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

Rongbin Hu⁎, Yinfeng Zhu⁎, Guoxin Shen⁎, and Hong Zhang⁎

⁎Department of Biological Sciences, Texas Tech University, Lubbock, TX, USA; ⁎Zhejiang Academy of Agricultural Sciences, Hangzhou, Zhejiang Province, China

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.

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 phenotype12 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
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. 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. The Cl\(^–\) concentration in the *pp2a-c5-1* mutant that overexpresses AtCLCc is similar to that of the *pp2a-c5-1* mutant. To maintain the charge neutrality inside vacuoles of AtCLCc-overexpressing plants, we expected that AtCLCc-overexpressing plants should 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,
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.

**References**

1. Hu R, Zhu Y, Wei J, Chen J, Shi H, Shen G, Zhang H. 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. Plant Cell Environ 2017; 40:150-64; PMID: 27676158; http://dx.doi.org/10.1111/pce.12837

2. Hu R, Zhu Y, Shen G, Zhang H. TAP46 plays a positive role in the abscisic acid insensitive5-regulated gene expression in Arabidopsis. Plant Physiol 2014; 164:721-34; PMID:24357600; http://dx.doi.org/10.1104/pp.113.233684

3. DeLong A. Switching the flip: protein phosphatase roles in signaling pathways. Cur Opin Plant Biol 2006; 9:470-7; PMID:16890477; http://dx.doi.org/10.1016/j.bbi.2006.07.015

4. He X, Anderson JC, Pozo OD, Gu Y, Tang X, Martin GB. Silencing of subfamily I of protein phosphatase 2A catalytic subunits results in activation of plant defense responses and localized cell death. Plant J 2004; 38:563-77; PMID:15125764; http://dx.doi.org/10.1111/j.1365-313X.2004.02073.x

5. Camilleri C, Azimzadeh J, Pastuglia M, Bellini C, Grandjean O, Boucher D. The Arabidopsis TONNEAU2 gene encodes a putative novel protein phosphatase 2A regulatory subunit essential for the control of the cortical cytoskeleton. Plant Cell 2002; 14:833-45; PMID:11971138; http://dx.doi.org/10.1104/pp.114.250563

6. Zhou HW, Nussbauer C, Chao Y, Delong A. Disparate roles for the regulatory A subunit isoforms in Arabidopsis protein phosphatase 2A. Plant Cell 2004; 16:709-22; PMID:14973165; http://dx.doi.org/10.1105/pp.101402

7. Chen J, Hu R, Zhu Y, Shen G, Zhang H. Arabidopsis phosphotyrosyl phosphatase activator is essential for protein phosphatase 2A holoenzyme assembly and plays important roles in hormone signaling, salt stress response, and plant development. Plant Physiol 2014; 166:1519-34; PMID:25281708; http://dx.doi.org/10.1104/pp.114.250563

8. Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signaling. Biochem J 2001; 353:417-39; PMID:11171037; http://dx.doi.org/10.1042/bj3530417

9. Farkas I, Dombradi V, Miskei M, Szabados L, Koncz C. Arabidopsis PPP family of serine/threonine phosphatases. Trend Plant Sci 2007; 12:169-76; PMID:23790269; http://dx.doi.org/10.1016/j.tplants.2007.03.003

10. Pernas M, García-Casado G, Rojo E, Solano R, Sánchez–Serrano Jj. A protein phosphatase 2A catalytic subunit is a negative regulator of abscisic acid signalling. Plant J 2007; 51:763-78; PMID:17617176; http://dx.doi.org/10.1111/j.1365-313X.2007.03179.x

11. Zhu Jk. Salt and drought stress signal transduction in plants. Ann Rev Plant Physiol Plant Mol Biol 2002; 53:247-73; PMID:12221975; http://dx.doi.org/10.1146/annurev.arplant.53.091401.143329

12. Jossier M, Kroniewicz L, Dalmas F, Le Thiec D, Ephritikhine G, Tho- meire S, Barbier-Brygoo H, Vavasseur A, Filleur S, Leonhardt N. The Arabidopsis TONNEAU2 gene encodes a putative novel protein phosphatase 2A regulatory subunit essential for the control of the cortical cytoskeleton. Plant Cell 2002; 14:833-45; PMID:11971138; http://dx.doi.org/10.1104/pp.114.250563

13. Sun W, Deng D, Yang L, Zheng X, Yu J, Pan H, et al. Overexpression of the chloride channel gene (GmCLC1) from soybean increases salt tolerance in transgenic *P. deltoides x P. euramerica* ‘Nanlin895’. Plant Omics 2013; 6:347-54