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ORIGINAL ARTICLE

The *Arabidopsis thaliana* Adenosine 5'-Phosphosulfate Reductase 2 (AtAPR2) Participates in Flowering Time and Glucose Response

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Abstract Sugars such as sucrose or glucose function both as building materials for biosynthesis, and as signaling molecules that modulate gene expression. Compared to studies of sugar signaling in bacteria, yeast and animals, knowledge of the signaling pathways in plants is still poorly understood. Here, we investigated the effect of the disruption and overexpression of an *Arabidopsis thaliana adenosine 5'-phosphosulfate reductase 2*, *AtAPR2*, on plant responses to glucose stresses. AtAPR2 encodes an enzyme of the sulfate assimilation pathway and it is a member of a three gene family that also includes *AtAPR1* and *AtAPR3*. Expression of *AtAPR1*, *AtAPR2* and *AtAPR3* were strongly induced by glucose treatment. Overexpression of *AtAPR2* resulted in enhanced cotyledon greening and fresh weight increase when plants were treated with high glucose. By contrast, a T-DNA insertion mutant (*atapr2-2*) line showed delayed greening and fresh weight growth inhibition in response to glucose and also the non-metabolizable analog 2-deoxyglucose. The expression of three glucose responsive genes, *Hexokinase 1* (*HXK1*), *Phenylalanine ammonia lyase* 1 (*PAL1*) and *Pathogenesis related gene 5* (*PR5*), was elevated in *AtAPR2*-overexpressing and WT plants in response to glucose treatment, but in the *atapr2-2* mutant line the transcript level for these genes decreased. Furthermore, *AtAPR2*-overexpressing plants displayed delayed flowering under long day condition. The data implicates AtAPR2 as a component controlling flowering time and glucose response in *Arabidopsis thaliana*, although the exact function of AtAPR2 is not clear.

Key words: Adenosine 5'-phosphosulfate reductase, 2-Deoxyglucose, Flowering time, Glucose

Introduction

Sugar signal transduction cascades are important components of regulatory networks in cells. However, such signaling pathways are poorly understood in plants compared with the situation in bacteria, yeast and animals. Nonetheless, changes in absolute sugar levels, sugar flux or sugar to nitrogen ratios have profound effects on plant metabolism, growth, development and stress response. In general, low sugar levels promote photosynthesis and mobilization of storage polysaccharides, whereas high sugar levels stimulate carbon import, storage and utilization (Koch 1996). Sugars modulate various developmental processes throughout the entire life cycle of plants, including seed development and germination, seedling and vegetative growth, floral induction, senescence and responses to environmental stimuli (Gibson 2005; Rolland et al. 2006). A number of genes involved in carbon and nitrogen metabolic processes have been shown to be regulated either by sugar excess or starvation conditions (Graham 1996; Koch 1996; Fujiki et al. 2000). Hesse et al. have reported that regulation of sulfate assimilation is regulated by sugar signaling likely for co-ordination of sulfate assimilation with nitrate and carbon assimilation (Hesse et al. 2003).

Plants assimilate sulfate for biosynthesis of many sulfur-containing compounds including cysteine, methionine, glutathionine and secondary metabolites such as glucosinolates (Leustek et al. 2000; Tsakraklides et al. 2002; Saito 2004; Zhang et al. 2014). Sulfur starvation is distinctly related with a decreased pathogen resistance of plants (Rausch and Wachter 2005; Kruse et al. 2007), thus, its deficiency leads

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to reduced growth and development, and decreased crop yield and quality. Therefore, uptake and assimilation of sulfur is necessary for cellular metabolism, plant growth and development, and response to various biotic and abiotic stresses (Leustek et al. 2000; Saito 2004; Rausch and Wachter 2005). Plants initiate the assimilation process by activating sulfate to adenosine 5'-phosphosulfate (APS) catalyzed by ATP sulfurylase. Adenosine 5'-phosphosulfate reductase (APR) catalyzes the reduction of APS to sulfite in a glutathione dependent reaction. Sulfide is formed from sulfite by ferredoxin-dependent sulfite reductase. *O*-acetylserine (OAS) thiol-lyase catalyzes the reaction of sulfide and OAS to form cysteine. OAS is produced by serine acetyltransferase (Brunold 1990; Leustek et al. 2000; Kopriva et al. 2002). Cysteine can directly incorporated into protein or further be metabolized into methionine or glutathione, a tripeptide with important functions in oxidative stress defense, regulation of sulfur assimilation, etc. (Noctor et al. 1998). Thus, cysteine synthesis is a central point of cellular metabolism as this reaction interconnects sulfate, nitrate, and carbon assimilation (Kopriva et al. 2002).

APS reductases play a key role in the reductive sulfate assimilation pathway of plants. This enzyme is composed of two domains, an amino terminal reductase domain and a C-terminal glutaredoxin (GRX)-like domain that serves as the entry point for electrons from glutathione. The N-terminal domain resembles bacterial APS reductases that, unlike the plant type enzymes, use reduced thioredoxin (TRX), rather than glutathione, as the electron donor for APS reduction (Lillig et al. 1999; Bick et al. 2000; Kim et al. 2005; Setya et al. 1996). It is clear that the N-terminal domains of all plant APS reductases, like the smaller bacterial APS reductases, contain a single [4Fe-4S] cluster as the sole prosthetic group (Weber et al. 2001; Kim et al. 2006). Thus, the APS reductases differ from *Escherichia coli* 3'-phosphoadenosine 5'-phosphosulfate (PAPS) reductase, which does not contain any prosthetic groups (Schwenn et al. 1988; Brenedt et al. 1995; Savage et al. 1997; Chartron et al. 2007). Recently evidence was obtained that the [4Fe-4S] cluster found in APS reductases does not serve as an electron carrier and its exact function remains unknown (Kim et al. 2004; Carroll et al. 2005; Chartron et al. 2006).

In this study, we report that AtAPR2 functions as a component in the response to glucose (Glc) treatment. The overexpression of *AtAPR2* in transgenic Arabidopsis plants produced increased tolerance toward Glc during seedling growth, whereas disruption in the expression of *AtAPR2* induced hypersensitivity to Glc. Additionally, the *AtAPR2* T-

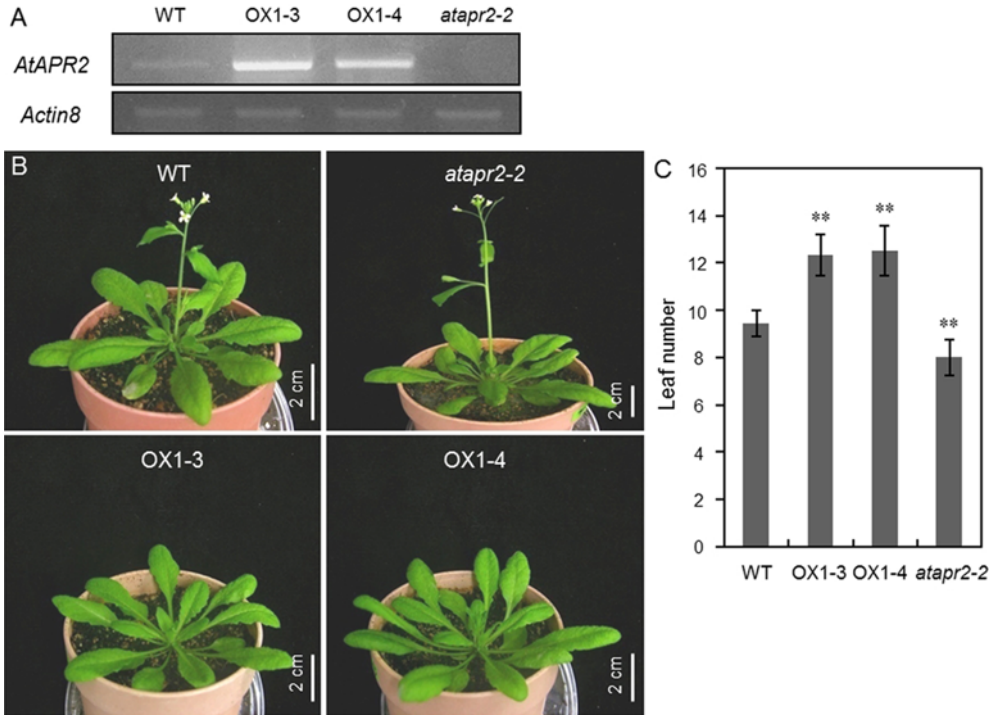

Fig. 1. Phenotypes of *AtAPR2*-overexpression plants. (A) Expression levels of *AtAPR2* in wild-type (WT), *atapr2-2* mutant and two independent transgenic lines overexpressing *AtAPR2* (OX1-3 and OX1-4) were determined by RT-PCR using total RNA isolated from 2-week-old seedlings. *Actin8* was used as an internal control in RT-PCR. (B) Delayed flowering in *AtAPR2*-overexpression plants (OX1-3 and OX1-4) and early flowering in *atapr2-2* mutants grown under a 16-h/8-h light/dark photoperiod. (C) *AtAPR2*-overexpression lines developed more leaves before flowering compared with WT plants; *atapr2-2* mutant flowered earlier with fewer leaves compared with WT plants. The data are means ± SE (n = 12 each) from three independent experiments. Differences in the leaf number of Arabidopsis plants are significant at the $P < 0.01$ (**) level.

DNA mutant (*atapr2-2*) plants showed altered flowering time. These results indicate that AtAPR2 is capable of modulating the Glc response and flowering time in Arabidopsis.

Results

Altered Flowering Timing in Mutant *atapr2-2* and *AtAPR2*-Overexpression Plants

In an effort to investigate the in vivo functions of AtAPR2, *AtAPR2* was overexpressed in Arabidopsis under the control of the 35S promoter. Using kanamycin resistance segregation and reverse transcription (RT)-PCR analysis (Fig. 1A), we selected plants homozygous for the 35S-*AtAPR2* T-DNA (hereafter referred to as OX1-3 and OX1-4). In an effort to further evaluate the function of AtAPR2 *in* Arabidopsis, an At1g62180-tagged T-DNA insertion mutant CS851804 was analyzed. The T-DNA inserted in exon 4 of the At1g62180 gene was verified via PCR and the cloning of the left T-DNA border (Fig. S1). Once homozygosity had been established, the absence of *AtAPR2* was verified via RT-PCR (Fig. 1A). The respective mutant was designated as *atapr2-2*.

The *AtAPR2*-overexpression plants showed a delayed flowering phenotype compared to the wild-type (WT) parental plants and *atapr2-2* plants. To observe this phenotype the plants were grown under long-day (16-h-light/8-h-dark) photoperiod (Fig. 1B). Additionally, the *AtAPR2*-overexpression plants developed on average about three or five more leaves than WT or *atapr2-2* mutant, respectively, at flowering (Fig. 1C). In contrast, under the same growth conditions the *atapr2-2* mutant produced an average of two leaves fewer than the WT (Fig. 1C). Aside from altered flowering time no other differences in growth or development were observed between WT, *atapr2-2* and *AtAPR2*-overexpressing plants. No differences in seed germination were observed on Murashige and Skoog (MS) medium between WT, *atapr2-2* and *AtAPR2*-overexpressing plants. These observations reveal that AtAPR2 function influences flowering time.

Expression of Three Arabidopsis *AtAPR* Homologous Genes After Glc Treatment

It has been relatively well established that flowering time is altered by Glc treatment (Funck et al. 2012). To determine how Glc treatment effects *AtAPR* expression level of mRNA for three different *AtAPR* homologous genes was measured in 14-d-old Arabidopsis seedlings threated with Glc treatment using quantitative real-time (qPCR). Fig. 2 shows that time-course transcript levels of *AtAPR1*, *AtAPR2* and *AtAPR3*, reached a peak within 6 h after Glc treatment then slightly decreased until 12 hours. The expression patterns of *AtAPR1*, *AtAPR2* and *AtAPR3* in Glc-treated Arabidopsis seedlings similar. These observations suggest that all three Arabidopsis *AtAPR* genes are Glc responsive.

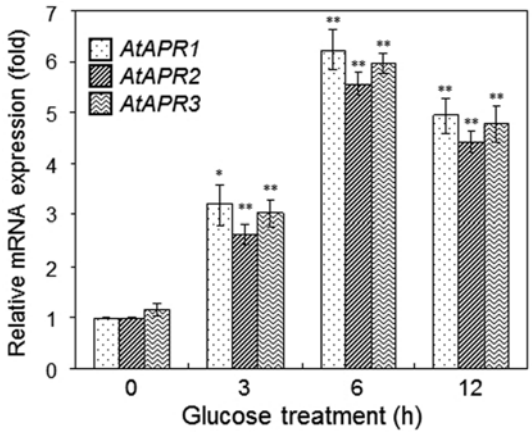

Fig. 2. Expression of the *AtAPR* isoforms in Arabidopsis under Glc stress. qPCR analysis showing the induction kinetics of *AtAPRs* in plants treated with Glc stress. Two-week-old Arabidopsis seedlings were exposed to 6% Glc, for 0-12 h. Error bars indicate standard deviations of three independent biological samples. Differences between the expression of *AtAPR1*, *AtAPR2* or *AtAPR3* in 14-d-old Arabidopsis seedlings untreated and treated with Glc stress are significant at the $0.05 > P > 0.01$ (*) or the $P < 0.01$ (**) levels.

Arabidopsis Plants Overexpressing AtAPR2 Show Increased Insensitivity to Glc and 2-deoxyglucose (2-DG)

Given the delayed flowering phenotype of *AtAPR2* overexpressing plants and the Glc responsiveness of *AtAPR2* expression, the Glc-response phenotype of *AtAPR2* overexpressing plants was assessed. WT Arabidopsis show a Glc response that is characterized by an inhibition of cotyledon greening. When germinated on MS medium lacking Glc WT, *atapr2-2*, and *AtAPR2*-overexpressing (OX1-3, OX1-4) showed similar germination rate and similar morphology (Fig. 3A). However, on MS supplemented with 6% (w/v) Glc the cotyledon greening rate of WT was only slightly above 45% 7 d after germination. By contrast, less than 24% of the *atapr2-2* mutant cotyledons expanded and turned green, but 73%-78% of the OX1-3 and OX1-4 expanded and produced chlorophyll (Figs. 3B and 3C).

After further growth for 14 d on Glc-supplemented medium the fresh weight of *atapr2-2* reached only 89% of that of WT, whereas the fresh weight of the *AtAPR2*-overexpressing plants reached to 114%-122% compared to WT (Fig. 3D). These results demonstrated that the *atapr2-2* mutant is more sensitive to Glc treatment than WT. However, the *AtAPR2*-overexpressing plants were less sensitive to Glc than WT and the *atapr2-2* mutant.

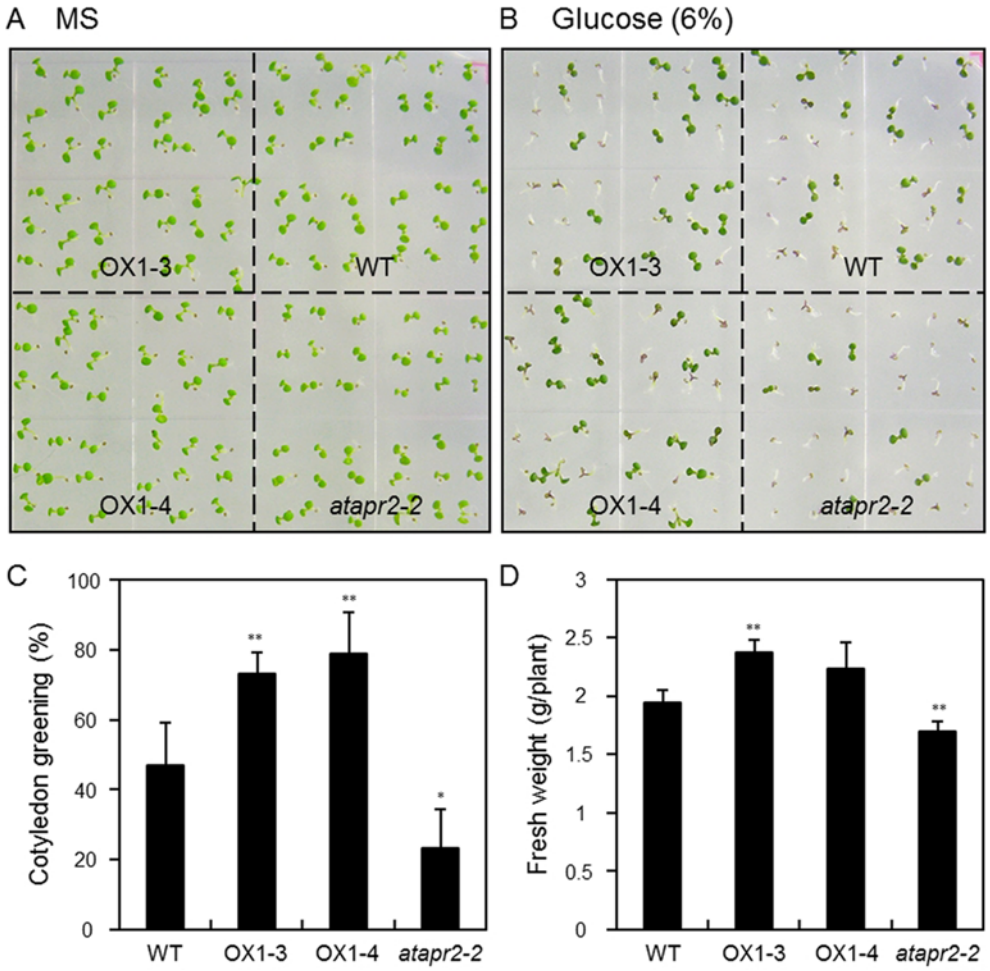

Fig. 3. Glc sensitivity of *atapr2-2* mutant and *AtAPR2*-overexpression transgenic plants. (A and B) Seeds were sown on MS agar plates supplemented (B) or not with 6% Glc (A) and allowed to grow for 7 d. The photograph shows that *AtAPR2*-overexpression transgenic lines (OX1-3 and OX1-4) show better development and greener than WT and *atapr2-2* mutant under Glc stress conditions. (C) Effect of Glc treatment on cotyledon greening. Seeds were sown on MS agar plates with 6% Glc and permitted to grow for 7 d, and seedlings with green cotyledons were counted (triplicates, n = 50 each). Error bars represent standard deviations. Differences among the WT, *atapr2-2*, and two *AtAPR2*-overexpressing (OX1-3 and OX1-4) plants grown in the same conditions are significant at the $0.05 > P > 0.01$ (*) or the $P < 0.01$ (**) levels. (D) Effect of Glc treatment on plant fresh weight. Seeds were germinated on MS medium containing 6% Glc for 14 d, and measured for the fresh weight (triplicates, n = 30 each). Error bars represent standard deviations. Differences among the WT, *atapr2-2*, and two *AtAPR2*-overexpressing (OX1-3 and OX1-4) plants grown in the same conditions are significant at the $P < 0.01$ (**) levels.

One possibility for the observed phenotype on Glc is that the plants show symptoms of osmotic stress, compared with a direct effect of Glc on growth. This possibility was examined by comparing plant response to mannitol, 3-*O*-methylglucose (3-OMG) and 2-DG in control experiments. Mannitol is a non-metabolizable osmoticum. 3-OMG and 2-DG are none metabolizable Glc analogs. No apparent difference was observed among the WT, *atapr2-2* and *AtAPR2*-overexpressing plants when they were germinated on 400 mM mannitol (Fig. 4C) or 25 mM 3-OMG (Fig. 4C), a Glc analog that is not phosphorylated by hexokinase, the first step in the utilization of glucose in glycolysis. However, the cotyledon greening rate of *atapr2-2* mutant was much more affected than that of WT and *AtAPR2*-overexpressing plants by treatment with low concentrations of 2-DG (Figs. 4B and 4C), a Glc analog that can be phosphorylated by hexokinase. As in the Glc assay, the cotyledon greening percentage of *AtAPR2*-overexpressing plants was higher than that of WT (Fig. 4). Conversely, AtAPR2-mediated suppression of the cotyledon greening inhibition of HXK1 phosphorylation-related signal in overexpressing plants might be responsible, at least in part, for the insensitivity to Glc that is observed in these plants.

To obtain different hexose information on how fructose (Frc) or mannose (Man) treatments act on transgenic cotyledon greening, the seeds of the WT, *atapr2-2* and *AtAPR2*-overexpressing plants were germinated in MS media supplemented with 6% Frc or 0.1% Man, and then permitted to grow

for 7 d. As shown in Supplementary Fig. S2, Frc or Man treatments resulted in no significant differences in the cotyledon greening rate after 7 d among WT, *atapr2-2* and *AtAPR2*-overexpressing plants. These results are consistent with the suggestion that AtAPR2 is a necessary component for the Glc -triggered developmental leaf growth process.

Effects of Glc on Stress-related Genes

It has been relatively well-established that the expressions of the *HXK1*, *PR5* and *PAL1* genes are induced by Glc treatment (Xiao et al. 2000; Nambara and Marion-Poll 2005). Fig. 5 revealed that the transcript levels of Glc-inducible genes including *HXK1*, *PR5* and *PAL1* were enhanced following induction in *AtAPR2*-overexpressing and WT plants following Glc treatment, rather than in *atapr2-2* mutant plants. The expressions of the *HXK1*, *PR5* and *PAL1* were slightly less reduced in the *atapr2-2* mutant compare with the WT and *AtAPR2*-overexpressing plants. Furthermore, the transcript levels of these three Glc-inducible genes were significantly induced under the control (H_2O) condition in *atapr2-2* mutant plant. While, the transcript levels of *Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit 1* (*RBCS1*), which is known to be down-regulated during Glc treatment (Krapp et al. 1993), was not significantly different between the control (H_2O) and Glc treatment in WT, *atapr2-2* mutant and *AtAPR2*-overexpressing plants. These observations support the notion

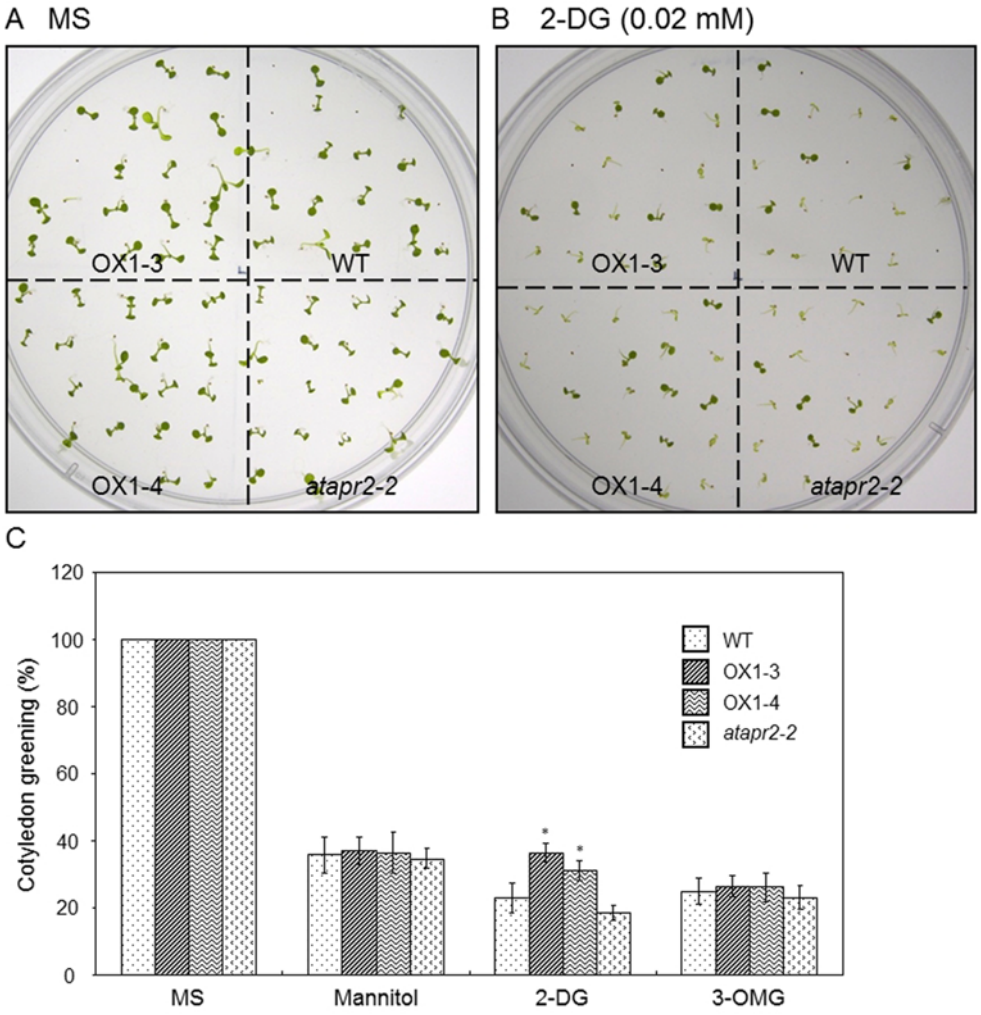

Fig. 4. Glc analogs sensitivity of *atapr2-2* mutant and *AtAPR2*-overexpressing transgenic seedlings. (A and B) Seeds were sown on MS agar plates supplemented (B) or not with 0.02 mM 2-deoxyglucose (2-DG) (A) and allowed to grow for 7 d. The photograph shows that *AtAPR2*-overexpression transgenic lines (OX1-3 and OX1-4) show better development and greener than WT and *atapr2-2* mutant plants under Glc stress conditions. (C) Effect of Glc analogs treatment on cotyledon greening. Seeds were sown on MS agar plates with 400 mM mannitol, 0.02 mM 2-DG or 20 mM 3-*O*-methylglucose (3-OMG) and permitted to grow for 7 d, and seedlings with green cotyledons were counted (triplicates, n = 50 each). Error bars represent standard deviations. Differences among the WT, *atapr2-2*, and two *AtAPR2*-overexpressing (OX1-3 and OX1-4) plants grown in the same conditions are significant at the $0.05 > P > 0.01$ (*) level.

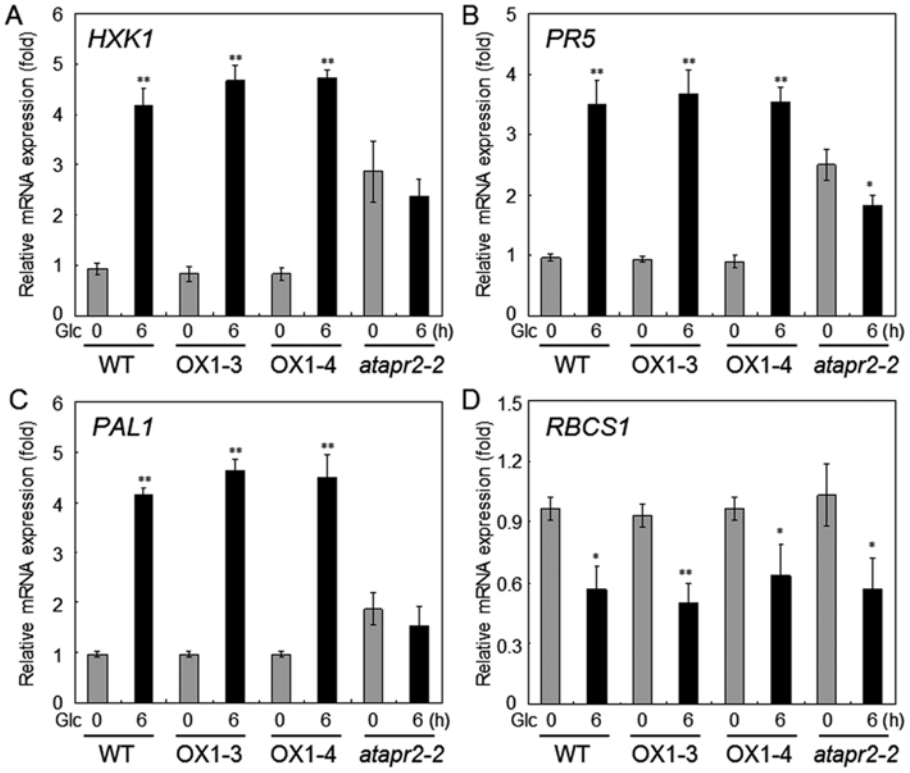

Fig. 5. Expression of sugar-regulated genes in *atapr2-2* mutant and *AtAPR2*-overexpression transgenic plants. mRNA levels were determined by qPCR using total RNA from 10-d-old seedlings, which were exposed in 6% Glc with gentle shaking for indicated time. *Actin8* was used as an internal control in qPCR. Differences between the expression of *HXK1* (A), *PR5* (B), *PAL1* (C) or *RBCS1* (D) in Arabidopsis seedlings untreated and treated with Glc stress are significant at the $0.05 > P > 0.01$ (*) or the $P < 0.01$ (**) levels.

that *AtAPR2* regulates the expression of these Glc marker genes under Glc condition. However, the expression levels of *HXK1*, *PR5* and *PAL1* in WT and *AtAPR2*-overexpressing plants seems to be very similar under Glc condition. This likely indicates that the overexpression of *AtAPR2* by itself is not sufficient for the induction of Glc-related genes, and may need additional components.

Discussion

APR catalyses a highly regulated step in sulfate reduction and plays an important role is control of the flux through the pathway (Mugford et al. 2011). Sulfur assimilation is one of three assimilative pathways that converge to form cysteine, the central sulfur-containing metabolite in plants. In addition to the thiol group of cysteine derived from sulfur assimilation, this compound also contains an amino group derived from nitrogen metabolism, and a carbon skeleton derived from carbon assimilation. These three pathways need to be coordinated to deliver the cysteine necessary for growth and development. In the context of the present discussion, it is especially important to note that expression of AtAPR2 is Glc-responsive. According to quantitative analysis of *AtAPR* isoforms expression during Glc treatment, *AtAPR* isoforms are up-regulated by Glc (Fig. 2). In this study, the transgenic plants overexpressing *AtAPR2* show late flowering and the *atapr2-2* T-DNA mutant show early flowering phenotype compared to its WT plants grown at a long-day condition (Fig. 1), suggesting that flowering time response in Arabidopsis involves AtAPR2.

The results of the present paper show that the *AtAPR2*-overexpressing plants were demonstrated to be more insensitivity to exogenous Glc but not mannitol than were the WT and *atapr2-2* mutant (Fig. 3). This clearly demonstrates that the carbohydrate effect is not mediated via an increase in the osmotic potential of the nutrient solution.

Glc is most likely the predominant hexose signal in gene regulation in plants and other organisms. Just like sucrose, Glc represses photosynthesis and germinative and postgerminative developmental programs in different plant systems (Smeekens 2000; Rolland et al. 2002). Glc is phosphorylated in plant cells by both unspecific hexokinases (HXKs) and Glc-specific HXKs (glucokinases). The Glc analog 2-DG and Man are transported into the plant cells and phosphorylated by HXK to 2-DG-6-phosphate (2-DG-6-P) and Man-6-phosphate (Man-6-P), respectively (Loreti et al. 2001). Two other Glc analogs, 6-deoxyglucose (6-DG) and 3-OMG, are transported

into the cells but not phosphorylated by HXK (Smeekens 2000; Loreti et al. 2001; Rolland et al. 2002). Generally, Glc-induced repression of photosynthesis and seed germination can be mimicked by 2-DG and Man, but not by 6-DG or 3-OMG, demonstrating that hexose transport as such does not suffice for gene repression, but that the sensor is intracellular and that hexose phosphorylation is essential. Frc is also phosphorylated by HXK and it is likely that this mediates signals via the same pathway as Glc. *AtAPR2*-overexpressing transgenic plants showed enhanced insensitivity to Glc in comparison to the WT, whereas the *atapr2-2* lines displayed enhanced sensitivity to Glc in cotyledon greening (Fig. 3), which implies that AtAPR2 is a component in the regulation of Glc or Glc-mediated stress response pathways in Arabidopsis. As in the Glc assay, T-DNA mutant plants were hypersensitive to low concentration of 2-DG, as shown by the inhibition of cotyledon greening (Fig. 4). The *AtAPR2*-overexpressing transgenic plants were 2-DG hyposensitive and appeared green (Fig. 4). By testing other Glc analogs, 3-OMG, Frc or Man treatments resulted in no significant differences in the cotyledon greening rate among WT, *atapr2-2* and *AtAPR2*-overexpressing plants (Figs. 4 and S2). These observations suggest that AtAPR2 is a necessary component for the Glc-triggered developmental leaf growth process.

The evidence does not clarify the exact function that AtAPR2 plays in flowering time and Glc response control, whether it is part of a signaling or response pathway or whether the phenotype results from a pleiotropic effect of changing AtAPR2 expression.

Materials and Methods

Plant Materials, Growth Conditions and Glc Induction

Arabidopsis (Col-0) plants were grown in growth chambers under intense light (120 μmol m^{-2} s^{-1}) at 22 °C, 60% relative humidity, and a 16 h d length. The *AtAPR2* T-DNA insertion line CS851804 (*atapr2-2*) was acquired from the Arabidopsis T-DNA insertion collection of the Arabidopsis Biological Resource Center (http://www.arabidopsis.org). In order to select plants homozygous for the T-DNA insertion, the gene-specific primers 5'-ATTAGGTTATCTGATCGAACCC-3' and 5'-GATGTTCCCTTTGTGTAGACC-3' (forward and reverse, respectively) were utilized for the *atapr2-2* line. Plants yielding no PCR products with the gene-specific primers were subsequently tested for the presence of the T-DNA insertion using the gene-specific forward primer in combination with the T-DNA left border specific primer 5'-AACGTCCGCAATGTGTTATTAAGTTGTC-3'. The plants were challenged with Glc via the submersion of 10-d-old Arabidopsis seedlings in a solution containing 6% Glc. Samples were obtained at 0, 3, 6 and 12 h of Glc treatment, frozen in liquid nitrogen, and stored at -80°C.

Overexpression Construct of *AtAPR2*

Total RNA was isolated from Arabidopsis leaves using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RT-PCR was utilized to obtain a full-length *AtAPR2* cDNA (At1g62180). The cDNA was then cloned into the pENTR1A vector (Invitrogen) for DNA sequence analysis. RT-PCR primers were: forward primer 5'-ACGTGGATCCAGATGGCTTTAGCTGTTACTTC-3' and reverse primer 5'-ACGTGAATTCAGTCACCGAAGAAGATTCACAAAC-3'. Amplification proceeded for 35 cycles as follows: 94°C, 30 sec; 57°C, 30 sec; 72°C, 1 min. After cloning into pENTR1A, the final construct was generated by LR-reaction with pEarleyGate100 vector (Earley et al. 2006). The resulting construct was introduced into *A. tumefaciens* strain GV3101 by *in planta* vacuum infiltration. Homozygous lines (T_3 generation) from 11 independent transformants were obtained and two lines (OX1-3 and OX1-4) evidencing high levels of transgene expression were selected for phenotypic characterization. Kanamycin resistance of the T_2 generation from these two selected lines segregated as a single locus.

Extraction of RNA and RT-PCR

Total RNA was extracted from the frozen samples using the Plant RNeasy extraction kit (Qiagen, Valencia, CA, USA). In order to remove any residual genomic DNA in the preparation, the RNA was treated with RNAse-free DNAse I in accordance with the manufacturer's instructions. The concentration of RNA was spectrophotometrically quantified and 5 μg of total RNA was separated on a 1.2% formaldehyde agarose gel to verify the concentration and monitor the extraction integrity. RT-PCR was employed to measure the levels of *AtAPR2* expression in WT, mutant and transgenic plants, using 500 ng of total RNA together with the following primers: *AtAPR2*: forward (5'-AACGCTGAGTCACATTCACGAAGCG-3') and reverse (5'-GAAAGTTCCACACATCAGCTCCTTC-3'); *Actin8* (*At1g49240*): forward (5'-TGCCTATCTACGAGGGTTTC-3') and reverse (5'-GTCCGTCGGGTAATTCATAG-3'). After 27 PCR amplification cycles, 20 μL of each RT-PCR product was loaded onto a 1.2% (w/v) agarose gel to visualize the amplified DNA.

Stress Tests

For the Glc, Frc and Man stress tests, seeds were sown on MS medium (Murashige and Skoog 1962) medium supplemented with 6% Glc, 6% Frc and 0.1% Man, respectively, grown in a growth chamber, and assessed for percentage of cotyledon greening after 7 d. Experiments were conducted in triplicate for each line (50 seeds each). To test osmotic stress, seeds were sown on MS medium supplemented with 400 mM mannitol. Growth and phenotypic assessment was as described for the sugars. For 2-DG stress test, seeds were sown on MS medium supplemented with 0 and 0.01 mM 2-DG. Growth and phenotypic assessment was as described for the sugars. For 3-OMG stress test, seeds were sown on MS medium supplemented with 0 and 25 mM 3-OMG. Growth and phenotypic assessment was as described for the sugars.

qPCR

Total RNA was extracted from the variously-treated 10-d-old Arabidopsis seedlings using an RNeasy Plant Mini kit (Qiagen). qPCR was carried out using the SensiMix One-Step kit (Quantance, London, UK) and a Rotor-Gene 6000 quantitative PCR apparatus (Corbett Research, Mortlake, NSW, Australia). Arabidopsis *Actin8* was used as the internal control. Results were analyzed using RG6000 1.7 software (Corbett Research). Quantitative analysis was carried out using the Delta Delta C_T method (Livak and Schmittgen 2001). Each sample was run in three independent experiments. The reaction primers utilized were: *AtAPR1* (*At4G04610*), forward 5'-CTCGTTTCGGTGTTTCATTGGAGCC-3' and reverse 5'-ACAATCCCTTGCTCTAACCAAACC-3'; *AtAPR2* (*At1G62180*), forward 5'-AACGCTGAGTCACATTCACGAAGCG-3' and reverse 5'-GAAAGTTCCACACATCAGCTCCTTC-3'; *AtAPR3*

(*At4G21990*), forward 5'-GGCTTCTCTGAGTTTGTCCGGGAAG-3' and reverse 5'-TCCAAGCACGTAAACCCTTCAACGC-3'; *HXK1* (*At4G29130*), forward 5'-GACGAACCCACCAAGCTCGAG-3' and reverse 5'-TGCATCTCAACGGTCATAGC-3'; *RBCS1* (*At1G67090*), forward 5'-CACGGATTTGTGTACCGTG-3' and reverse 5'-CTTTA-GCGACTCATGGTTC-3'; *PR5* (*At1G75040*), forward 5'-CACTCT-GGCTGAATTCACTC-3' and reverse 5'-ACCTCTCACAGGCAC-TCTTG-3'; *PAL1* (*At2G37040*), forward 5'-GGAACAAATACAATT-CCTTAAC-3' and reverse 5'-GCACCAAAACCAGTAGTAAC-3'; *Actin8*, forward 5'-TGCCTATCTACGAGGGTTTC-3' and reverse 5'-GTCCGTCGGGTAATTCATAG-3'.

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Author's Contributions

The work presented here was carried out in collaboration between all authors; JS Chung and CS Kim designed research; JS Chung and HN Lee performed research; JS Chung, T Leustek, DB Knaff and CS Kim analyzed data; JS Chung, T Leustek, DB Knaff and CS Kim wrote the paper.

Supporting Information

Fig. S1. Scheme of the T-DNA insertion in the *AtAPR2* gene.
Fig. S2. Hexose sensitivity of *atapr2-2* mutant and *AtAPR2*-overexpressing transgenic seedlings.

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