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SHORT COMMUNICATION

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Overexpression of the *PP2A-C5* gene confers increased salt tolerance in *Arabidopsis thaliana*

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ABSTRACT

Protein phosphatase 2A (PP2A) was shown to play important roles in biotic and abiotic stress signaling pathways in plants. PP2A is made of 3 subunits: a scaffolding subunit A, a regulatory subunit B, and a catalytic subunit C. It is believed that the B subunit recognizes specific substrates and the C subunit directly acts on the selected substrates, whereas the A subunit brings a B subunit and a C subunit together to form a specific PP2A holoenzyme. Because there are multiple isoforms for each PP2A subunit, there could be hundreds of novel PP2A holoenzymes in plants. For an example, there are 3 A subunits, 17 B subunits, and 5 C subunits in Arabidopsis, which could form 255 different PP2A holoenzymes. Understanding the roles of these PP2A holoenzymes in various signaling pathways is a challenging task. In a recent study,[1] we discovered that PP2A-C5, the catalytic subunit 5 of PP2A, plays an important role in salt tolerance in Arabidopsis. We found that a knockout mutant of *PP2A-C5* (i.e. *pp2a-c5–1*) was very sensitive to salt treatments, whereas *PP2A-C5*-overexpressing plants were more tolerant to salt stresses. Genetic analyses between *pp2a-c5–1* and *Salt-Overly-Sensitive* (*SOS*) mutants indicated that *PP2A-C5* does not function in the same pathway as *SOS* genes. Using yeast 2-hybrid analysis, we found that PP2A-C5 interacts with several vacuolar membrane bound chloride channel proteins. We hypothesize that these vacuolar chloride channel proteins might be PP2A-C5's substrates *in vivo*, and the action of PP2A-C5 on these channel proteins could increase or activate their activities, thereby result in accumulation of the chloride and sodium contents in vacuoles, leading to increased salt tolerance in plants.

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Protein phosphatase 2A (PP2A) plays numerous roles in plants such as in cell cycle progression, root cortical cell elongation, tissue development, and plant responses to biotic and abiotic stresses.[2-7] The PP2A holoenzyme comprises 3 subunits: a scaffolding subunit A, a regulatory subunit B, and a catalytic subunit C.[8] In Arabidopsis, there are 5 catalytic subunits: PP2A-C1 to PP2A-C5.[9] A previous study reported that PP2A-C2 was negatively involved in salt signaling pathway, as the *pp2a-c2* mutant displayed enhanced salt sensitivity.[10] Interestingly, in our recent publication,[1] we discovered that PP2A-C5 plays a positive role in plant response to salt stresses, as *PP2A-C5*-overexpression leads to enhanced tolerance to several salt treatments at both seedling and vegetative stages in Arabidopsis development, whereas a loss-of-function mutant of *PP2A-C5* (i.e., *pp2a-C5–1*) displays salt hypersensitive phenotypes in comparing to wild-type plants.[1] These results clearly indicate that PP2A plays important roles in plant salt tolerance.

To explore how *PP2A-C5* is involved in salt response in plants, we analyzed its relationship with the Salt Overly Sensitive (SOS) genes in Arabidopsis. The SOS genes mediated salt response had been well studied previously,[11] and its 3 genes, *SOS1, SOS2*, and *SOS3*, are involved in regulating Na^+ homeostasis in plant cells. We generated double knockout mutants of *pp2a-c5–1 sos1–1, pp2a-c5–1 sos2–2*, and *pp2a-c5–1 sos3–1*, and analyzed the performance of these double mutants under salt stresses.[1] We found that these double mutants displayed more severe sensitivity in seedling growth and root growth to salt treatments than their parental single gene mutants, indicating that *PP2A-C5* functions independent of the SOS pathway.[1] To elucidate the molecular mechanism of how PP2A-C5 might be involved in the salt signaling, we conducted a yeast-2 hybrid screening to identify PP2A-C5's interacting proteins (hopefully they might be PP2A-C5's substrates *in vivo*). One of the PP2A-C5 interacting proteins, the vacuolar membrane bound chloride channel protein C, i.e., AtCLCc, appeared to be the most relevant protein,[1] as loss of function in *AtCLCc* resulted in salt sensitive phenotype[12] and overexpression of *AtCLCc* orthologous genes from soybean enhanced salt tolerance in transgenic plants.[13] Indeed we confirmed that overexpression of *AtCLCc* increases salt tolerance in transgenic Arabidopsis,[1] a phenotype comparable to overexpression of *PP2A-C5* in transgenic Arabidopsis.[1]

PP2A-C5 not only interacts with AtCLCc, but also with AtCLCa, AtCLCb, and AtCLCg in the yeast 2-hybrid system.[1] These 4 chloride channel proteins are all vacuolar membrane bound, a result that might not be accidental. To demonstrate if

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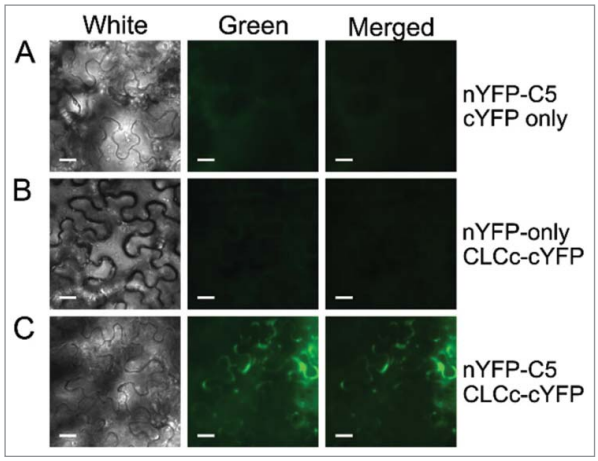

Figure 1. Protein-protein interaction *in vivo* between PP2A-C5 and AtCLCc as demonstrated by the bimolecular fluorescence complementation experiment. (A) The nYFP-C5 fusion construct and the cYFP construct were introduced into tobacco leaf cells and no green fluorescence signal was observed. (B) The AtCLCc-cYFP fusion construct and the nYFP construct were introduced into tobacco leaf cells and no green fluorescence signal was observed. (C) The nYFP-C5 fusion construct and the AtCLCc-cYFP fusion construct were introduced into tobacco leaf cells and green fluorescence signal was observed. The bar represents 10 μm.

PP2A-C5 interacts with AtCLCc *in vivo*, we performed bimolecular fluorescence complementation (BiFC) experiments using *N. benthamiana* leaves. In this system, we first fused PP2A-C5 to nYFP (the N-terminal part of the yellow fluorescence protein) to form the nYFP-C5 fusion construct, and fused AtCLCc to cYFP (i.e., the C-terminal part of YFP) to form the CLCc-cYFP fusion construct. Then we introduced Agrobacterial cells into tobacco leaf cells through the infiltration technique and the Agrobacterial cells contained our gene constructs in 3 combinations: nYFP-C5 with cYFP constructs, nYFP and CLCc-cYFP constructs, and nYFP-C5 and CLCc-cYFP constructs. Only in the third combination we observed fluorescence signals in the infiltrated leaf tissues (Fig. 1C), indicating that it was the interaction between PP2A-C5 and AtCLCc that brought nYFP and cYFP together to produce green fluorescence in the tobacco leaf cells.

The physical interaction between PP2A-C5 and AtCLCc and similar salt tolerant phenotype of *PP2A-C5*-overexpressing plants and *AtCLCc*-overexpressing plants suggested a functional correlation. We then investigated the genetic relationship between *PP2A-C5* and *AtCLCc* by overexpressing *AtCLCc* in the *pp2a-c5–1* mutant background and we could not rescue the salt sensitive phenotype of the *pp2a-c5–1* mutant, indicating that *PP2A-C5* and *AtCLCc* function in the same pathway and *AtCLCc* functions downstream of *PP2A-C5*.[1] Our data suggest that increasing *PP2A-C5* expression might lead to higher activities of chloride channel proteins. This assumption appears consistent with the biochemical analysis of chloride (Cl^-) concentrations in these plants. We observed the highest Cl^- concentration in *PP2A-C5*-overexpressing plants and *AtCLCc*-overexpressing plants, and the lowest concentration in the *pp2a-c5-1* mutant.[1] The Cl^- concentration in the *pp2a-c5–1* mutant that overexpresses *AtCLCc* is similar to that of the *pp2a-c5–1* mutant.[1] To maintain the charge neutrality inside vacuoles of *AtCLCc*-overexpressing plants, we expected that *AtCLCc*-overexpressing plants should

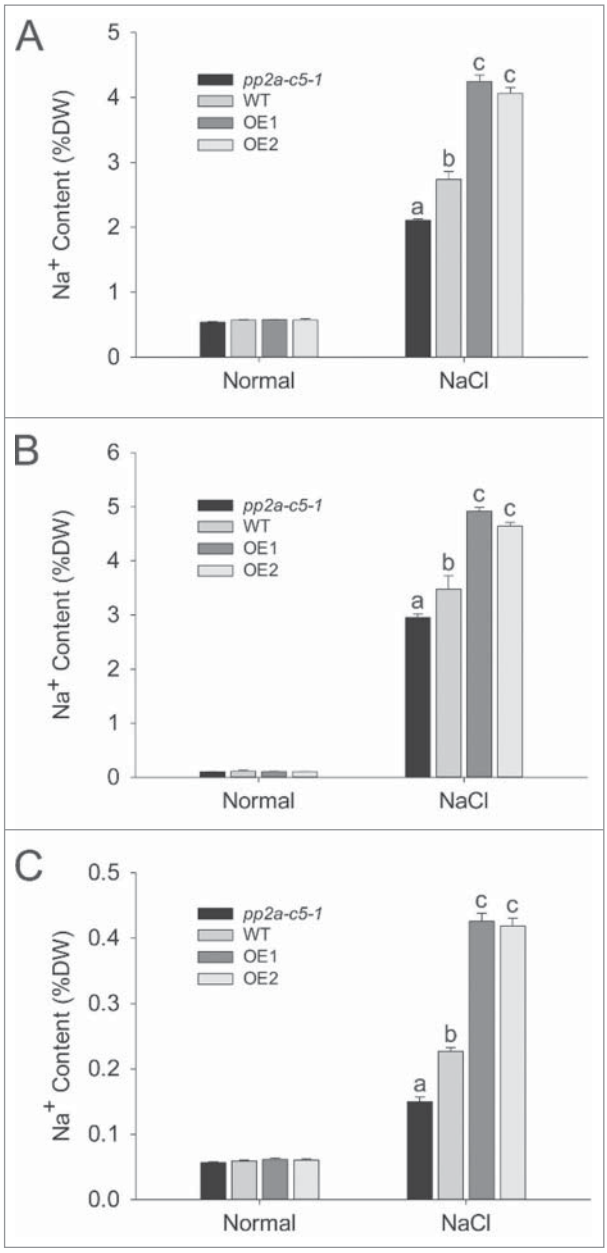

Figure 2. Na^+ content in *pp2a-C5-1*, wild-type, and 2-independent *PP2A-C5*-overexpressing plants in the absence or presence of 100 mM NaCl for 7 d. (A) Na^+ content in whole seedlings. (B) Na^+ content in up-ground tissues. (C) Na^+ content in root tissues. Three biologic replications and 3 technical replications were performed (n = 20 plants from 3 individual plates). Statistical significances at 1% between samples are indicated by different letters according to the Student *t*-test.

have higher levels of cations. Indeed our analyses of Na^+ contents indicate similar results as Cl^- contents: *PP2A-C5*-overexpressing plants and *AtCLCc*-overexpressing plants contain the highest amount of Na^+, whereas the *pp2a-c5–1* mutant contains the least (Fig. 2).

Based on our study, we propose a working model to show how PP2A might participate in the salt signaling pathway in plant cells (Fig. 3). We believe that AtCLCc and AtCLCa are substrates of PP2A-C5 in plant cells and these vacuolar membrane bound chloride channel proteins exist in 2 forms: dephosphorylated form (active or high activity form) and phosphorylated form (inactive or low activity form). When *PP2A-C5* is overexpressed in transgenic Arabidopsis plants,

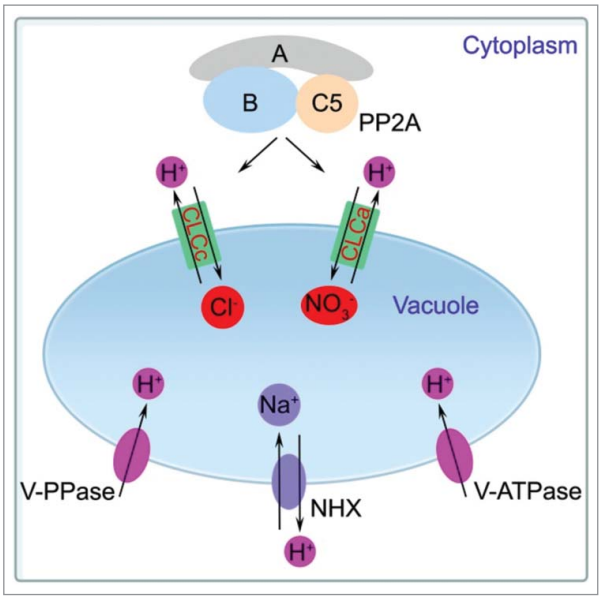

Figure 3. A working model on how PP2A-C5 might be involved in salt signaling pathway in Arabidopsis. The specific PP2A holoenzyme containing the C5 subunit up-regulates activities of AtCLCc and/or AtCLCa on vacuolar membrane by removing phosphates from its substrate proteins, leading to more anions (i.e., Cl^- and NO_3^-) to move into vacuole, thereby resulting in increased salt tolerance or better growth and development under treatment of NaCl, KCl, and KNO_3. AtCLCc, H^+/Cl^- antiporter; AtCLCa, H^+/NO_3^- antiporter; NHX, H^+/Na^+ antiporter; V-ATPase, vacuolar ATPase; V-PPase, vacuolar pyrophosphatase.

more AtCLCc would be in the dephosphorylated form, which leads to higher concentrations of Cl^- and Na^+ in plant vacuoles, thereby leading to higher salt tolerance. In contrast, all or more AtCLCc would be in the phosphorylated form in the *pp2a-c5–1* mutant (depending on whether PP2A-C5 is the only phosphatase that recognizes AtCLCc), which leads to less Cl^- and Na^+ accumulation in plant vacuoles, leading to increased salt sensitivity in the *pp2a-c5–1* mutant. This model looks logic and is supported by physiologic and genetic data, but it lacks direct biochemical evidence. It is imperative to biochemically demonstrate that PP2A (with the C5 subunit) can dephosphorylate AtCLCc and AtCLCa *in vivo* and *in vitro*, and the phosphorylated and dephosphorylated AtCLCc and AtCLCa are the inactive and active forms of these chloride channel proteins, respectively. In addition, identifying which B subunit is involved in salt response or which B subunit interacts with PP2A-C5 is also needed to gain more insight into PP2A's mode of action in plant salt response. Major challenges in the study of the function, regulation, and mode of action of plant PP2A holoenzymes still lie ahead.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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