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AtPP2CG1, a protein phosphatase 2C, positively regulates salt tolerance of *Arabidopsis* in abscisic acid-dependent manner ☆

Xin Liu [a,1,2], Yanming Zhu [a,1,2], Hong Zhai [b], Hua Cai [a,2], Wei Ji [a,2], Xiao Luo [a,2], Jing Li [c,2], Xi Bai [a,*]

[a] Plant Bioengineering Laboratory, Northeast Agricultural University, Harbin 150030, PR China
[b] Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin 150040, PR China
[c] Plant Secondary Metabolism Laboratory, Northeast Agricultural University, Harbin 150030, PR China

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ABSTRACT

AtPP2CG1 (*Arabidopsis thaliana* protein phosphatase 2C G Group 1) was predicted as an abiotic stress candidate gene by bioinformatic analysis in our previous study. The gene encodes a putative protein phosphatase 2C that belongs to Group G of PP2C. There is no report of Group G genes involved in abiotic stress so far. Real-time RT-PCR analysis showed that *AtPP2CG1* expression was induced by salt, drought, and abscisic acid (ABA) treatment. The expression levels of *AtPP2CG1* in the ABA synthesis-deficient mutant *abi2–3* were much lower than that in WT plants under salt stress suggesting that the expression of *AtPP2CG1* acts in an ABA-dependent manner. Over-expression of *AtPP2CG1* led to enhanced salt tolerance, whereas its loss of function caused decreased salt tolerance. These results indicate that AtPP2CG1 positively regulates salt stress in an ABA-dependent manner. Under salt treatment, *AtPP2CG1* up-regulated the expression levels of stress-responsive genes, including *RD29A, RD29B, DREB2A* and *KIN1*. GUS activity was detected in roots, leaves, stems, flower, and trichomes of *AtPP2CG1* promoter–GUS transgenic plants. AtPP2CG1 protein was localized in nucleus and cytoplasm via AtPP2CG1:eGFP and YFP:AtPP2CG1 fusion approaches.

1. Introduction

Salt stress is a negative factor that greatly impacts plant development and restricts crop production [1]. Plants synthesize numerous hormonal signals that work in concert to integrate cellular physiology to adverse environmental cues. A key abiotic stress signal is the carotenoid-derived molecule abscisic acid (ABA) [2]. ABA affects seed development and plays a critical role in regulation of plant responses to stress [3–7]. Abiotic stresses trigger a dramatic increase in ABA, which induces expression of stress-responsive genes in plants through a variety of second messengers and regulators [8].

Reversible protein phosphorylation is a major mechanism of ABA signal transduction, and mediates many processes through a broad array of protein kinases and protein phosphatases [9]. PP2Cs are monomeric protein phosphatases which are found in human, yeast and, plant [10]. The *Arabidopsis thaliana* genome contains 76 PP2C-type phosphatase candidate genes that have been clustered into 10 groups (A–J), with the exception of six genes that have distinctive features [11]. PP2Cs of Group A have been confirmed as negative regulators of ABA early signal transduction; the most prominent members appear to be ABI1, ABI2, HAB1, HAB2, AHG1 and PP2CA/AHG3 [12–15]. The *abi1–1* and *abi2–1* dominant mutants caused ABA-resistant seed germination and seedling growth, reduced seed dormancy, abnormal stomatal regulation, and defects in various responses to drought stress. The *hab1–1* mutants were sensitive to ABA of seed germination while 35S:HAB1 over-expression plants were not sensitive to ABA, compared to wild-type (WT) plants.

Recently, the regulatory mechanisms of Group A phosphatases have begun to be elucidated. The PYR1-PYL/RCAR family was identified as ABA receptors, and is comprised of 14 genes in *Arabidopsis*. PYR1 binds to ABA and in turn binds to and inhibits ABI1, ABI2 and HAB1 [16]. Other PP2Cs of the same group from *Arabidopsis* have been shown to be involved in mitogen-activated protein kinase (MAPK) signaling, flower development, pathogen resistance, and mediating phytochrome signaling [11,17,18].

We previously predicted *AtPP2CG1* as a stress-responsive candidate gene [19]. In this work, we showed evidences for the

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* Corresponding author. Fax: +86 451 86668757.
E-mail addresses: fangfei6073@126.com (X. Liu), ymzhu2001@neau.edu.cn (Y. Zhu), Zhai.h@neigaehrb.ac.cn (H. Zhai), small-big@sohu.com (H. Cai), iwei_j@hotmail.com (W. Ji), luoxiao2010@yahoo.cn (X. Luo), lijing@neau.edu.cn (J. Li), baixi@neau.edu.cn (X. Bai).
[1] These authors contributed equally to this work.
[2] Fax: +86 451 55191776.

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positive role of *AtPP2CG1* involving in response to salt stress in ABA-dependent manner in *Arabidopsis*. First, we studied the expression patterns of *AtPP2CG1* in WT plants under salt, cold, drought and ABA treatments. The expression patterns of *AtPP2CG1* in *abi2–3* mutant plants under salt treatment were also analyzed. Second, we investigated the functions of *AtPP2CG1* in salt stress by using *pp2cg1* mutant and 35S:*AtPP2CG1* over-expression plants. Third, we showed the expression patterns of stress-responsive genes in WT, *pp2cg1* and 35S:*AtPP2CG1* over-expression plants. In addition, we investigated the tissue localization and subcellular localization of *AtPP2CG1*.

2. Materials and methods

2.1. Plant materials and growth

All the *Arabidopsis* (*A. thaliana*) plants used in this study were in the Columbia (Col-0) ecotype background. The *pp2cg1* T-DNA inserted mutant line (SALK_036544c) and ethylmethane sulfonate-induced *abi2–3* mutant line were obtained from The European Arabidopsis Stock Center (NASC, Loughborough, United Kingdom). Seeds were vernalized at 4 °C for 2 days to disrupt dormancy, and then were sowed on the 1/2 Murashige and Skoog (MS) medium with 0.8% agar [20] or in pots filled with soil in a controlled environmental chamber at 22 °C. All plants were grown in a 16/8 h day/night cycle at a light intensity of 100 μmol photons m^{-2} s^{-1}. Seedlings used for stress treatment were grown in 1/2 MS liquid medium.

2.2. Generation of AtPP2CG1 transgenic Arabidopsis

To obtain *AtPP2CG1* transgenic plants, the *AtPP2CG1* (At2g33700) coding sequence was cloned from cDNA using the following gene-specific primers: *AtPP2CG1* (At2g33700)-F 5′-TCACCGATGAGTATGGATTT-3′ and *AtPP2CG1* (At2g33700)-R 5′-GGCAATAGTCTCACAAAGTAAC-3′. Then, PCR products were inserted into the pGEM-T vector and the integrity of the construct was verified by sequencing. The *AtPP2CG1* cDNA was digested by *Nco*I and *Spe*I and was inserted between the CaMV 35S promoter and nopaline synthase terminator (NOS-Ter) in the plant expression vector pFGC1008. The vector 35S:*AtPP2CG1* was introduced into the *Agrobacterium tumefaciens* strain, LBA4404, and transformed into WT (wild type) plants by the floral-dip method [21]. Homozygote seeds were selected by hygromycin (25 mg/L) in two generations screening. The expression levels of *AtPP2CG1* were confirmed by reverse transcription PCR using the following primers: *AtPP2CG1* (At2g33700)-F, 5′-TTCTCGCTCCTCTTGCTTACT-3′; *AtPP2CG1* (At2g33700)-R, 5′-GTTCCCAAATACCCCTGCT-3′.

2.3. Genotyping of T-DNA insertion mutant plants

The T-DNA insertion point of the *pp2cg1* homozygous mutant was identified by PCR with the following primer pairs: F, 5′-TCACTATCACCGCAGCTAGAAT-3′ and R, 5′-TTTCATTAATTTCCGGCAGG-3′ and F, 5′-AGGATCTGTTGATTTTGGGTA-3′ and R, 5′-GTGTTATTAAGTTGTCTAAGCGTC-3′. The *pp2cg1* homozygous mutant plants were confirmed by RT-PCR with the gene-specific primers described above.

2.4. Salt tolerance assay

For salt tolerance assay at germination stage, seeds were grown on 1/2 MS medium with 120 mM NaCl after vernalization. Seeds were considered germinated when radicles completely penetrated the seed coat [22]. Generation rates were scored every day up to 7 days after seeds were placed on medium. Cotyledon opening rates were counted on the 7th day after sowing.

For salt tolerance assay at seedling stage, seeds were placed on 1/2 MS medium for about 7 days, after which the seedlings were carefully transferred onto the medium with 150 mM NaCl. Root lengths and fresh weights of the plants were measured after growing on the vertical agar plates for 15 days. All experiments were repeated at least three times.

2.5. Real-time RT-PCR analysis

The stress treatment method, RNA extraction, cDNA preparation, and RT-PCR were performed as previously described [23]. Gene-specific primers used in RT-PCR analysis were: *Actin2* (At3g18780)-F, 5′-TTACCCGATGGGCAAGTC-3′ and *Actin2* (At3g18780)-R, 5′-GCTCATACGGTCAGCGATAC-3′; and primers of *AtPP2CG1* as described above. Relative quantity of gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method [24]. We also analyzed the expression levels of *AtPP2CG1* in the *abi2–3* mutant plants under salt treatment.

To determine the expression levels of stress-related genes in WT, *pp2cg1* and *AtPP2CG1* over-expression plants in response to salt stress, total RNA was isolated from 3-week-old plants treated with 250 mM NaCl for 0, 3, and 12 h. The gene-specific primers used were: *RD29A* (At5g52310)-F, 5′-GGCGTAACAGGTAAACCTAGAG-3′ and *RD29A* (At5g52310)-R, 5′-TCCGATGTAAACGTCGTCC-3′; *RD29B* (At5g52300)-F, 5′-GCGCACCAGTGTATGAATCCTC-3′ and *RD29B* (At5g52300)-R, 5′-TGTGGTCAGAAGACACGACAGG-3′; and primers of *DREB2A* (At5g05410) and *KIN1* (At5g15960) described previously [25]. Every assay was performed as three fully independent biological replicates. The gene expression levels were standardized as previously described [26].

2.6. Tissue localization of AtPP2CG1

The −1.6-kb promoter region of *AtPP2CG1* was amplified with the primer pair: F, 5′-AACAACCCACGTTATGTATATTC-3′ and R, 5′-CGGTGAGTTCTTCTCTGAATC-3′ and cloned into pCAMBIA 3301, containing the reporter gene for the enzyme β-glucuronidase (GUS). Transformation was performed as previously described. At least four *AtPP2CG1* promoter–GUS transgenic plants were analyzed. GUS staining was performed as previously described [27]. Seedlings had been washed in 70% ethanol at 56 °C. Tissues were observed with a stereomicroscope (Olympus, Japan).

2.7. Subcellular localization of AtPP2CG1

Expression vectors with the fluorescent protein tags, enhanced green fluorescent protein (eGFP) and yellow fluorescent protein (YFP), were constructed for subcellular localization analysis. The *AtPP2CG1* coding region was fused to the C-terminal end of eGFP in the vector PBSK, under the control of the CaMV 35S promoter. *AtPP2CG1* coding domain sequence (CDS) was amplified from pGEM-T–AtPP2CG1 with primer pairs that contained *Hind*III-compatible restriction site at their 3′-end and *Spe*I-compatible restriction site at 5′-end. The primer pairs used were: F, 5′-CCCAAGCTTGGGATGAGTATG-3′ and R, 5′-GACTAGTCGGATAGCCATCGAG-3′. In addition, the coding region of *AtPP2CG1* was fused to the N-terminal end of YFP in the vector pPS48. *AtPP2CG1* was amplified from pGEM-T–AtPP2CG1 with primer pairs: F, 5′-GGCTTAAUATGAGTATGGATTTTTC-3′ and R, 5′-GGTTTAAUTCACGGATAGCCAT-3′. Following sequence verification of the inserts, the AtPP2CG1:eGFP, YFP:AtPP2CG1 and the control vectors were transiently expressed into onion epidermal cells independently by particle bombardment. Onion epidermal cells were transferred to 1/2 MS medium and kept at 28 °C for 16 h in darkness after bombardment. Cells were observed with the 488 and 513 nm argon laser, respectively,

using a confocal laser-scanning microscope (LSM 700; Zeiss Microimaging, Jena, Germany).

3. Results

3.1. AtPP2CG1 gene is inducible by abiotic stress

To examine how *AtPP2CG1* in response to stress conditions, the expression levels of *AtPP2CG1* were detected in shoots and roots of WT *Arabidopsis* under salt, drought, cold and ABA treatments by Real-time RT-PCR. *AtPP2CG1* expression in shoots was up-regulated under salt and drought treatments (Fig. 1A), and was up-regulated in roots under salt and ABA treatments (Fig. 1B). *AtPP2CG1* expression patterns under salt and ABA treatments were similar which indicated that salt-induced *AtPP2CG1* expression might be ABA-dependent (Fig. 1A and B).

3.2. The expression pattern of AtPP2CG1 under salt stress is in an ABA-dependent manner

We further analyzed the expression of *AtPP2CG1* in the ABA synthesis-deficient mutant, *abi2–3*, to determine whether *AtPP2CG1* expression is ABA-dependent under salt treatment. Under salt treatment in WT plants, the transcripts of *AtPP2CG1* started to increase at 3 h, peaked at 6 h with a level of 8-fold compared to the control (0 h) in shoots and roots (Fig. 1A and B). In the *abi2–3* mutant, the transcripts of *AtPP2CG1* peaked at 6 h with a level of 2-fold compared to the control in shoots and roots (Fig. 1C). The expression levels of *AtPP2CG1* were much lower in *abi2–3* mutant plants than that in WT plants under salt stress, suggesting that the salt-induced expression of *AtPP2CG1* is ABA-dependent.

3.3. AtPP2CG1 positively modulates salt response in plants

To understand the function of *AtPP2CG1* in salt stress, homozygous mutant and over-expression transgenic plants were evaluated. *AtPP2CG1* was not expressed in homozygous *pp2cg1* while was highly expressed in 35S: *AtPP2CG1* (Fig. 2A). The over-expression line1 and line2 were used in the study.

WT, *pp2cg1* and the two independent *AtPP2CG1* transgenic lines were plated on 1/2 MS media supplemented with 130 mM NaCl. Germination rates of the three types of plants showed no differences at any of the days examined (Fig. 2C). However, the cotyledon opening rates of the over-expression lines were higher than WT plants, and the rates of the *pp2cg1* mutants were lower than WT plants (Fig. 2B and E).

WT, mutant and over-expression seedlings were grown in plane 1/2 MS agar plates until the four-leaf-stage when they were transferred to vertical agar plates supplemented with 150 mM NaCl. Root lengths and fresh weights of seedlings were measured after 14 days of treatment. Fresh weights of 35S:*AtPP2CG1* over-expression plants were higher than WT, while fresh weights of *pp2cg1* plants were lower than WT under salt treatment (Fig. 2D and F). In contrast, root lengths of WT, *pp2cg1* and 35S:*AtPP2CG1* over-expression plants did not show remarkable differences under salt stress (Fig. 2G). Thus, the results indicated that *AtPP2CG1* positively regulated salt tolerance in *Arabidopsis*.

3.4. AtPP2CG1 regulates stress-responsive gene expression

Induction of stress-responsive genes is one of the most important mechanisms for adaptation in plants [28]. To determine whether *AtPP2CG1* could induce expression of stress-responsive genes under salt treatment, we measured several stress-responsive genes, including *RD29A*, *RD29B*, *KIN1* and *DREB2A*. Under salt treatment, all of the marker genes were up-regulated in WT, *pp2cg1* and 35S:*AtPP2CG1* over-expression plants. *KIN1*, *RD29A*, *RD29B* and *DREB2A* expression levels in over-expression plants were higher than WT, while the expression levels of these genes in mutant plants were lower than WT (Fig. 3), except for *DREB2A* at 12 h. Therefore, *AtPP2CG1* could up-regulate the expression of *RD29A*, *RD29B*, *KIN1* and *DREB2A* under salt treatment.

3.5. Tissue and subcellular localization of AtPP2CG1

We generated *AtPP2CG1* promoter–GUS transgenic plants to analyze the expression profile of *AtPP2CG1* in *Arabidopsis*. As shown in Fig. 4A, a and c, GUS activity was detected in cotyledons, hypocotyls and roots at seedling stage. At the adult stage, GUS activity was detected mainly in flower, stem, stigma base, vein, seed coat, and trichomes; whereas, weaker straining was observed in mesophyll cells (Fig. 4A, b and d–h). The vascular tissues and trichomes were more intensely stained than other tissues in *AtPP2CG1* promoter–GUS transgenic plants.

We used AtPP2CG1:eGFP and YFP:AtPP2CG1 fusion proteins to investigate the subcellular localization of AtPP2CG1 protein in cells. AtPP2CG1:eGFP, YFP:AtPP2CG1 and control vectors were transiently expressed in onion epidermal cells. Cells expressing GFP/YFP alone exhibited signal in nucleus and cytoplasm (Fig 4B, a and c). AtPP2CG1:eGFP and YFP:AtPP2CG1 fusion proteins were distributed in nucleus and cytoplasm (Fig. 4B, b and d), indicating that *AtPP2CG1* might function in the nucleus and cytoplasm.

4. Discussion

Plants have a large number of PP2Cs, which play essential roles in signal transduction under abiotic and biotic stress. Previous studies showed that PP2Cs were involved in responses to stress through the ABA, MAPK pathway or interacting with some ion channels directly, such as *AtPP2CA*, *AP2C1* and *ABI2* [29–31]. Here,

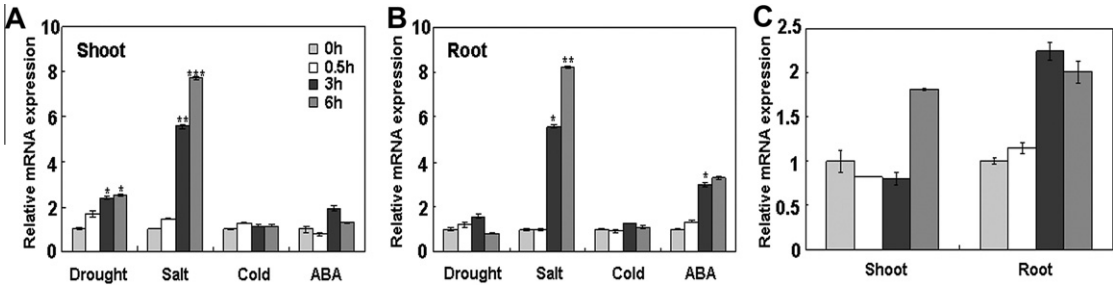

Fig. 1. (A) Evaluation of *AtPP2CG1* expression levels by Real-time RT-PCR analysis under stress conditions of cold, dehydration, salt, and, ABA in shoots of 3-week-old WT plants. (B) Expression levels of *AtPP2CG1* under stress treatments in roots of WT plants. (C) Expression levels of *AtPP2CG1* under salt treatments in shoots and roots of 3-week-old *abi2–3* plants. Values represent means of three biological replicates, and error bars indicate standard deviation. Significant differences from WT are denoted by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

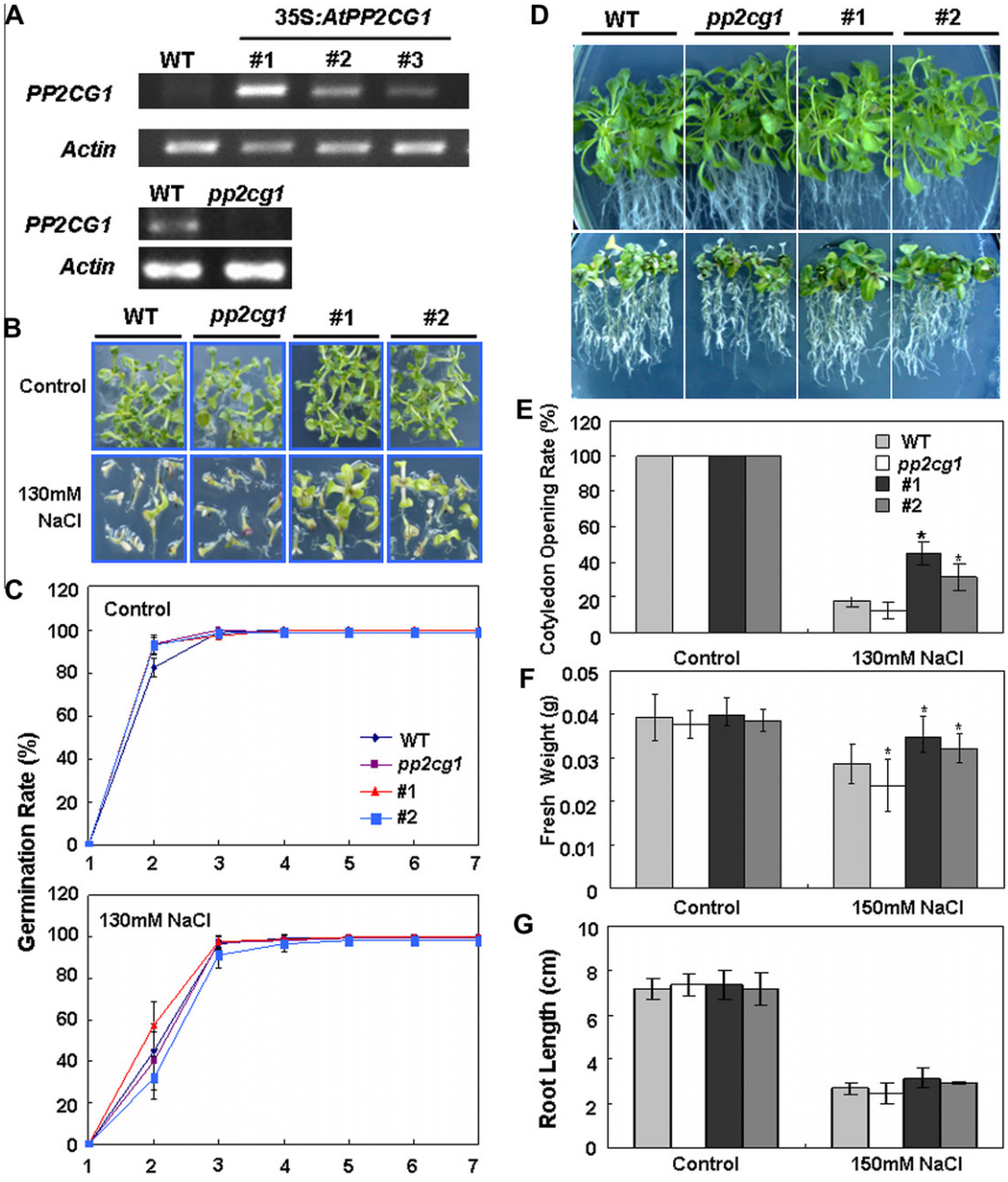

Fig. 2. (A) Semi-quantitative RT-PCR analysis of *AtPP2CG1* expression levels in WT, *pp2cg1* mutants and homozygous 35S:*AtPP2CG1* over-expression plants. The *Actin2* gene was used as an internal standardizing control. (B) Image showing differences in germination of WT, *pp2cg1* mutant, and *AtPP2CG1* over-expression seeds under 130 mM NaCl treatment. (C) Percentage of seeds that germinated on 130 mM NaCl. (*X*-axis) Time (day) of salt treatment (130 mM NaCl); data shown represent the means (± standard error of the mean) of four replicates ($n = 36$ seeds for each line). (*Y*-axis) Germination rates statistics. (D) Salt sensitive assay. The image was taken on day 14 after transfer of 7-day-old seedlings from 1/2 MS medium to plates containing 150 mM NaCl. (E) Cotyledon opening rates of WT, mutant, and transgenic plants grown on 1/2 MS medium supplemented with 130 mM NaCl. (F) Fresh weights of plants grown on 150 mM NaCl. All values are means (± standard error of the mean) from three independent experiments ($n = 28$ seedlings per experiment). (G) Root lengths of seedlings under 150 mM NaCl treatment. Significant differences from WT are denoted by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

we characterized the *AtPP2CG1* gene encoding a PP2C of Group G and determined its involvement in salt tolerance of *Arabidopsis*.

Most ABA-inducible genes contain an ABA-responsive element (ABRE) in their promoters. The presence of ABRE and/or ABRE-CE together as ABA-Responsive Complex or ABRC is essential for abiotic stress inducibility through the ABA-dependent pathway [32]. An ABRC is located −0.61 kb upstream region of the initiation codon for *AtPP2CG1*. Real-time PCR results revealed that *AtPP2CG1* was induced by salt, drought, and ABA. Expression levels of *AtPP2CG1* in the *abi2–3* mutant plants demonstrated that *AtPP2CG1* expression changes under salt stress occur in an ABA-dependent manner. Taken together, all these results suggest that *AtPP2CG1* responses to and regulates salt stress in *Arabidopsis* in an ABA-dependent manner.

Phenotype analysis of *AtPP2CG1* in salt tolerance demonstrated that *AtPP2CG1* positively regulated salt stress in *Arabidopsis*, but did not involve the root tissues, as evidenced by lack of difference in root lengths of WT, *pp2cg1* or 35S:*AtPP2CG1* over-expression plants at the seedling stage (Fig. 2G). There are two possible reasons for this finding: (1) *AtPP2CG1* responsiveness to salt stress may principally function in shoots. In fact, a similar result was reported for SCABP8/CBL10 (a putative calcium sensor), mutants of which exhibit hypersensitivity to salt stress in shoot tissues [33]. (2) There may be functional redundancy of *AtPP2CG1* in root. Three

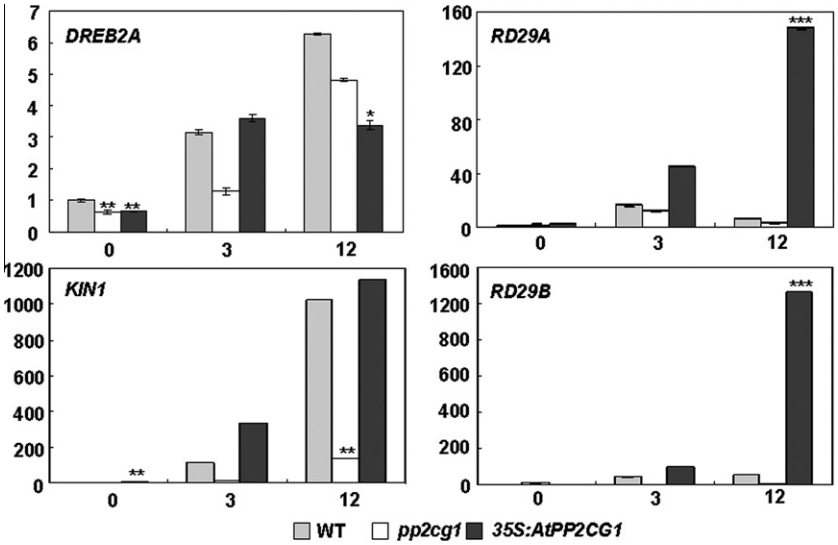

Fig. 3. Expression patterns of stress-responsive genes in WT, *pp2cg1* and 35S:AtPP2CG1 over-expression plants. (*X*-axis) Time (h) of salt treatment (250 mM NaCl). (*Y*-axis) expression levels. Expression levels of *DREB2A*, *RD29A*, *RD29B* and *KIN1* under normal condition and under 250 mM NaCl treatment were measured by Real-time RT-PCR. Values represent means of three biological replicates, and error bars indicate standard deviation. Significant differences from WT are denoted by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

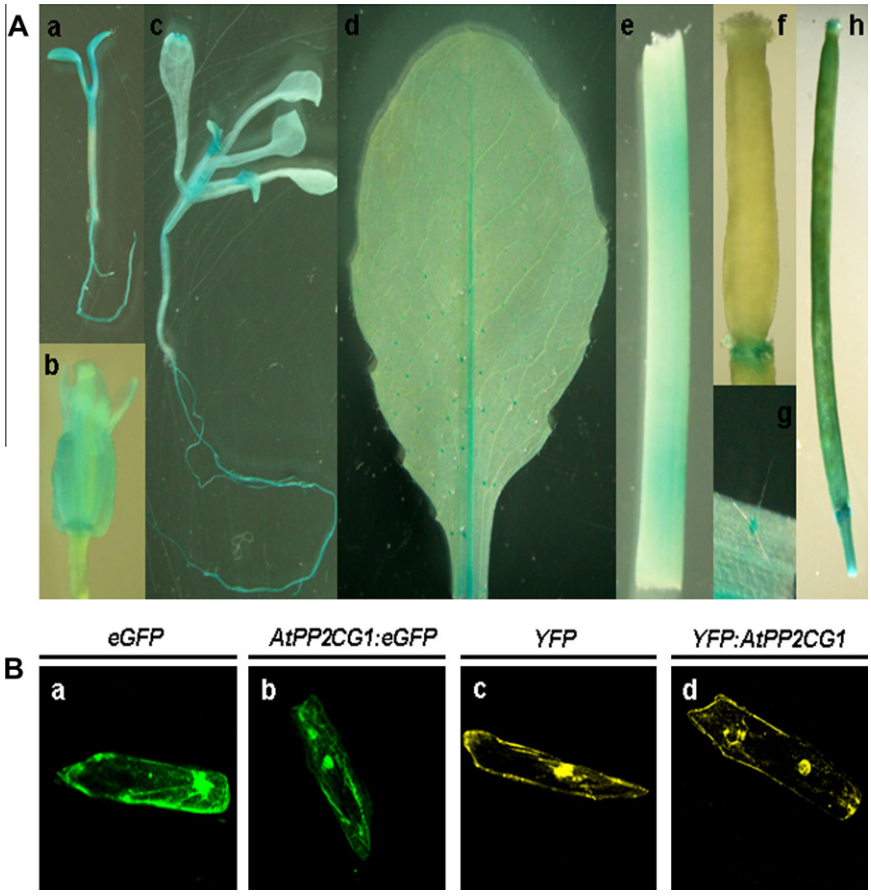

Fig. 4. (A) Tissue localization of AtPP2CG1 promoter–GUS expression in transgenic *Arabidopsis* (a–h). AtPP2CG1 promoter–GUS expression in (a) 5-day-old seedling; (b) flower from a 35-day-old plant; (c) 8-day-old seedling; parts of a 42-day-old plant, including (d) rosette leaf, (e) stem, (f) stigma base, (g) trichomes, and (h) silique. (B) Subcellular localization of AtPP2CG1 fused to the C-terminal end of eGFP or N-terminal end of YFP in onion epidermal cells. (a) eGFP (controls); (b) AtPP2CG1:eGFP; (c) pPS48–YFP (controls); (d) YFP:AtPP2CG1. The subcellular localization was visualized by fluorescence microscopy.

of the known PP2C genes of Group G have been shown to be up-regulated by salt, but their functions in the salt response have not been elucidated [34].

There are at least two signal regulatory systems involved in stress-responsive gene expression, namely ABA-dependent and -independent signal transduction cascades [28]. Salt-induced

expression of *AtPP2CG1* is ABA-dependent. We analyzed regulation functions of the *AtPP2CG1* in ABA-dependent and -independent pathways by Real-time RT-PCR. The drought and salt-induced expression of *DREB2A* occur via the ABA-independent pathway. The salt-induced expression of *RD29B* occurs via the ABA-dependent pathway. In addition, *RD29A* is involved in both ABA-dependent and -independent pathways, and can regulate *DREB2* expression. *KIN1* can be induced by low temperature, exogenous ABA, and dehydration [35–37]. Our study showed that the expression levels of four stress-responsive genes in 35S:*AtPP2CG1* transgenic plants increased more substantially in response to salt than in the WT and *pp2cg1* mutant plants. The expression level of *DREB2A* did not follow this trend at 12 h of salt stress, which may reflect the fact that *DREB2A* is an early stress-responsive gene [38]. Thus, *AtPP2CG1* may up-regulate expression of stress-responsive genes in both ABA-dependent and -independent pathways under salt stress.

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