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Research article

Chromatin remodeling for the transcription of type 2C protein phosphatase genes in response to salt stress

Nguyen Hoai Nguyen[a], Choonkyun Jung[b], Jong-Joo Cheong[a,*]

[a] Center for Food and Bioconvergence, Seoul National University, Seoul, 08826, Republic of Korea
[b] Department of International Agricultural Technology, Institutes of Green Bio Science and Technology, Seoul National University, Pyeongchang, 25354, Republic of Korea

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ABSTRACT

Type 2C protein phosphatases (PP2Cs) counteract protein kinases, thereby inhibiting the abscisic acid (ABA)-mediated response to abiotic stress in *Arabidopsis thaliana*. In the absence of stress, the promoters of PP2C genes (e.g., *ABI1*, *ABI2*, and *HAI1*) are negatively regulated by repressors that suppress gene transcription in a signal-independent manner. Quantitative reverse transcription PCR (RT-qPCR) and chromatin immunoprecipitation (ChIP) assays revealed that the levels of PP2C gene transcripts and RNA polymerase II (RNAPII) that stalled at the transcription start sites (TSS) of PP2C gene loci were increased under salt stress. The salt-induced increases in RNA polymerase-mediated transcription were reduced in *35S:AtMYB44* plants, confirming that AtMYB44 acts as a repressor of PP2C gene transcription. ChIP assays revealed that AtMYB44 repressors are released and nucleosomes are evicted from the promoter regions in response to salt stress. Under these conditions, histone H3 acetylation (H3ac) and methylation (H3K4me3) around the TSS regions significantly increased. The salt-induced increases in PP2C gene transcription were reduced in *abf3* plants, indicating that ABF3 activates PP2C gene transcription. Overall, our data indicate that salt stress converts PP2C gene chromatin from a repressor-associated suppression status to an activator-mediated transcription status. In addition, we observed that the Arabidopsis mutant *brm-3*, which is moderately defective in SWI2/SNF2 chromatin remodeling ATPase BRAHMA (BRM) activity, produced more PP2C gene transcripts under salt stress conditions, indicating that BRM ATPase contributes to the repression of PP2C gene transcription.

1. Introduction

The clade A type 2C protein phosphatases (PP2Cs) counteract sucrose non-fermenting 1-related protein kinases (SnRKs), allowing plant cells to maintain the phosphorylation balance needed to control abscisic acid (ABA) signaling processes (Ma et al., 2009; Umezawa et al., 2010). It has been suggested that PP2Cs physically interact with SnRK2s to form complexes, inactivating the kinases via dephosphorylation (Umezawa et al., 2009). Moreover, under abiotic stress conditions, the soluble ABA receptors PYR/PYL/RCAR capture ABA and then bind to PP2Cs, inhibiting phosphatase activity (Ma et al., 2009; Park et al., 2009). SnRK2s are released from the PP2C–SnRK2 complex and phosphorylate ABA-responsive element-binding factors (AREB/ABFs), activating the expression of ABA-responsive genes (Fujita et al., 2013).

Expression of PP2C genes is suppressed by a number of MYB transcription factors in a signal-independent manner. Cui et al. (2013) showed that expression of a group of PP2C genes, such as *ABI1*, *ABI2* and *AtPP2CA*, was suppressed in *AtMYB20*-overexpressing Arabidopsis transgenic lines but induced in repression lines in response to salt treatment. We previously performed microarray and northern blot analyses and observed that salt-induced expression of a group of PP2C genes, such as *ABI1*, *ABI2*, *AtPP2CA*, *HAB1*, and *HAB2*, was significantly suppressed in *AtMYB44*-overexpressing transgenic Arabidopsis (Jung et al., 2008). Recently, we reported experimental data indicating that AtMYB44 physically interacts with a TOPLESS (TPL)-RELATED (TPR) corepressor through the putative ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR-ASSOCIATED AMPHIPHILIC REPRESSION (EAR) motif in the C-terminal catalytic domain (Nguyen and Cheong, 2018a). The AtMYB44-TPR complex represses transcription of PP2C genes via the promotion of histone deacetylation at the genes' loci.

Expression of PP2C genes is induced in response to abiotic stresses such as salinity and drought (*Singh* et al.; 2016). To initiate transcription of a gene in eukaryotes, the chromatin structure must be modified to facilitate the accessibility of DNA to RNA polymerase II (RNAPII)

* Corresponding author.
E-mail address: cheongjj@snu.ac.kr (J.-J. Cheong).

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(Jonkers and Lis, 2015). Thus, gene transcription is accompanied by nucleosome eviction or replacement with other nucleosomes with different compositions (Venkatesh and Workman, 2015). Wang et al. (2018) reported that ABF1, ABF2/AREB1, ABF3 and ABF4/AREB2 act as activators for ABA-induced expression of group A PP2C genes, suggesting a role in negative feedback regulation of ABA signaling.

Chromatin remodeling ATPase BRAHMA (BRM) is a SWI2/SNF2 subfamily ATPase that hydrolyze ATP to obtain the energy necessary to alter the interaction of nucleosomes with DNA, in order to change the position and occupancy (Clapier and Caines, 2009; Hargreaves and Crabtree, 2011; Narlikar et al., 2013). The Arabidopsis BRM plays a role in the ABA-mediated drought stress response (Han et al., 2015). Peirats-Llobet et al. (2016) showed that core components of the ABA signaling pathway physically interact with BRM remodelers and modify them post-translational through phosphorylation and dephosphorylation. Yang et al. (2015) showed that Arabidopsis BRM directly targets the *PIN-FORMED* (*PIN*) gene locus, which is required for root stem cell niche maintenance. Additionally, Zhang et al. (2017) reported that BRM regulates chlorophyll biosynthesis in Arabidopsis.

We investigated chromatin modifications at PP2C gene loci in response to salt stress, following a gene transcription mechanism. Our data showed that PP2C gene chromatins were converted from a repressor-associated suppression status to an activator-mediated transcription status. In response to salt stress, PP2C gene promoters release the AtMYB44 repressor. Nucleosomes are evicted and their histone components are modified during the transition to an activated status. Then, ABF3 transcription factor binds to the promoters of an open structure and acts as an activator of PP2C gene transcription. During this process, the chromatin remodeler BRM ATPase contributes to maintaining the suppression of PP2C gene expression.

2. Materials and methods

2.1. Plant materials and growth conditions

The *abf3* mutant seeds (SALK-096965) (Yoshida et al., 2010) were donated by Professor Kazuko Yamaguchi-Shinozaki (University of Tokyo, Japan). The *brm-3* seeds (SALK-088462) (Farrona et al., 2007) were donated by Professor Marta Kamila Koblowska (University of Warsaw, Poland) and Professor Sara Farrona (National University of Ireland Galway, Ireland). The seeds were surface-sterilized and stored at 4 °C for 3 days for stratification, then grown on half-strength Murashige and Skoog (1/2 × MS) medium supplemented with 2% sucrose. The growth conditions included a 16-h light/8-h dark cycle, 23 ± 1 °C, and white light intensity of ~100 μmol photons $m^{-2}s^{-1}$. Two-week-old plants were transferred to fresh 1/2 × MS medium supplemented with 0 or 250 mM NaCl and allowed to grow for a further 6 h.

2.2. Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Two-week-old plants (100 mg for each sample) were used for total RNA extraction with the Easy-spin™ IIp Plant RNA Extraction kit (iNtRON Biotechnology, Sungnam, Korea). The RevertAid First Strand cDNA Synthesis kit (Thermo Scientific™, Waltham, Massachusetts, USA) was used for first-strand cDNA synthesis. Real-time PCR ingredients included SolGent™ 2x Real-Time Smart Mix (SolGent Co., Daejon, Korea), cDNA samples, and specific primers (Fig. S1 and Table S1). For all real-time PCR experiments, *ACTIN2* (At3g18780) was used as an internal control. The real-time PCR thermal cycling conditions and the calculation of relative transcript levels followed the methods of a previous study (Nguyen and Cheong, 2018b). The RT-qPCR assays were performed using three independent replicates and statistical analysis was conducted with Duncan's test at the 95% confidence level (Duncan, 1955).

2.3. Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation (ChIP) assays were carried out using the EpiQuik™ Plant ChIP kit (Epigentek, Farmingdale, NY, USA) and following the manufacturer's instructions. Antibodies (2 μg per reaction) used were anti-RNA polymerase II CTD repeat YSPTSPS (4H8) (RNAPII; Abcam ab5408, Cambridge, UK), anti-histone H3 (H3; Abcam ab1791), anti-histone H3 trimethyl lysine 4 (H3K4me3; Abcam ab8580), and anti-acetyl-histone H3 (H3ac; Merck Millipore 06–599, Burlington, Massachusetts, USA). In this assays, 1 μg per reaction of normal rabbit IgG (Merck Millipore 12–370) was used to determine non-specific binding of antibodies. Precipitated DNA samples were used for real-time PCR with specific primers (Fig. S1 and Table S1). ChIP-qPCR assays were performed using three independent replicates and statistical analysis was conducted with Duncan's test at the 95% confidence level (Duncan, 1955).

For green fluorescence protein (GFP) binding assay, 5 μg of anti-GFP (Abcam ab290) was used per reaction. The GFP binding signal was calculated as the percent input (IP/IN%). Because 5% of the extracted chromatin was used as the “input sample” for these assays, the IP/IN% was calculated using formula $100 \times 2^{[(Ct-Input\ -\ 4.322)\ -\ Ct-ChIP]}$. The *GYPSY-LIKE RETROTRANSPOSON* (At4g07700) primer set was used in the qPCR assay to determine non-specific binding of AtMYB44 proteins, and the signals were subtracted from the total binding.

3. Results and discussion

3.1. Suppression of PP2C gene expression by AtMYB44 repressor

Previously, we showed that AtMYB44 binds to gene promoters and represses the expression of clade A PP2C genes such as *ABI1*, *ABI2*, and *HAI1* under normal conditions (Nguyen and Cheong, 2018a). A RT-qPCR assay using primers (Fig. S1 and Table S1) for *ABI1* (region B), *ABI2* (region C), and *HAI1* (region C) showed that PP2C gene transcript levels increased in response to salt treatment (Fig. 1a). However, the increases were significantly smaller in *35S:AtMYB44* transgenic plants than in wild-type plants in response to salt stress, confirming our previous observations based on northern blot and microarray analyses (Jung et al., 2008). Under salt-stress conditions, the levels of RNA polymerase stalled at the transcription start sites of PP2C gene loci increased substantially (Fig. 1b). In *35S:AtMYB44* transgenic Arabidopsis, however, the salt-induced increase of RNA polymerase stalling was significantly reduced.

These results confirm that AtMYB44 acts as a repressor of PP2C gene transcription. In relation to the repressive function of transcription factor AtMYB44, we previously observed that AtMYB44 protein binds to the promoters of its own gene *AtMYB44* (Nguyen and Cheong, 2018c) and the late embryogenesis abundant protein gene *AtLEA4-5* (Nguyen et al., 2019) to repress RNA polymerase-mediated gene expression.

3.2. Release of repressors from PP2C gene promoters in response to salt stress

ChIP-qPCR assays of *35S:AtMYB44–GFP* plants (Jung et al., 2008) using a green fluorescent protein (GFP) antibody revealed that AtMYB44–GFP fusion proteins bind directly to the promoters, including the TSS proximal regions, of the *ABI1*, *ABI2*, and *HAI1* genes. The levels of AtMYB44-GFP proteins bound to PP2C gene promoter regions were significantly reduced in response to 250 mM salt treatment (Fig. 2). We previously observed similar results using ChIP assays of the *AtMYB44* (Nguyen and Cheong, 2018c) and *AtLEA4-5* (Nguyen et al., 2019) gene promoters.

The C-terminal catalytic domain (motif 22.2) of AtMYB44 contains a putative small ubiquitin-like modifier (SUMO) acceptor site, IKAE (Jung et al., 2012). The consensus SUMOylation site is known as Ψ-K-x-

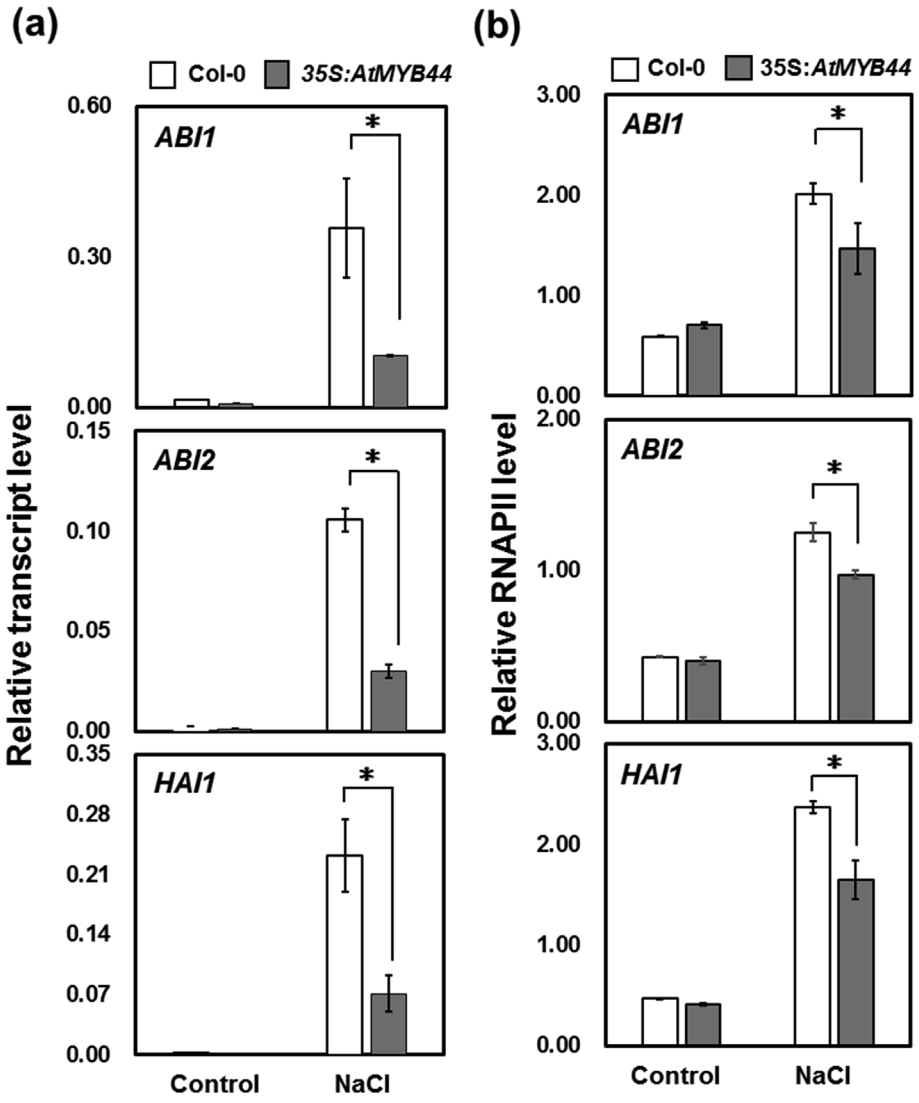

Fig. 1. PP2C gene transcription in *AtMYB44*-overexpressing (*35S:AtMYB44*) transgenic plants. (a) Quantitation of PP2C gene transcripts in *35S:AtMYB44* plants. RT-qPCR was performed using specific primers targeting *ABI1* (region B), *ABI2* (region C), and *HAI1* (region C) (Fig. S1 and Table S1), and ACTIN2 was used as an internal control. (b) Quantitation of RNA polymerase II (RNAPII) stalled at PP2C gene loci. The DNA samples from RNAPII-ChIP assays were subjected to real-time PCR using specific primers for region B of each tested PP2C gene. The ChIP signal was normalized to the internal control (*ACTIN2*). Data represent the mean ± standard error of values obtained from three independent experiments performed in triplicate. Columns marked with an asterisk (*) differ significantly ($P < 0.05$).

D/E, where Ψ indicates a large hydrophobic residue, K represents a modified lysine residue, x is any amino acid, and D/E represents aspartic acid or glutamic acid (Rodriguez et al., 2001; Rosonina et al., 2017). SUMO inhibits transcription by promoting the recruitment of transcriptional corepressor complexes and alters the levels of transcription factors associated with target binding sites on chromatin by influencing their DNA-binding abilities or promoting the clearance of the transcription factors from chromatin (Flotho and Melchior, 2013; Rosonina et al., 2017). Additionally, SUMO promotes histone deacetylase-mediated transcriptional repression (Yang and Sharrocks, 2004). For example, Miller et al. (2010) found that SUMO targeted histone 2B, components of LEUNIG/TPL corepressor complexes, and proteins that control histone acetylation and DNA methylation. SUMO proteins have been reported to function in the regulation of many physiological processes, including the abiotic stress response in plants (Castro et al., 2012). Therefore, it is possible that SUMO modifiers are involved in post-translational modification of AtMYB44 to alter its binding levels on PP2C gene promoters in response to salt stress. However, this possibility was not verified in the present study and requires further analysis.

3.3. Histone modifications of PP2C gene promoters in response to salt stress

ChIP-qPCR assays using histone H3 antibody showed that nucleosome density at the PP2C gene promoter regions was strongly reduced in response to salt stress (Fig. 3). Therefore, salt stress induces changes in nucleosome occupancy and may facilitate the replacement of repressors, including AtMYB44, with activators of PP2C gene expression.

Previously, we reported that AtMYB44 forms a complex with TPR corepressors and recruits histone deacetylases to suppress PP2C gene transcription under normal conditions (Nguyen and Cheong, 2018a). In response to salt stress, the proportion of acetylated (H3ac) histones among total histones remaining at the PP2C gene loci increased significantly (Fig. 4a). These results suggest that the histone deacetylases recruited by TPR corepressors are eliminated from PP2C gene chromatins under salt-stress conditions. Additionally, activator proteins that replace AtMYB44 may recruit histone acetyltransferases (Stockinger et al., 2001; Weiste and Dröge-Laser, 2014) to PP2C gene loci. Unacetylated histones repress transcription, but acetylated histones are weaker repressors of transcription. Devaiah et al. (2016) showed that a histone acetyltransferase acetylates an amino acid residue critical for nucleosome stability, thus resulting in nucleosome eviction and chromatin decompaction at target genes and thereby activating transcription.

For each histone remaining at PP2C gene loci, the proportion of methylated (H3K4me3) histones increased in response to salt stress (Fig. 4b). H3K4me3 is a marker found at open and potentially active promoters; therefore, it has been described as playing an essential role in transcription activation (Howe et al., 2017). H3K4me3 levels at TSS regions of PP2C gene loci were significantly lower in *35S:AtMYB44*

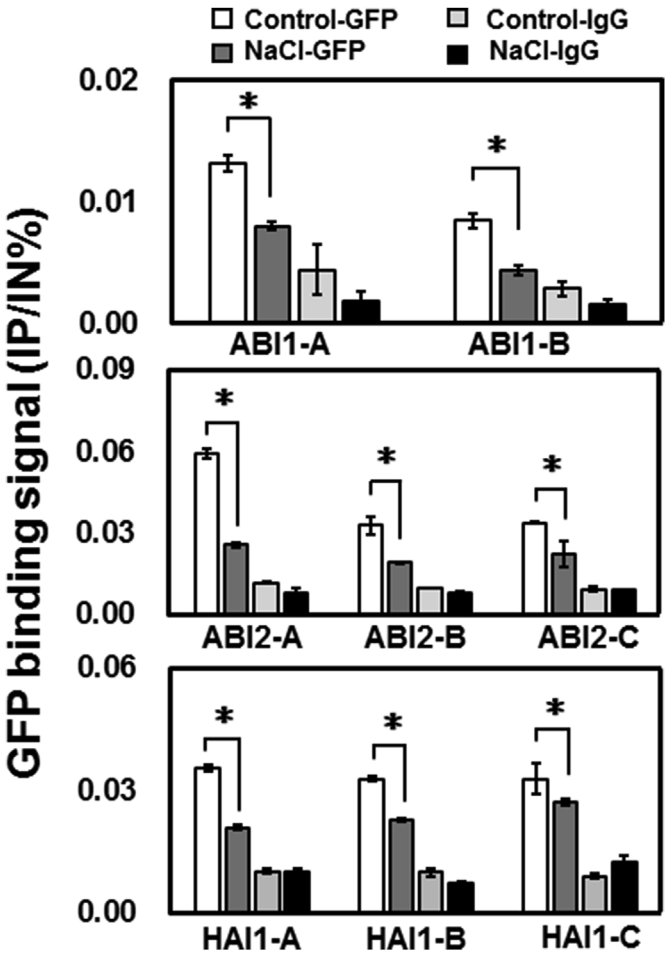

Fig. 2. Binding of AtMYB44-GFP fusion proteins to PP2C gene loci. The ChIP assays were carried out using antibodies against GFP. Real-time PCR was performed using specific primers for *ABI1*, *ABI2*, and *HAI1* (Fig. S1 and Table S1). IgG antibody was used to determine non-specific binding of antibodies. The GFP binding signal was calculated as percent input (IP/IN%). Data are reported as mean ± standard error of values obtained from three independent experiments performed in triplicate. Columns marked with an asterisk (*) differ significantly ($P < 0.05$).

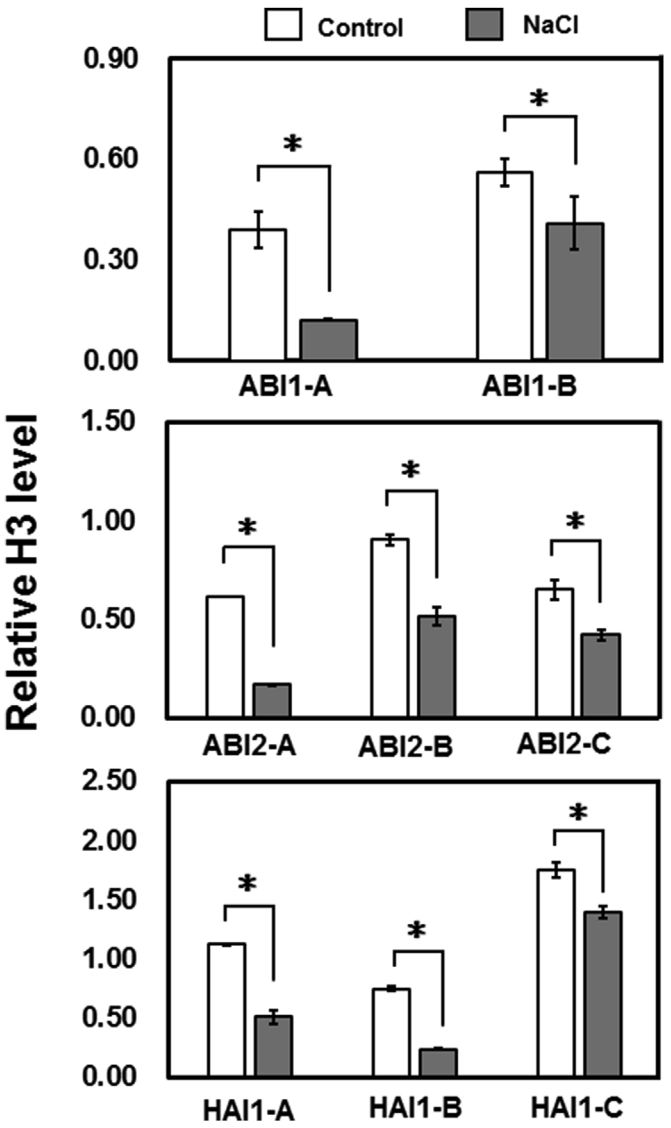

Fig. 3. Nucleosome density at PP2C gene loci. ChIP assays were conducted using antibodies against histone H3. Real-time PCR was performed using specific primers (Fig. S1 and Table S1). The ChIP signal was normalized to the internal control (*ACTIN2*). Data are reported as mean ± standard error of values obtained from three independent experiments performed in triplicate. Columns marked with an asterisk (*) differ significantly ($P < 0.05$).

plants than in wild-type plants (Fig. S2). Thus, it is plausible that the AtMYB44–TPR complex may recruit H3K4 demethylase or reduce H3K4 methyltransferase activity at these gene loci. However, no evidence of direct interaction between TPL/TPR and H3K4 demethylase or methyltransferase has yet been found. Recent studies support the hypothesis that H3K4me3 deposition on chromatin is a consequence of transcription, rather than a cause, which records the memory of previous states and promotes transcriptional consistency among cells (Fromm and Avramova, 2014; Howe et al., 2017). Thus, the reduced levels of H3K4me3 observed at PP2C gene loci likely result from PP2C gene transcription in response to salt stress.

3.4. Activation of PP2C gene transcription by ABF3

Under salt stress, PP2C gene promoters may be occupied by alternative transcription factors that activate gene expression. Numerous bZIP-type transcription factors, including AREB1, AREB2, and ABF3, trigger gene expression downstream of SnRK2 kinases during ABA signaling under abiotic stress conditions (Yoshida et al., 2010, 2015). In particular, under salt or drought stress, transcript levels of some PP2C genes, including *HAI1*, *HAI2*, and *HAI3*, were significantly lower in the *areb1/areb2/abf3* triple mutant than in wild-type plants (Yoshida et al., 2010). Recently, Wang et al. (2019) reported that ABFs mediate rapid induction of group A PP2C gene transcription, playing a role in negative feedback regulation of ABA signaling. Thus, ABFs may occupy PP2C gene promoters and regulate gene transcription in response to salt stress, helping to maintain balanced phosphorylation levels in the cell.

Analysis with the Plant Promoter Analysis Navigator (PlantPAN; http://PlantPAN2.itps.ncku.edu.tw) revealed that a number of ABF3-binding sites, including TCACGttt and ACACGgtt (Weirauch et al., 2014), are present in the promoter regions of PP2C genes (~1.5 kb), including four in the *ABI1* promoter, four in *ABI2*, and three in *HAI1* (Fig. S1). In fact, Song et al. (2016) showed that ABF3 directly associates with the promoters of these genes. Under salt-stress conditions, transcript levels of PP2C genes in *abf3* mutant plants were markedly lower than those in wild-type plants (Fig. 5), supporting ABF3's role in the activation of PP2C gene expression.

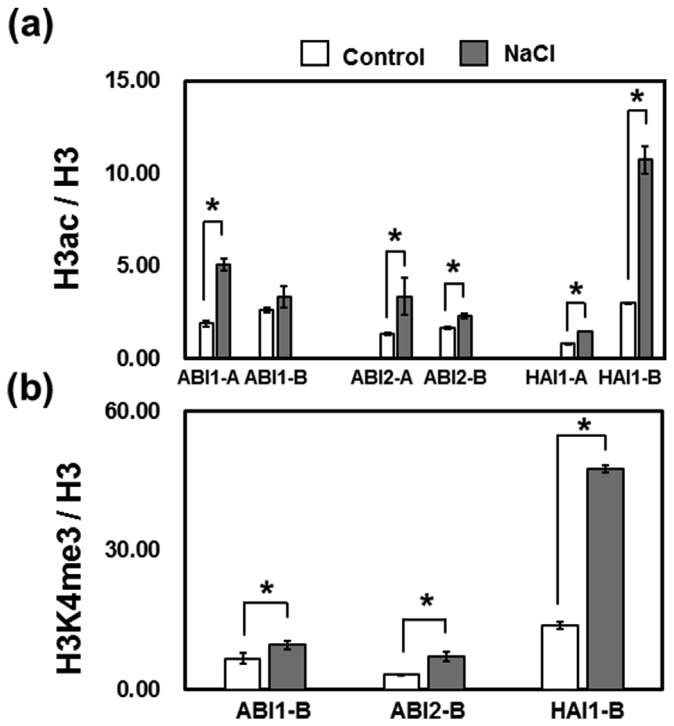

Fig. 4. Histone modifications at PP2C gene loci in response to salt stress. (a) Relative levels of acetylated histones (H3ac) to total histones (H3). (b) Relative levels of methylated histones (H3K4me3) to total histones (H3). ChIP assays were conducted using antibodies against histone H3, H3ac, and H3K4me3. Real-time PCR was performed using specific primers (Fig. S1 and Table S1). The ChIP signal was normalized to the internal control (*ACTIN2*). Data are reported as mean ± standard error of values obtained from three independent experiments performed in triplicate. Columns marked with an asterisk (*) differ significantly ($P < 0.05$).

3.5. Role of chromatin remodeling ATPase in PP2C gene expression

Using genome-wide analyses, Li et al. (2016) showed that the chromatin remodeling ATPase BRM is localized to many genomic sites, including the *ABI1*, *ABI2*, and *HAI1* loci. Therefore, we measured the PP2C gene transcript levels in the weak allele mutant *brm-3* (Farrona et al., 2007) under stress conditions. The PP2C gene transcript levels in the *brm-3* mutant were higher than those in wild-type plants under salt stress (Fig. 6). This result indicates that chromatin remodeling ATPase activity contributes to repressing PP2C gene transcription.

BRM ATPase uses energy derived from ATP hydrolysis to alter the non-covalent interaction between DNA and the histone octamer and change the density or positioning of nucleosomes (Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011; Narlikar et al., 2013). Han et al. (2012) reported that Arabidposis BRM represses ABA responses in the absence of the stress stimulus, by stabilizing the nucleosome occupation at the TSS proximal region of *ABI5* gene. Regarding the biochemical activity of BRM, Peirats-Llobet et al. (2016) suggested that phosphorylation of BRM leads to its inhibition, and dephosphorylation restores the ability of BRM to repress ABA responses.

4. Conclusions

Under stress conditions, PP2C gene chromatins were converted from a repressor-associated suppression status into an activator-mediated transcription status to enable gene expression. AtMYB44 repressor proteins bound to the PP2C gene promoters under normal conditions were released in response to salt stress. During the transition to activated status, nucleosomes are evicted from the promoter regions and their histones are acetylated. The transcription factor ABF3 occupies the open promoters of PP2C genes and acts as an activator of gene transcription. The chromatin remodeling ATPase BRAHMA (BRM) contributes to the repression of PP2C gene transcription. These observations are summarized in a working model to explain how PP2C gene regulation occurs with chromatin changes (Fig. S3).

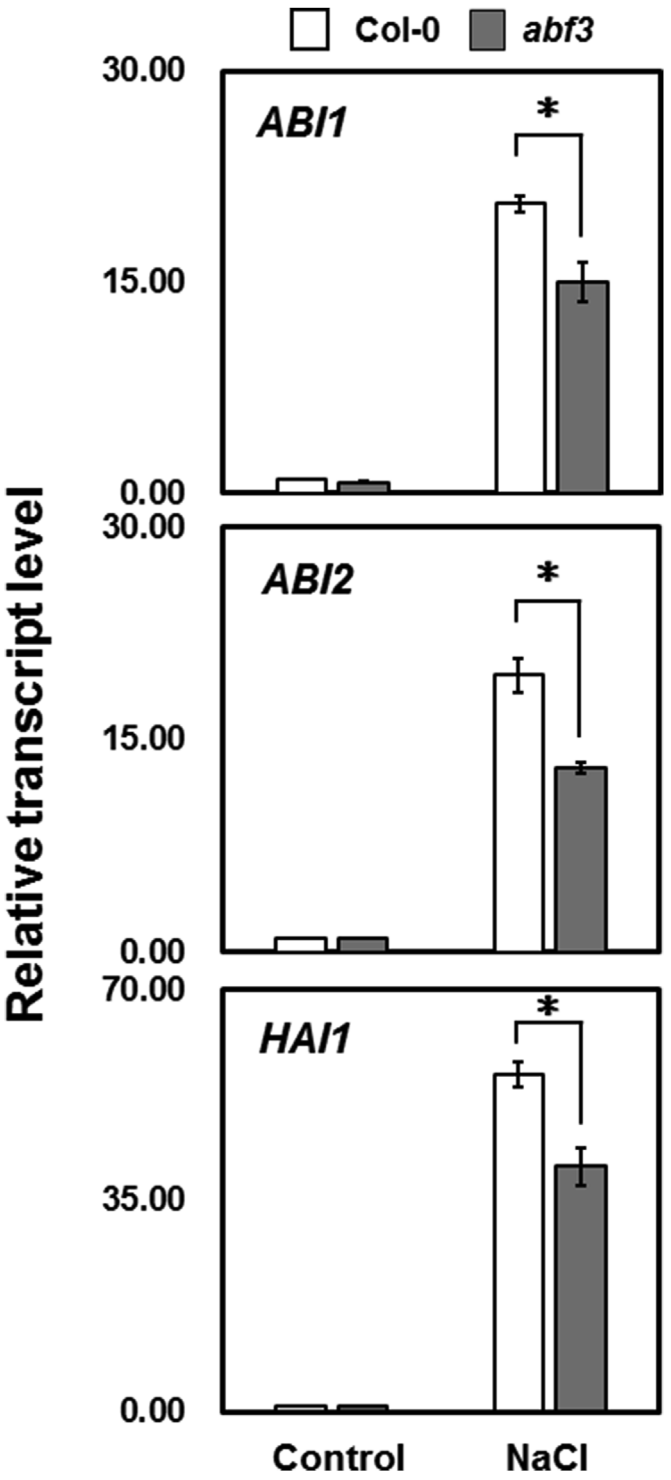

Fig. 5. PP2C gene transcription in *abf3* mutant plants. RT-qPCR assays were performed using specific primers targeting *ABI1* (region B), *ABI2* (region C), and *HAI1* (region C) (Fig. S1 and Table S1). *ACTIN2* was used as an internal control. The data represent mean ± standard error of values obtained from three independent experiments performed in triplicate. Columns marked with an asterisk (*) differ significantly ($P < 0.05$).

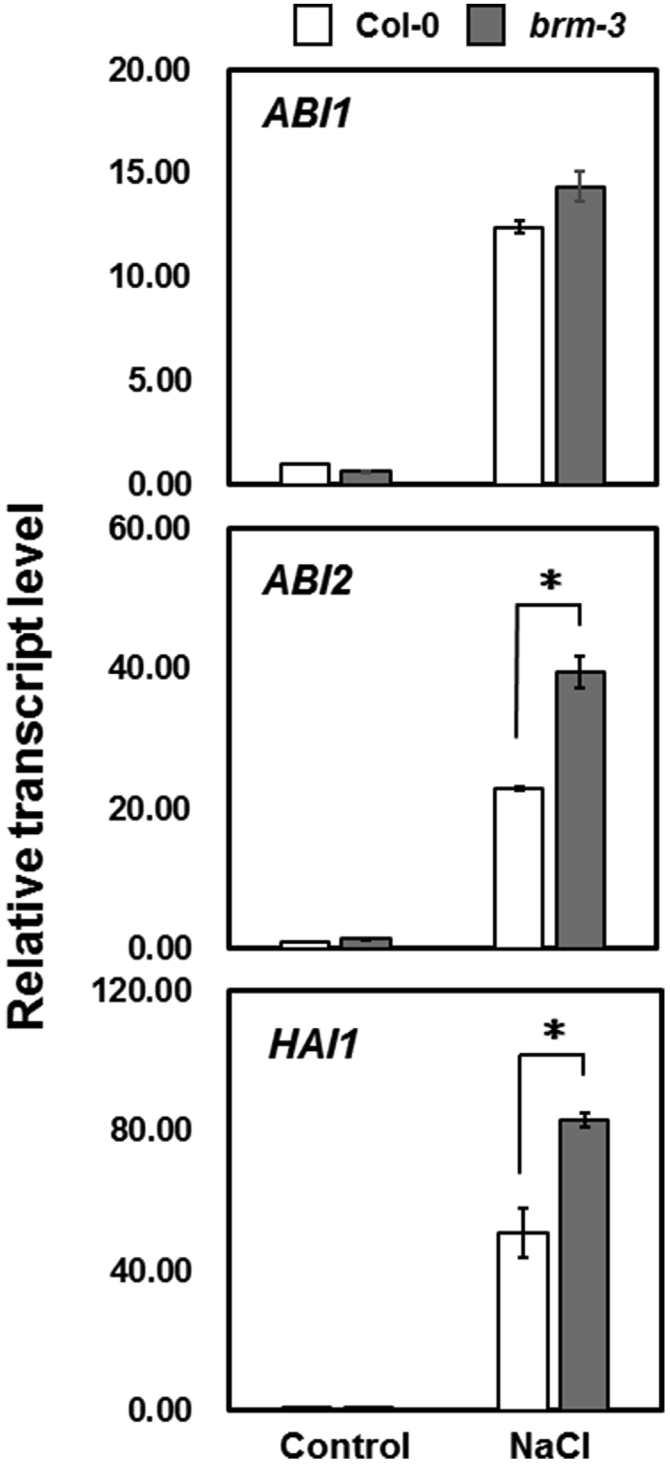

Fig. 6. PP2C gene transcription in *brm-3* mutant Arabidopsis. RT-qPCR was performed using specific primers targeting *ABI1* (region B), *ABI2* (region C), and *HAI1* (region C) (Fig. S1 and Table S1). *ACTIN2* was used as an internal control. Data represent mean ± standard error of values obtained from three independent experiments performed in triplicate. Columns marked with an asterisk (*) differ significantly ($P < 0.05$).

Conflicts of interest

The authors declare no conflicts of interest.

Author's contributions

NHN conducted the experiments and wrote the manuscript. CJ designed and supervised the experiments. JJC designed the research and edited the manuscript.

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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.2019.06.012.

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