gpa1-4 plants ($P = 0.0037$) and similar to levels observed in wild-type plants (26.8-fold induction, $P = 0.7787$); thus, over-expression of *At*GPA1–Q222L rescues the *At*gpa1-null phenotype in our assay, consistent with previous findings that examined root growth phenotypes following chronic D-glucose exposure in these genotypes [32]. However, the *At*GPA1–Q222L mutant lacks the intrinsic GTPase activity, leading to the conclusion that, while an interaction between *At*RGS1 and *At*GPA1 is critical for the At4g01080 transcript level increase, the intrinsic GAP activity *At*RGS1, per se, is not critical for glucose induction of *At4g0 1080* via *At*GPA1. It does not preclude a role for the GAP function by *At*RGS1 at later times in this signaling pathway.

To test this further we examined in vivo interaction between *At*RGS1 and *At*GPA1 using bimolecular fluorescence complementation (BiFC) [61]. As shown in Fig. 2, *At*RGS1 and *At*GPA1-split YFP tagged proteins complement to reconstitute a fluorescent YFP. Interestingly, a tagged *At*RGS1 (E320K) mutant also interacts with *At*GPA1 using BiFC. Since this mutation has been shown to disrupt the interaction between *At*GPA1 and the C-terminal RGS-box-containing-domain of *At*RGS1, we conclude that the interaction occurs through the 7TM domain. It should be noted that BiFC is not a quantitative measure of interaction and that weak or strong transient interactions can drive stably-reconstituted YFP molecules [61].

3.6. The AtGPA1 interactor THF1 is involved indirectly in At4g01080 transcriptional regulation

RGS proteins attenuate Gα signaling via their GAP activities, but also can act as scaffolding proteins to bring together various components of a G-protein signaling complex [62,63]. Our earlier finding that *At*GPA1 has rapid nucleotide exchange and is by default active at steady state [36] suggests an alternate form of regulation for the protein in *Arabidopsis*, possibly through selective localization via interaction with scaffolds such as the 7TM domain of *At*RGS1. Furthermore, treatment with high concentrations of D-glucose promotes a transient change in conformation between *At*GPA1 and *At*RGS1, leading to increased FRET efficiency between fluorescently-labeled versions of these proteins [36]. A stable interaction between *At*GPA1 and *At*RGS1 via a scaffolding-like association where signaling is briefly allowed to proceed between the active Gα and its effector is consistent with the requirement of both *At*RGS1 and *At*GPA1 for glucose-enhanced *At4g01080* transcript levels. The observed glucose-induced change in conformation between *At*RGS1 and ATGPA1 is transient but this does not preclude the possibility that these two proteins are stably associated. We speculate that *At*RGS1 would first promote signaling through a prebound

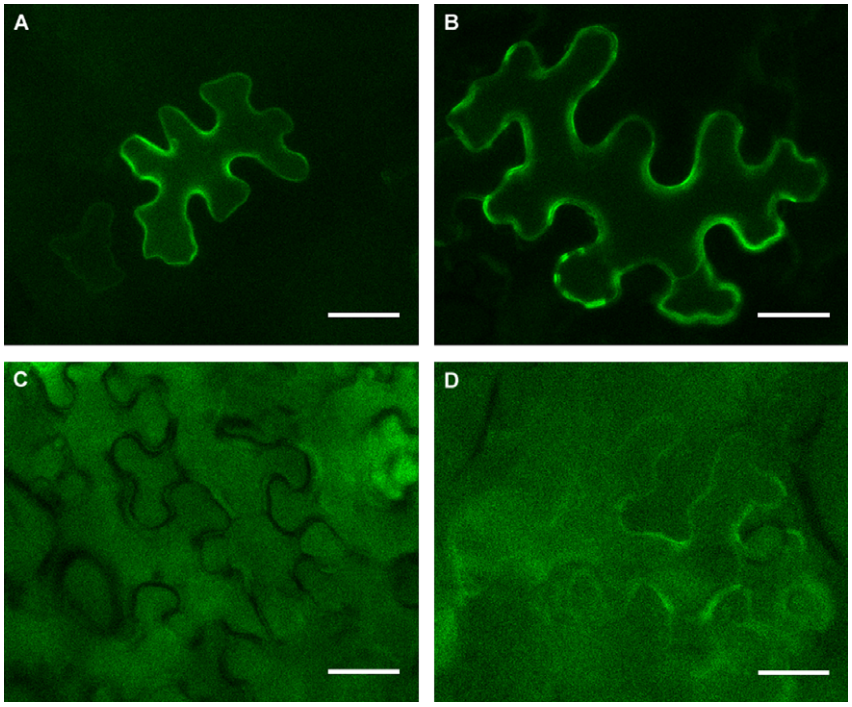

Fig. 2. Interaction between *At*RGS1 and *At*GPA1 through the 7-transmembrane (7TM) domain. *Agrobacteria* harboring RGS1-sYFP-N and GPA1-sYFP-C (A) and RGS1(E320K)-sYFP-N and GPA1-sYFP-C (B) were co-infiltrated into *Nicotiana benthamiana* leaves. Because it was previously shown that the E320K mutation in *At*RGS1 disrupts interaction between the C-terminal-located RGS box and *At*GPA1 [36], the in vivo interaction between *At*GPA1 and *At*RGS1 (E320K) shown here is likely occurring through the 7TM domain. The cell membrane proteins PIP2A-C-YFP (C) and p31-C-YFP (D) together with *At*RGS1-N-YFP were used as negative controls to monitor spontaneous re-association between N and C terminal halves of YFP. Because no fluorescence was visible, the images in C and D were taken at higher gain setting of camera than for A and B in order to visualize the cell outline. Bar = 30 μm.

*At*GPA1 by facilitating the association of *At*GPA1 with downstream partners. *At*RGS1 would also deactivate *At*GPA1 subunit by acting as a GAP protein to promote *At*GPA1 GTP hydrolysis. The inactive *At*GPA1 might remain associated with *At*RGS1 during long-term treatments with glucose, albeit in a conformation that is not conducive to FRET [16], or that the scaffold-like docking site on *At*RGS1 is only transiently available to *At*GPA1 or its effector following glucose treatment. This would explain the opposing sugar sensitivity phenotypes displayed by *Atrgs1*-null and *At*gpa1-null plants under conditions of chronic glucose exposure.

*At*GPA1 associates with at least one other protein with a predicted scaffolding role, the plastid membrane protein THF1, which is itself regulated by D-glucose levels [32]. THF1 interacts with AtGPA1 in a nucleotide-independent manner at sites were plastids abut the plasma membrane. *thf1-null* mutants display variegated leaves and are hypersensitive to chronic exposure of glucose, while THF1-overexpressing plants are resistant to glucose [32,64]. Furthermore, THF1 protein levels are regulated by glucose, with high glucose concentrations leading to a proteasome-dependent degradation of the protein in roots [32]. The results observed here are consistent with the structure of *At*RGS1 acting as a scaffold to facilitate an interaction between effector proteins and *At*GPA1, perhaps newly released from its interaction with THF1 following D-glucose-mediated degradation of that protein. Equally plausibly, THF1 could be part of a glucose-mediated signaling complex with *At*GPA1, aiding in or prolonging a transient scaffold-like association between *At*GPA1 with *At*RGS1 before THF1 is degraded. The hypersensitivity to chronic glucose treatment of both *Atgpa1-null* and *thf1-null* mutant plants would support the idea that THF1 acts to promote or prolong *At*GPA1 signaling.

To distinguish among these possibilities for the involvement of THF1 in *At*RGS1-mediated glucose sensing, *At4g01080* mRNA levels were assessed in *thf1-null* mutant plants before and after glucose treatment. In *thf1-1* seedlings, there was a trend towards slightly higher basal level of *At4g01080* mRNA compared to wild-type, but the difference was not significant (1.2-fold increase in *thf1-1* vs. wild-type, $P = 0.3827$), suggesting that any increase in the pool of *At*GPA1 available to interact with *At*RGS1 resulting from deletion of *THF1* had only a minor impact upon At4g01080 steady-state transcript levels under sugar-starved conditions. Increased availability of *At*GPA1 to interact with *At*RGS1 through sugar-mediated degradation of THF1, therefore, does not appear to be the primary mode of regulation for this glucose signaling pathway.

Instead, we speculate that THF1 may stabilize *At*GPA1 interactions. Following glucose treatment of *thf1-1* seedlings, the increase in *At4g01080* transcription was less relative to wild-type, although the difference was again not considered significant by our criterion (Fig. 1D; 20.1-fold induction, $P = 0.3023$). However, in plants over-expressing a THF1–GFP construct, *At4g01080*mRNA levels were found to be significantly increased over wild-type levels following glucose treatment (43.6-fold induction, $P = 0.0035$). Thus, while not being absolutely required for glucose-mediated transcriptional regulation of *At4g01080*, THF1 does appear to play a role in this process. With regard to the scaffolding model for *At*RGS1 proposed above, the minor decrease in *At4g01080* mRNA levels in *thf1-1* seedlings compared to wild-type following glucose treatment argues against THF1 recruitment of *At*GPA1. The effects seen in THF1–YFP-overexpressing plants would instead suggest that THF1 promotes *At*GPA1 signaling by enhancing a scaffold-like interaction between the active *At*GPA1 and *At*RGS1, either by inhibiting *At*GPA1 deactivation via *At*RGS1 GAP activity or by prolonging the availability of the docking site for *At*GPA1 or its effector following glucose treatment.

3.7. The D-glucose increase in At4g01080 steady-state transcript level requires AGB1

Another aspect of the mechanism of *At4g01080* transcript up-regulation is that the process may require either the formation of a Gαβγ heterotrimer or a close association of an active Gα with the Gβγ dimer via a mechanism facilitated by *At*RGS1. Based upon the in vitro rate constants observed for *At*GPA1, which suggest that GTP hydrolysis rather than GDP/GTP exchange is the rate limiting step in the cycling between the active and inactive forms of the protein, it is predicted that more than 99% of *At*GPA1 molecules would be present in the active form under steady state conditions [36]. Under such conditions, and in contrast to the case in metazoan systems, negative regulation of *At*GPA1 through the GAP activity of *At*RGS1 may be required to allow for the formation of appreciable amounts of αβγ heterotrimer. Therefore, in mutant *Arabidopsis* plants lacking *At*RGS1 or *At*GPA1, heterotrimer formation would not occur; thus, if normal regulation of *At4g01080* transcripts requires the activity of the αβγ heterotrimer, a similar phenotype might be expected in both *Atrgs1*- and *At*gpa1-null backgrounds. If either αβγ heterotrimer formation or the recruitment of Gα and the Gβγ dimer by *At*RGS1 is required for regulation of *At4g01080* gene expression, AGB1 expression would be necessary for this regulation, and *agb1-null* plants should display a phenotype for *At4g0*

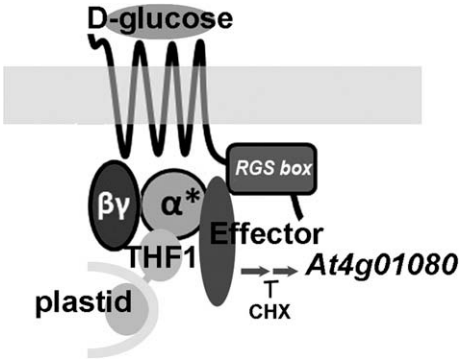

Fig. 3. A proposed physical model for a *At*RGS1-G-protein sugar sensor based on the genetic data of Fig. 1D. *At*RGS1 is indicated by the 7-transmembrane protein containing the regulator of G signaling (RGS) motif in its carboxy-terminal cytoplasmic domain. The heterotrimeric G protein complex is associated with *At*RGS1 and is represented by its G alpha (α) subunit and the G beta (β) and G gamma (γ) dimer. The activated form of a (α*) is known to be part of the heterotrimeric complex. A physical association between α and *At*RGS1, between α and βγ, and between α* and THF1 have been shown previously. THF1 is a protein of the outer membrane of root cell plastids. The interaction interface has been mapped to the globular cytoplasmic domain on THF1. The effector has yet to be identified but is added here assuming that the effect of glucose activation of α* on gene transcription is not direct. CHX, cyclohexamide; *At4g01080* encodes a plasma membrane protein of unknown function and the steady state level of its mRNA is used here as a rapid reporter of *At*RGS1-mediated sugar sensing.

1080 induction similar to that seen in the *Atrgs1-2* and *Atgpa1-4* mutants. In *agb1-2* seedlings, the increase in *At4g01080* transcript levels was attenuated relative to wild-type (Fig. 1D; 5.4-fold induction, $P = 0.0048$). A similar phenotype was seen in *Atgpa1-4, agb1-2* double mutant plants (11.9-fold induction, $P = 0.0035$).

Taken together, these results demonstrate the involvement of AGB1 in glucose-mediated *At4g01080* transcript level control, and lend support to the idea of signaling through the αβγ heterotrimer or through the combined signaling of Gα and the Gβγ dimer. The presence of a robust up-regulation of *At4g01080* levels in plants with the constitutively active *At*GPA1–Q222L mutant is still consistent with signaling through the heterotrimer as it has been shown that *At*GPA1–Q222L remains a part of the heterotrimeric complex [65]. If signaling through both Gα and Gβ are important for glucose-induced *At4g01080* gene induction while Gα GTPase is not necessary (as demonstrated by *At*GPA1–Q222L), the critical role for *At*RGS1 would again seem to be that of a networking protein, facilitating the interaction of Gα and the Gβγ dimer or, in the case that the Gαβγ heterotrimer does not dissociate, enhancing that interaction between the G protein heterotrimer and its downstream effector(s). In conclusion, the work here enables the assembly of some of the components of a novel glucose sensing complex at the plasma membrane (Fig. 3).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.08.038.

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