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Original Article

Overexpression of *PP2A-C5* that encodes the catalytic subunit 5 of protein phosphatase 2A in *Arabidopsis* confers better root and shoot development under salt conditions

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ABSTRACT

Protein phosphatase 2A (PP2A) is an enzyme consisting of three subunits: a scaffolding A subunit, a regulatory B subunit and a catalytic C subunit. PP2As were shown to play diverse roles in eukaryotes. In this study, the function of the *Arabidopsis PP2A-C5* gene that encodes the catalytic subunit 5 of PP2A was studied using both loss-of-function and gain-of-function analyses. Loss-of-function mutant *pp2a-c5-1* displayed more impaired growth during root and shoot development, whereas overexpression of *PP2A-C5* conferred better root and shoot growth under different salt treatments, indicating that PP2A-C5 plays an important role in plant growth under salt conditions. Double knockout mutants of *pp2a-c5-1* and salt overly sensitive (*sos*) mutants *sos1-1*, *sos2-2* or *sos3-1* showed additive sensitivity to NaCl, indicating that *PP2A-C5* functions in a pathway different from the SOS signalling pathway. Using yeast two-hybrid analysis, four vacuolar membrane chloride channel (CLC) proteins, AtCLCa, AtCLCb, AtCLCc and AtCLCg, were found to interact with PP2A-C5. Moreover, overexpression of *AtCLCc* leads to increased salt tolerance and Cl^- accumulation in transgenic *Arabidopsis* plants. These data indicate that PP2A-C5-mediated better growth under salt conditions might involve up-regulation of CLC activities on vacuolar membranes and that *PP2A-C5* could be used for improving salt tolerance in crops.

Key-words: chloride channel; salt signalling; salt tolerance.

INTRODUCTION

Protein phosphatase 2A (PP2A) is one of the major serine/threonine protein phosphatases in plants and plays important roles in cellular processes in plants (DeLong 2006; Farkas *et al.* 2007). As a trimeric protein complex, PP2A consists of a scaffolding subunit A, a regulatory subunit B and a catalytic subunit C (Janssens and Goris 2001). In *Arabidopsis*, there are 3 genes coding for A subunits, 17 genes for B subunits and 5 genes for C subunits (Farkas *et al.* 2007). Among the three A subunits in *Arabidopsis*, PP2A-A1 was shown to be involved in salt stress response (Blakeslee *et al.* 2008) and plays more important roles than PP2A-A2 and PP2A-A3, as its mutant (i.e. *pp2a-a1*) displays disrupted gravitropism, ABA insensitivity and enhanced ethylene and blue light responses (Garbers *et al.* 1996; Rashotte *et al.* 2001; Kwak *et al.* 2002; Larsen and Cancel 2003; Tseng and Briggs 2010), whereas other A mutant such as *pp2a-a2* or *pp2a-a3* does not display similar phenotype as *pp2a-a1* (Zhou *et al.* 2004). The B subunits were grouped into three subfamilies based on the functional domains they contain: WD-40 repeat (B subfamily), B56 domain (B′ subfamily) and EF-hand domain (B″ subfamily) (Farkas *et al.* 2007). Although there are more B subunits found in plants, very little is known about their functions except in a few cases where they were shown to be involved in plant development, salt stress response and regulating enzyme activities (Camilleri *et al.* 2002; Heidari *et al.* 2011; Leivar *et al.* 2011).

The five PP2A catalytic subunits in *Arabidopsis*, PP2A-C1 to PP2A-C5, were grouped into two subfamilies: the subfamily I that includes PP2A-C1 (At1g59830), PP2A-C2 (At1g10430) and PP2A-C5 (At1g69960), and the subfamily II that includes PP2A-C3 (At2g42500) and PP2A-C4 (At5g58500) (Farkas *et al.* 2007). One study showed that PP2A-C2 was negatively involved in the ABA signalling pathway (Pernas *et al.* 2007), and furthermore, the *pp2a-c2* mutant displayed enhanced sensitivity to NaCl, indicating a possible role of *PP2A-C2* in salt signalling pathway (Pernas *et al.* 2007). Yet another study showed that activation of plant defence response and localized cell death was observed in *Nicotiana benthamiana* when the catalytic subunits of the subfamily I were silenced (He *et al.* 2004). Furthermore, the *Arabidopsis* double mutant of *pp2a-c3 pp2a-c4* showed altered auxin distribution and plant development, while single mutant of *pp2a-c3* or *pp2a-c4* was phenotypically normal, suggesting that PP2A-C3 and PP2A-C4 share redundant roles in controlling root and embryo development (Ballesteros *et al.* 2013). These studies indicate that PP2A plays diverse roles in plant cellular metabolisms and plant development.

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Soil salinity is one of the major environmental stresses that greatly inhibit plant growth and development (Munns and Testers 2008). Plants have evolved three major strategies to cope with salt stress: restriction of Na^+ from entering cells (influx), compartmentalization of Na^+ into vacuoles to reduce the toxicity of Na^+ in cytoplasm and increasing Na^+ export from cytoplasm (efflux) (Zhu 2003; Munns and Testers 2008). Compartmentalization of Na^+ into vacuoles is mediated by the NHX family antiporters. Using the H^+ gradient across the vacuolar membrane as the driving force generated by the vacuolar H^+-ATPase and H^+-pyrophosphatase, NHX-type antiporters transport Na^+ into the vacuoles (Blumwald *et al.* 2000). The *AtNHX1* gene was the first vacuolar Na^+/H^+ antiporter gene cloned in plants (Gaxiola *et al.* 1999), and overexpression of *AtNHX1* in *Arabidopsis* and in other plants leads to increased salt tolerance (Apse *et al.* 1999; He *et al.* 2005; Banjara *et al.* 2011). Export of Na^+ from cytoplasm could be mediated by the salt overly sensitive (SOS) signalling pathway in *Arabidopsis* (Zhu 2002 and Zhu 2003). There are three major components in the SOS pathway, SOS1, SOS2 and SOS3. The SOS1 is a plasma membrane-bound Na^+/H^+ antiporter (Shi *et al.* 2002), SOS2 is a serine/threonine protein kinase (Liu *et al.* 2000) and SOS3 is a calcineurin-like calcium sensor protein (Liu and Zhu 1998). Under high salt environment, the immediate elevation of Ca^{2+} can be sensed by the calcium binding protein SOS3, which facilitates the binding of SOS3 to SOS2, consequently releasing the self-inhibition of SOS2 (Halfter *et al.* 2000; Ishitani *et al.* 2000; Zhu 2002). The SOS3–SOS2 complex is then recruited to the plasma membrane where the activated SOS2 activates the activity of SOS1, leading to an increased efflux of Na^+ from cytoplasm (Quintero *et al.* 2002; Shi *et al.* 2002; Qiu *et al.* 2003 and Zhu 2003).

Accumulation of ions in vacuoles, not only cation, but anion as well, leads to increased salt tolerance in plants, as this approach appears to be used by some salt tolerant citrus and grapevine (Storey and Walker 1999; Storey *et al.* 2003). The vacuolar membrane bound chloride channel (CLC) proteins have been reported to move Cl^- and NO_3^- into vacuole in exchange for protons (Barbier-Brygoo *et al.* 2011). For example, a mutant in one of the CLC genes in *Arabidopsis*, *atclcc-1*, is sensitive to NaCl (Jossier *et al.* 2010), whereas overexpression of *atclcc-1* orthologous genes led to increased salt tolerance in transgenic plants (Li *et al.* 2006; Zhou and Qiu 2010; Sun *et al.* 2013), confirming the important roles of CLC proteins in salt tolerance.

Here, we provide a study on the function of the PP2A catalytic subunit 5, PP2A-C5, in *Arabidopsis*. Our data showed that PP2A-C5 is required for salt response in a pathway that is independent of the *SOS* pathway in *Arabidopsis*. In particular, PP2A-C5 is critical for enhancing root and shoot development under salt conditions, and PP2A-C5 might regulate the activities of CLC proteins to increase sequestration of anions into vacuoles, thereby increasing salt tolerance in plant.

RESULTS

Characterization of the pp2a-c5-1 mutant

To elucidate the biological function of the catalytic subunit 5 of PP2A, a mutant allele of *PP2A-C5* (At1g69960) was obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University, and this mutant was designated as *pp2a-c5-1*. The T-DNA was inserted into the first intron of *PP2A-C5* (Fig. 1a), which was confirmed by using the PCR-based genotyping method (Fig. 1b). Because of the big size of the T-DNA insertion, it was predicted that the expression of *PP2A-C5* would be affected. To analyse the transcript of

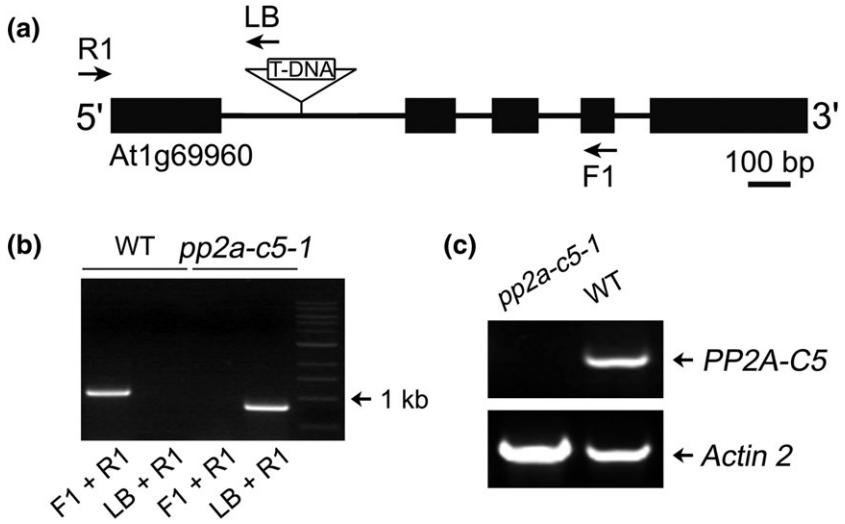

Figure 1. Confirmation of the *pp2a-c5-1* mutant. (a). Genomic structure of *pp2a-c5-1* mutant (SALK_139822). Black boxes and lines indicate exons and introns of *PP2A-C5*, respectively. T-DNA insertion site is indicated by the triangle containing the T-DNA left border (LB) sequence. F1 and R1, PCR primers used for amplifying a *PP2A-C5* fragment. (b). PCR genotyping shows that the *pp2a-c5-1* mutant is homozygous for the T-DNA insertion. F1, R1 and LB, PCR primers used for amplifying DNA fragments from wild-type (WT) plant and *pp2a-c5-1* mutant. C. RT-PCR analysis of the *PP2A-C5* transcript in WT and *pp2a-c5-1* mutant plants. The *Actin 2* transcript was used as the RNA internal control.

PP2A-C5, we conducted reverse transcription-polymerase chain reaction (RT-PCR) with primer pairs that cover the full-length of *PP2A-C5* coding sequence. While there was ample amplification product from the mRNAs of wild-type plants, no transcript was amplified from the mRNAs of the *pp2a-c5-1* mutant (Fig. 1c). The RT-PCR result indicated that *pp2a-c5-1* is likely a null mutant. The *pp2a-c5-1* mutant was crossed with wild-type plants, and the F_1 progenies were selfed to produce F_2 seeds. The ratio of salt sensitive plants (phenotype of *pp2a-c5-1*, see next section) to normal plants in the F_2 population is 1:3, and all salt sensitive plants were homozygous T-DNA insertion plants based on PCR analysis (Supp. Fig. 1). This result indicates that the T-DNA insertion mutation in *pp2a-c5-1* is a recessive mutation in the *PP2A-C5* locus.

The pp2a-c5-1 mutant is hypersensitive to salt treatment

One of our major interests in studying PP2A was to study if PP2A is involved in plant response to abiotic stresses, so we subjected the *pp2a-c5-1* mutant to ABA and salt stress treatments. We did not see major phenotypic differences between wild-type and the *pp2a-c5-1* mutant after ABA treatment (Supp. Fig. 2), but we found that the *pp2a-c5-1* mutant was sensitive to salt. In the root bending assay, 4-day-old *pp2a-c5-1* mutant and wild-type plants were transferred onto MS plates that contained no or 75 mM NaCl. After one week of growth, the root length of *pp2a-c5-1* was about the same as wild-type plants in the absence of salt, whereas in the presence of salt, the *pp2a-c5-1* mutant showed greater growth inhibition in comparing to wild-type plants (Fig. 2a). When considering the relative root length, the *pp2a-c5-1* mutant displayed greater root growth inhibition than that of wild-type plants. For example, at 75 mM NaCl concentration, the root length of wild-type plants was about 56% of that under MS medium, whereas the root length of *pp2a-c5-1* mutant under the salt treatment was less than 30% of that under MS medium (Fig. 2b). Similar phenotype was also observed at a higher concentration of NaCl (i.e. 100 mM). Besides the shorter root length, the *pp2a-5-1* mutant appeared pale and chlorotic, and developed fewer lateral roots (Fig. 2a).

The salinity stress includes two phases: a rapid osmotic stress and a long-time ion toxicity stress (Munns and Testers 2008). To determine whether ion toxicity or osmotic stress is the main reason for the salt sensitivity in the *pp2a-c5-1* mutant, mannitol was used to induce osmotic stress. Four-day-old seedlings of wild-type and the *pp2a-c5-1* mutant plants were transferred onto mannitol containing media (50 mM, 100 mM, 300 mM and 400 mM, respectively). After growing on the mannitol-containing media for a week, all plants displayed similar root lengths (Fig. 2c). These experiments indicated that the salt sensitive phenotype of *pp2a-c5-1* is because of ion toxicity, not osmotic stress.

To determine whether the loss of function mutant *pp2a-c5-1* is compromised in salt tolerance in germination and early seedling development, the *pp2a-c5-1* mutant was germinated and grown on MS plates in the presence or absence of salt. In the absence of NaCl, there were no obvious differences between *pp2a-c5-1* and wild-type plants in germination and early seedling development (Supp. Fig. 3). Under salt treatment, no germination difference was found between wild-type and the mutant; however, early seedling development was more inhibited in the *pp2a-c5-1* mutant than in wild-type plants (Supp. Fig. 4). Root development was clearly slower in the *pp2a-c5-1* mutant on the 75 mM NaCl medium for 2 weeks of growth (Supp. Fig. 4).

Salt tolerance was also tested for plants growing in soil. Plants were grown side by side in the same pot, and salt treatment was applied by flood irrigating the pot with salt solutions, 75 mM and 100 mM, respectively, every 2 d. The *pp2a-c5-1* mutant did not show any obvious difference in comparing with wild-type plants when irrigated with water (Fig. 2d). However, under salt treatment for 2 weeks, the *pp2a-c5-1* mutant plants were smaller in size, and half of their leaves became chlorotic, yet wild-type plants were still green (Fig. 2d).

Salt sensitivity of the pp2a-c5-1 mutant is because of mutation in the PP2A-C5 gene

To determine if the salt sensitivity of the *pp2a-c5-1* mutant is indeed because of a mutation in the *PP2A-C5* gene, a wild-type *PP2A-C5* gene was overexpressed in the *pp2a-c5-1* mutant background. The coding sequence of *PP2A-C5* from a wild-type cDNA library was fused to the 35S promoter in the binary vector pFGC5941 (McGinnis *et al.* 2005) and then transformed into the *pp2a-c5-1* mutant. Twelve independent transgenic lines were obtained, and two homozygous transgenic lines were used for the root bending assay. Real time-PCR analysis showed that the two *PP2A-C5* overexpressing lines in the *pp2a-c5-1* mutant background, C5-Com1 and C5-Com2, expressed *PP2A-C5* transcript at levels that are slightly higher than that of wild-type plants, but significantly lower than those from *PP2A-C5*-overexpressing plants in the wild-type background (Supp. Fig. 5). When treated with 75 mM NaCl in the medium, the *pp2a-c5-1* mutant developed shorter roots, and produced smaller leaves (Fig. 2e), whereas the *pp2a-c5-1* mutants harbouring the P_{35S} :: *PP2A-C5* transgene produced longer root systems that were similar to or slightly longer than those of wild-type plants (Fig. 2e). This experiment indicates that the T-DNA insertion into the *PP2A-C5* gene is responsible for the observed salt sensitivity in the *pp2a-c5-1* mutant.

PP2A-C5 is up-regulated by salt

Because the *pp2a-c5-1* mutant is more sensitive to salt stress, it prompted us to test if expression of *PP2A-C5* is regulated by salt. Eight-day-old *Arabidopsis* seedlings on MS medium were transferred to filter paper saturated with 200 mM NaCl, and plant samples were collected at 0 h, 3 h, 6 h, 9 h and 12 h for total RNA isolation, which were then used for RT-PCR analysis. Our data indicated that the *PP2A-C5* transcript was up-regulated by salt treatment, and the highest accumulation of

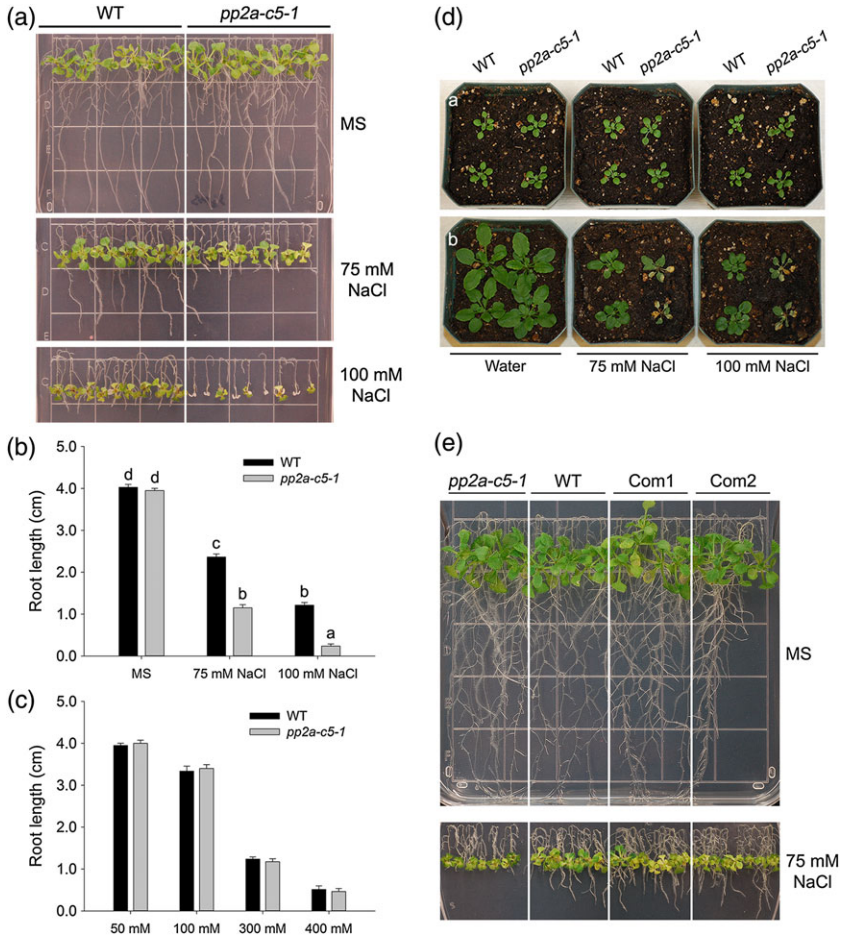

Figure 2. The *pp2a-c5-1* mutant is sensitive to salt treatment. (a). Phenotypes of wild-type (WT) and *pp2a-c5-1* mutant plants growing vertically on MS plates containing no salt or 75 mM and 100 mM NaCl, respectively. Pictures were taken 6 d after plants were transferred to salt plates. (b). Root lengths of wild-type and *pp2a-c5-1* mutant plants under salt conditions ($n = 20$ plants from three individual plates). Statistical significance between samples was indicted by different letters according to the Student *t*-test. (c). Root lengths of wild-type and *pp2a-c5-1* mutant plants grown on MS medium containing various concentrations of mannitol (50 mM, 100 mM, 300 mM and 400 mM, respectively) ($n = 20$ plants from three individual plates). (d). Phenotypes of wild-type and *pp2a-c5-1* mutant before and after salt treatment in soil. (a). Wild-type and *pp2a-c5-1* mutant plants before salt treatment. (b). Wild-type and *pp2a-c5-1* mutant plants after salt treatment. Plants were firstly grown on MS medium until they reach six-leaf stage; then they were transferred into soil side by side. Plants were flood irrigated with water or salt solutions every other day. Pictures were taken two weeks after salt treatment. E. *PP2A-C5* overexpression rescues the *pp2a-c5-1* mutant phenotype. Four-day-old *Arabidopsis* seedlings were transferred onto MS plates containing no or 75 mM NaCl and allowed to grow for 7 d. The transgenic *pp2a-c5-1* mutant plants containing a wild-type *PP2A-C5* gene (Com1 and Com2) displayed a phenotype that is similar to, or slightly better than, wild-type's phenotype, whereas *pp2a-c5-1* mutant displayed sensitive phenotype after salt treatment ($n = 20$ plants from three individual plates).

PP2A-C5 transcript was found at the 3 h after plants were transferred to salty paper (Fig. 3a). Plant samples treated on filter paper containing different concentrations of salt (50 mM, 100 mM and 150 mM, respectively) were also collected after 6 h of salt treatment. Our RT-PCR results indicated that the *PP2A-C5* transcript was increased by all three salt concentrations (Fig. 3a). It appeared that higher salt concentrations increased higher levels of *PP2A-C5* transcript (Fig. 3a). Interestingly, the NaCl concentration that induces the highest *PP2A-C5* transcript is 150 mM, not 200 mM (Fig. 3b), based on the quantitative analysis of the PCR data in Fig. 3a, which is likely because of that the 200 mM concentration of NaCl is more damaging to cellular metabolisms. Nevertheless, it is clear that the expression of *PP2A-C5* is up-regulated by salt.

Ectopic overexpression of PP2A-C5 confers better vegetative growth under salt conditions

Because the *pp2a-c5-1* mutant showed higher sensitivity to salt, hinting a possibility that a gain of function in *PP2A-C5* might increase salt tolerance. To test this possibility, we overexpressed *PP2A-C5* using the 35S promoter in *Arabidopsis* plants. More than 30 independent transgenic lines were obtained, and seven homozygous lines were analysed by real-time quantitative PCR and Western blot techniques. We found that five transgenic lines expressed *PP2A-C5* transcript at higher levels (Fig. 3c), and these five lines also expressed PP2A-C proteins at relatively higher levels as revealed in the Western blot analysis using PP2Ac antibodies that recognize all C subunits of PP2A (Fig. 3d).

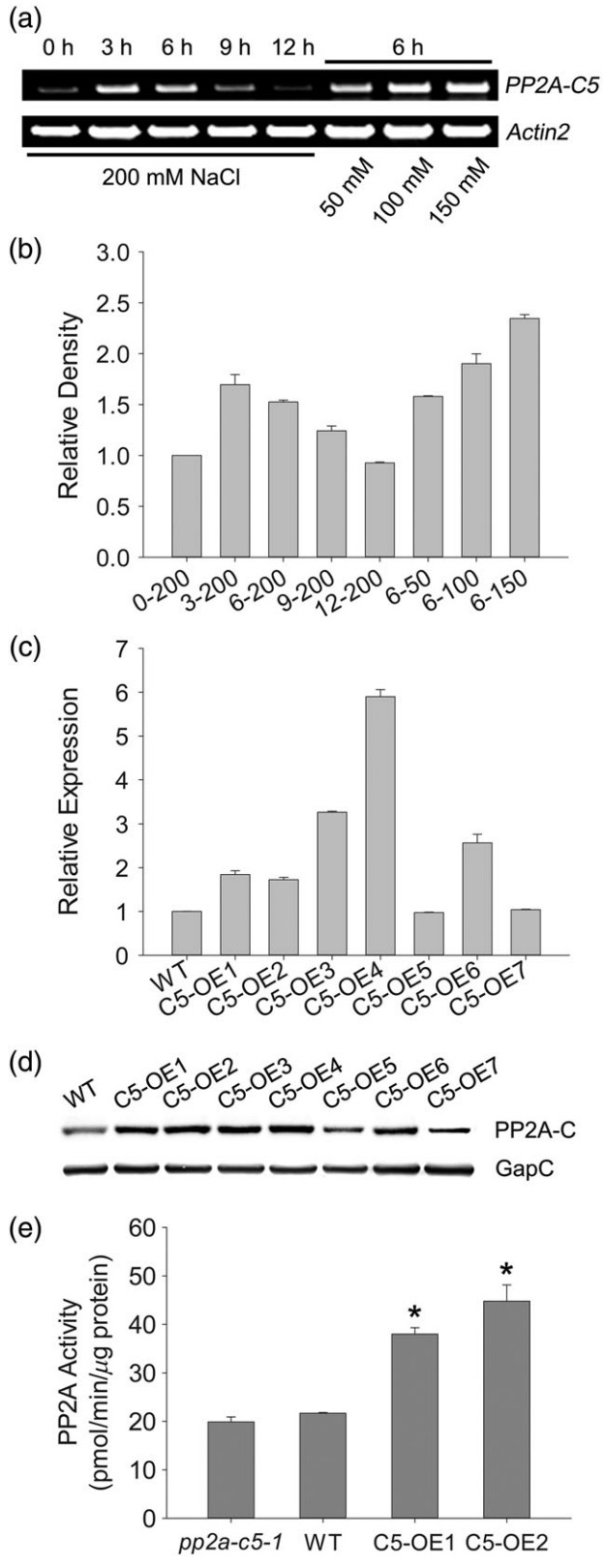

Figure 3. Expression of *PP2A-C5* under salt conditions and molecular characterizations of *PP2A-C5*-overexpressing plants. (a). Expression of *PP2A-C5* is salt inducible. Eight-day-old *Arabidopsis* seedlings were transferred onto a filter paper saturated with 200 mM NaCl for various times (3, 6, 9 and 12 h, respectively) or to filter papers saturated with various concentrations of NaCl (50 mM, 100 mM and 150 mM, respectively); then, mRNAs were isolated for RT-PCR analyses. The *Actin 2* transcript was used as the internal control. (b). Quantitative analysis of the PCR data in (a). The *PP2A-C5* transcript from plants before salt treatment (0 h) was set as value 1, and transcripts from NaCl treated plants were compared to the 0 h sample value; 0–200 to 12–200 were plant samples from 0 h to 12 h after 200 mM NaCl treatment; 6–50, 6–100 and 6–150 were plant samples treated for 6 h under 50 mM, 100 mM and 150 mM NaCl, respectively. (c). Real-time quantitative PCR analysis of *PP2A-C5*-overexpressing plants. WT, wild-type; C5-OE1 to C5-OE7, seven independent *PP2A-C5*-overexpressing plants. Expression levels are expressed as the relative ratios to the transcript level of *PP2A-C5* in wild-type plants under normal growth condition ($n = 15$ from five plants with three technical replicates). (d). Western blot analysis of *PP2A-C5*-overexpressing plants. PP2Ac antibody that recognizes all 5 C subunits of PP2A in Arabidopsis was used in the blot. GapC, cytosolic glyceraldehyde-3-phosphate-dehydrogenase used as the protein loading control. (e). PP2A activities in *pp2a-c5-1*, wild-type and two *PP2A-C5*-overexpressing plants. *, significant at 1% according to Student *t*-test. Three biological replicates and three independent experiments were performed.

Because there are five PP2A catalytic subunits in *Arabidopsis*, it was important to know if loss of function or gain of function in *PP2A-C5* would have an impact on the overall PP2A activity in *Arabidopsis*. We therefore determined the PP2A activities in the *pp2a-c5-1* mutant and two *PP2A-C5*-overexpressing plants (i.e. C5-OE1 and C5-OE2). With the peptide substrate RRAC(pT)VA for phosphatase assay (Promega, Madison, Wisconsin), there was just a little reduction, from around 22.1 (pmol/min/μg protein) to around 19.4 (pmol/min/μg protein), in the overall PP2A activity in the *pp2a-c5-1* mutant when compared with the wild-type *Arabidopsis* using cellular extracts of 10-day-old seedlings, yet it was clear that overexpression of *PP2A-C5* led to at least onefold increase in PP2A activities in the two *PP2A-C5*-overexpressing plants (Fig. 3e). These two transgenic lines, C5-OE1 and C5-OE2, were selected for further studies. In the root bending assay, we found that both C5-OE1 and C5-OE2 developed longer primary roots and showed better seedling growth than wild-type plants after transferring to MS plates containing 75 mM NaCl (Fig. 4a). However, transgenic plants and wild-type plants showed no differences in primary root growth on MS plate in the absence of NaCl (Fig. 4a). Moreover, the number of lateral roots of C5-OE1 and C5-OE2 plants was not significantly different from that of wild-type plants on MS plate (Supp. Fig. 6). Quantitative data showed that the primary root lengths and fresh weights of C5-OE1 and C5-OE2 were significantly larger than those of wild-type plants after salt treatment (Fig. 4b,c).

In the absence of salt, wild-type plants displayed slightly better growth than *PP2A-C5*-overexpressing plants in soil (Fig. 4d,e). However, after irrigation with 250 mM NaCl solution for two weeks, *PP2A-C5*-overexpressing plants displayed much better growth than wild-type plants (Fig. 4e). The growth of wild-type plants was severely inhibited after the addition of 250 mM NaCl in soil, yet transgenic plants grew much better than wild-type plants (Fig. 4e). The enhanced salt

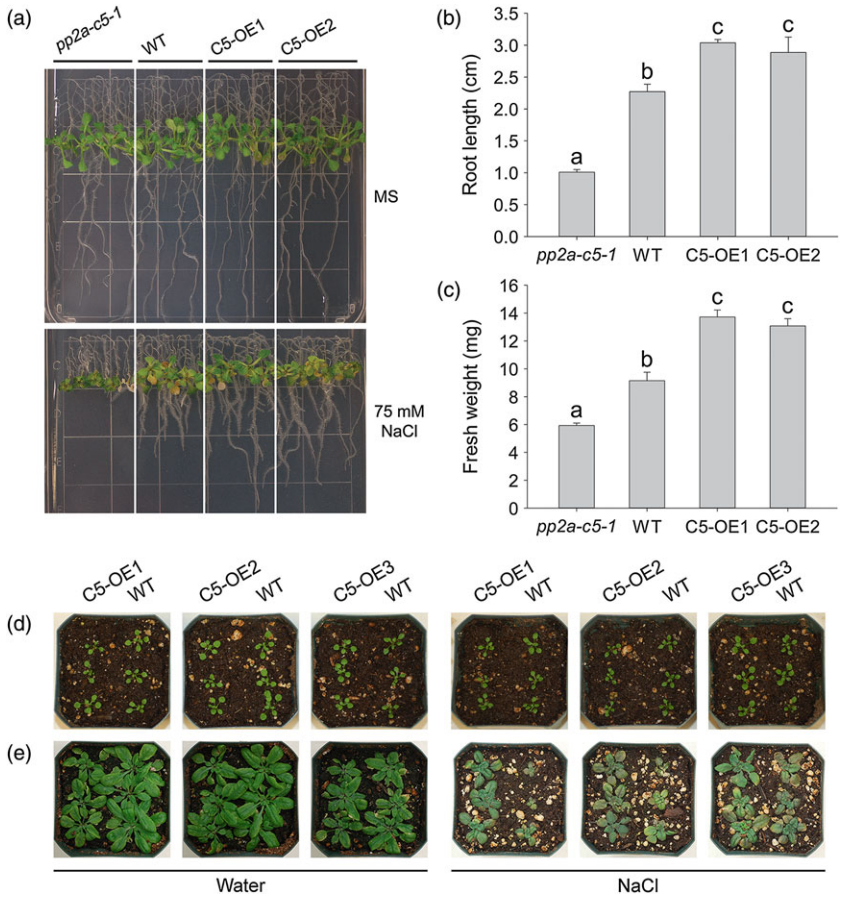

Figure 4. Overexpression of *PP2A-C5* increases salt tolerance in transgenic plants. (a). Phenotypes of *pp2a-c5-1* mutant, wild-type (WT) and *PP2A-C5*-overexpressing plants (C5-OE1 and C5-OE2) under normal and salt (75 mM NaCl) conditions on MS plates. (b). Analysis of root length in *pp2a-c5-1* mutant, wild-type and *PP2A-C5*-overexpressing plants after salt treatment on MS plates ($n = 25$ plants from four individual plates). Statistical significance between samples was indicated by different letters according to the Student *t*-test. (c). Analysis of fresh weight in *pp2a-c5-1* mutant, wild-type and *PP2A-C5*-overexpressing plants after salt treatment on MS plates. Fresh weights of *pp2a-c5-1* mutant, wild-type and *PP2A-C5*-overexpressing plants were obtained 10 d after plants were allowed to grow under 75 mM NaCl condition ($n = 50$ plants from five individual plates for each experiment and three repeats were performed). (d). Phenotypes of wild-type and *PP2A-C5*-overexpressing plants before salt treatment in soil. (e). Phenotypes of wild-type and *PP2A-C5*-overexpressing plants after salt treatment for two weeks in soil. Plants were irrigated with water or 250 mM NaCl solution every other day for 2 weeks, then the pictures were taken.

tolerance by overexpressing *PP2A-C5* in transgenic plants clearly indicates that *PP2A-C5* plays a critical role in regulating salt tolerance in plants.

Genetic relationship between PP2A-5C and the SOS signalling pathway

Much efforts have been made to dissect the complex salt signalling pathways in plants, and many genes that play important roles in regulatory networks of salt tolerance have been identified (Munns 2005; Munns and Testers 2008). Among the identified genes involved in salt signalling pathways, the SOS genes are the best characterized (Zhu 2002). To test the potential genetic interactions between *PP2A-C5* and components of the SOS pathway, the *pp2a-c5-1* mutant was crossed with *sos1-1*, *sos2-2* and *sos3-1*, respectively, and double mutants were obtained (Supp. Figs 7 to 9). We then compared the three double mutants with their parental mutant lines in the salt sensitivity assay. The *pp2a-c5-1 sos1-1* double mutant displayed significantly higher sensitivity to salt stress than the two parental mutants (Fig. 5a). For example, root growth in the *pp2a-c5-1 sos1-1* double mutant was more severely inhibited than either *pp2a-c5-1* or *sos1-1* mutant at 10 mM NaCl, a concentration that usually does not harm *Arabidopsis* plants (Fig. 5b). The relative root lengths of the double mutant, *sos1-1* mutant and *pp2a-c5-1* mutant are 36%, 65% and 90% of that of wild-type plants, respectively (Fig. 5c). At 20 mM NaCl, the relative root lengths of the double mutant, *sos1-1* mutant and *pp2a-c5-1* are 16%, 35% and 75% of that of wild-type plants, respectively (Fig. 5c). The double mutant displayed a more severe phenotype than the two parental mutants under both salt concentrations. The double mutants *pp2a-c5-1 sos2-2* and *pp2a-c5-1 sos3-1* displayed a similar phenotype, which is more sensitive to salt treatment than either parental mutants (Supp. Figs 10 and 11). Taken these data, we conclude that *PP2A-C5* functions in a pathway that is independent of the SOS pathway.

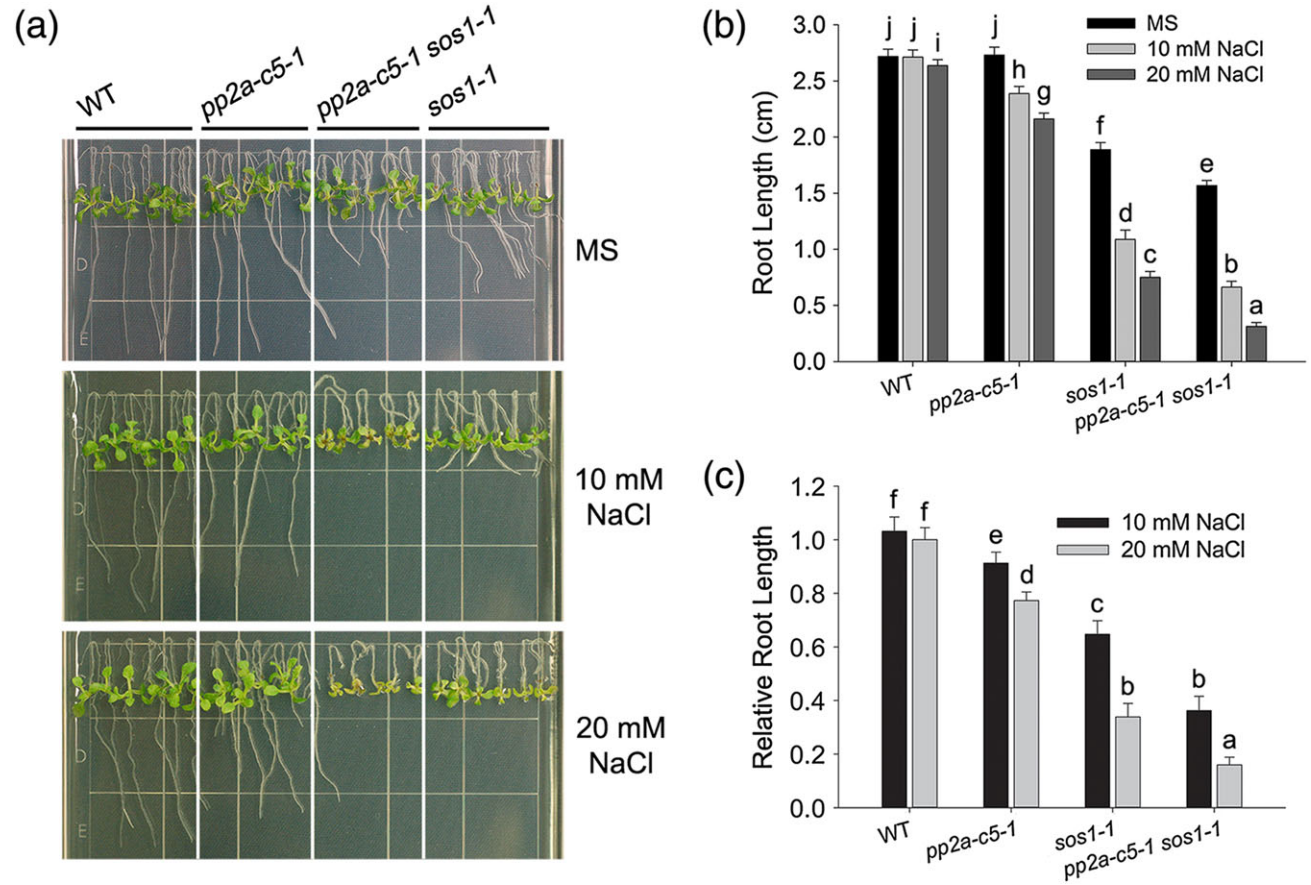

Figure 5. Analysis of the *pp2a-c5-1 sos1-1* double mutant. (a). Phenotypes of wild-type (WT), *pp2a-c5-1* mutant, *pp2a-c5-1 sos1-1* double mutant and *sos1-1* mutant in the absence of salt or after salt treatments. (b). Root lengths of wild-type, *pp2a-c5-1* mutant, *sos1-1* mutant and *pp2a-c5-1 sos1-1* double mutant in the absence or presence of NaCl. Three biological replications and three technical experiments were performed ($n = 20$ plants from three individual plates). Statistical significance between samples was indicated by different letters according to the Student *t*-test. (c). Relative root lengths of wild-type, *pp2a-c5-1* mutant, *sos1-1* mutant and *pp2a-c5-1 sos1-1* double mutants after salt treatments.

The pp2a-c5-1 mutant is also more sensitive to other salts

To study if the salt sensitivity of the *pp2a-c5-1* mutant is specific to NaCl only, we studied how the *pp2a-c5-1* mutant would perform in the presence of 75 mM of KCl and 75 mM KNO_3 in media. We found that the *pp2a-c5-1* mutant was also more sensitive to these two salts than wild-type plants (Fig. 6). Interestingly, overexpression of *PP2A-C5* made transgenic plants grew better than wild-type plants in the presence of these two salts (Fig. 6 and Supp. Fig. 12). In particular, *PP2A-C5*-overexpressing plants grew the best in the medium containing nitrate salt (KNO_3), suggesting that overexpression of *PP2A-C5* not only makes plant more tolerant to sodium chloride, but also makes plants grow better in the presence of 75 mM of potassium chloride and potassium nitrate.

PP2A-C5 interacts with chloride channel proteins in the yeast two-hybrid system

To explore the potential molecular mechanism of PP2A-C5's involvement in salt response, we sought for proteins that could interact with PP2A-C5 by using the yeast two-hybrid technique (Golemis *et al.* 1996). We used the PP2A-C5 as the bait and screened an *Arabidopsis* prey cDNA library. Out of about 50 000 yeast colonies, we identified several potential PP2A-C5-interacting proteins (Table 1). Among these candidate proteins, a chloride channel protein, AtCLCc, appeared the most relevant protein, because it was previously shown that AtCLCc plays important roles in salt tolerance in *Arabidopsis* (Jossier *et al.* 2010). Because there are seven CLC proteins in *Arabidopsis*, we tested if PP2A-C5 would interact with the other six CLC proteins by using the yeast two-hybrid analysis. We found that PP2A-C5 also interacted with AtCLCa, AtCLCb and AtCLCg, weakly with AtCLCe, but not with AtCLCd and AtCLCf (Supp. Fig. 13a). Among the seven CLC proteins, AtCLCa, AtCLCb, AtCLCc and AtCLCg are localized on the vacuolar membranes (Lv *et al.* 2009), indicating a possibility that the four vacuolar membrane-bound proteins are substrates of PP2A-C5. We then performed another set of yeast two-hybrid assays to test if other PP2A-C subunits could interact with AtCLCc. We only found interaction between

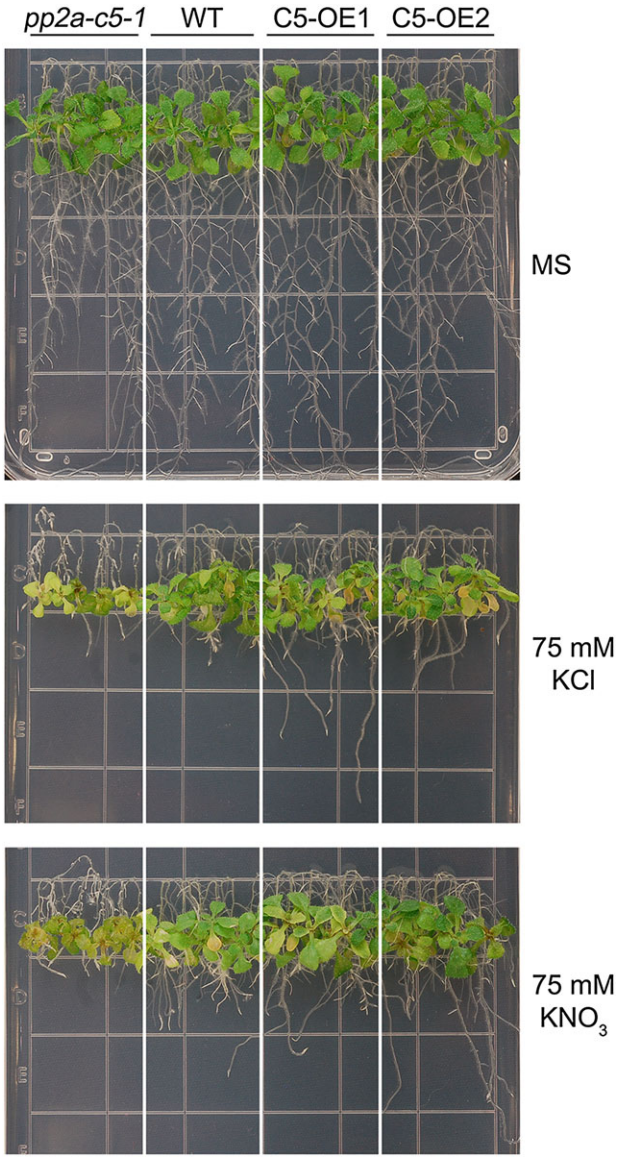

Figure 6. Overexpression of *PP2A-C5* increases tolerance to potassium salts in transgenic plants. Four-day-old *Arabidopsis* seedlings were transferred onto MS plates containing 75 mM KCl and 75 mM KNO_3, respectively, and allowed to grow for 10 d before the pictures were taken. WT, wild-type; C5-OE1 and C5-OE2, two independent *PP2A-C5*-overexpressing plants.

PP2A-C5 and AtCLCc, not the other four PP2A-C subunits (Supp. Fig. 13b), indicating that PP2A-C5 is the only C subunit potentially involved in the interaction with the vacuolar membrane-bound chloride channel proteins. We then analysed which part of AtCLCc is responsible for the interaction with PP2A-C5 by conducting yeast two-hybrid analysis, and we found that the C-terminal sequence (residues 562 to 779), and the N-terminal sequence (residues 1 to 92) of AtCLCc could interact with PP2A-C5 (Supp. Fig. 13c).

Table 1. PP2A-C5-interacting proteins identified from yeast two-hybrid screening

AGI number	Name	Description
At1g29910	CAB3	Chlorophyll *a*/*b* binding protein 3
At1g29930	CAB1	Chlorophyll *a*/*b* binding protein 1
At3g06200	N/A	P-loop containing nucleoside triphosphate hydrolases superfamily protein
At3g16420	PBP1	PYK1-binding protein 1
At3g26060	PRXQ	Peroxiredoxin Q
At3g26520	SITIP	Salt-stress inducible tonoplast intrinsic protein
At5g16050	GRF5	General regulatory factor 5
At5G49890	AtCLCc	*Arabidopsis thaliana* chloride channel C

Overexpression of AtCLCc leads to increased salt tolerance

Literatures indicate that AtCLCc functions as a H^+/Cl^- antiporter on the vacuolar membranes, using the H^+ gradient generated by the vacuolar membrane bound proton pumps such as H^+-ATPase and V-PPase (Harada *et al.* 2004; Zifarelli and Pusch 2010; Barbier-Brygoo *et al.* 2011). Decreased import of Cl^- into vacuole is likely the reason for the observed salt sensitivity in the *atclcc-1* mutant (Jossier *et al.* 2010), and overexpression of an *AtCLCc* ortholog from soybean in *Populus deltoides* × *P. euramericana* and *Arabidopsis* increased salt tolerance (Zhou and Qiu 2010; Sun *et al.* 2013). To test if overexpression of *AtCLCc* could lead to increased salt tolerance in *Arabidopsis*, we created transgenic plants that overexpress *AtCLCc* (Supp. Fig. 14). Two *AtCLCc*-overexpressing plants, CLCc-OE1 and CLCc-OE2, were chosen for salt tolerance test, and our data showed that these two *AtCLCc*-overexpressing plants were indeed significantly more salt tolerant than wild-type plants based on the root growth assay in the presence of 100 mM NaCl (Fig. 7a). To test the relationship between *PP2A-C5* and *AtCLCc*, we also overexpressed *AtCLCc* in the *pp2a-c5-1* mutant. Three *AtCLCc*-overexpressing plants, c5-CLCc-OE1 to c5-CLCc-OE3, were identified (Supp. Fig. 14), and two of them were used for salt tolerance test (Fig. 7b). Our data showed that these two c5-*AtCLCc*-overexpressing plants were just as sensitive to 100 mM NaCl as the *pp2a-c5-1* mutant (Fig. 7b,c), indicating that *AtCLCc* might function downstream of *PP2A-C5*. Because both *PP2A-C5*-overexpressing and *AtCLCc*-overexpressing plants displayed better primary root growth than other genotypes, it is likely that *PP2A-C5*-overexpression leads to increased AtCLCc activity, thereby increasing Cl^- import into vacuoles. To test this hypothesis, we measured Cl^- concentrations in *PP2A-C5*-overexpressing plants, wild-type plants, *pp2a-c5-1* mutant, *AtCLCc*-overexpressing plants and c5-*AtCLCc*-overexpressing plants. As expected, we found significantly higher Cl^- concentrations in *PP2A-C5*-overexpressing plants and *AtCLCc*-overexpressing plants than wild-type plants, and lower Cl^- concentration in the *pp2a-c5-1* mutant (Fig. 7d). As expected, the Cl^- concentrations in

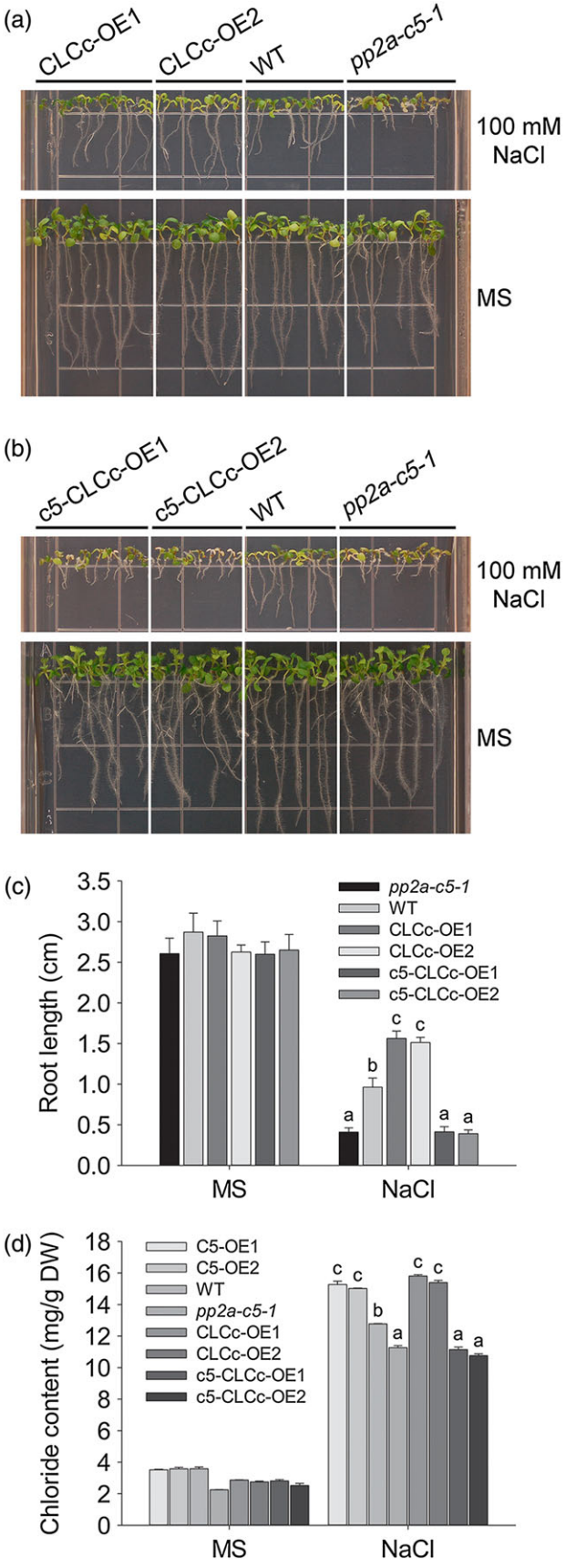

Figure 7. Overexpression of *AtCLCc* increases salt tolerance in transgenic plants, but cannot rescue the salt sensitive phenotype of the *pp2a-c5-1* mutant. (a). Phenotypes of *AtCLCc*-overexpressing, wild-type and *pp2a-c5-1* plants in the presence of 100 mM or absence of NaCl. CLCc-OE1 and CLCcOE2, two independent *AtCLCc*-overexpressing plants; WT, wild-type. (b). Phenotypes of c5-*AtCLCc*-overexpressing, wild-type and *pp2a-c5-1* plants in the presence of 100 mM or absence of NaCl. c5-CLCc-OE1 and c5-CLCc-OE2, two independent *AtCLCc*-overexpressing plants in the *pp2a-c5-1* mutant background. (c). Root lengths of *pp2a-c5-1*, wild-type, *AtCLCc*-overexpressing and c5-*AtCLCc*-overexpressing plants in the presence of 100 mM or absence of NaCl. $n = 24$ plants from three individual plates. Statistical significance between samples was indicated by different letters according to Student *t*-test. D. The Cl^- contents in *PP2A-C5*-overexpressing, wild-type, *pp2a-c5-1*, *AtCLCc*-overexpressing and c5-*AtCLCc*-overexpressing plants in the presence of 100 mM or absence of NaCl. Three biological replications and three technical experiments were performed. More than 50 plants were used for each Cl^- content measurement.

c5-*AtCLCc*-overexpressing plants were similar to the Cl^- concentration in the *pp2a-c5-1* mutant (Fig. 7d), which is consistent with the salt sensitivity data that overexpression of *AtCLCc* in the *pp2a-c5-1* mutant background does not rescue the salt sensitive phenotype of the *pp2a-c5-1* mutant, indicating that *PP2A-C5* functions upstream of *AtCLCc* in *Arabidopsis*.

DISCUSSION

In this report, we demonstrated that *PP2A-C5* plays an important role in plant response to environmental salt stimuli in *Arabidopsis*. Loss of function in the *PP2A-C5* gene leads to increased sensitivity to salt treatment (Figs 2, 4, 6 and 7), whereas overexpression of *PP2A-C5* increases the resistance to salt in transgenic plants (Figs 4 and 6). There is a direct causal relationship between *pp2a-c5-1* and salt sensitivity, as the salt sensitive phenotype of the *pp2a-c5-1* mutant could be rescued by expressing a wild-type copy of the *PP2A-C5* gene in the mutant background (Fig. 2e). Our study indicates that PP2A-C5 acts independent of the SOS signalling pathway, as shown by the double mutant analyses (Fig. 5 and Supp. Figs 10 and 11). Although Cl^- is an essential micronutrient for plants, it can become toxic at high concentrations in cytoplasm (Teakle and Tyerman 2010). When NaCl concentration in soil increases, plant cells face Na^+ toxicity as well as Cl^- toxicity. Therefore, vacuole sequestration of Cl^- could be an effective way to prevent accumulation of Cl^- in the cytoplasm. We found that PP2A-C5 might interact with a chloride channel protein AtCLCc and its closely related CLC proteins on vacuolar membranes based on the yeast two-hybrid analysis (Supp. Fig. 13). The potential interactions between PP2A-C5 and CLC proteins still need to be confirmed in planta by using other approaches, but the implication of these interactions leads us to a hypothesis on the mode of action of PP2A-C5. Two sites in CLC proteins, the serine 27 in the N-terminal side and the serine 672 in the C-terminal side, were shown to be phosphorylated and dephosphorylated by kinases and phosphatases (Whiteman *et al.* 2008; Jones *et al.* 2009; Reiland *et al.*, 2009; Vialaret and Maurel 2014), indicating that the possible interaction between AtCLCc and PP2A-C5 might

not be accidental, but with functional implication. Overexpression of *AtCLCc* in *Arabidopsis* leads to increased salt resistance in root growth (Fig. 7a) that is similar to the phenotype of *PP2A-C5*-overexpressing plants. However, overexpression of *AtCLCc* in the *pp2a-c5-1* mutant could not rescue the salt sensitive phenotype of the *pp2a-c5-c1* mutant (Fig. 7b), indicating that *PP2A-C5* might function upstream of *AtCLCc* in response to salt stress. We then found that Cl^- contents in *PP2A-C5*-overexpressing and *AtCLCc*-overexpressing plants were higher than that in wild-type plants, and the lowest Cl^- contents were found in the *pp2a-c5-1* mutant and c5-*AtCLCc*-overexpressing plants (Fig. 7d), suggesting that PP2A-C5's role in salt response network might involve CLC-mediated anion transport into vacuoles in plants. Based on our data, we are tempted to propose a model on how PP2A-C5 might regulate salt stress response in plants. The PP2A-C5 might up-regulate CLC proteins on the vacuolar membranes, which in turn increases the active transport of anions such as Cl^- and NO_3^- into vacuoles using H^+ as the driving force, leading to increased tolerance under salt conditions (Supp. Fig. 15).

Although more experiments will be needed to test this model, it is supported by existing literatures. For examples, overexpression of a soybean CLC gene, *GmCLC1*, in transgenic plants or tobacco bright yellow (BY)-2 cells led to significantly increased salt tolerance (Li *et al.* 2006; Zhou and Qiu 2010; Sun *et al.* 2013). Loss of *AtCLCc*, the *GmCLC1* ortholog in *Arabidopsis*, led to salt sensitive phenotype in *Arabidopsis* (Jossier *et al.* 2010). Our *pp2a-c5-1* mutant is very sensitive to NaCl, KCl and KNO_3 (Figs 2 and 6), indicating that the sensitivity is not specific to Na^+. The AtCLCa is a major nitrate/proton antiporter in *Arabidopsis*, as it imports two NO_3^- at the expense of one H^+ (De Angeli *et al.* 2006). A recent report showed that AtCLCa accumulates anions in the vacuole during stomatal opening in response to light and it releases anions during stomatal closure in response to ABA (Wege *et al.* 2014). The dual roles of AtCLCa are likely controlled by phosphorylation/dephosphorylation events, as when AtCLCa is phosphorylated by a protein kinase called OST1 (i.e. SnRK2.6), it releases anions, K^+ and water from vacuole, guard cells close; however, when AtCLCa is dephosphorylated by a phosphatase, it imports anions, K^+ and water, guard cells open (Wege *et al.* 2014). The phosphatase that dephosphorylates AtCLCa could be PP2A. If *PP2A-C5*-overexpression activates AtCLCa, it would facilitate nitrate import into vacuoles, which could help plants utilize nitrate more efficiently. Our *PP2A-C5*-overexpressing plants grow much better in the presence of 75 mM KNO_3 (Fig. 6), supporting the idea that PP2A-C5 might regulate other CLC proteins like AtCLCa on vacuolar membranes. This possibility is supported by the yeast two-hybrid data (Supp. Fig. 13a). A recent report by Vialaret & Maurel (2014) indicated that the level of phosphorylation at the serine 672 of AtCLCc was induced in response to NaCl, which seems in conflict with our model, as their data hints that the phosphorylated AtCLCc at serine 672 should be the active form instead of the dephosphorylated AtCLCc as the active form. This discrepancy might be because of (1) the phosphorylated serine 672 is not the target of PP2A-C5, but other phosphorylated serine(s) or (2) the different experimental systems used: they used hydroponic culture system to treat plants with NaCl for a short time (i.e. 45 min or 2 h), and we grew plants on MS plate or in soils and treated plants with salt for a much longer time (usually one week to three weeks); therefore, the phosphorylation status at serine 672 might be different in the two experimental systems. Other PP2A-C5-interacting proteins from yeast two-hybrid screening (Table 1), such as SITIP (salt-stress inducible tonoplast intrinsic protein) and GRF5 (general regulatory factor 5), might be substrate proteins of PP2A-C5; therefore, PP2A-C5's role in salt response pathway may involve other mechanisms as well.

Because PP2A's subunit is encoded by multiple genes, for example, 3 A genes, 17 B genes and 5 C genes in *Arabidopsis* (Farkas *et al.* 2007), at least 255 novel forms of PP2A could be formed, which explains the functional diversity of PP2A in plants. The individual members of a highly conserved subunit family might have a conserved function, but each member may still have non-overlapping functions. For example, all A subunits can function as a scaffolding protein to bring a B subunit and a C subunit together, yet the A1 is more important than A2 and A3, as mutations in A1 lead to pleiotropic consequences, but mutations in A2 or A3 do not show detectable phenotypes (Zhou *et al.* 2004). Like A subunits, the five catalytic C subunits are also highly conserved. In this study, however, we found that the total PP2A activity decreased just a little in *pp2a-c5-1*, yet the mutant phenotype can still be detected under salt condition, which indicates that the function of PP2A-C5 could not be compensated by its closest subunits PP2A-C1 and PP2A-C2. Although the subunit redundancy could be a protective mechanism that insures plants to have PP2A activity in all tissues and under most environmental conditions, a subunit-specific function could still be revealed by studying mutants under specific conditions. It is clear that the C subunits have distinct functions. For examples, the *pp2a-c2* mutant is more sensitive to high salt treatment, and hypersensitive to ABA and sugar stress (Pernas *et al.* 2007); in contrast, *pp2a-c1* does not show similar phenotype under similar salt treatments, and *pp2a-c5-1* mutant responded to ABA treatment similarly as wild-type plants did (Supp. Fig. 2). In addition, the *PP2A-C5* gene plays regulatory roles in brassinosteroid signalling pathway, as the *pp2a-c5-1* mutant displayed slightly reduced response to brassinosteroid treatment (Tang *et al.* 2011). Earlier studies also showed that certain members of the C subunit family might function differently from other members. Members of the subfamily I of PP2A C subunits are involved in pathogen infection response, whereas members of the subfamily II are not, as silencing the activities of the C subunits in the subfamily I would lead to activation of localized cell death and increased resistance to bacterial pathogen infection in tobacco (He *et al.* 2004). Based on these results, it appears that members of the PP2A subfamily I are likely involved in BR, ABA, salt and pathogen response in plants (He *et al.* 2004; Pernas *et al.* 2007; Tang *et al.* 2011).

B subunits are considered as regulatory subunits, as they are responsible for selecting substrates, controlling PP2A enzyme activities and determining subcellular localizations for PP2A holoenzymes in eukaryotic cells (Janssens and Goris 2001). In most cases, mutations in a specific B subunit gene lead to a

specific defect in cellular metabolism (Camilleri *et al.* 2002; Heidari *et al.* 2011; Leivar *et al.* 2011). Is B subunit involved in salt stress response? Probably yes. To address the molecular mechanism of PP2A-C5's involvement in salt tolerance, knowing which B subunit is involved in salt stress response is necessary, so is biochemically confirming if AtCLCc and AtCLCa are PP2A-C5's substrates. While elucidation of PP2A's involvement in salt signalling pathway at the molecular level will be extremely valuable in our understanding of PP2A's critical roles in eukaryotic cells, the PP2A-C5's potential role in improving salt tolerance and nitrogen use in crops should be tested.

MATERIAL AND METHODS

Plant materials

The T-DNA insertion mutant *pp2a-c5-1* (Salk_139822) was obtained from ABRC at Ohio State University. The diagnostic primers pp2a-c5-1-F1 and pp2a-c5-1-R1 for confirming the T-DNA insertion site by PCR were designed with the online tool (http:signal.salk.edu/cgi-bin/tdnaexpress). Mutants *sos1-1*, *sos2-2* and *sos3-1* were provided by Dr. Huazhong Shi.

Generation of double mutants

To generate double mutants between *pp2a-c5-1* and the *sos* mutants, crossings were made between *pp2a-c5-1* and the three *sos* mutants, *sos1-1*, *sos2-2* and *sos3-1*, respectively. The F_1 hybrids were self-crossed, and F_2 progenies were tested individually by PCR to confirm the genotypes of the homozygous double mutants. The *sos1-1* mutant contains a 7 bp deletion, and the PCR product of the mutant allele using primers SOS1-F1 and SOS1-R1 can be separated on a 3% agarose gel with a small mobility shift change. For *sos2-2*, the SOS2-F1 and SOS2-R1 primers were used for the identification of the wild-type *SOS2* gene, and the SOS2-F2 and SOS2-R1 primers were used for the identification of the *sos2-2* mutant allele (Halfter *et al.* 2000). For the *sos3-1* mutant identification, the SOS3-F1 and SOS3-R1 primers were used in PCR to produce 112 and 121 bp fragments in mutant and wild-type plants, respectively (Halfter *et al.* 2000).

Plant growth conditions

Arabidopsis seeds were surface sterilized with 15% bleach for 10 min, followed by 3 times of wash with distilled water. After stratification at 4 °C in darkness for 3 d, seeds were put on plates containing MS medium (Murashige and Skoog 1962), 1% (w/v) sucrose and 0.7% (w/v) agar, pH 5.8. *Arabidopsis* plants were grown at 22 °C in a controlled environmental growth chamber with a 16 h-light/8 h-dark photoperiod (light intensity 120 mE $s^{-1}m^{-2}$) after treatment at 4 °C in darkness for 3 d.

For salt treatment on MS plate, after vertical growth on normal condition for 4 d, young seedlings were transferred to MS plates that were supplemented with different concentrations of salts (10 mM, 20 mM, 30 mM, 40 mM, 75 mM or 100 mM, respectively), and these plates were inverted (upside down), so that plant roots would make a u turn and grow downward according to Zhu *et al.* (1998). Newly elongated root length, from the top to the primary root end, was measured as a way to test plant salt sensitivity. For osmotic treatment, 4-day-old seedlings were transferred to MS medium containing mannitol with the concentrations as indicated. Pictures were taken one week after treatment. Data for root length and fresh weight were collected. Each experiment was repeated at least 3 times.

To test plant salt tolerance in soil, seeds were firstly germinated and grown on MS plate until they reach six-leaf age. Then, young seedlings were transferred into soil; each pot contains two mutants and two wild-type plants side by side. Every 2 d, plants were flood irrigated with 50 ml of salt solutions, with the NaCl concentration of 75 mM and 100 mM, respectively. For controls, plants were irrigated with water only. Pictures were taken 2 weeks after salt treatment started.

Vector construction and plant transformation

Full-length cDNAs of *PP2A-C5* and *AtCLCc* were amplified from an 8-day-old *Arabidopsis* seedling cDNA library with primers OC5-F1 and OC5-R1, and with OCLCc-F1 and OCLCc-R1, respectively. The PCR reaction condition was 95 °C 30 s, 56 °C 30 s, 72 °C 60 s and 35 cycles. The PCR products were digested with *Nco* I and *Bam* HI, and cloned into the corresponding sites in the binary expression vector pFGC5941 (McGinnis *et al.* 2005) under the control of the cauliflower mosaic virus 35S promoter. The resultant vectors were then transformed into the *Agrobacterium tumefaciens* strain GV3101 cells, which were then used to transform wild-type *Arabidopsis* and *pp2a-c5-1* mutant plants using the floral-dip method (Clough and Bent 1998). Transgenic plants were selected based on the Basta resistance. More than 30 independent transgenic plants for *PP2A-C5* overexpression study, 10 dependent transgenic plants for *pp2a-c5-1* complementation study and more than 20 independent transgenic plants for *AtCLCc* overexpression studies in wild-type and *pp2a-c5-1* mutant plants were obtained, and transcript levels of the single-copy transgenic plants for each construct were analysed using the real time-PCR method. Two to three high expression lines for each construct were used for physiological and salt stress experiments.

Reverse transcription-PCR and real-time quantitative PCR experiments

For reverse transcription (RT)-PCR experiments, eight-day-old *Arabidopsis* seedlings were transferred onto filter papers soaked with 50 mM, 100 mM and 150 mM NaCl solutions for 6 h, or 200 mM for various times (3 h, 6 h, 9 h and 12 h, respectively), then samples were collected and stored in −80 °C freezer until use. Total RNAs were extracted using the PureLink™ Plant RNA Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For reverse transcription, the first-strand cDNA was synthesized from 1 μg of total RNAs using superscript reverse transcriptase (Invitrogen,

Carlsbad, CA) with the oligo(dT)$_{18}$ as primer according to the manufacturer's instructions. PCR was then performed with the condition of 95 °C 30 s, 56 °C 30 s, 72 °C 60 s for 28 cycles using primers PP2A-C5-RT-F1 and PP2A-C5-RT-R1, and final products were applied to gel electrophoresis. The *Actin 2* gene was used as the internal control for the RT-PCR analysis using primers Actin 2-F1 and Actin 2-R1.

For real-time quantitative PCR experiments, in a total 25 μl reaction solution, 5 μl of the cDNA products (from 10 times diluted RT product) was used as the template. PCR was performed in the real-time PCR machine 7500 sequence detection system from Applied Biosystems (Foster city, California, USA) using the SYBR Green supermix from Bio-Rad Laboratories (Hercules, California, USA). The reaction conditions are as follows: pre-incubation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s and extension at 55 °C for 40 s using primers PP2A-C5-qRT-F1 and PP2A-C5-qRT-R1. The *Actin 8* gene was used as the internal control for the real-time quantitative PCR analysis using primers Actin 8-F1 and Actin 8-R1. Three biological and three technical replicates were performed for each experiment.

Western blot analysis

Plant materials were collected and grounded into powder in the presence of liquid nitrogen. Total proteins were then extracted with the protein extraction buffer (10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium lauryl sarcosine, 40 mM sodium phosphate buffer, pH 7.0, 10 mM β-mercaptoethanol, 1 μg/mL leupeptin, 1 μg/mL aprotonin, 1 mM PMSF). Protein concentration was determined using the Bradford method (Bradford 1976). For each sample, 20 μg of proteins was loaded into each lane and separated on 10% SDS-PAGE gel. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane with the transfer buffer containing 20% methanol. The membrane was firstly incubated with TBSTM (5% milk in TBS buffer with 0.1% Tween-20) for 1 h to block unspecific sites. The membrane was then washed 3 times with TBST, before it was incubated with the first antibody (PP2Ac total antibody) for 2 h. Followed by 3 times of wash with TBST buffer, the second antibody (alkaline phosphatase-conjugated goat anti-rabbit secondary antibody) was added in a 1:5000 ratio. Signals were obtained by using the alkaline phosphatase system from Bio-Rad Laboratories (Hercules, California, USA). The PP2Ac total antibody was purchased from EMD (Billerica, Massachusetts, USA; Cat No. 07-324) and was used in a dilution of 1:1000. This commercial antibody was previously used by others (Wu *et al.* 2011; Chen *et al.* 2014; Hu *et al.* 2014).

PP2A enzyme activity assay

PP2A activity assay was conducted using the Protein Ser/Thr Phosphatase Assay System from Promega (Madison, Wisconsin, USA) according to the manufacture's instruction. In this system, a peptide substrate, RRA(pT)VA, was used for the activity assay. And the Molybdate Dye/Additive mixture was used to react with free phosphate to develop the colour. The final absorbance density was read at 600 nm wavelength. The PP2A enzyme activity was calculated according to the standard curve. In this system, the phosphopeptide is not a substrate of protein phosphatase 1, and the Inhibitor-II from New England Biolabs (Beverly, Massachusetts, USA) also was used to inhibit the potential affection of PP1. The reaction buffer was made specifically for PP2A activity assay.

Yeast two-hybrid screening and yeast two-hybrid analysis of protein–protein interactions between PP2A-C5 and CLC proteins and between AtCLCc and PP2A-C subunits

The full-length *PP2A-C5* cDNA was amplified from an eight-day-old *Arabidopsis* cDNA prey library (Luo *et al.* 2006) with the C5-YF1 and C5-YR1, and the PCR fragment was then digested with *Eco* RI and *Xho* I, and cloned into the bait vector pEG202 (Golemis *et al.* 1996). Then, the bait was used to screen an *Arabidopsis* cDNA prey library using the procedures as outlined in the interaction trap yeast two-hybrid system (Golemis *et al.* 1996). Candidate proteins are listed in Table 1. To test the interactions between PP2A-C5 and CLC proteins in *Arabidopsis*, all seven *Arabidopsis* CLC cDNAs, AtCLCa to AtCLCg, were amplified from an *Arabidopsis* cDNA library using primers CLCa-YF1 and CLCa-YR-1, and others as listed in Supp. Table 1, cut with *Eco* RI and *Xho* I, and then cloned into the bait vector pEG202. These CLC proteins were used as baits, and the PP2A-C5 was used as the prey in yeast two-hybrid analyses. To test the interactions between AtCLCc and PP2A C subunits, all C subunit cDNAs, PP2A-C1 to PP2A-C5, were amplified from an *Arabidopsis* cDNA library using primers C1-YF1 and C1-YR1, and others as listed in Supp. Table 1, and cut with *Eco* RI and *Xho* I, then cloned into the prey vector pJG4-5 (Golemis *et al.* 1996). These C subunits were used as preys, and the AtCLCc was used as the bait in yeast two-hybrid analyses. An unrelated membrane protein TOM20 was used as the negative control in these experiments, and it was amplified by using primers Tom20-YF1 and Tom20-YR1, and cut with *Eco* RI and *Xho* I, then cloned into the prey vector pJG4-5 or the bait vector pEG202 (it was used as bait and prey, respectively). To test which part of CLCc interacts with PP2A-C5, the N-terminal sequence, residues 1 to 92, and C-terminal sequence, residues 562 to 779, of CLCc were amplified from a cDNA library using the primers CLCc-YF1 and CLCc-YCR1, and CLCc-YNF1 and CLC-YR1, respectively, then cloned into the bait vector (pEG202) with restriction enzymes *Eco* RI and *Xho* I, and then used as preys to interact with the PP2A-C5 bait.

Determination of chloride content in plant tissues

Soil grown 3-week-old plants were treated with water (control) or 100 mM NaCl (treatment) for another week. The plants were collected and dried for 2 d in an 80 °C oven. Around 100 mg dry samples were then grounded and shaken in 0.5 M nitric acid for 2 d at room temperature. The supernatant ion

solutions were used for chloride content analysis with the Ferricyanide method according to the description of Munns *et al.* (2010).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Oligonucleotide primers for PCR and cloning experiments

Fig. S1. Genotyping PCR results confirm that *pp2ac5-1* is recessive to *PP2A-C5*. The *pp2a-c5-1* mutant was crossed to wild-type plants, and the F_1 progenies were selfed to produce F_2 seeds. All salt sensitive F_2 plants proved to be homozygous *pp2a-c5-1* mutant based on the PCR results. The genotyping PCR result of four salt sensitive F_2 plants is shown here.

Fig. S2. Phenotypes of wild-type (WT) and *pp2a-c5-1* mutant plants in the absence and presence of different concentrations of ABA on MS plates 8 d after germination.

Fig. S3. Phenotypes of wild-type (WT), *pp2a-c5-1* and *PP2A-C5*-overexpressing plants (C5-OE1 and C5-OE2) on MS plate.

Fig. S4. Phenotypes of wild-type (WT) and *pp2a-c5-1* mutant plants on salt-containing MS plate and root lengths of these plants two weeks after germination on MS or salt-containing MS plate (75 mM NaCl). $n = 20$ plants from three individual plates; *, significant at 1% according to Student *t*-test.

Fig. S5. Real-time PCR analysis of *PP2A-C5* transcript in wild-type and *PP2A-C5*-overexpressing plants. WT, wild-type; C5-OE1 and C5-OE2, two independent transgenic lines with *PP2A-C5* overexpression in wild-type background; C5-Com1 and C5-Com2, two independent transgenic lines with *PP2A-C5* overexpression in the *pp2ac5-1* mutant background. Three biological replications and three independent experiments were performed ($n = 15$ from five plants × three technical replicates). Statistical significance between samples was indicated by different letters according to the Student *t*-test.

Fig. S6. Numbers of lateral roots in wild-type (WT), *pp2a-c5-1* and *PP2A-C5*-overexpressing plants (C5-OE1 and C5-OE2) in the presence of salt (75 mM NaCl) or absence of salt (MS). Four-day-old plants were transferred to MS plates containing NaCl to grow vertically for a week before the numbers of lateral roots were counted. $n = 50$ plants from three individual plates; * significant at 1% according to Student *t*-test.

Fig. S7. Molecular confirmation of the *pp2a-c5-1 sos1-1* double mutant. Left panel: because *sos1-1* is a deletion mutant, a smaller DNA fragment was amplified by PCR in the *pp2ac5-1 sos1-1* double mutant when compared to a DNA fragment amplified from wild-type (WT) plant. Right panel: genotyping PCR confirmed the homozygous state of *pp2a-c5-1* in the double mutant. F1, R1 and LB, PCR primers used for amplifying DNA fragments from WT and *pp2a-c5-1 sos1-1* double mutant.

Fig. S8. Molecular confirmation of the *pp2a-c5-1 sos2-2* double mutant. Left panel: with *sos2-2* specific primers, a smaller PCR product was amplified from the *pp2a-c5-1 sos2-2* double mutant. Right panel: genotyping PCR confirmed the homozygous state of *pp2a-c5-1* in the double mutant. F1, R1 and LB, PCR primers used for amplifying DNA fragments from wild-type (WT) and the *pp2a-c5-1 sos2-2* double mutant.

Fig.S9. Molecular confirmation of the *pp2a-c5-1 sos3-1* double mutant. Left panel: with *sos3-1* specific primers, a smaller PCR product was amplified in the *pp2a-c5-1 sos3-1* double mutant. Right panel: genotyping PCR confirmed the homozygous state of *pp2a-c5-1* in the *pp2a-c5-1 sos3-1* double mutant. F1, R1 and LB, PCR primers used for amplifying DNA fragments from wild-type (WT) and the *pp2a-c5-1 sos3-1* double mutant.

Fig. S10. Analysis of the *pp2a-c5-1 sos2-2* double mutant. A. Phenotypes of wild-type (WT), *pp2a-c5-1* mutant, *pp2a-c5-1 sos2-2* double mutant and *sos2-2* mutant in the absence of salt or after salt treatments. B. Root lengths of wild-type, *pp2a-c5-1* mutant, *sos2-2* mutant and *pp2a-c5-1 sos2-2* double mutant in the absence or presence of NaCl. Three biological replications and three technical experiments were performed ($n = 20$ plants from three individual plates). Statistical significance between samples was indicated by different letters according to the Student *t*-test. C. Relative root lengths of wild-type, *pp2a-c5-1* mutant, *sos2-2* mutant and *pp2a-c5-1 sos2-2* double mutant after salt treatments.

Fig. S11. Analysis of the *pp2a-c5-1 sos3-1* double mutant. A. Phenotypes of wild-type (WT), *pp2a-c5-1* mutant, *pp2a-c5-1 sos3-1* double mutant and *sos3-1* mutant in the absence of salt or after salt treatments. B. Root lengths of wild-type, *pp2a-c5-1* mutant, *sos3-1* mutant and *pp2a-c5-1 sos3-1* double mutant in the absence or presence of NaCl. Three biological replications and three technical experiments were performed ($n = 20$ plants from three individual plates). Statistical significance between samples was indicated by different letters according to the Student *t*-test. C. Relative root lengths of wild-type, *pp2a-c5-1* mutant, *sos3-1* mutant and *pp2a-c5-1 sos3-1* double mutant after salt treatments.

Fig. S12. Root lengths of wild-type, *pp2ac5*-1 mutant, and *PP2A-C5*-overexpressing plants in the presence of 75 mM of KCl (A) or 75 mM of KNO_3 (B). WT, wild-type; C5-OE1 and C5-OE2, two independent *PP2A-C5*-overexpressing plants. Three biological replications and three independent experiments were performed. $n = 20$ plants from three individual plates; statistical significance between samples was indicated by different letters according to the Student *t*-test.

Fig. S13. Protein–protein interactions between PP2A-C5 and *Arabidopsis* CLC proteins. A. Protein–protein interaction analysis between PP2A-C5 and the seven *Arabidopsis* CLC proteins using the yeast two-hybrid system. B. Protein–protein interaction analysis between AtCLCc and the five *Arabidopsis* PP2A C subunits using the yeast two-hybrid system. C. Protein–protein interaction analysis between PP2A-C5 and the N-terminal sequence (residues 1 to 92) and the C-terminal sequence of CLCc (residues 562 to 779), respectively, using the yeast two-hybrid system.

Fig. S14. Expression of *AtCLCc* in wild-type (WT), *AtCLCc*-overexpressing (CLCc-OE1 to CLCc-OE3), *pp2a-c5-1* mutant and c5-*AtCLCc*-overexpressing (c5-CLCc-OE1 to c5-CLCc-OE3) plants. Total RNAs were isolated from eight-day-old Arabidopsis plants for real-time quantitative PCR analyses. Three biological replications and three independent experiments were performed ($n = 15$ from five plants × three technical replicates). The *Actin 2* transcript was used as the internal control, and the expression levels are expressed as the relative ratios to the transcript level of *AtCLCc* in wild-type plants.

Fig. S15. A model on how PP2A-C5 mediates salt tolerance in plants. PP2A-C5 might positively regulate the vacuolar membrane-bound CLC proteins such as AtCLCa and CLCc, which then increases nitrate and chloride import into vacuoles, leading to increased salt tolerance and better growth under the conditions of 75 mM of KCl or KNO_3. AtCLCa is a major H^+/NO_3^- antiporter (De Angeli *et al.* 2006; Wege *et al.* 2014), whereas AtCLCc is an H^+/Cl^- antiporter (Barbier-Brygoo *et al.* 2011).