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Research article

A basic helix-loop-helix 104 (bHLH104) protein functions as a transcriptional repressor for glucose and abscisic acid signaling in *Arabidopsis*

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ABSTRACT

Transduction of glucose (Glc) signaling is critical for plant development, metabolism, and stress responses. However, identifying initial Glc sensing and response stimulating mechanisms in plants has been difficult due to dual functions of glucose as energy sources and signaling component. A basic Helix-Loop-Helix 104 (bHLH104) protein is a homolog of bHLH34 previously isolated from *Arabidopsis* that functions as a transcriptional activator of Glc and abscisic acid (ABA) responses. In this study, we characterized bHLH104 as a transcription factor that binds to the regulatory region of *Arabidopsis Plasma membrane Glc-responsive Regulator* (*AtPGR*) gene. The bHLH104 binds to 5′-AANA-3′ element of the promoter region of *AtPGR in vitro* and represses beta-glucuronidase (GUS) activity in *AtPGR* promoter-GUS transgenic plants. Genetic approaches show that bHLH104 positively regulates Glc and abscisic acid (ABA) response. These results suggest that bHLH104 is involved in Glc- and ABA-mediated signaling pathway. Taken together, these findings provide evidence that bHLH104 is an important transcription regulator in plant-sensitivity to Glc and ABA signaling.

1. Introduction

Sessile plant needs to adapt to changing availability of nutrients in the environment. As a result, distinct plant developmental, physiological, biochemical and metabolic processes are regulated in response to changing levels of glucose (Mishra et al., 2009). Glucose (Glc) has profound effects on all stages of plant life, including germination, development, photosynthesis, stress response, and senescence (Rolland et al., 2002; Lee et al., 2015). Since Glc affects the expression of various genes involved in different cellular processes, diverse signaling pathways that can control these genes have been suggested. At least three Glc signal transduction pathways have been found in plants (Xiao et al., 2000; Price et al., 2003), including *Arabidopsis thaliana* hexokinase (AtHXK)-dependent pathways, AtHXK-independent pathways, and glycolysis-dependent pathways that require the catalytic activity of AtHXK.

Previous studies have also provided important evidence of Glc interactions with phytohormone response and other metabolic pathways (Price et al., 2004; Yuan and Wysocka-Diller, 2006). Among phytohormones, abscisic acid (ABA) is important for seed germination and seedling development. A few studies on *Arabidopsis* have provided evidence that Glc signaling is involved in ABA biosynthesis and signaling (Finkelstein and Gibson, 2002; Eckardt, 2002; Rook et al., 2006). High concentrations of Glc can increase the accumulation of ABA in seedlings (Arenas-Huertero et al., 2000). Many Glc-insensitive mutants displaying abnormal seedling development have been found to be allelic to genes involved in ABA biosynthesis or ABA signaling pathways (Laby et al., 2000; Gibson, 2005). For example, *Arabidopsis* sucrose uncoupled-6 gene has been found to be identical to *ABI4*, one of ABA-insensitive genes (Huijser et al., 2000).

Abbreviations: EMSA, electrophoresis mobility shift assay; GUS, β-glucuronidase; GFP, green fluorescent protein; qPCR, quantitative real-time polymerase chain reaction; WT, wild-type

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Expression of various genes involved in Glc signaling is generally regulated at transcriptional level. A number of *cis*-regulatory elements responsible for the regulation by Glc have been determined (Rolland et al., 2006). Several *cis*-elements in plant Glc-regulated promoters have also been identified, including G-box (Giuliano et al., 1988), sugar-response element (SURE) (Grierson et al., 1994), GCCT element (Chung et al., 2016), and GAGA element (Min et al., 2017).

Chung et al. (2011) have demonstrated Glc-regulated AtPGR is a plasma membrane-localized protein that contains seven predicted transmembrane motifs. *AtPGR*-overexpressing transgenic plants can regulate the induction of Glc and 2-deoxyglucose insensitivity under stress conditions whereas *atpgr* RNAi mutant appear to show increased sensitivity to Glc and 2-deoxyglucose during cotyledon greening, implying that AtPGR regulates Glc or Glc-mediated responses in *Arabidopsis* (Chung et al., 2011). Recently, we have performed functional characterization of a nuclear-localized basic Helix-Loop-Helix protein named bHLH34 (Min et al., 2017). Functional studies have demonstrated that bHLH34 is an activator of *AtPGR* transcription and a transcription factor in Glc-mediated signaling pathway. In addition, our previous genetic data have suggested that the function of bHLH34 is different from that of bHLH104, which is epistatic to bHLH34 in Glc response.

Here, we characterized the function of bHLH104 (At4g14410). We found that bHLH104 acted as a transcriptional repressor to bind 5′-AANA-3′ *cis*-element in *AtPGR* promoter. Overexpression of *bHLH104* in transgenic plants showed increased sensitivity to Glc and ABA in early seedling stage. These results suggest that bHLH104 and its highly homologous protein bHLH34 have different functions in Glc response.

2. Materials and methods

2.1. Plant materials, growth conditions, and stress induction

Arabidopsis thaliana (Col-0) seedlings were grown in a growth room under intense light at 22 °C with 60% relative humidity and long day conditions (16 h light/8 h dark). *bhlh104* T-DNA mutant was confirmed as previously described (Min et al., 2017) and used in this study. *AtPGR* promoter 999 (P999)-GUS construct was generated as described previously (Chung et al., 2016). It was used to construct the overexpression of *bHLH104* in P999-GUS transgenic lines. Full-length *bHLH104* cDNA (*At4g14410)* was amplified using primers shown in Supplementary Table S1. PCR product was then cloned into pDONR/ZEO vector followed by DNA sequence analysis. PCR amplification was performed with 35 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 0.5 min. DNA fragment was then cloned into plant expression vector pEarlyGate202 (Earley et al., 2006) using Gateway system (Invitrogen) according to the manufacturer's instruction. After that, the plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101. Transformants were then introduced into P999-GUS transgenic plants to generate *bHLH104*-overexpressing/P999-GUS lines via *in planta* vacuum infiltration (Bechtold and Pelletier, 1998). T_3 homozygous transgenic lines (OX2-1/P999-GUS, OX3-5/P999-GUS) were selected for GUS analysis. Phosphinothricin (Duchefa, Haarlem, Netherlands) resistance of T_2 generation from these selected lines segregated as a single locus. Constitutive CaMV35S promoter (35S PRO)-GUS served as a positive control for analysis of GUS activity. We obtained 35S PRO-GUS transgenic seeds from Dr. J.I. Kim (Han et al., 2015).

For Glc treatment, 12-day-old *Arabidopsis* seedlings were submerged in 6% Glc and sampled at 0, 6, and 12 h. For ABA treatment, 12-day-old *Arabidopsis* seedlings were submerged in 100 μM ABA at 0, 6, and 12 h. In each case, collected seedlings were promptly frozen in liquid nitrogen and stored at −80 °C.

2.2. Construction of recombinant bHLH104 protein

For DNA-binding analysis of bHLH104 protein, full-length cDNA fragments were PCR amplified using primers shown in Supplementary Table S1. These DNA fragments were then introduced into pJET1.2 vector (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) for DNA sequence analysis. DNA fragment was then cloned into pMAL-p2x vector (New England BioLabs, Beverly, MA, USA) according to the manufacturer's instruction. Recombinant protein was expressed in *Escherichia coli* (*E. coli*) strain BL21 (DE3) codon$^+$ (Stratagene, La Jolla, CA, USA) and purified using maltose binding protein (MBP) resin (New England BioLabs, Beverly, MA, USA).

2.3. Electrophoretic mobility shift assay (EMSA)

Four DNA fragments derived from the 5′-upstream region of *AtPGR* [P1, between nucleotide (nt) positions −999 and −875 relative to the *AtPGR* start codon site; P2, -874 to −735 nt; P3, -734 to −595 nt; and P4, -594 to −456 nt] were synthesized with a method described previously (Min et al., 2017). bHLH104 DNA binding elements (DBEs) oligonucleotides 104-DBE1 (−999 to −988 nt), 104-DBE2 (−888 to −877 nt), 104-DBE3 (−675 to −664 nt), 104-DBE4 (−506 to −495 nt), and E-box element were prepared using two complementary primers listed in Supplementary Table S1. These double-stranded complementary primers were radiolabeled with γ-^{32}P-ATP and T4 polynucleotide kinase (New England BioLabs) as probes. Reactions for DNA–protein binding were performed at 25 °C for 20 min in 20 μL volume containing 100 ng of each purified MBP-bHLH104 fusion protein, 1 μg of poly(dI-dC), 0.3 pmol of DNA fragments end-labeled with γ-^{32}P-ATP, and DNA-binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 2.5% glycerol, 1 mM EDTA, and 1 mM DTT). The mixture was subjected to 5% non-denaturing PAGE in TBE buffer (44.5 mM Tris-hydroxymethyl aminomethane, 44.5 mM boric acid, and 1 mM EDTA) at 100 V for 2 h at 4 °C. The gel containing DNA–protein complexes was dried and exposed to X-ray film.

2.4. Chromatin immunoprecipitation (ChIP)-qPCR analysis

0.8 g-1 g leaves of bHLH104-GFP overexpressing plants (see Supplementary Materials and methods) were cross-linked under vacuum in 1.0% (w/v) formaldehyde for 10 min, and was incubated in 125 mM glycine solution for 5 min to quench the cross-liking reaction. The cross-linked plant material was frozen in liquid nitrogen and ground to a fine powder. For all the following steps, the ChIP Kit-Plants (Abcam, Cambridge, MA, USA) was used according to the manufacturer's instructions. Nuclei was extracted and chromatin was sonicated on ice to obtain 0.5- to 1.5-kb fragments of DNA. The sonicated chromatin suspension was centrifuged for 10 min at 12,000 g at 4 °C to remove cell debris. 100 μL of the supernatant was immunoprecipitated with 2 μg of anti-GFP antibody (Thermo Fisher Scientific, Rockford, IL, USA) or mouse lgG (negative control) for overnight at 4 °C. Input chromatin (5 μL) was taken prior to immunoprecipitation and used as a DNA input for subsequent PCR. Reverse cross-linking was performed and DNA was extracted and purified using columns from the kit. Immunoprecipitated DNA was quantified by quantitative real-time PCR (qPCR) using specific primers. The primers used to amplify the promoter regions of *AtPGR* are shown in Supplementary Table S1. The ChIP-qPCR result was presented as percentage of input DNA.

2.5. Construction of bHLH104 promoter-GUS transgenic plants

To generate *bHLH104* promoter (PRO)-driven GUS construct, 1713-bp upstream genomic DNA fragment from the bHLH104 translation start codon was amplified by PCR with primers shown in Supplementary Table S1 and digested with *Pst*I and *Bam*HI. Fragments were then cloned into pCAMBIA1391 vector, resulting in a translational fusion of *bHLH104* promoter with GUS-coding region. Nucleotide sequences of the new construct were confirmed by DNA sequencing.

2.6. Generation of bHLH104-overexpressing transgenic lines

Total RNA samples were isolated from *Arabidopsis* leaves using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RT-PCR was used to obtain full-length *bHLH104* cDNA with primers shown in Supplementary Table S1. Amplification proceeded for 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min. The amplicon was then cloned into pDONR/ZEO vector and confirmed by DNA sequencing. The DNA fragment was then cloned into plant expression vector pGWB514 (Nakagawa et al., 2007) using the Gateway system (Invitrogen) according to the manufacturer's instruction. After that, the construct was introduced into *A. tumefaciens* strain GV3101 via *in planta* vacuum infiltration. Homozygous lines (T_3 generation) from 12 independent transformants were obtained and two lines of *bHLH104*-overexpressing transgenic plants (OX7-4 and OX8-5) showing high levels of transgene expression were selected for phenotypic characterization. Hygromycin (AG Scientific, San Diego, CA, USA) resistance of T_2 generation from these selected lines segregated as a single locus.

2.7. Phenotypic analysis

For Glc germination rate or cotyledon greening test, seeds were sown on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 5% Glc and grown in a growth chamber under intense light at 22 °C with long day conditions (16 h light-8 h dark). Germination was defined as an obvious protrusion of the root radicle through the seed coat. Germination rate of each line was measured for 1–9 days. Cotyledon greening was defined when a cotyledon was fully expanded and turned green. Green cotyledon was counted at 7 or 9 days. Experiments were carried out in triplicates for each line (50 seeds each).

For ABA germination rate or cotyledon greening tests, seeds were sown on MS medium supplemented with 0 or 1 μM ABA and allowed to grow in the growth chamber under the same conditions as Glc phenotypic assay. Germination rate of each line was measured for 1–7 days. Cotyledon greening of each seedling was measured at 10 or 12 days. Experiments were carried out in triplicate for each line (50 seeds each).

3. Results

3.1. bHLH104 represses the expression of AtPGR gene

bHLH104 and bHLH34 sequences are highly homologous, sharing 56% identities at amino acid (aa) level (Min et al., 2017). Furthermore, bHLH104 and bHLH34 harbor a conserved DNA-binding domain within the basic helix-loop-helix (bHLH) motif in their central region that is 88% identical between the two proteins (Fig. 1). Recently, we have demonstrated that bHLH34 can bind to GAGA Glc-response *cis*-element of the promoter upstream region of *AtPGR* and activates the expression of *AtPGR* gene during Glc treatment (Min et al., 2017). To determine whether bHLH104 could regulate transcriptional activity of *AtPGR* under Glc treatment condition, we constructed *AtbHLH104*-overexpressing plants under the control of CaMV35S (Cauliflower Mosaic Virus 35S) promoter in P999-GUS transgenic plants (see Materials and methods). Two individual lines (OX2-1/P999-GUS and OX-3-5/P999-GUS) showing high levels of *bHLH104* transgene expression (Supplementary Fig. S1A) were selected for the analysis of *AtPGR* transcriptional activity. As shown in Fig. 2A, P999-GUS expression was induced by Glc. However, under H_2O treatment condition, GUS expression in OX2-1/P999-GUS and OX3-5/P999-GUS lines was decreased approximately 1.49- and 2.19-fold, respectively, compared to that in P999-GUS plant. When 6% Glc was applied to seedlings for 12 h, GUS expression levels were significantly decreased (1.42- to 1.94-fold) in OX2-1/P999-GUS and OX3-5/P999-GUS lines compared to those in P999-GUS plants (Fig. 2A, Supplementary Figs. S1B and S1C) Particularly, the two P999-GUS transgenic lines with overexpressing *bHLH104* constructs did not further reduce the expression of P999-GUS after Glc treatment (Fig. 2A), indicating that other components may be require to repress Glc-mediated *AtPGR* expression. Constitutive CaMV35S promoter (35S PRO)-GUS served as a positive control for analysis of GUS

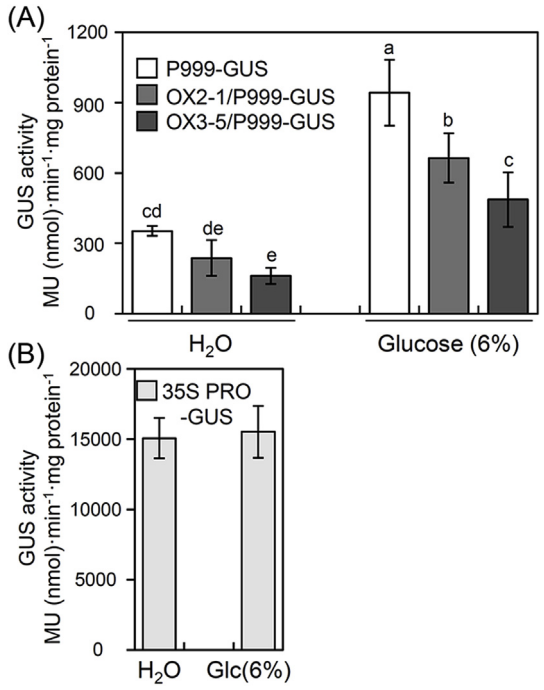

Fig. 2. bHLH104 represses *AtPGR* expressions during glucose condition. Activities of the 999-bp upstream promoter site (P999) of *AtPGR* were measured using GUS reporter in transgenic plants (P999-GUS). These P999-GUS transgenic lines overexpressing *bHLH104* (OX2-1/P999-GUS, OX3-5/P999-GUS) were analyzed histochemically after treated with H_2O or 6% glucose for 12 h. P999-GUS, OX2-1/P999-GUS, OX3-5/P999-GUS (A), and 35S PRO-GUS (B) seedlings grown on MS medium for 12 days were carefully taken out and treated with H_2O or 6% Glc for 12 h. Subsequently, seedlings were subjected to GUS staining and GUS activity was measured. 35S PRO-GUS served as a positive control to determine GUS activity. Values of GUS activities are averages of three independent enzymatic assays. Each assay was performed with extracts obtained from four individual seedlings of each transgenic plant. Error bars indicate standard deviations (ANOVA, $P < 0.05$).

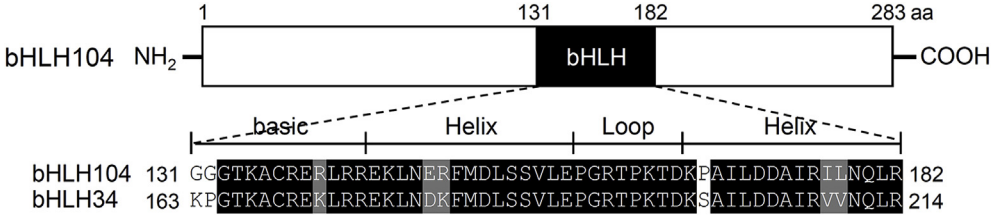

Fig. 1. Basic Helix Loop Helix (bHLH) motif of bHLH104 protein. The primary structure of bHLH104 harbors bHLH motif site (131–182) as shown in the black box. Sequences of bHLH104 (At4g14410) and bHLH34 (At3g23210) are shown. Alignment of bHLH motif deduced amino acid sequences of bHLH104 and bHLH34 from *Arabidopsis*. Black and gray shading indicate identical and similar amino acids, respectively.

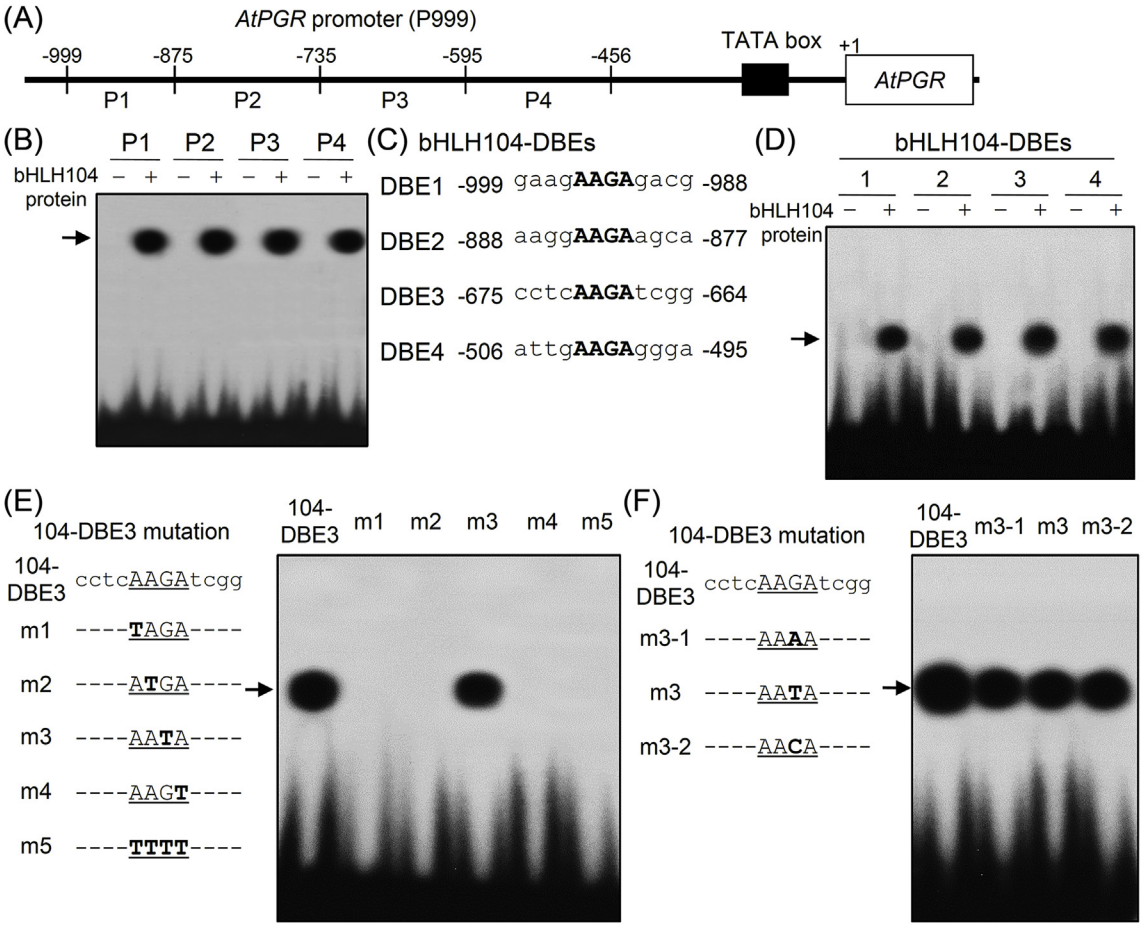

Fig. 3. EMSA analyses of bHLH104. (A) A schematic diagram showing the four (P1, P2, P3, and P4) fragments derived from the promoter 5′ upstream region (P999) of *AtPGR* gene. Numbers indicate nucleotide positions relative to the translation start site, ATG (A as +1). (B) Identification of DNA-binding promoter fragment of bHLH104 protein. Experiments were performed two times and similar results were obtained. ^{32}P-radiolabeled P1, P2, P3, and P4 DNA fragments were incubated in the absence (−) or presence (+) of MBP-bHLH104 (arrow). (C) Sequences of the four DNA binding elements (DBEs) in *AtPGR* promoter. AAGA motifs are shown in bold. (D) Identification of the DNA-binding promoter site in bHLH104 protein. Experiments were carried out two times and similar results were obtained. Lane (−), ^{32}P-radiolabeled bHLH104 DBE1-4 oligonucleotides incubated in the absence of MBP-bHLH104; lane (+), ^{32}P-radiolabeled DBE1-4 oligonucleotides incubated in the presence of MBP-bHLH104 (arrow). (E) bHLH104 binds to AAGA and AATA elements. A list of sequences of 104-DBE3 and m1 to m4 point-mutated or four-nucleotide-mutated m5 probes. Mutated nucleotides are boldfaced. The binding sequence of bHLH104 was determined using various mutated sequences. The arrow indicates the position of DBE3 probe- and m3 probe-MBP-bHLH104 complex. (F) bHLH104 binds to AANA elements. A list of sequences of 104-DBE3, m3-1, m3, and m3-2 probes. Mutated nucleotides are boldfaced. The binding sequence of bHLH104 was determined using various mutated sequences. The arrow indicates the position of DBE3 probe- and several m3 mutated probe-MBP-bHLH104 complex.

activity (Fig. 2B, Supplementary Figs. S1B and S1C). These results indicate that bHLH104 can regulate the expression of *AtPGR* gene under Glc condition and function as a transcriptional repressor.

3.2. bHLH104 binding to the AANA cis-element of AtPGR promoter in vitro

Previously, we have reported that bHLH34 acts as an activator of *AtPGR* gene in Glc signaling (Min et al., 2017). As shown in Fig. 2, bHLH104 acted as a repressor of *AtPGR* gene in Glc signaling, implying that bHLH104 and bHLH34 might bind to different *cis*-elements for Glc signaling, although bHLH104 and bHLH34 amino acids sequences were highly homologous. Therefore, we need to further study *cis*-element of bHLH104 for Glc signaling. We attempted to express and purify bHLH104 protein in *E. coli* to undertake electrophoretic mobility shift assay (EMSA) with promoter in the region −999 to −456 of *AtPGR* (Fig. 3A). First, EMSA was carried out to test whether bHLH104 could interact with each of six bHLH34-DNA binding elements (34-DBEs) of *AtPGR* promoter region having conserved *cis*-elements (5′-GAGA-3′) (Supplementary Fig. S2A). Purified bHLH104 protein could only bind to one oligonucleotides 34-DBE1 (Supplementary Fig. S2B, lane 1). This indicates that bHLH104 is a DNA-binding protein that can bind to different DNA sites having bHLH34 *cis*-element (5′-GAGA-3′) of *AtPGR* promoter region. To investigate bHLH104's binding site, EMSA was carried out to test whether bHLH104 could interact with each of the four DNA fragments (P1: −999 to −875 nt, P2: −874 to −735 nt, P3: −734 to −595 nt, and P4: −594 to −456; Fig. 3A). Purified bHLH104 protein could bind to all four fragments (P1, P2, P3, and P4) as shown in Fig. 3B.

To further investigate bHLH104's binding sites, we aligned sequences of the P1-P4 and 34-DBE1 fragments and found that there were conserved 5′-AAGA-3′ *cis*-elements at four regions of three DNA fragments (P1, P3, and P4, but not P2) and 34-DBE1 sequences in the alignment (Fig. 3C). These four bHLH104 DNA binding elements (104-DBEs) were named 104-DBE1 (P1: −999 to −988 nt), 104-DBE2 (P1: −888 to −877 nt), 104-DBE3 (P3: −675 to −664 nt), and 104-DBE4 (P4: −506 to −495). They were employed as probes in EMSA (Fig. 3C). Purified bHLH104 protein could bind strongly to all four oligonucleotides (104-DBE1 to 104-DBE4) (Fig. 3D).

To investigate whether bHLH104 could recognize these oligonucleotides, excess unlabeled 104-DBE3 was added. An addition of a molar excess of the unlabeled 104-DBE3 oligonucleotide probe gradually diminished shifted bands of bHLH104 protein in a dose-dependent

manner (Supplementary Fig. S3A). These findings indicate that bHLH104 can recognize these four 104-DBEs containing the consensus AAGA sequence.

To verify the specificity of binding between bHLH104 and the AAGA element, an EMSA was performed using various mutated 104-DBE3 oligonucleotide fragments (Fig. 3E). Mutations introduced in the AAGA sequence inhibited the binding of bHLH104 (Fig. 3E, lanes m1, m2, m4, and m5) except m3 oligonucleotide (Fig. 3E). To further verify the specificity of binding between bHLH104 protein and m3 oligonucleotide, an EMSA was performed using two mutated m3-1 and m3-2 oligonucleotide fragments (Fig. 3F). Mutations introduced into m3-1 and m3-2 element sequences could bind to bHLH104 (Fig. 3F, lanes m3-1 and m3-2). These results demonstrate that bHLH104 can bind to P2 DNA fragment which contains one 5′-AAAA-3′ element (P2: −845 to 842 nt), implying that bHLH104 has sequence-specific binding affinity for 5′-AANA-3′ element *in vitro*.

On the other hand, consensus DNA sequence recognized by bHLH protein is well known as the E-box (5′-CANNTG-3′) (Robinson et al., 2000; Min et al., 2017). Single copy E-box (between positions −793 and −778 nt) exists in P999 site of the *AtPGR* gene. To determine whether bHLH104 protein could recognize the E-box, we conducted EMSA assay with probe containing the E-box sequence (Supplementary Fig. S3B). Our results showed that bHLH104 could bind to E-box sequence (Supplementary Fig. S3B). This result indicates that bHLH104 not only can bind to AANA *cis*-element, but also can bind to E-box sequence.

To examine whether bHLH104 interacts with the *AtPGR* promoter *in vivo*, a chromatin immunoprecipitation (ChIP)-qPCR analysis was carried out. In ChIP-qPCR analysis, bHLH104-GFP (Green Fluorescent Protein) was immunoprecipitated using an anti-GFP antibody after bHLH104-GFP was cross-linked with DNA. *AtPGR* promoter fragments were observed only as bHLH104-GFP was precipitated (Supplementary Fig. S3C), indicating that bHLH104 binds the *AtPGR* promoter *in vivo*.

3.3. bHLH104 localizes in the nucleus

To determine the subcellular localization of bHLH104, we constructed *Arabidopsis* transgenic plants expressing bHLH104-GFP (Green Fluorescent Protein) under control of a cauliflower mosaic virus 35S promoter. As shown in Fig. 4A, the fluorescent signal of the bHLH104-GFP construct was relatively strong in the nuclei of root cells of transgenic seedlings. This result indicates that bHLH104 is a nuclear-localized protein, consistent with its predicted function as a transcription factor.

3.4. bHLH104 expression in different organs and developmental stages

To gain insights into tissue expression pattern of *bHLH104*, we analyzed expression patterns of *bHLH104* in various organs and at different developmental stages of *Arabidopsis* seedlings by quantitative real-time PCR (qPCR). Results of qPCR experiments showed that *bHLH104* was expressed relatively strongly in roots and leaves but weakly in stems and flowers (Fig. 4B). Next, to analyze expression level of *bHLH104* during leaf developmental stage from 1 week after germination (WAG) to 5 WAG, qPCR assay was performed. As shown in Fig. 4C, *bHLH104* transcripts were similar between 1 WAG and 5 WAG stages. Its expression level was higher at 2, 3, and 4 WAG stages than that at 1 or 5 WAG.

3.5. bHLH104 is regulated by glucose and ABA

To determine *in vivo* functions of bHLH104, accumulation of *bHLH104* transcript was assessed in *Arabidopsis* seedlings using qPCR during Glc and ABA treatment. *bHLH104* level was decreased in seedlings at indicated time after Glc treatment (Fig. 5A). In contrast, the expression level of *bHLH104* was increased at 6 h and 12 h after ABA

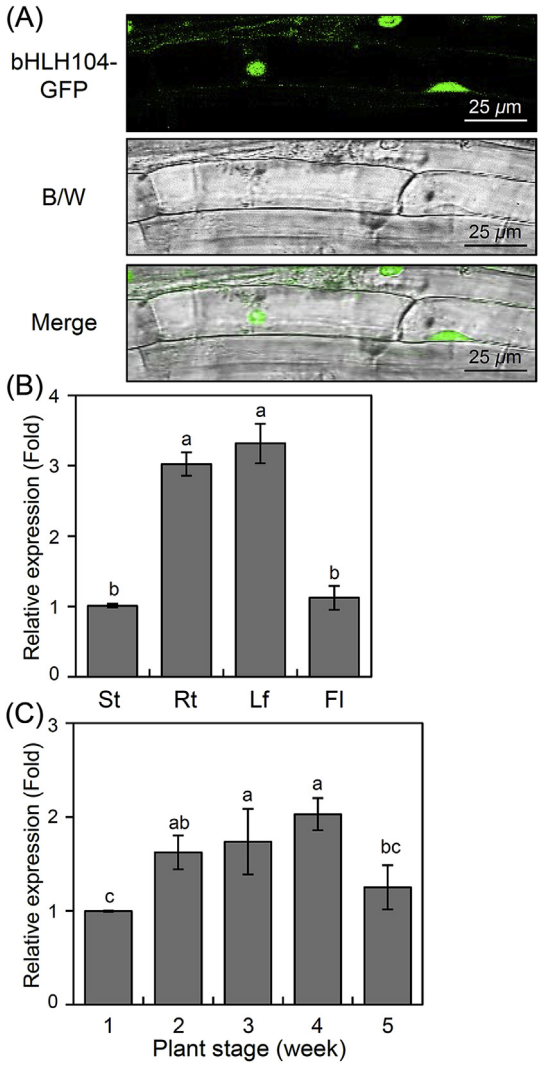

Fig. 4. Nuclear localization and expression profiles of *bHLH104* in *Arabidopsis*. (A) Seven-day-old transgenic plants grown on sterile MS medium were analyzed for GFP expression by confocal microscopy. Green fluorescent signal of bHLH104-GFP was observed in the nuclei of root cells. GFP, green fluorescent protein; B/W, black and white. Scale bars = 25 μm. (B and C) Expression profiles of *bHLH104* gene in various organs and at different plant stages. Results represent the average of three independent biological replicates (n = 4-20, mean values ± SD, ANOVA, $P < 0.05$). (B) RNA levels were confirmed by qPCR using total RNA isolated from stems (St), roots (Rt), leaf (Lf), and flowers (Fl). (C) RNA levels were determined by qPCR using total RNA isolated at indicated plant sampling stage. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

treatment (Fig. 5B). Stress-inducible *Arabidopsis thaliana Hexokinase 1* (*AtHXK1*) and *Responsive to ABA 18* (*RAB18*) genes were used as references for Glc and ABA stress treatment (Fig. 5A and B). These results demonstrate that *bHLH104* is regulated by Glc and ABA.

To further determine the tissue specificity of bHLH104, its Glc-reducible and ABA-inducible expression patterns were examined by histochemical GUS staining of *bHLH104* promoter (*bHLH104* PRO)-GUS transgenic plants. GUS activity of 12 day-old-transgenic seedlings was detected in leaves and roots, but not in hypocotyl under normal condition (H_2O) (Fig. 5C). After treatment with 6% Glc, GUS expression was strongly down-regulated in rosette leaves (Fig. 5C and D). However, GUS activity was slightly up-regulated in leaves and hypocotyl under 100 μM ABA treatment (Fig. 5C and D). Since histochemical GUS staining experiments could not provide a quantitative measure of GUS expression level, quantitative experiment of GUS activity was

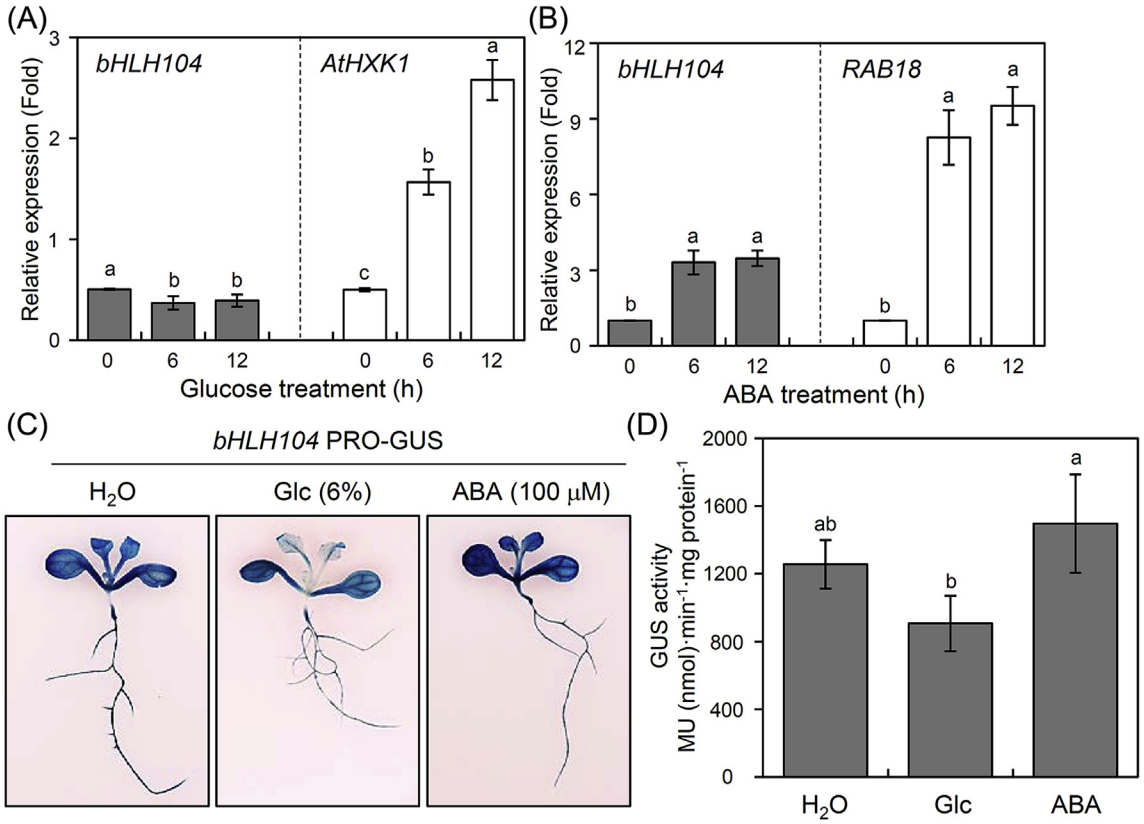

Fig. 5. Expression levels of *bHLH104* in *Arabidopsis* seedlings and *bHLH104* PRO-GUS transgenic lines under normal, glucose, and ABA conditions. (A and B) Expression of *bHLH104* in *Arabidopsis* under glucose and ABA conditions. Expression levels of *bHLH104* involved in glucose (A) and ABA (B) responses were determined by qPCR analyses. Total RNA samples were obtained from 12-day-old seedlings treated with 6% glucose or 100 μM ABA at indicated time point. Error bars indicate standard deviations of three independent experiments (ANOVA, $P < 0.05$). Each experiment was performed with total RNA of each sample obtained from fifteen seedlings. *Arabidopsis Actin 1* was used as the internal control. *AtHXK1* (A) or *RAB18* (B) gene served as a control for glucose or ABA treatment, respectively. (C and D) Activities of 1713-bp regulatory promoter region of *bHLH104* gene were examined by means of GUS as a reporter in transgenic plants (*bHLH104* PRO-GUS). These transgenic plants were analyzed histochemically after treatment with H_2O, 6% glucose, or 100 μM ABA for 6 h (C). Subsequently, seedlings were subjected to GUS staining and GUS activity was measured (D). Values of GUS activities are averages of three independent enzymatic assays. Each assay was performed using extracts obtained from five individual seedlings of each transgenic plant. Error bars indicate standard deviations (ANOVA, $P < 0.05$).

performed (Fig. 5D). Overall, data of these assays were consistent with those obtained from qPCR assay of *bHLH104* transcript.

3.6. bHLH104 decreases the expression of AtPGR gene

To investigate whether bHLH104 also regulated the transcriptional activity of *AtPGR* under ABA condition, we analyzed GUS activities in P999-GUS, OX2-1/P999-GUS, and OX-3-5/P999-GUS transgenic plants after ABA treatment. As shown in Fig. 6, GUS expressions levels in two OX2-1/P999-GUS, and OX-3-5/P999-GUS transgenic lines were decreased approximately 1.49- and 2.19-fold, respectively, compared to those in P999-GUS lines under untreated condition (Fig. 6A and C). When 100 μM ABA was applied to seedlings for 12 h, GUS expression levels were significantly decreased (1.3- to 1.7-fold) in OX2-1/P999-GUS and OX3-5/P999-GUS lines in comparison with those in P999-GUS plant (Fig. 6B and C). These results indicate that the expression of *AtPGR* gene is decreased by ABA hormone and that bHLH104 acts as a repressor of *AtPGR* transcription under ABA condition. However, the ABA treatment did not resulted in the significant differences of the GUS expressions (Fig. 6C), indicating that bHLH104 requires additional components in the suppression of ABA-mediated *AtPGR* expression.

3.7. Glucose and ABA responses in bHLH104 transgenic plants

To determine whether *bHLH104* was related to the ABA and Glc responses, *bHLH104* was overexpressed in *Arabidopsis* transgenic plants under the control of CaMV 35S promoter. Fifteen homozygous lines (T_3 generation) were obtained and two independent lines (OX7-4 and OX8-5) showing high levels of transgene expression (Supplementary Fig. S4A) were selected for phenotypic characterization. To further evaluate functional consequences of destruction of *bHLH104*, we obtained *At4g14410*-tagged T-DNA insertion mutant SALK_005802. The absence of *bHLH104* transcript was confirmed by qPCR (Supplementary Fig. S4A). The mutant was designated as *bhlh104*. No growth differences were observed among WT, *bHLH104*-overexpressing plants, and *bhlh104* mutant when they were grown on MS medium (Supplementary Fig. S4B).

To characterize the effect of *bHLH104* expression on Glc and ABA response, seeds of WT, *bhlh104*, and *bHLH104*-overexpressing plants were germinated on MS medium containing 5% Glc and 1 μM ABA. Germination percentage of *bHLH104*-overexpressing transgenic lines was much more affected than that of WT and *bhlh104* mutant by treatment with 5% Glc and 1 μM ABA. The germination rate of *bhlh104* mutant was slightly higher than that of the WT in the Glc treatment (Fig. 7A), while nearly no difference was observed in the presence of ABA (Fig. 7C). Glc- or ABA-induced sensitivity was also evaluated by measuring cotyledon greening rate. The relative reduction in cotyledon greening of *bHLH104*-overexpressing lines in response to Glc was more profound than that of the WT and *bhlh104* at 7 or 9 days after germination. The cotyledon greening efficiency was 31% for WT and 41.2% for *bhlh104* during treatment with Glc for 9 days. In contrast, the cotyledon greening efficiency for *bHLH104*-overexpressing lines was

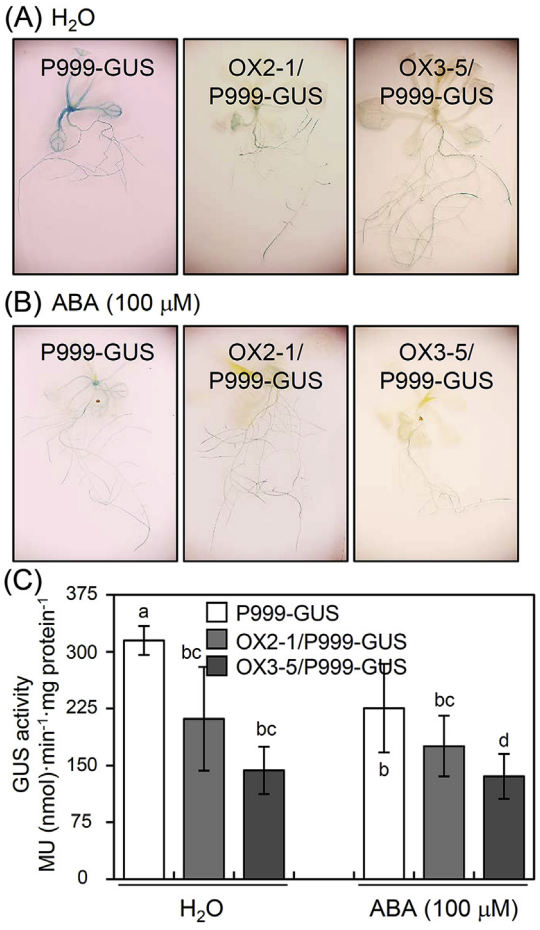

Fig. 6. bHLH104 represses *AtPGR* expressions during ABA condition. (A and B) Activities of 999-bp upstream promoter site (P999) of *AtPGR* were measured using GUS reporter in transgenic plants (P999-GUS). These P999-GUS transgenic lines overexpressing *bHLH104* (OX2-1/P999-GUS, OX3-5/P999-GUS) were analyzed histochemically after treatment with H_2O (A) or 100 μM ABA (B) for 12 h. (C) P999-GUS, OX2-1/P999-GUS, and OX3-5/P999-GUS seedlings grown on MS medium for 12 days were carefully taken out and treated with H_2O or 100 μM ABA for 12 h. Subsequently, seedlings were subjected to GUS staining and GUS activity was measured. Values of GUS activities are averages of three independent enzymatic assays. Each assay was performed with extracts obtained from three individual seedlings of each transgenic plant. Error bars indicate standard deviations (ANOVA, $P < 0.05$).

19.8–22.8% (Fig. 7B and Supplementary Fig. S4C). These results demonstrate that bHLH104 is required for Glc-modulated seed germination and cotyledon greening in *Arabidopsis*.

To further characterize effects of ABA on *bHLH104*-overexpressing and *bhlh104* plants, we evaluated plant response to treatment with 1 μM ABA. The cotyledon greening rate of the WT was slightly above 40% at 12 days after germination. Fewer than 60% of *bhlh104* remained cotyledon greening compared to 31.5–33.1% in *bHLH104*-overexpressing lines (OX7-4 and OX8-5) (Fig. 7D and Supplementary Fig. S4D). These results indicate that *bHLH104*-overexpressing lines are more likely to be sensitive to ABA than WT and *bhlh104* mutant. Taken together, the genetic characterization show that bHLH104 positively regulates responses to Glc and ABA.

3.8. *AtPGR expression in bHLH34 transgenic plants upon Glc treatment*

To determine *AtPGR* expression in *bHLH104* transgenic lines after 6% Glc treatment, qPCR experiment was conducted. Fig. 8 shows that *AtPGR* transcript levels in *bHLH104*-overexpressing transgenic lines without Glc treatment appear to be lower than those in WT and *bhlh104* plants. As expected, *AtPGR* expressions were reduced by Glc treatment in *bHLH104*-overexpressing transgenic (OX7-4 and OX8-5) lines compared to those in WT and *bhlh104*. However, transcript accumulation of *AtPGR* showed slightly induction in *bhlh104* compared to that in WT during Glc treatment. These data suggest that AtPGR is involved in Glc response through bHLH104-mediated signaling transduction.

4. Discussion

Previously, we have reported that bHLH34 acts as an activator of the *AtPGR* gene in Glc signaling (Min et al., 2017). The predicted bHLH104 protein possessed basic helix-loop-helix motif in its central region (Fig. 1). Amino acid sequence analysis indicated that bHLH104 shared high homology with bHLH34 (Fig. 1). Considering the great similarity between bHLH104 and bHLH34 (Fig. 1), it was appropriate to consider that bHLH104 and bHLH34 might perform similar function in Glc response. Here, our results suggest that bHLH104 regulates *AtPGR* expression different from bHLH34 transcriptional activator during normal and Glc conditions. As shown in Fig. 2A and Supplementary Fig. S1, *bHLH104*-overexpressing constructs in P999-GUS lines more decreased in GUS activity under Glc untreated or treated conditions than P999-GUS alone lines. In addition, transcript levels of *AtPGR* gene were decreased in *bHLH104*-overexpressing lines than those in WT and *bhlh104* mutant under Glc untreated or treated conditions. However, *AtPGR* expression in *bhlh104* mutant was slightly higher than that in WT plants (Fig. 8). These data suggest that bHLH104 acts as a repressor of *AtPGR* in Glc response. The P999-GUS transgenic lines with overexpressing *bHLH104* constructs did not more decrease the expression of P999-GUS under Glc condition (Fig. 2A), suggesting that other redundant factors requires to repress Glc-mediated *AtPGR* expression. Previously, AtSTKL1 (for *A. thaliana* Storekeeper-like 1) and AtSTKL2 function as both repressors of *AtPGR* transcription and transcription factors in the Glc signaling pathway (Chung et al., 2016). Thus, other repressors, such as AtSTKL1 and AtSTKL2, may be involved in the complex expression of *AtPGR* gene in Glc response. Overall, bHLH104 and bHLH34 displayed different regulation in *AtPGR* expression during Glc condition. This implies that bHLH104 and bHLH34 are likely to interact with different *cis*-elements of *AtPGR* upstream promoter. Our EMSA experiments showed that the preferred DNA-binding site of bHLH104 *in vitro* was a 5′-AANA-3′ containing element and E-box (5′-CACTTG-3′) sequences in the *AtPGR* upstream promoter (Fig. 3, Supplementary Figs. S2 and S3). Furthermore, ChIP-qPCR analyses showed that bHLH104 binds to the promoter of *AtPGR in vivo* (Supplementary Fig. S3C), indicating bHLH104 directly targets *AtPGR*. Previously, Min et al. (2017) have reported that bHLH34 can bind to both 5′-GAGA-3′ and E-box element sequences. Therefore, bHLH104 and bHLH34 require different coupling *cis*-elements to regulate the transcriptional activity of *AtPGR* gene. These results suggest that the E-box element might serve as a basic Glc-responsive *cis*-element sequence and that its combinatorial interaction with other *cis*-element sequences might determine the expression level of *AtPGR* gene.

In the present study, *bHLH104*-overexpressing lines displayed enhanced sensitivity to Glc response in comparison with WT plants whereas *bhlh104* mutant displayed enhanced insensitivity to Glc condition during seed germination and cotyledon greening (Fig. 7 and Supplementary Fig. S4). These results imply that bHLH104 is a regulatory component for Glc signaling pathway. Min et al. (2017) previously demonstrated that *bhlh104/bhlh34* double mutant phenocopied the Glc insensitivity of *bhlh104* single mutant, indicating that bHLH104 is epistatic to bHLH34 in Glc response. Recently, Li et al. (2016) reported that bHLH104 and bHLH34 positively regulate iron homeostasis in *Arabidopsis* as a homo- and hetero-dimer, and we found that bHLH104 and bHLH34 bind to different *cis*-elements in response to Glc. Thus, it is possible that the bHLH104-bHLH34 complex cooperatively regulates the *AtPGR* expression by binding on the different *cis*-elements in Glc signaling.

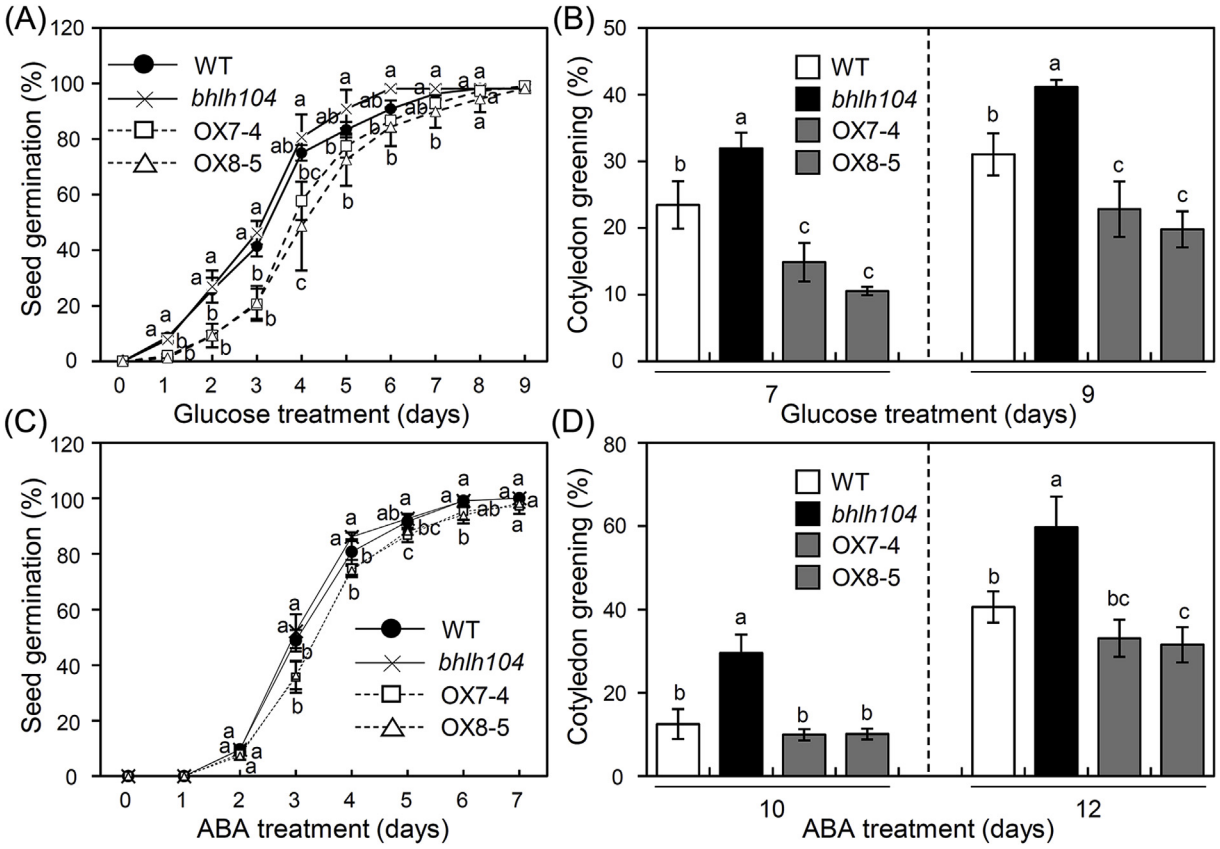

Fig. 7. Genetic analysis of *bHLH104* transgenic plants on glucose and ABA sensitivity. (A) Seed germination assays in glucose condition. Seeds of WT, *bhlh104*, *bHLH104*-overexpressing (OX7-4 and OX8-5) plants were sown on sterile MS medium supplement with 5% glucose and permitted to grow for indicated days. Germination was then scored (triplicates, n = 50 each). Error bars indicate standard deviations for three independent experiments (50 seeds per point, ANOVA, $P < 0.05$). (B) Effects of glucose treatment on cotyledon greening. Seeds were sown on MS medium supplement with 5% glucose and permitted to grow for 7 or 9 days. Green cotyledons were then counted (triplicates, n = 50 each). Error bars indicate standard deviations for three independent experiments (ANOVA, $P < 0.05$). (C) Sensitivity of germination to ABA. Seeds were sown on MS agar plates supplement with 1 μM ABA and allowed to grow for indicated days. Germination was then counted (triplicates, n = 50 each). Error bars indicate standard deviations for three independent experiments (ANOVA, $P < 0.05$). (D) Sensitivity of cotyledon greening to ABA. Seeds were sown on MS medium containing 1 μM ABA and permitted to grow for 10 or 12 days. Seedlings with green cotyledons were then counted (triplicates, n = 50 each). Error bars indicate standard deviations for three independent experiments (ANOVA, $P < 0.05$).

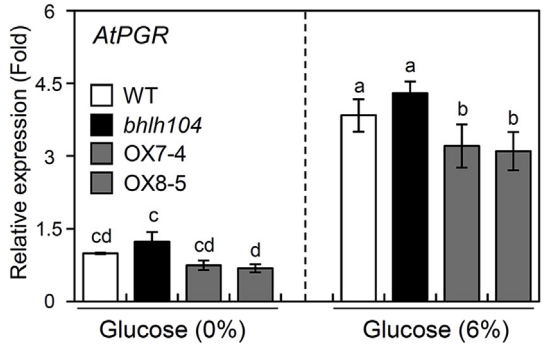

Fig. 8. Expression levels of glucose-inducible *AtPGR* gene in WT, *bhlh104*, and *bHLH104*-overexpressing transgenic plants. mRNA levels of *AtPGR* were measured by qPCR using total RNA from 12-day-old WT, *bhlh104*, and two independent *bHLH104*-overexpressing (OX7-4 and OX8-5) seedlings treated with 6% Glc for 6 h. The mean value of three technical replicates was normalized to the level of *Actin* 1 mRNA as an internal control. Error bars indicate standard deviations (n = 15 each, ANOVA, $P < 0.05$).

We have previously shown that *bHLH34* expression is regulated not only by Glc but also by ABA (Min et al., 2017), implying that *bHLH104* possibly regulates ABA response. To test this possibility, GUS activity was investigated in *bHLH104*-overexpressing/P999-GUS transgenic plants with or without ABA treatment. As shown in Fig. 6, GUS activity appeared to be more reduced in *bHLH104*-overexpressing/P999-GUS lines compared to that in P999-GUS transgenic plants with or without ABA application. These data indicate that the bHLH104 can also serve as a repressor of the *AtPGR*, such as Glc signaling in ABA response. Additionally, *bHLH104*-overexpressing transgenic lines showed enhanced sensitivity to ABA in comparison with WT and *bhlh104* whereas *bhlh104* mutant showed enhanced insensitivity to ABA condition during cotyledon greening (Fig. 7 and Supplementary Fig. S4), implying that bHLH104 displayed similar physiological phenotypes between glucose and ABA response in early seedling stage.

In conclusion, we found that bHLH104 transcription factor could bind to the AANA containing regulatory sequences of *AtPGR* upstream promoter *in vitro*. Furthermore, we showed that Glc- and ABA-reduced GUS expression driven by AANA *cis*-element sequences of *AtPGR* promoter was dependent upon the presence of bHLH104 transcriptional repressor *in vivo*. This mechanism may cause the sensitive phenotype in Glc and ABA responses in the presence of *bHLH104* gene during early seedling stage which may regulate the expression of Glc- and ABA-responsive genes. To clarify the precise molecular functions of bHLH104 transcription factor in Glc and ABA response, Chip-assay needs to be performed in further experiments. Particularly interesting challenges are to identify bHLH104-interacting proteins by yeast two-hybrid screening approach. Results of such experiment will provide a better understanding of cellular functions of bHLH104 transcriptional repressor in Glc or ABA responses of plants.

Contributions of authors

The work presented here was carried out in collaboration between all authors; JH Min, CR Park, YH Jang and CS Kim designed research; JH Min, CR Park, YH Jang, HW Ju, KH Lee and SB Lee performed research; JH Min, CR Park and CS Kim analyzed data; JH Min and CS Kim wrote the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.01.008.

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