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Research Article

Arabidopsis ACT DOMAIN REPEAT9 represses glucose signaling pathways

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Abstract

Nutrient sensing and signaling are critical for plants to coordinate growth and development in response to nutrient availability. Plant ACT DOMAIN REPEAT (ACR) proteins have been proposed to serve as nutrient sensors, but their functions remain largely unknown. Here, we showed that Arabidopsis (*Arabidopsis thaliana*) ACR9 might function as a repressor in glucose (Glc) signaling pathways. *ACR9* was highly expressed in the leaves, and its expression was downregulated by sugars. Interestingly, the *acr9-1* and *acr9-2* T-DNA insertion mutants were hypersensitive to Glc during seedling growth, development, and anthocyanin accumulation. Nitrogen deficiency increased the mutants' sensitivity to Glc. The expression of sugar-responsive genes was also significantly enhanced in the *acr9* mutants. By contrast, the *35S:ACR9* and *35S:ACR9-GFP* overexpression (OE) lines were insensitive to Glc during early seedling development. The Glc signaling pathway is known to interact with the plant hormone abscisic acid (ABA). Notably, the *acr9* mutants were also hypersensitive to ABA during early seedling development. The Glc sensor HEXOKINASE1 (HXK1) and the energy sensor SUCROSE NON-FERMENTING1 (SNF1)-RELATED PROTEIN KINASE1 (SnRK1) are key components of the Glc signaling pathways. The *acr9-1/hxk1-3* and *acr9-1/snrk1* double mutants were no longer hypersensitive to Glc, indicating that functional HXK1 and SnRK1 were required for the *acr9-1* mutant to be hypersensitive to Glc. Together, these results suggest that ACR9 is a repressor of the Glc signaling pathway, which may act independently or upstream of the HXK1-SnRK1 signaling module.

Introduction

The ACT domain, named after aspartate kinase (AK), chorismate mutase (CM), and TyrA (prephenate dehydrogenase, PPDH), is a loosely conserved structural motif of 60 to 80 amino acids commonly found in feedback-regulated amino acid biosynthetic enzymes (Aravind and Koonin 1999; Chipman and Shaanan 2001; Liberles et al. 2005; Grant 2006; Curien et al. 2008; Lang et al. 2014). In addition to AK, CM, and PPDH, the other amino acid metabolic enzymes, including phosphoglycerate dehydrogenase, acetohydroxyacid synthase, threonine deaminase, and phenylalanine hydroxylase, also contain the ACT domain (Chipman and Shaanan 2001; Flydal et al. 2019). In these amino acid metabolic enzymes, the ACT domain functions as an allosteric amino acid-binding domain to regulate the production of the end products.

The purine metabolic enzyme formyltetrahydrofolate hydrolase (PurU), the glutamine sensor GlnD (uridylyl transferase), the stringent response SpoT/RelA protein (GTP pyrophosphokinase/phosphohydrolase), and serine/threonine/tyrosine protein kinases also contain the ACT domain (Cashel et al. 1996; Chipman and Shaanan 2001; Martin et al. 2006; Zhang et al. 2010). In addition to metabolic enzymes, the ACT domain is present in the aromatic amino acid-regulated transcription factor TyrR, the nickel-responsive transcription factor NikR, the basic helix-loop-helix transcription factors, and the thiamin-binding protein YkoF (Wilson et al. 1995; Schreiter et al. 2003; Devedjiev et al. 2004; Feller et al. 2006; Grant 2006). The ACT domain has been proposed

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to function as a small-molecule-binding domain, and its ligands are not limited to amino acids (Grant 2006).

In humans, the ACT domain-containing protein CASTOR1 (cellular arginine sensor for mTORC1) is one of the nutrient-signaling hubs that regulates adaptive immunity and tumorigenesis (Chantranupong et al. 2016; Saxton et al. 2016; Long et al. 2021; Li et al. 2021b). Interestingly, human CASTOR1 contains 4 copies of the ACT domain and no recognizable catalytic domain, similar to the previously identified ACT DOMAIN REPEAT (ACR) proteins in plants (Hsieh and Goodman 2002; Sung et al. 2011; Liao et al. 2020). The CASTOR1 homologs are conserved in metazoans, which share limited sequence identity with plant ACRs (Saxton et al. 2016; Liao et al. 2020). Nevertheless, the composition and arrangement of the ACT domains in animal CASTORs are similar to plant ACRs (Liao et al. 2020).

The ACR protein family in most, if not all, plants can be divided into 3 distinct groups (Liao et al. 2020). The reference plant Arabidopsis (*Arabidopsis thaliana*) has 12 ACR proteins (Sung et al. 2011). Group I ACRs, e.g. ACR1 to ACR8, contain 4 ACT domains and an extra C-terminal domain (Hsieh and Goodman 2002). Group II ACRs, including ACR9 and ACR10, also consist of 4 ACT domains but do not have an extra sequence after the 4th ACT domain (Liao et al. 2020). By contrast, group III ACRs, ACR11 and ACR12, have an N-terminal targeting peptide followed by 2 ACT domains. The ACR11 and ACR12 proteins are localized to the chloroplast (Sung et al. 2011). Notably, sequence and phylogenetic analyses revealed that human CASTOR1 was most similar to group II ACRs (Liao et al. 2020).

ACR proteins are highly conserved in plants; however, their functions are largely unknown (Liao et al. 2020). In Arabidopsis, plant hormones and abiotic stresses differentially regulate the expression of *ACR1* to *ACR8*, and the importance of these regulations is unclear (Hsieh and Goodman 2002). ACR11 interacts and forms a protein complex with Fd-dependent glutamine oxoglutarate aminotransferase1 (Fd-GOGAT1; Takabayashi et al. 2016). The Fd-GOGAT enzyme activity was significantly reduced in the *acr11* mutants (Takabayashi et al. 2016). ACR11 also interacts and activates chloroplastic glutamine synthetase2 (GS2; Osanai et al. 2017). In addition, ACR11 is involved in regulating reactive oxygen species production and salicylic acid-associated immune responses (Singh et al. 2018). ACR11 has been proposed to function as a glutamine sensor (Sung et al. 2011; Osanai et al. 2017; Singh et al. 2018); however, the evidence showing that ACR11 can bind glutamine is still lacking. The molecular mechanisms of ACR11 in regulating GS2/Fd-GOGAT1 and defense responses have yet to be elucidated.

In addition to Arabidopsis, the group I ACRs have also been characterized in rice (*Oryza sativa*). Rice has 13 ACRs, including 9 in group I and 2 in groups II and III (Liu 2006; Liao et al. 2020). OsACR7 was shown to interact with HSP18.0-CII in the nucleus (Hayakawa et al. 2006). OsACR9 was also localized to the nucleus (Kudo et al. 2008). The functions of these rice ACRs are unknown. The tomato (*Solanum lycopersicum*) ACR11 homolog, SlACR11A, interacted with an F-box protein, and OE of *SlACR11A* could enhance cold tolerance in tomatoes (Song et al. 2021).

None of the plant group II ACRs have been characterized to date. Given that the human CASTOR1 protein plays a vital role in arginine sensing, plant group II ACRs may also participate in nutrient sensing and signaling (Liao et al. 2020). In this report, we took a reverse genetic approach to study the function of Arabidopsis ACR9 in nutrient sensing and signaling. The results suggest that ACR9 is involved in regulating early seedling growth, development, and anthocyanin biosynthesis in response to high glucose (Glc) concentrations.

Glc is the primary carbon and energy source for plants. In addition, Glc is a signaling molecule involved in regulating seedling growth, development, and a diverse array of cellular responses (Jang et al. 1997; Sheen et al. 1999; Moore et al. 2003). Tremendous efforts in the past decades have established Glc signaling as one of the most studied nutrient-signaling pathways in plants (Rolland et al. 2006; Sheen 2014; Li et al. 2021a). The Glc sensor HEXOKINASE1 (HXK1), the energy sensor SUCROSE NON-FERMENTING1 (SNF1)-RELATED PROTEIN KINASE1 (SnRK1), and the target of rapamycin kinase are 3 master regulators of plant Glc signaling networks (Jang et al. 1997; Moore et al. 2003; Baena-Gonzalez et al. 2007; Xiong et al. 2013; Li et al. 2021a). Still, many important components of the Glc signaling pathways have yet to be identified.

Notably, the Arabidopsis *acr9* knockout mutants were hypersensitive to Glc, whereas the *35S:ACR9* and *35S:ACR9-GFP* OE lines were insensitive to Glc during early seedling development. Thus, ACR9 may function as a repressor in the Glc signaling pathway. Glc signaling may interact with plant hormone abscisic acid (ABA) signaling to regulate early seedling development in Arabidopsis (Rolland et al. 2006). The *acr9* mutants were also hypersensitive to ABA. Genetic analyses revealed that the repressing effect of ACR9 in the Glc signaling pathway required functional HXK1 and SnRK1. These results suggest that ACR9 may act upstream of the HXK1-SnRK1 signaling module to regulate the Glc responses in Arabidopsis.

Results

ACR9 is highly expressed in leaves

Arabidopsis ACR9 was predicted to contain 4 ACT domains (Fig. 1A). RNA gel blot analysis revealed that steady-state mRNA levels of *ACR9* were high in leaves, medium in roots, and low to undetectable in stems, flowers, and siliques (Supplemental Fig. S1A). We generated *ACR9* promoter (p)-GUS transgenic lines to examine the tissue-specific expression of *ACR9*. The ACR9p-GUS activities were low in 3- and 5-d-old seedlings (Supplemental Fig. S1, B and C), and higher ACR9p-GUS activity was detected in the emerging leaves of 8-d-old seedlings (Supplemental Fig. S1D). In 14-d-old seedlings, the ACR9p-GUS activity was mainly detected in the leaves (Supplemental Fig. S1E). During the reproductive stage, high levels of ACR9p-GUS activity were

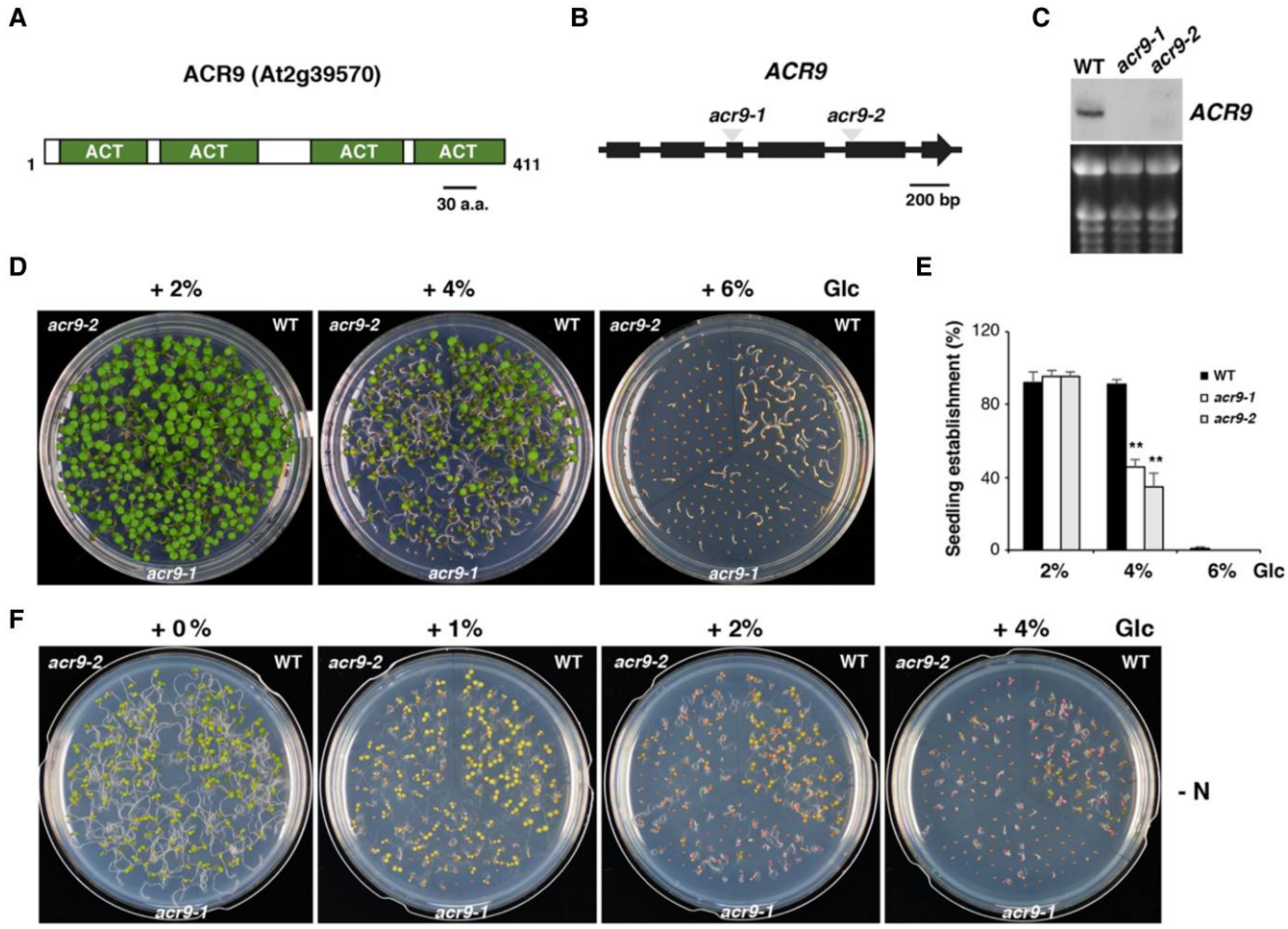

Figure 1. The Arabidopsis *acr9-1* and *acr9-2* mutant seedlings are hypersensitive to Glc. **A**) Schematic diagram of the ACR9 protein containing 4 ACT domains. **B**) The positions of T-DNA insertion in the *acr9-1* and *acr9-2* mutants. **C**) RNA gel blot analysis. The *ACR9* transcripts were undetectable in the *acr9-1* and *acr9-2* mutants. **D**) Images of 10-d-old Arabidopsis WT, *acr9-1*, and *acr9-2* seedlings grown on 1/2 MS plus 2%, 4%, and 6% Glc. **E**) Effects of Glc on seedling establishment in WT, *acr9-1*, and *acr9-2*. **F**) Images of seedlings grown on N-free (−N) media containing 0% to 4% Glc. The *acr9* mutants are hypersensitive to Glc under N deficiency. Data in (**E**) are means ± SD from 3 biological replicates. Significant differences compared with the WT were determined using Student's *t*-test: $^{**}P < 0.01$.

detected in the rosette and cauline leaves (Supplemental Fig. S1, F and G). ACR9p-GUS was also detected in the flower buds and pedicels of young siliques (Supplemental Fig. S1H), the anther connective tissue and style (Supplemental Fig. S1I), and the pedicel of a mature silique (Supplemental Fig. S1J). These results suggested that *ACR9* was primarily expressed in the leaves.

The *acr9* mutants are hypersensitive to Glc

To study the functions of ACR9, we isolated 2 independent T-DNA insertion mutants, *acr9-1* and *acr9-2* (Fig. 1B). RNA gel blot analysis revealed that the *ACR9* transcripts were undetectable in the mutants (Fig. 1C). The *acr9-1* and *acr9-2* mutants did not have aberrant phenotypes when grown in the soil under standard conditions (Supplemental Fig. S2). The ACT domain is known to bind amino acids. We tested whether amino acids affected the *acr9* mutants. The results showed that the wild-type (WT), *acr9-1*, and *acr9-2* seedlings had similar phenotypes in response to exogenous amino acids (Supplemental Fig. S3). Sugars are essential nutrients and energy sources for plants. We next examined if the expression of *ACR9* was regulated by sucrose (Suc). Notably, the expression of *ACR9* was downregulated by Suc regardless of the light or dark treatments (Supplemental Fig. S4).

We further examined the effects of sugars on the growth of *acr9-1* and *acr9-2* mutant seedlings. The *acr9* mutant cotyledons were smaller than the WT under 2% Glc (Fig. 1D). Interestingly, high Glc concentrations, e.g. 4% and 6% Glc, significantly inhibited the establishment of *acr9* mutant seedlings compared to the WT (Fig. 1, D and E). Treatments of 2%, 4%, and 6% Suc and the osmotic controls sorbitol and mannitol (Man) did not affect *acr9-1* and *acr9-2* mutants (Supplemental Fig. S5), indicating that the effects of Glc were not caused by osmotic stress. The *acr9-1* and *acr9-2* mutant seedlings were hypersensitive to 6% but not 4% fructose (Supplemental Fig. S5). Carbon and nitrogen (N) metabolism are closely related, and the N status often affects plants' responses to sugars (Liao et al. 2022). Under N-deficient

conditions, the *acr9-1* and *acr9-2* mutant seedlings were also hypersensitive to 1%, 2%, and 4% Glc (Fig. 1F).

Since the *acr9* mutants were more sensitive to Glc than fructose, we thus focused on the effects of Glc in this study. In the presence of 4% Glc, the growth and development of *acr9-1* and *acr9-2* mutant seedlings were significantly arrested compared to the WT (Supplemental Fig. S6). The full-length *ACR9* cDNA driven by the cauliflower mosaic virus *35S* promoter, e.g. *35S:ACR9*, could complement the *acr9-1* mutant (Supplemental Fig. S6), confirming that the Glc-hypersensitive phenotype of the *acr9* mutants was indeed caused by loss-of-function in ACR9.

The *acr9* mutants are hypersensitive to Glc-induced anthocyanin biosynthesis

It is well-known that sugars can induce anthocyanin biosynthesis (Liao et al. 2022). We next examined if the *acr9* mutants were hypersensitive to Glc in anthocyanin accumulation. When grown on vertical plates containing 0.5% to 4% Glc, the primary root length of the *acr9-1* and *acr9-2* mutants was significantly shorter than the WT (Fig. 2A; Supplemental Fig. S7). These results supported that the *acr9* mutants were hypersensitive to Glc in seedling growth inhibition. Increasing concentrations of Glc would enhance anthocyanin accumulation in Arabidopsis seedlings. It was visible that the *acr9-1* and *acr9-2* mutant seedlings accumulated more anthocyanins than the WT under high Glc concentrations (Fig. 2A). Both *acr9-1* and *acr9-2* mutant seedlings had significantly higher anthocyanin concentrations than the WT in response to 1% to 4% Glc (Fig. 2B).

Expression of anthocyanin biosynthetic genes is enhanced in the *acr9* mutants

The anthocyanin biosynthetic and regulatory genes are well characterized in plants (Liao et al. 2022). We compared the expression of anthocyanin biosynthetic and regulatory genes in the WT and *acr9* mutants under 2% and 4% Glc (Fig. 2C). The expression levels of most anthocyanin biosynthetic and regulatory genes were similar between the WT and *acr9* mutants under 2% Glc (Fig. 2C). Compared to 2% Glc, treatment of 4% Glc enhanced the expression of most anthocyanin biosynthetic and regulatory genes in the WT and *acr9* mutants. The late anthocyanin biosynthetic genes *DFR*, *LDOX*, and *UFGT*, and the transcription activator genes *PAP1*, *PAP2*, and *TT8* were strongly induced by 4% Glc in the WT (Fig. 2C). Notably, the expression levels of these genes except *TT8* in the *acr9-1* and *acr9-2* mutants were even higher than the WT under 4% Glc (Fig. 2C). In addition, the expression of most early anthocyanin biosynthetic genes and the positive transcription factor genes *GL3*, *EGL3*, and *TTG1* was further enhanced by 4% Glc in the *acr9-1* and *acr9-2* mutants compared to the WT (Fig. 2C). In the *acr9-1* and *acr9-2* mutants, the expression levels of *MYBL2*, encoding a transcriptional repressor of the anthocyanin biosynthetic genes, were significantly lower than the WT under 2% and 4% Glc (Fig. 2C).

We also examined the expression of anthocyanin biosynthetic and regulatory genes in the WT and *acr9* seedlings grown on 2% and 4% Suc and Man, respectively. Under 2% and 4% Suc treatments, none of the anthocyanin biosynthetic genes had higher expression levels in the *acr9* mutants than the WT (Supplemental Fig. S8A). In the regulatory genes, only the *PAP1* expression levels in the *acr9* mutants were slightly higher than the WT under 4% Suc (Supplemental Fig. S8A). In contrast to Glc and Suc, the expression of several anthocyanin biosynthetic and regulatory genes was downregulated in the *acr9* mutants compared to the WT under 2% and 4% Man (Supplemental Fig. S8B). These results suggested that Glc had specific effects on the enhancement of anthocyanin biosynthesis in the *acr9-1* and *acr9-2* mutants, and these effects were not caused by osmotic stress.

Expression of sugar-responsive genes is enhanced in the *acr9* mutants

We further characterized the molecular phenotypes of the *acr9* mutants by analyzing the expression of genes involved in sugar response and signaling. *APL3*, encoding the large subunit of ADP-GLUCOSE PYROPHOSPHORYLASE, and *BAM5*, encoding β-AMYLASE5, are sugar-responsive marker genes (Mita et al. 1995; Sokolov et al. 1998). 4% Glc strongly induced the expression of *APL3* and *BAM5* in the WT and *acr9* mutants compared to 2% Glc (Fig. 3A). There was significantly greater induction of *APL3* and *BAM5* in the *acr9-1* and *acr9-2* mutants compared with the WT in response to 4% Glc (Fig. 3A). These molecular phenotypes were consistent with the Glc-hypersensitive phenotypes observed in the *acr9* mutant seedlings. No differences in *APL3* and *BAM5* expression were found between the WT and *acr9* mutants in either 2% or 4% Suc (Fig. 3A). Transcript levels of *APL3* in 2% Man and *BAM5* in either 2% or 4% Man in the *acr9* mutants were lower than the WT (Fig. 3A). These molecular phenotypes also supported that the *acr9* mutants were specifically hypersensitive to Glc, and these effects were not due to osmotic stress.

Expression of sugar signaling genes is not affected in the *acr9* mutants

In addition to *APL3* and *BAM5*, we also compared the expression of genes involved in different sugar signaling pathways in the WT and *acr9* mutants in response to 2% and 4% Glc, Suc, and Man. The examined sugar signaling genes include *HXK1* and *TREHALOSE-6-PHOSPHATE SYNTHASE1* (*TPS1*) of the hexokinase-dependent pathway; *REGULATOR OF G-PROTEIN SIGNALING1* (*RGS1*) and *G PROTEIN ALPHA SUBUNIT1* (*GPA1*) of the RGS pathway; *SNF1 KINASE HOMOLOG10* (*AKIN10*, *SnRK1.1*), *AKIN11* (*SnRK1.2*), and *PLEIOTROPIC REGULATORY LOCUS1* (*PRL1*) of the SnRK1 pathway; and *SUGAR-INSENSITIVE3* (*SIS3*) of an independent sugar-responsive pathway. Compared to 2% Glc, the expression of *HXK1* and *TPS1* was induced, and *ACR9* was repressed by 4% Glc in the WT (Fig. 3B). In the *acr9-1* and *acr9-2* mutants, transcript levels of *HXK1* and *TPS1* in 4% and 2% Glc,

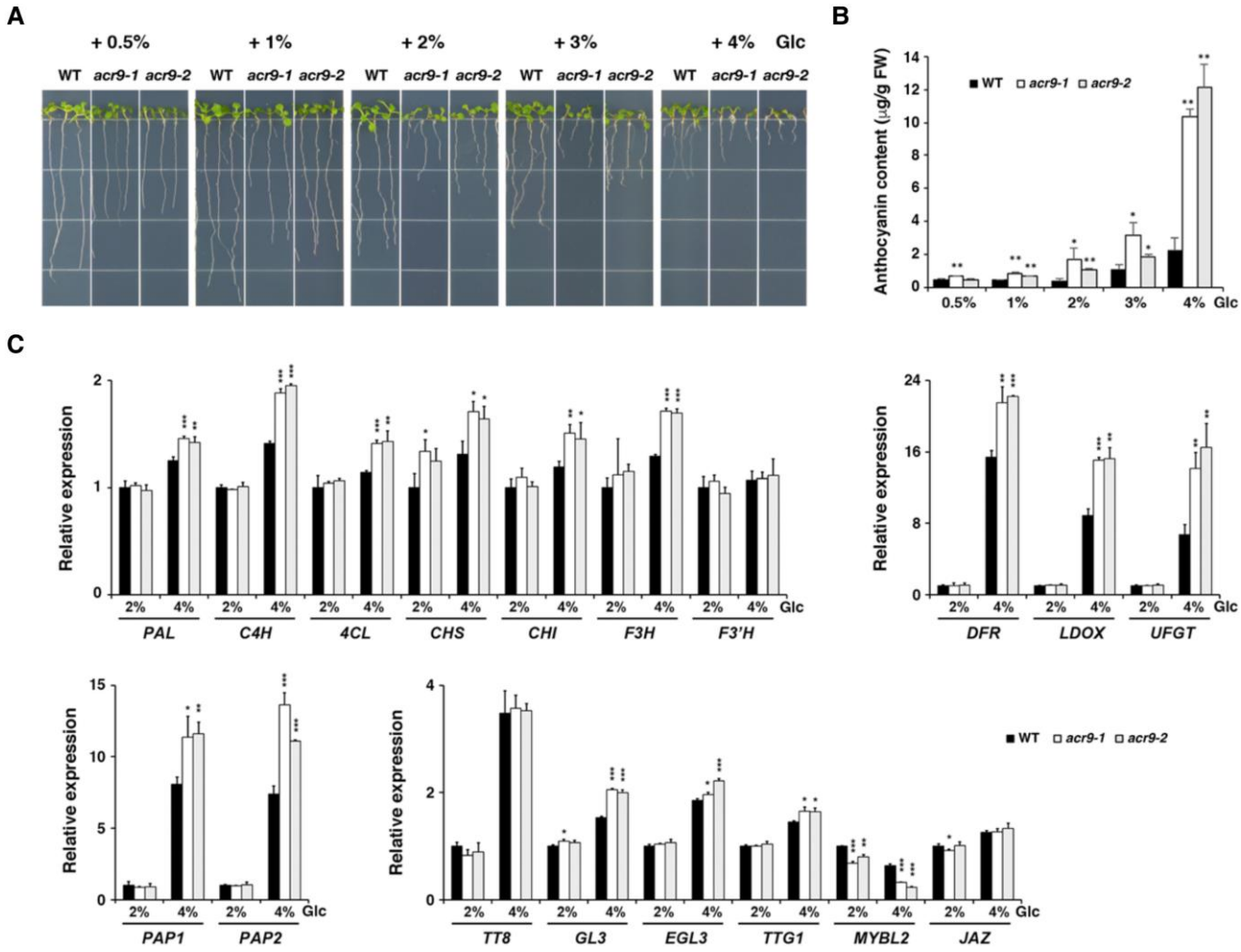

Figure 2. Glc-induced anthocyanin biosynthesis is enhanced in the *acr9* mutants. **A**) Ten-day-old Arabidopsis WT, *acr9-1*, and *acr9-2* seedlings grown on media containing 0.5% to 4% Glc. **B**) Anthocyanin content from plants in (**A**). **C**) RT-qPCR analysis of anthocyanin biosynthetic and regulatory genes. Expression is relative to that in the WT with 2% Glc, the value of which was set at 1. Data in **(B)** and **(C)** are means ± SD from 3 biological replicates. Significant differences compared with the WT were determined using Student's *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *PAL, PHENYLALANINE AMMONIA LYASE; C4H, CINNAMATE 4-HYDROXYLASE; 4CL, 4-COUMARATE:COA LIGASE; CHS, CHALCONE SYNTHASE; CHI, CHALCONE ISOMERASE; F3H, FLAVANONE 3-HYDROXYLASE; F3′H, FLAVANONE 3′-HYDROXYLASE; DFR, DIHYDROFLAVONOL-4-REDUCTASE; LDOX, LEUCOANTHOCYANIDIN DIOXYGENASE; UFGT, URIDINE DIPHOSPHATE-GLUCOSE:FLAVONOID 3-O-GLUCOSYLTRANSFERASE; PAP1/2, PRODUCTION OF ANTHOCYANIN PIGMENTS1/2; TT8, TRANSPARENT TESTA8; GL3, GLABRA3; EGL3, ENHANCER OF GL3; TTG1, TRANSPARENT TESTA GLABRA1; MYBL2, MYB-LIKE2; JAZ1, JASMONATE-ZIM DOMAIN.*

respectively, were slightly higher than in the WT (Fig. 3B). The effects of 2% and 4% Suc and Man on the expression of sugar signaling genes were shown in Supplemental Fig. S9. Unlike the sugar-responsive genes *APL3* and *BAM5*, the expression of sugar signaling genes had no major differences between the WT and *acr9* mutants in either 2% or 4% Glc.

Expression of sugar transport genes is not affected in the *acr9* mutants

Members of the SUGAR TRANSPORT PROTEIN (STP) and the SWEET protein families have been shown to transport Glc (Chen et al. 2010; Yamada et al. 2011). We examined the expression of representative *STP* and *SWEET* genes in the WT and *acr9* mutants in response to 2% and 4% Glc, Suc, and Man. The expression levels of *STP1*, *STP4*, *STP7*, *STP13*, *SWEET1*, *SWEET4*, *SWEET5*, *SWEET7*, and *SWEET13* were similar between the WT and *acr9* mutants in Glc (Fig. 3C) and Suc (Supplemental Fig. S10A). These results suggested that the Glc-hypersensitive phenotype of the *acr9* mutants was not caused by perturbation of the sugar transport genes. In contrast, Man repressed *STP1*, *STP4*, and *STP7* and enhanced *SWEET1* expression in the *acr9* mutants compared to the WT (Supplemental Fig. S10B). The importance of these Man effects on the *acr9* mutants requires further study.

ACR9-GFP is localized to the cytosol and nucleus

The ACR9 protein was predicted to localize to the cytosol (https://suba.live/). However, transient expression of *35S:*

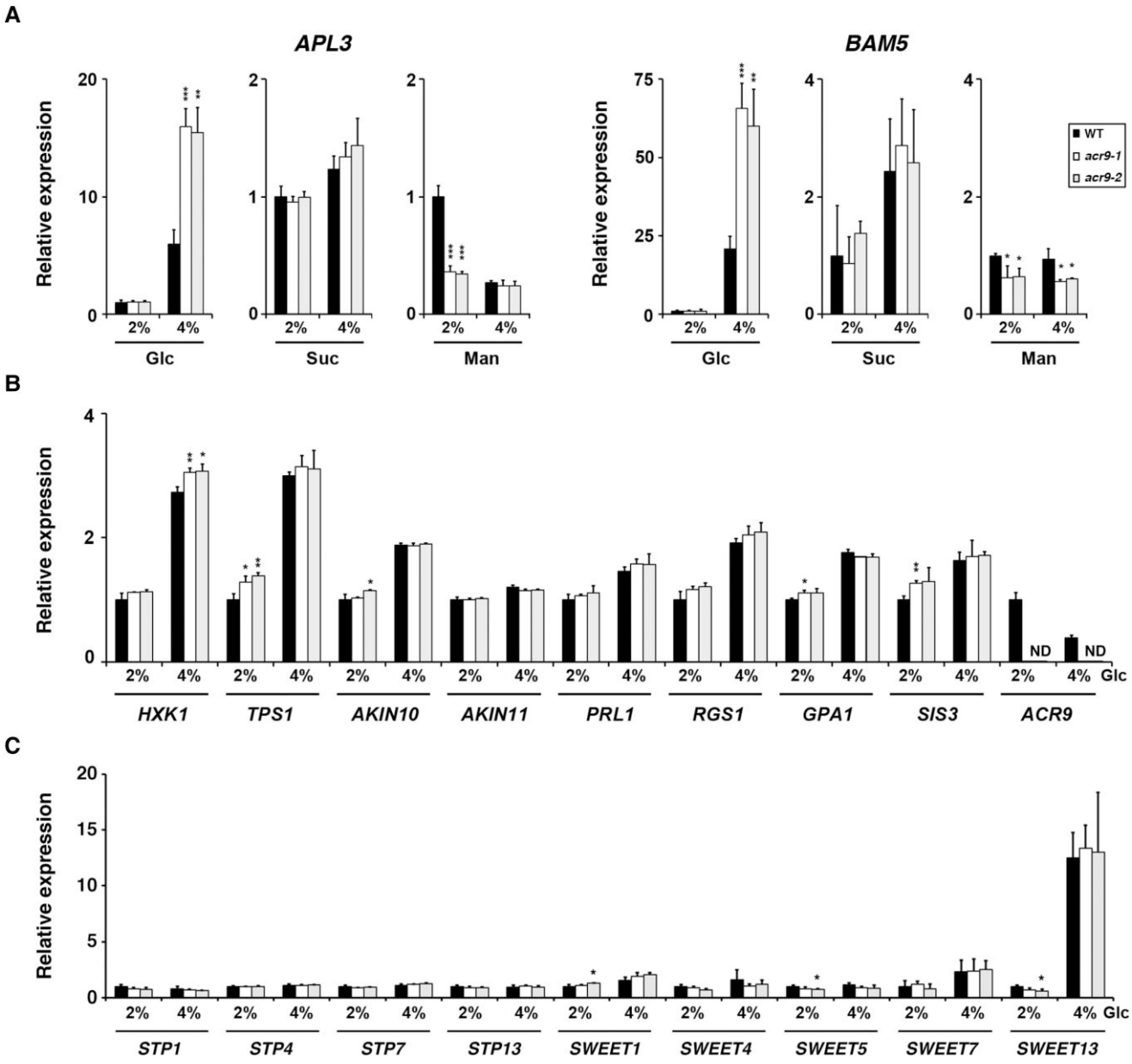

Figure 3. The expression of Glc-responsive genes is enhanced in the *acr9* mutants. **A**) RT-qPCR analysis of Glc-responsive marker genes. The expression of *APL3* and *BAM5* was significantly enhanced in the *acr9* mutants under 4% Glc. **B**) RT-qPCR analysis of genes involved in sugar signaling. Four percent Glc repressed the expression of *ACR9* in the WT compared to 2% Glc. **C**) RT-qPCR analysis of representative sugar transport genes. Expression is relative to that in the WT with 2% Glc, Suc, or Man, the value of which was set at 1. Data are means ± SD from 3 biological replicates. Significant differences compared with the WT were determined using Student's *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *APL3*, *ADP-GLUCOSE PYROPHOSPHORYLASE3*; *BAM5*, *β-AMYLASE5*; *HXK1*, *HEXOKINASE1*; *TPS1*, *TREHALOSE-6-PHOSPHATE SYNTHASE1*; *AKIN10/11*, *SNF1 KINASE HOMOLOG10/11*; *PRL1*, *PLEIOTROPIC REGULATORY LOCUS1*; *RGS1*, *REGULATOR OF G-PROTEIN SIGNALING1*; *GPA1*, *G PROTEIN α-SUBUNIT1*; *SIS3*, *SUGAR-INSITIVE3*; *ACR9*, *ACT DOMAIN REPEAT9*; *STP*, *SUGAR TRANSPORT PROTEIN*.

ACR9-GFP in the protoplast indicated that the ACR9-GFP was localized to the cytosol and nucleus (Fig. 4A). We further transformed *35S:ACR9-GFP* into the *acr9-1* mutant for complementation assays. The *35S:ACR9-GFP* construct could complement the *acr9-1* mutant (Fig. 4B), indicating that the ACR9-GFP was functional in planta. In the root of a *35S:ACR9-GFP/acr9-1* complemented plant, the fluorescent signals of ACR9-GFP were detected in the cytosol and nucleus (Fig. 4C). These results suggested that the ACR9-GFP is localized to the cytosol and nucleus.

35S:ACR9 and 35S:ACR9-GFP transgenic plants are insensitive to Glc

Since *35S:ACR9* and *35S:ACR9-GFP* complemented the *acr9-1* mutant, we transformed these constructs into WT

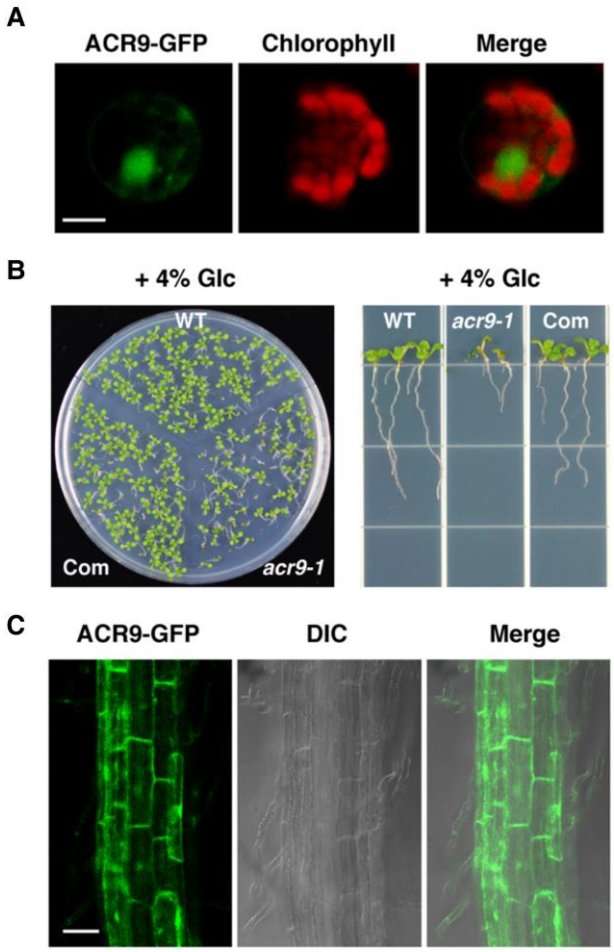

Figure 4. The ACR9-GFP is localized to the cytosol and nucleus. **A**) Transient expression of *35S:ACR9-GFP* in a protoplast. **B**) *35S:ACR9-GFP* complemented the *acr9-1* mutant. The Glc-hypersensitive phenotype of the *acr9-1* mutant was rescued in the *35S:ACR9-GFP/acr9-1* complementation (Com) line. **C**) Localization of ACR9-GFP in the root of a *35S:ACR9-GFP/acr9-1* complemented plant. The scale bar is 20 μm in (**A**) and 50 μm in (**C**). WT, wild type; DIC, differential interference contrast.

Arabidopsis to generate *ACR9* and *ACR9-GFP* OE lines. RNA gel blot analysis showed that high levels of *ACR9* mRNA accumulated in 3 independent *35S:ACR9* OE lines (Fig. 5A). When grown on 4% and 6% Glc, the *35S:ACR9* OE seedlings were less sensitive to Glc inhibition than the WT (Fig. 5, B and C). These differences were specific to high Glc because the WT and *35S:ACR9* OE lines had similar seedling establishment rates under 1% Suc or 2% Glc (Fig. 5, B and C).

Similarly, the *35S:ACR9-GFP* OE lines and WT had similar phenotypes when grown on the standard tissue culture plate containing 1% Suc (Supplemental Fig. S11A). The seedlings of 3 independent *35S:ACR9-GFP* OE lines showed a Glc-insensitive phenotype in response to 4% and 6% Glc (Supplemental Fig. S11, A and B). In the root of a *35S:ACR9-GFP* OE plant, the ACR9-GFP was localized to the cytosol and nucleus (Supplemental Fig. S11C). We further confirmed that the enriched bright dots of the ACR9-GFP signals co-localized with the fluorescent signals stained by DAPI (Supplemental Fig. S11D). Similar to the *acr9* mutants, the *35S:ACR9* and *35S:ACR9-GFP* OE lines did not show aberrant phenotypes when grown in soils under standard growth conditions (Supplemental Fig. S12).

ACR9 acts upstream of HXK1-SnRK1 in the Glc signaling pathway

HXK1 is a Glc sensor in plants (Jang et al. 1997; Moore et al. 2003). To examine if ACR9 acts in the HXK1 signaling pathway, we crossed *acr9-1* to *hxk1-3* to generate the *acr9-1/hxk1-3* double mutant. RT-PCR analysis confirmed that the *ACR9* and *HXK1* transcripts were undetectable in the double mutant (Fig. 6A). The WT, *acr9-1*, *hxk1-3*, and *acr9-1/hxk1-3* seedlings had similar phenotypes when grown on the medium containing 1% Suc (Fig. 6B). Under high Glc treatments, in contrast to *acr9-1*, which was Glc-hypersensitive, the *hxk1-3* mutant was Glc-insensitive (Fig. 6, B and C). Notably, loss of function in *HXK1* significantly attenuated the Glc-hypersensitive phenotype of the *acr9-1* mutant. The *acr9-1/hxk1-3* double mutant and WT had similar phenotypes in early seedling development in responses to high concentrations of Glc (Fig. 6, B and C). These results suggested that the Glc-hypersensitive phenotype of the *acr9-1* mutant required functional HXK1.

SnRK1 is an energy sensor downstream of the Glc signaling pathways (Baena-Gonzalez et al. 2007). We generated the *acr9-1/snrk1* double mutant by crossing *acr9-1* to the *snrk1* mutant. RT-PCR analysis was used to verify that the *acr9-1/snrk1* double mutant had no detectable *ACR9* and *SnRK1* transcripts (Fig. 7A). The *acr9-1*, *snrk1*, and *acr9-1/snrk1* mutants shared similar growth phenotypes and seedling establishment rates with the WT when grown on the control medium containing 1% Suc (Fig. 7, B and C). In contrast to *acr9-1*, the *snrk1* mutant was Glc-insensitive in response to high concentrations of Glc (Fig. 7, B and C). Interestingly, loss of function in *SnRK1* also significantly attenuated the Glc-hypersensitive phenotype of the *acr9-1* mutant. The *acr9-1/snrk1* double mutant shared similar phenotypes with the WT rather than the *acr9-1* or *snrk1* single mutant under 4% to 6% Glc (Fig. 7, B and C). These results implicated that functional SnRK1 was critical for the *acr9-1* mutant to show the Glc-hypersensitive phenotype.

The *acr9* mutants are hypersensitive to ABA

It is known that the Glc and ABA signaling pathways may interact to regulate Arabidopsis early seedling development (Rolland et al. 2006). We found that the Arabidopsis *acr9* mutants were also hypersensitive to ABA regardless of the Suc concentrations (Supplemental Fig. S13). Notably, the addition of ABA significantly enhanced the Glc-hypersensitive response of the *acr9* mutants (Fig. 8A). Without ABA treatment, the *acr9* mutant seedling establishment was significantly reduced in 4% Glc, but not in 0.5%, 1%, or 2% Glc, compared with the WT (Fig. 8B). In the presence of 1 μM

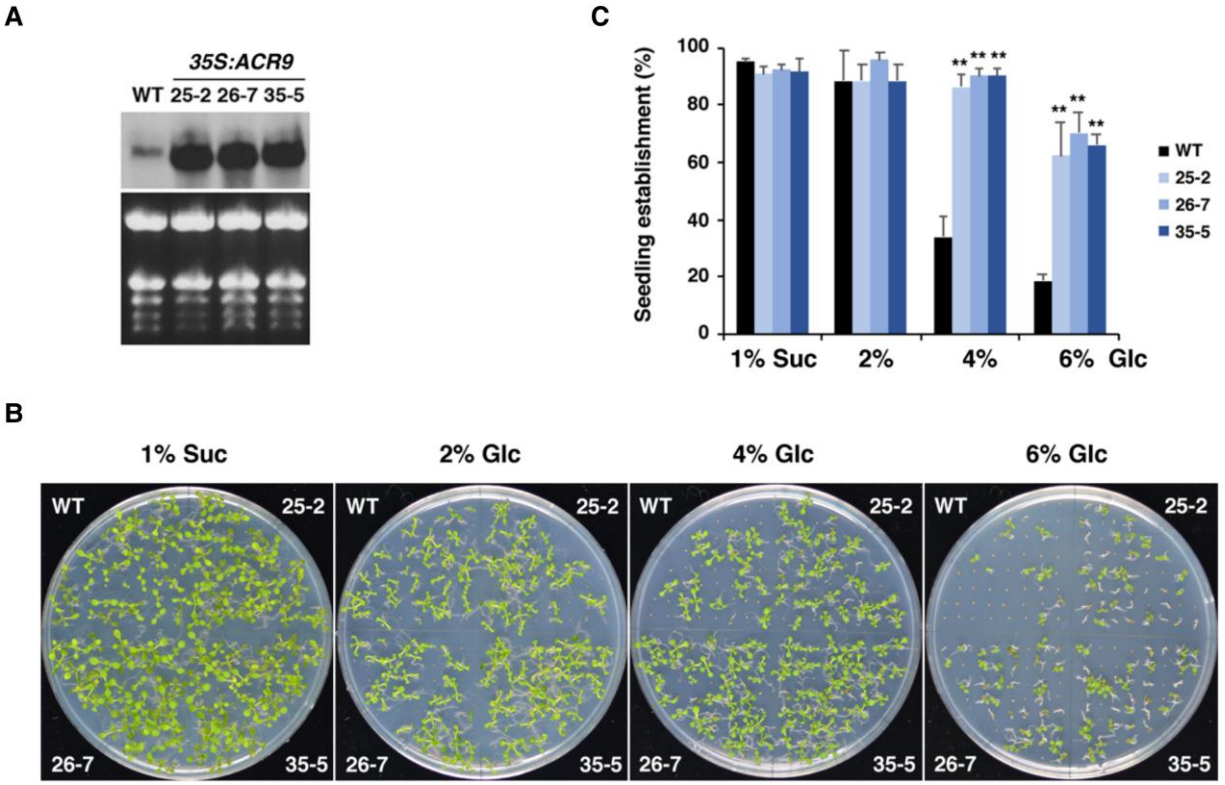

Figure 5. The *35S:ACR9* OE lines are Glc-insensitive. **A**) RNA gel blot analysis of *ACR9* in the WT and *35S:ACR9* OE lines 25-2, 26-7 and 35-5. **B**) Images of 10-d-old WT and *35S:ACR9* OE seedlings. **C**) Effects of Glc on seedling establishment. The *35S:ACR9* OE lines were less sensitive to 4% and 6% Glc than the WT. Data are means ± SD from 3 biological replicates. Significant differences compared with the WT were determined using Student's *t*-test: $^{**}P < 0.01$.

ABA, the seedling establishment rate of the *acr9* mutants was significantly lower than that of the WT under 1% and 2% Glc (Fig. 8B). None of the WT or *acr9* mutant seedlings could grow under the treatment of 1 μM ABA plus 4% Glc (Fig. 8, A and B). These results suggested that ABA and Glc had additive effects on repressing the *acr9* mutant seedling establishment.

A proposed working model of ACR9 in Glc signaling

We integrated the results reported here with the known Glc signaling pathways (Rolland et al. 2006; Carvalho et al. 2016) to propose a working model of ACR9 (Fig. 9). The Glc-hypersensitive phenotype of the *acr9-1* mutant was lost in the *acr9-1*/*hxk1-3* and *acr9-1*/*snrk1* double mutants, indicating ACR9 might act upstream of the HXK1-SnRK1 module in the Glc signaling pathway (Fig. 9). However, loss-of-function in *ACR9* also attenuated the Glc insensitive phenotype of the *hxk1-3* and *snrk1* mutants. Under high Glc conditions, the *acr9-1*/*hxk1-3* and *acr9-1*/*snrk1* double mutants shared similar phenotypes with the WT rather than the *hxk1-3* or *snrk1* single mutant (Figs. 6 and 7). These results suggested that ACR9 might partly repress the Glc signaling independent of HXK1 and SnRK1 (Fig. 9). Arabidopsis ACR9 might use both the HXK1-SnRK1 dependent and independent signaling pathways to regulate early seedling development in response to high Glc. In addition, the *acr9* mutants were hypersensitive to ABA, suggesting that ACR9 might function as a repressor in the ABA signaling pathway. ABA may use the ACR9-dependent and ACR9-independent pathways to inhibit early seedling development in Arabidopsis (Fig. 9).

Discussion

Glc and Suc are important energy sources for the cell. In addition to metabolism, Glc and Suc also function as signaling molecules to affect plant growth and development. Glc and Suc have distinct signaling functions despite their close relationship in metabolism. The inhibition of early seedling growth and development by high concentrations of Glc is one of the best-characterized Glc signaling pathways in plants (Jang et al. 1997; Moore et al. 2003). The Arabidopsis *acr9* knockout mutants did not have aberrant phenotypes under standard growth conditions. The *acr9* mutants were hypersensitive to Glc but not Suc in early seedling development, primary root growth, anthocyanin biosynthesis, and expression of sugar-responsive genes. These phenotypes suggest that ACR9 is a negative regulator specifically involved in Glc rather than a general sugar signaling pathway.

Glc signaling pathways are involved in various cellular processes during the entire plant life cycle, from embryogenesis and germination to reproduction and senescence (Li et al.

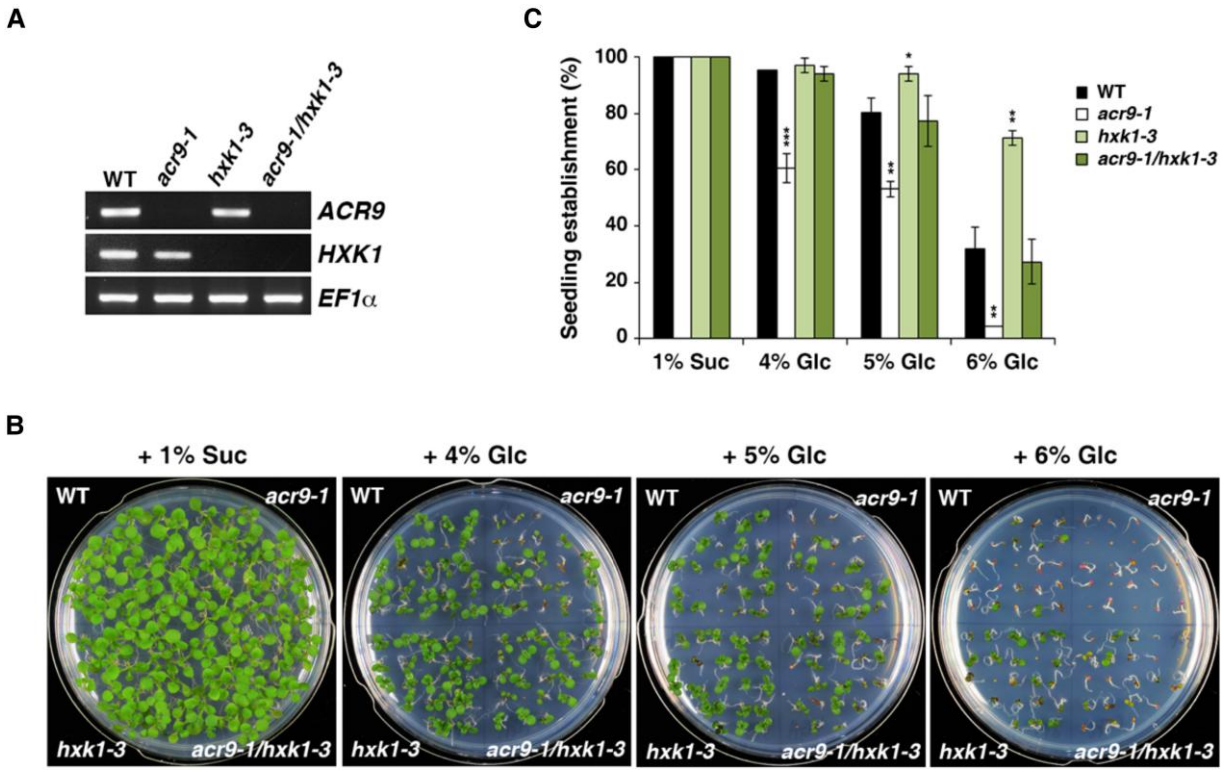

Figure 6. The Glc-hypersensitive phenotype of *acr9-1* is HXK1 dependent. **A**) RT-PCR analysis. **B**) Images of 10-d-old WT, *acr9-1*, *hxk1-3*, and *acr9-1/hxk1-3* seedlings. **C**) Effects of Glc on seedling establishment. The *acr9-1* and *hxk1-3* mutants were hypersensitive and insensitive to Glc, respectively. The *acr9-1/hxk1-3* double mutant and WT seedlings had similar responses to 4% to 6% Glc. Data are means ± SD from 3 biological replicates. Significant differences compared with the WT were determined using Student's *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

2021a). The discovery that ACR9 is involved in Glc signaling is unexpected, as amino acids rather than sugars are the predominant ligands for the ACT domain-containing proteins identified to date. The most characterized function of the ACT domain is amino acid-binding in allosteric regulation of feedback inhibition of amino acid biosynthetic enzymes. Upon binding to a specific amino acid in excess, the regulatory ACT domain will repress the catalytic activity of the same protein, usually a rate-limiting enzyme, to inhibit amino acid biosynthesis. Thus, the ACT domain functions as a repressor in feedback-regulated amino acid biosynthetic enzymes. In this aspect, the repressor role of ACR9 in Glc signaling is consistent with the known characteristics of the ACT domain.

The involvement of ACR9 in the Glc signaling pathways raises an interesting question as to whether ACR9 can function as a Glc sensor in plants. The Glc sensor HXK1 and the energy sensor SnRK1 are key components of the Glc signaling pathways (Jang et al. 1997; Moore et al. 2003; Baena-Gonzalez et al. 2007). The Glc hypersensitive response of the *acr9* mutant seedlings was significantly attenuated in the *acr9-1/hxk1-3* and *acr9-1/snrk1* double mutants. One of the possible explanations for these results is that ACR9 acts upstream of the HXK1-SnRK1 signaling module to regulate early seedling development. Still, part of the negative effects of ACR9 in Glc signaling was HXK1-SnRK1 independent because the *hxk1-3* and *snrk1* single mutants were more insensitive to Glc than the *acr9-1/hxk1-3* and *acr9-1/snrk1* double mutants. We have proposed a working model that ACR9 may use both HXK1-SnRK1 dependent and independent pathways to regulate early seedling development in response to Glc (Fig. 9). Consistent with this working model, overexpressing ACR9 and ACR9-GFP further represses the Glc signaling in the arrest of early seedling development.

The low sequence identity among the ACT domains may enable the ACT domain-containing proteins to bind to various ligands in the cell. The ACT domain has been proposed to bind small molecules, not limited to amino acids (Grant 2006). Although none of the ACT domain-containing proteins are known to bind sugars, we cannot exclude the possibility that ACR9 binds Glc and functions as a Glc sensor in Arabidopsis. HXK1 is a Glc sensor in plants (Jang et al. 1997; Moore et al. 2003). In contrast to *acr9*, the *hxk1* mutants are Glc insensitive, indicating that HXK1 is an activator of the Glc signaling pathway (Moore et al. 2003). By sensing the endogenous levels of Glc, HXK1 positively regulates the Glc signaling in early seedling growth and development (Moore et al. 2003). In seedling development, the *acr9* mutants are hypersensitive to Glc, a phenotype opposite to the *hxk1* mutants. ACR9 may sense Glc and antagonize the effects of HXK1 in Glc sensing and signaling. The existence of a Glc sensor that functions as a repressor may balance and fine-tune

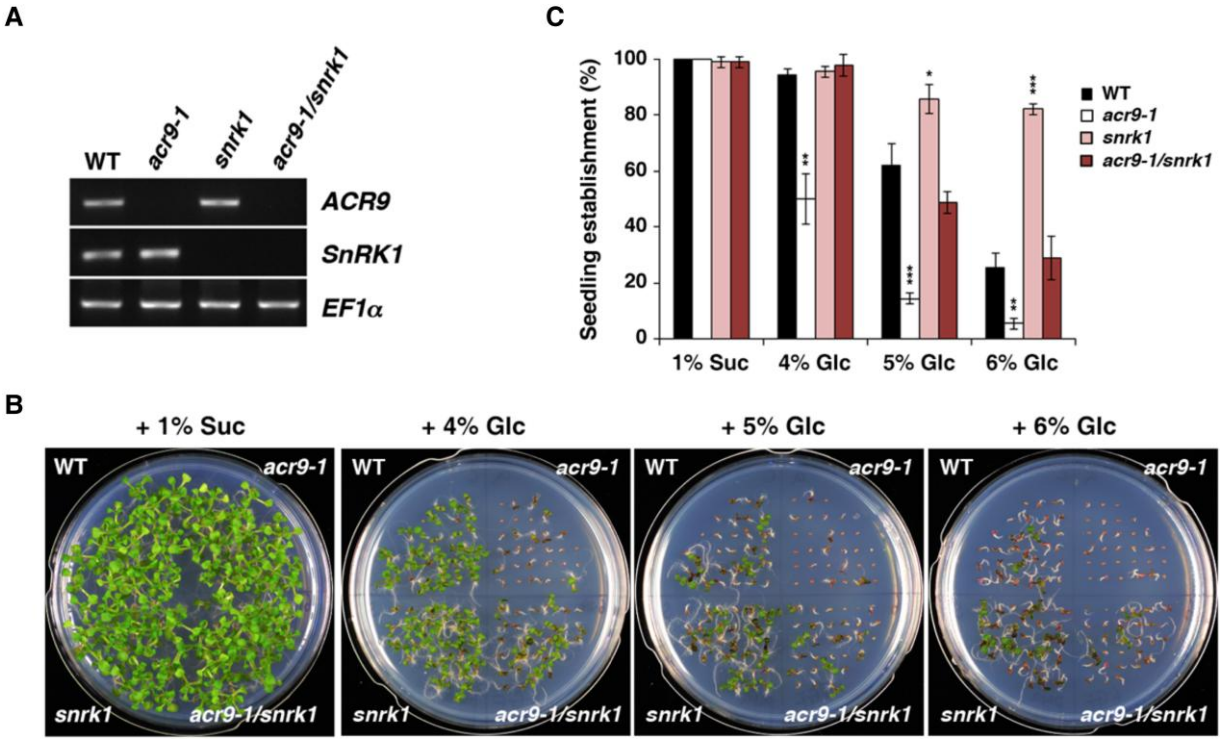

Figure 7. The Glc-hypersensitive phenotype of *acr9-1* is SnRK1 dependent. **A**) RT-PCR analysis. **B**) Images of 10-d-old WT, *acr9-1*, *snrk1*, and *acr9-1*/*snrk1* seedlings. **C**) Effects of Glc on seedling establishment. The *acr9-1* and *snrk1* mutants were hypersensitive and insensitive to Glc, respectively. The *acr9-1*/*snrk1* double mutant and WT seedlings had similar responses to 4% to 6% Glc. Data are means ± SD from 3 biological replicates. Significant differences compared with the WT were determined using Student's *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the responses mediated by the Glc sensor HXK1 in the Glc signaling pathways. It will be interesting to investigate if ACR9 can bind Glc and function as a Glc sensor in Arabidopsis.

Alternatively, the primary function of ACR9 may still be an amino acid sensor. The metabolism of C and N is highly interdependent. Maintaining C/N balance is essential for plants to reach optimal growth and development. In addition to metabolism, crosstalk between the C and N signaling pathways may also widely exist in plants. However, studies on C and N signaling are usually conducted separately. Integrating the coordinated regulation of C and N responses is still challenging. Regulatory proteins that interconnect the C and N signaling pathways are largely unknown. Furthermore, most plant N sensing and signaling studies focused on nitrate (Vidal et al. 2020). We know very little about amino acid sensing and signaling in plants. Amino acid anabolism and catabolism are directly linked to C and N metabolism. It is not surprising if the amino acid signaling pathways interact with Glc metabolism and signaling. Under high Glc conditions, the metabolic status of amino acids and hence their signaling responses will be affected. It is intriguing that ACR9, an amino acid sensor candidate, genetically interacts with HXK1 and SnRK1 of the Glc and energy signaling pathways. ACR9 may be one of the amino acid sensors that mediates the responses between the C and N signaling pathways.

In addition to defects in early seedling development, the *acr9* mutants also accumulated more anthocyanins than the WT under high Glc. These results suggest that ACR9 functions as a repressor in Glc-induced anthocyanin biosynthesis. Genes involved in the biosynthesis and regulation of anthocyanins are well-studied in Arabidopsis. However, the molecular mechanism involved in Glc-induced anthocyanin biosynthesis remains largely unknown. The induction of the late anthocyanin biosynthesis genes, including *DFR*, *LDOX*, and *UFGT*, by the MYB-bHLH-WD40 transcriptional activator (MBW) complex, is the primary mechanism affecting the accumulation of anthocyanins (Tohge et al. 2017). The *acr9* mutants accumulate more anthocyanins than the WT by enhancing the expression of biosynthetic and activator genes and repressing the expression of repressor genes in response to high Glc (Fig. 2). The expression of sugar-responsive genes *APL3* and *BAM5* was also enhanced in the *acr9* mutants under high Glc (Fig. 3). Interestingly, the ACR9 protein is localized to the cytosol and nucleus, although it does not contain a recognizable nucleus localization signal. It is unknown if the nuclear ACR9 protein can directly regulate the expression of these genes.

The cytosolic and nuclear localization of ACR9 is reminiscent of the molecular mechanism of the Glc sensor HXK1. Arabidopsis HXK1 forms a protein complex with the vacuolar H^+-ATPase subunit B1 (VHA-B1) and the proteasome 19S

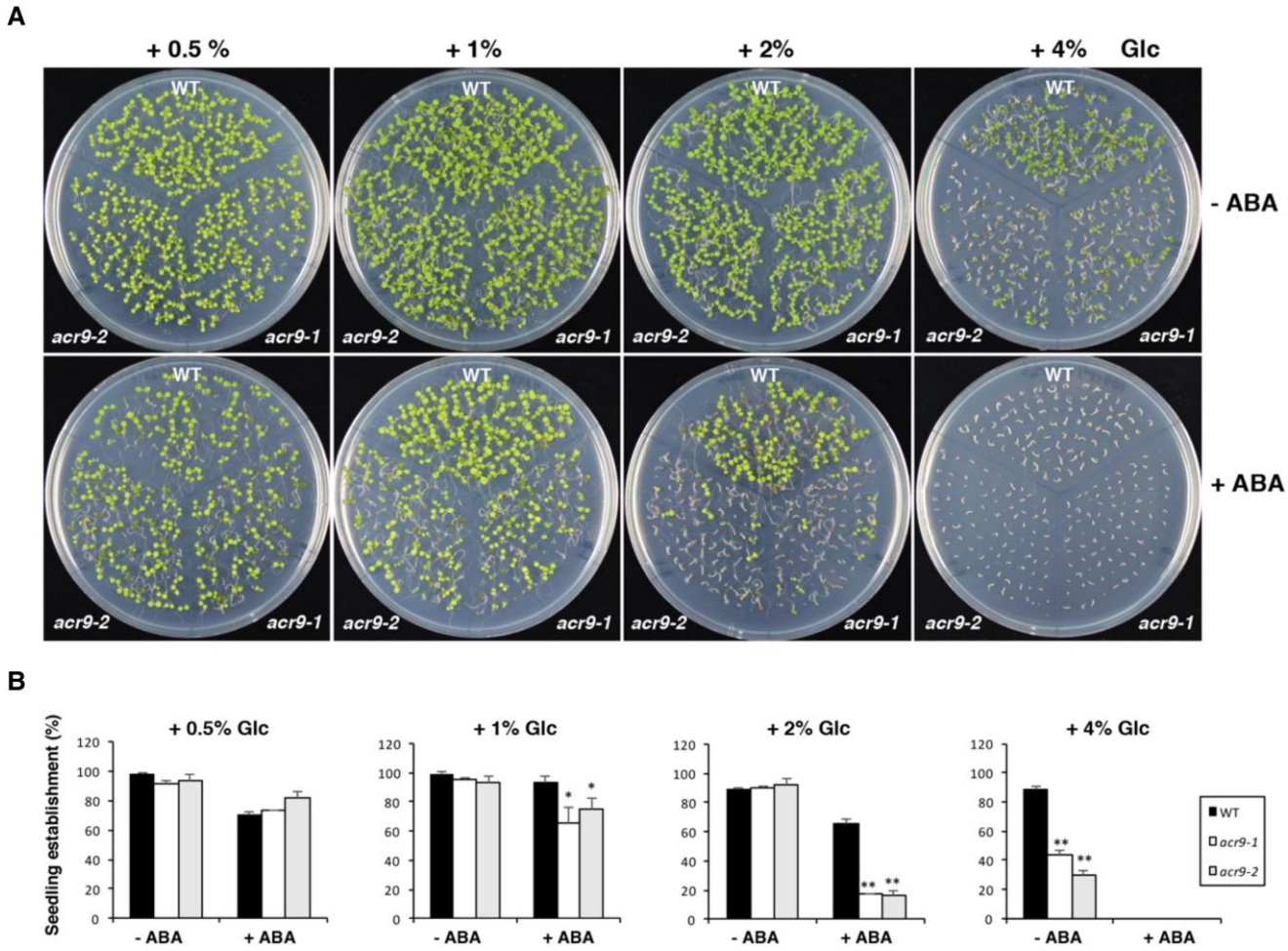

Figure 8. The Arabidopsis *acr9-1* and *acr9-2* mutant seedlings are hypersensitive to Glc and ABA. **A**) Images of 10-d-old Arabidopsis seedlings grown on 0.5%, 1%, 2%, and 4% Glc without or with 1 μM ABA. **B**) Effects of Glc and ABA on seedling establishment in WT, *acr9-1*, and *acr9-2*. Data are means ± SD from 3 biological replicates. Significant differences compared with the WT were determined using Student's *t*-test: $^*P < 0.05$, $^{**}P < 0.01$.

regulatory subunit RPT5B in the nucleus to regulate the expression of target genes in response to Glc (Cho et al. 2006). The apple bHLH3 transcription factor is a member of the MBW complex that activates the expression of anthocyanin biosynthetic genes. HXK1 directly phosphorylates and stabilizes bHLH3 to promote Glc-induced anthocyanin biosynthesis in apples (Hu et al. 2016). ACR9 may act upstream of HXK1 to regulate the expression of the target genes in the nucleus. Alternatively, ACR9 may form a distinct protein complex to regulate its target genes in response to Glc. Interestingly, the expression of *HXK1* is up-regulated by Glc, and HXK1 is a positive regulator in the Glc signaling pathway. By contrast, the expression of *ACR9* is downregulated by Glc, and ACR9 functions as a repressor of the Glc signaling pathway. The ACR9 repressor complex and the HXK1 activator complex may coordinate to balance the expression of the target genes of the Glc signaling pathways in the nucleus.

It is known that Glc and ABA signaling pathways may interact to regulate Arabidopsis seedling development (Rolland et al. 2006; Dekkers et al. 2008). Exogenous Glc has been shown to activate the expression of ABA biosynthesis and signaling genes and increase endogenous ABA levels (Cheng et al. 2002). Glc may use both HXK1-dependent and HXK1-independent pathways to interact with the ABA signaling pathway to regulate Arabidopsis seedling growth and development (Rolland et al. 2006; Carvalho et al. 2016). In addition to Glc, the *acr9* mutants are also hypersensitive to ABA in seedling development when grown on media containing 0.5%, 1%, or 2% Suc (Supplemental Fig. 13). Notably, Glc and ABA have additive effects on the developmental arrest of the *acr9* mutant seedlings (Fig. 8). ACR9 is likely involved in ABA-mediated post-germination developmental arrest, partly independent of the Glc signaling pathway. ABA may use the ACR9-dependent and ACR9-independent pathways to inhibit early seedling development in Arabidopsis (Fig. 9).

Exogenous Glc at low to intermediate concentrations, e.g. lower than 3% or 167 mM, can relieve the ABA-induced inhibition of seed germination (Price et al. 2003). In contrast, high levels of exogenous Glc and ABA will synergistically inhibit seed germination and seedling development (Arenas-Huertero et al. 2000). The *acr9* mutants had similar seedling establishment rates as the WT when grown on a

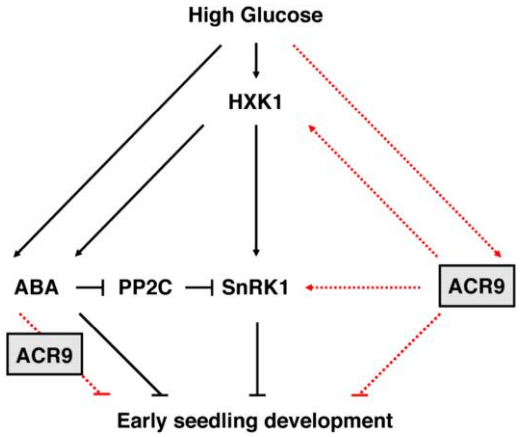

Figure 9. A proposed working model of ACR9 in Glc sensing and signaling. ACR9 may act upstream of the HXK1-SnRK1 signaling module to regulate early seedling development in response to high Glc concentrations. ACR9 may also function as a repressor for Glc signaling in inhibiting early seedling development independent of the HXK1-SnRK1 pathway. The plant hormone ABA may use the ACR9-dependent and ACR9-independent pathways to repress Arabidopsis seedling development. The interaction between ACR9 and the ABA signaling pathway requires further study. The dashed line indicates the genetic interaction of ACR9, ABA, and the Glc signaling pathways derived from this study.

medium containing 0.5% Glc plus ABA (Fig. 8). Interestingly, the *acr9* mutants were already hypersensitive to ABA in the presence of 1% Glc (Fig. 8). It is possible that the *acr9* mutants have accumulated higher levels of ABA than the WT under 1% Glc treatment. Alternatively, ACR9 may act as a negative regulator of the ABA response. The involvement of ACR9 in the ABA signaling pathways requires further study. For instance, measurements of ABA levels in the *acr9* mutants under various Glc concentrations and studies on the genetic interaction between the *acr9* and ABA biosynthetic and signaling mutants may elucidate the role of ACR9 in ABA-regulated responses.

The regulatory ACT domain is an evolutionarily conserved amino acid-binding motif that distributes widely across different domains of life. The ACR protein might incorporate the ACT module from a more ancient amino acid metabolic enzyme and evolve into a novel regulatory protein. Amino acid-binding is probably the primary function of plant ACR proteins. Nevertheless, amino acids are not the only small molecules that bind the ACT domain (Grant 2006). ACR proteins may bind and sense the status of amino acids or other small molecules to regulate various plant cellular processes. Identifying ligands that bind ACR9 and proteins that interact with ACR9 is critical for understanding the function of this novel protein.

Materials and methods

Plant materials and growth conditions

Arabidopsis (*A. thaliana*) ecotype Col-0 was used as the WT, and the *acr9-1* (SALK_082851), *acr9-2* (SALK_130534), *hxk1-3* (SALK_070739), and *snrk1* (SALK_127939) mutants obtained from the Arabidopsis Biological Resource Center were genotyped to isolate homozygous lines. For plants grown on tissue culture medium, seeds surface-sterilized by bleach containing 0.05% Tween-20 were sown on half-strength Murashige and Skoog (1/2 MS) plates (M524, PhytoTechnology Laboratories, Lenexa, KS, USA) containing 0.05% (w/v) 2-(*N*-morpholine)-ethanesulfonic acid, 1% (w/v) Suc, and 0.8% (w/v) agar, and adjusted to pH 5.7 with KOH. The plates were sealed with parafilm, kept at 4 °C for 2 d, and then transferred to a growth room with a 16-h light/8-h dark cycle at 22 °C. For sugar treatments, plants were grown on 1/2 MS plus the indicated concentrations of Suc, Glc, fructose, sorbitol, or Man. For amino acid treatments, 10 to 12 Arabidopsis WT and *acr9-1* seedlings in triplicate were grown vertically on a 9 × 9 cm square plate containing 1/2 MS plus 0.5% Suc supplemented with the indicated concentrations of amino acids (Pratelli et al. 2010). For plants grown in soil, 7-d-old seedlings on tissue culture plates were transferred to the soil and placed in a growth chamber with a 16-h light/8-h dark cycle at 22 °C.

Seedling establishment analysis

For seedling establishment analysis, 80 to 100 seeds of each genotype were sown in triplicate on 1/2 MS containing the indicated concentrations of sugars or ABA. Seven- or 10-d-old seedlings grown horizontally on a tissue culture plate were photographed, and the image was analyzed manually by Adobe Photoshop CS5 Extended (Adobe Inc., San Jose, CA, USA). Cotyledon greening, cotyledon expansion, and radical emergence were often used to arbitrarily score the response of Arabidopsis seedlings to Glc (Carvalho et al. 2010; Huang et al. 2010; Zheng et al. 2015). Seedling establishment rates were calculated as the number of seedlings displaying green and expanded cotyledons over the number of seeds sown on the same plate. Non-germinated seeds were considered a failure in seedling establishment in this study. It is noteworthy that Arabidopsis seedlings often have heterogeneous responses to high Glc concentrations. Furthermore, the seed quality changes from batch to batch, which may affect the germination rate and responses to exogenous Glc and ABA. Thus, we routinely used the same batch of seeds harvested from WT and mutant plants grown in the same environment and stored under the same conditions in the sugar and ABA response assays. Although the seedling establishment rate might vary between different experiments, we consistently observed that the *acr9* mutants were hypersensitive to Glc and the *ACR9* OE lines were less sensitive to Glc in different seed batches.

RNA gel blot, RT-PCR, and RT-qPCR analyses

Arabidopsis total RNA was extracted as described (Tseng et al. 2013). Ten micrograms of total RNA isolated from 2-wk-old WT, *acr9-1*, and *acr9-2* seedlings grown on 1/2 MS plus 1% Suc were used for RNA gel blot analysis. RNA

gel blot analyses used to examine the expression of *ACR9* in response to light/dark, Suc, and Man and in different organs from 6-wk-old plants were performed as described (Sung et al. 2011). Digoxigenin (DIG)-labeled single-stranded DNA probes of *ACR9* were generated by PCR using the following primers: 5′-TCCAAAGACTGCGATGTCCA-3′, 5′-TCACCAA CCCATAAGTGTCT-3′. RNA gel blot analyses, including probe labeling, prehybridization, hybridization, wash conditions, and detection, were performed according to the DIG application manual for filter hybridization (Roche Diagnostics GmbH, Mannheim, Germany). Total RNA extracted from the indicated samples was digested with DNase I using a TURBO DNA-free Kit (Thermo Fisher Scientific, Waltham, MA, USA) for RT-PCR and RT-qPCR analyses. The first-strand cDNA was synthesized with oligo$(dT)_{18}$ using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The primer sequences used for RT-PCR to detect the expression of *ACR9*, *HXK1*, *SnRK1*, and *EF1α* were listed in Supplemental Table S1. Transcript levels of genes involved in anthocyanin biosynthesis and regulation and sugar responses and signaling were determined by RT-qPCR with Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) on a Step-One Plus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). All RT-qPCRs were performed with 3 biological replicates, and the expression data were normalized to the nuclear gene *ACTIN2* (Huang et al. 2010; Zheng et al. 2015). The primers used for RT-qPCR analysis were listed in Supplemental Table S1.

Anthocyanin quantification

The extraction and quantitation of anthocyanin were performed as described (Mita et al. 1997). Ten-day-old Arabidopsis seedlings were weighed and then incubated in extraction buffer (1% HCl dissolved in methanol) overnight at 4 °C in the dark. The absorbance of the extract was measured at 530 and 657 nm. The anthocyanin content was quantified as (A_{530}−0.25×A_{657}) per gram of fresh weight.

Generation of *ACR9* promoter-GUS lines and histochemical GUS assay

The intergenic region between *ACR9* (*At2g39570*) and its upstream gene *At2g39560* is approximately 1.7 kb (https://bar.utoronto.ca/thalemine/report.do?id=24024720). Thus, about 1.5 kb upstream of the *ACR9* start codon would likely cover the promoter region. We arbitrarily used the −1 to −1,433 DNA sequence to construct the *ACR9p-GUS* reporter because a convenient restriction site is located nearby −1,433. Arabidopsis genomic DNA was used as a template to amplify the putative promoter region (−1 to −1,433) of the *ACR9* gene by PCR with primers 5′-GATTGTCGACAACCACGGG ATGGATGATGG-3′ and 5′- GGGAGGATCCTTTCGTTTTA GAGCCAATGAATCAA-3′. The PCR product carrying the *Sal*I and *Bam*HI restriction sites at both ends was cloned into the pCR2.1-TOPO vector (K450002, Thermo Fisher Scientific, Waltham, MA, USA). After sequence verification, the *Sal*I/*Bam*HI DNA fragment containing the *ACR9* promoter was subcloned into the pBI101 binary vector. The resulting *ACR9p-GUS* fusion construct was transformed into the *Agrobacterium tumefaciens* strain GV3101 and then transformed into the WT Arabidopsis plants by floral dip. Several independent *ACR9p-GUS* Arabidopsis transgenic lines were carried to T3 homozygosity for GUS assays. Arabidopsis *ACR9p-GUS* transgenic plants were stained for GUS activity by submerging in a freshly prepared solution containing 1 mg mL^{-1} X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronidase), 100 mM sodium phosphate buffer (pH 7.0), 500 μM $K_3Fe(CN)_6$, 500 μM $K_4Fe(CN)_6$, and 0.05% Triton X-100. The samples were kept under vacuum for 15 min at room temperature and then incubated at 37 °C for 20 min to several hours. After staining, 75% ethanol was used to remove chlorophyll from the samples and for further preservation. Similar GUS staining results were obtained in 3 independent lines.

Complementation of *acr9-1* by *35S:ACR9* and *35S:ACR9-GFP*

The full-length *ACR9* cDNA containing the entire coding region and part of the 5′- and 3′-UTR sequences amplified by RT-PCR with primers 5′-TGTTGTTGATTCATTGGCTC-3′ and 5′-AGTAGTAGATGAATATATTG-3′ was cloned into the pCR2.1-TOPO vector (K450002, Thermo Fisher Scientific, Waltham, MA, USA) and verified by sequencing. The resulting *ACR9* cDNA was used as a template to amplify the coding sequence by PCR with primers 5′-CACGTCTAG AATGGGAATTCTCAACGACGA-3′ and 5′-CTATCTCGAG TCACCAACCCATAAGTGTCT-3′. The PCR products were digested with *Xba*I and *Sac*I and ligated to a binary vector, pSMAB704, digested with the same restriction enzymes. The resulting construct harboring the entire *ACR9* coding region driven by the CaMV 35S promoter, e.g. *35S:ACR9*, was transformed into *A. tumefaciens* GV3101. For the construction of *35S:ACR9-GFP*, full-length *ACR9* cDNA without the stop codon was produced by RT-PCR with primers 5′-CACCATGGGAATTCTCAACGACGAC-3′ and 5′-CCAA CCCATAAGTGTCTTT-3′. The PCR products were cloned into a Gateway pENTR/D-TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA). After sequence verification, the cDNA clone was recombined into the destination vector pGWB505, and the resulting *35S:ACR9-GFP* construct was transformed into *A. tumefaciens* GV3101. The *A. tumefaciens* strains containing the *35S:ACR9* and *35S:ACR9-GFP* constructs were transformed into the Arabidopsis *acr9-1* mutant for complementation assay. Several independent lines from both transformation events were carried to T3 homozygosity for further analysis.

Generation of *35S:ACR9* and *35S:ACR9-GFP* OE lines

The *35S:ACR9* and *35S:ACR9-GFP* constructs complementing the *acr9-1* mutant were transformed into Arabidopsis Col-0 to generate *ACR9* and *ACR9-GFP* OE lines in the WT

background. Three independent lines for the *35S:ACR9* and *35S:ACR9-GFP* constructs were carried to T3 homozygosity for further analyses.

Subcellular localization of ACR9-GFP

The protoplast transient expression assay was performed as previously described (Yoo et al. 2007). Protoplasts and seedlings of the *35S:ACR9-GFP* complementation and OE lines were observed under the Zeiss LSM 880 with Airyscan (https://www.zeiss.com/microscopy/). The fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) (D1306; Thermo Fisher Scientific, Waltham, MA, USA) was used to stain the nucleus.

Accession numbers

Sequence data from this article can be found in The Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org/) under the following accession numbers: ACR9, AT2G39570; PAL, AT2G37040; C4H, AT2G30490; 4CL, AT1G51680; CHS, AT5G13930; CHI, AT3G55120; F3H, AT3G51240; F3′H, AT5G07990; DFR, AT5G42800; LDOX, AT4G22880; UF3GT, AT5G54060; PAP1, AT1G56650; PAP2, AT1G66390; TT8, AT4G09820; GL3, AT5G41315; EGL3, AT1G63650; TTG1, AT5G24520; MYBL2, AT1G71030; JAZ1, AT1G19180; APL3, AT4G39210; BAM5, AT4G15210; HXK1, AT4G29130; TPS1, AT1G78580; AKIN10 (SnRK1.1), AT3G01090; AKIN11 (SnRK1.2), AT3G29160; PRL1, AT4G15900; RGS1, AT3G26090; GPA1, AT2G26300; SIS3, AT3G47990; STP1, AT1G11260; STP4, AT3G19930; STP7, AT4G02050; STP13, AT5G26340; SWEET1, AT1G21460; SWEET4, AT3G28007; SWEET5, AT5G62850; SWEET7, AT4G10850; SWEET13 AT5G50800; ACT2, AT3G18780; EF1α, AT1G07940.

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Author contributions

M.H.H. designed research; H.S.L., Y.J.C., W.Y.H., and Y.C.L. performed research; H.S.L. and Y.J.C. analyzed data; and M.H.H. wrote the paper.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Arabidopsis *ACR9* is highly expressed in leaves.

Supplemental Figure S2. Arabidopsis *acr9-1* and *acr9-2* mutants do not have aberrant phenotypes under standard growth conditions.

Supplemental Figure S3. The Arabidopsis WT and *acr9-1* mutant seedlings responded similarly to exogenous amino acids.

Supplemental Figure S4. *ACR9* expression was downregulated by Suc.

Supplemental Figure S5. Phenotypic analysis of Arabidopsis *acr9* mutants grown on tissue culture plates containing different sugars.

Supplemental Figure S6. *35S:ACR9* complements the *acr9-1* mutant.

Supplemental Figure S7. The Arabidopsis *acr9* mutants are hypersensitive to Glc in root growth.

Supplemental Figure S8. Expression of anthocyanin biosynthetic and regulatory genes in the *acr9* mutants.

Supplemental Figure S9. Expression of sugar signaling genes in the *acr9* mutants.

Supplemental Figure S10. Expression of sugar transport genes in the *acr9* mutants.

Supplemental Figure S11. *35S:ACR9-GFP* OE lines are Glc insensitive.

Supplemental Figure S12. *35S:ACR9* and *35S:ACR9-GFP* OE plants do not have aberrant phenotypes under standard growth conditions.

Supplemental Figure S13. The Arabidopsis *acr9* mutants are hypersensitive to ABA.

Supplemental Table S1. List of primers used for RT-qPCR and RT-PCR analysis.

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Conflict of interest statement. None declared.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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