Plant Biotechnology 32, 337–341 (2015)
DOI: 10.5511/plantbiotechnology.15.0813b

Note

Physical interaction between SnRK2 and PP2C is conserved in *Populus trichocarpa*

Xueqing Song[1,2,†], Misato Ohtani[2,3,†], Chiaki Hori[2], Arika Takebayasi[2], Ryoko Hiroyama[2], Nur Ardiyana Rejab[3], Takaomi Suzuki[3], Taku Demura[2,3], Tongming Yin[1], Xiang Yu[2,*], Qiang Zhuge[1,*]

[1] Co-Innovation Center for Sustainable Forestry in Southern China, Key Laboratory of Forest Genetics & Biotechnology, Ministry of Education, Nanjing Forestry University, Nanjing 210037, People's Republic of China; [2] Biomass Engineering Program Cooperation Division, RIKEN Center for Sustainable Resource Science, Yokohama, Kanagawa 230-0045, Japan; [3] Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0192, Japan
* E-mail: qzhuge@njfu.edu.cn; yuxiang@riken.jp
Tel: +86-25-85428701; +81-45-503-9605 Fax: +86-25-85428701; +81-45-503-9609

Received July 2, 2015; accepted August 13, 2015 (Edited by M. Otani)

Abstract Type 2C protein phosphatase (PP2C) is a central player in abscisic acid (ABA) signaling transduction, which is required for plant growth, development and stress responses. In Arabidopsis, group A PP2Cs inhibit activity of the SNF1-related protein kinases 2 (SnRK2) family via physical interaction. To clarify whether this scheme is conserved in woody plants, we experimentally isolated the genes homologous to three members of group A PP2Cs (PtABI1, PtAHG1 and PtAHG3) and 12 SnRK2s (PtSnRK2.1–2.12) from a model tree *Populus trichocarpa*, and examined their interaction using a yeast two-hybrid assay. Our results showed that only three PtSnRK2 proteins had a positive interaction with PtPP2Cs: PtSnRK2.10 possessed strong interaction activity with all three PtPP2Cs, while significant, but relatively weak, interactions were observed with PtSnRK2.6 and PtSnRK2.9. These three PtSnRK proteins are grouped into subclass 2 or 3, which are considered to be ABA-dependent kinases in Arabidopsis. These findings suggest that physical interaction between SnRK2 and PP2C is also conserved in poplars and may be involved in the ABA signaling pathway in tree plants.

Key words: ABA signaling, physical interaction, *Populus trichocarpa*, PP2C, SnRK2, yeast two-hybrid.

Abscisic acid (ABA) signaling plays a pivotal role in many developmental and physiological processes, including seed dormancy, germination and seedling growth, as well as biotic and abiotic stress responses (Hirayama and Umezawa 2010; Ng et al. 2014; Yamaguchi-Shinozaki et al. 2006). Recent key studies of *Arabidopsis thaliana* (Arabidopsis) have provided many experimental data indicating that protein phosphatases type 2C (PP2C), which can interact with ABA receptors and/or subfamily SNF1-related protein kinase 2 (SnRK2), are global negative regulators of the ABA signaling pathway (Cutler et al. 2010; Leung et al. 1994, 1997; Meyer et al. 1994; Ng et al. 2014; Nishimura et al. 2007; Saez et al. 2004; Umezawa et al. 2009). In the absence of ABA, group A PP2Cs physically bind to plant-specific SnRK2s and dephosphorylate the kinase activation loop of SnRK2s, resulting in the inhibition of ABA signal transduction (Umezawa et al. 2009; Vlad et al. 2009).

In Arabidopsis, nine group A PP2Cs (ABI1, ABI2, HAB1, HAB2, AHG1, PP2CA/AHG3, etc.) have been implicated as negative regulators of ABA responses (Fujii et al. 2007, 2009; Fujita et al. 2009; Umezawa et al. 2010; T. Yoshida et al. 2006). Only the subclass 2 and 3 SnRKs are activated by ABA, and subclass 3 SnRK2s (SnRK2.2, SnRK2.3, and SnRK2.6) were shown to be major positive regulators of ABA signaling (Fujii et al. 2009; Fujita et al. 2009; Nakashima et al. 2009; Umezawa et al. 2009). The SnRK2 protein possesses a catalytic domain that is well-conserved among all subclasses of SnRKs and a relatively diverse C-terminal domain. The C-terminal domain can be separated into two subdomains, Domain 1 and Domain 2 (R. Yoshida et al. 2006). Domain 2, also known as the ABA box, is a highly negatively charged segment critical for the physical interactions between SnRK2s and PP2Cs (Belin et al. 2006; R. Yoshida et al. 2006; Yunta et al. 2011; Zhou et al. 2012). The characteristics of Domain 2 differ by subclass; Domain 2 in subclasses 2 and 3 is rich in aspartate, but that in subclass 1 is rich in glutamate. It was shown that ABA can activate only aspartate-rich SnRKs (Boudsocq et al. 2004; Kobayashi et

Abbreviations: ABA, abscisic acid; PP2C, Type 2C protein phosphatase; SnRK2, SNF1-related protein kinases 2.
† These authors contributed equally to this work.
This article can be found at http://www.jspcmb.jp/
Published online October 3, 2015

al. 2004), and deletion analysis of Domain 2 showed that it is necessary for ABA responsiveness (R. Yoshida et al. 2006).

Comparative genome analysis revealed that SnRK and PP2C genes are well conserved among a wide range of plant species (Umezawa et al. 2010), and heterologous expression of the Arabidopsis *ABI1* gene suggests possible conservation of the ABA signaling pathway between Arabidopsis and the model tree *Populus trichocarpa* (Arend et al. 2009). In woody plants, endogenous ABA levels show seasonal alteration, and ABA is involved in freezing tolerance and dormancy (Welling and Palva 2006). Interestingly, sensitivity to exogenous ABA is not stable, but changes seasonally (Welling and Palva 2006), suggesting that ABA signaling regulation plays critical roles in the perennial life cycle of woody plants. However, information on the SnRK–PP2C complex in woody plants is limited.

To clarify whether the SnRK2–PP2C physical interaction is conserved in tree species, we examined the genes homologous to SnRK and PP2C in the model tree *P. trichocarpa* (Tuskan et al. 2006). The *P. trichocarpa* genome database Phytozome (Goodstein et al. 2012) was searched via TBLASTN using the amino acid sequences of Arabidopsis SnRK2s and PP2Cs (ABI1, AHG1 and AHG3) as queries. We identified 12 *PtSnRK2* genes and three *PtPP2C* genes (Supplementary Table 1). Using the full-length amino acid sequences of SnRK2s and PP2Cs from poplar and Arabidopsis, phylogenetic trees were constructed using the neighbor-joining method (Figure 1, Figure 2 and Supplementary Table 1). The *PtSnRK* genes can be classified into subclasses 1 (PtSnRK2.1–2.4), 2 (PtSnRK2.10–2.12) and 3 (PtSnRK2.5-2.9), based on the classification of Arabidopsis SnRKs (Figure 1; Yu et al., submitted). We named the three PtPP2Cs PtABI1, PtAHG1 and PtAHG3, according to their sequence similarity with Arabidopsis homologs (Figure 2). These results suggest that the basic molecular components for ABA signaling are conserved in poplars.

Next, we experimentally isolated the cDNA sequences of these PtSnRK2 and PtPP2C genes to further examine the SnRK2–PP2C physical interaction using yeast two-hybrid analysis. Based on the putative cDNA sequence, specific primers were designed to amplify the open reading frame regions of these genes using first-strand cDNAs prepared from total RNA obtained from young *P. trichocarpa* shoots grown in pots, according to the methods described in Ohtani et al. (2011) (for primer information, please see Supplementary Table 2). Amplified PCR fragments were cloned into the Gateway entry vector pENTR™/D-TOPO® or pCR®8/GW/TOPO® (Invitrogen), and their sequences were checked to confirm they were identical to the presumed cDNA sequences. To construct the plasmids for yeast two-hybrid analysis, the PtSnRK2 and PtPP2C cDNA sequences were transferred to the pBD-GAL4-GWRFC

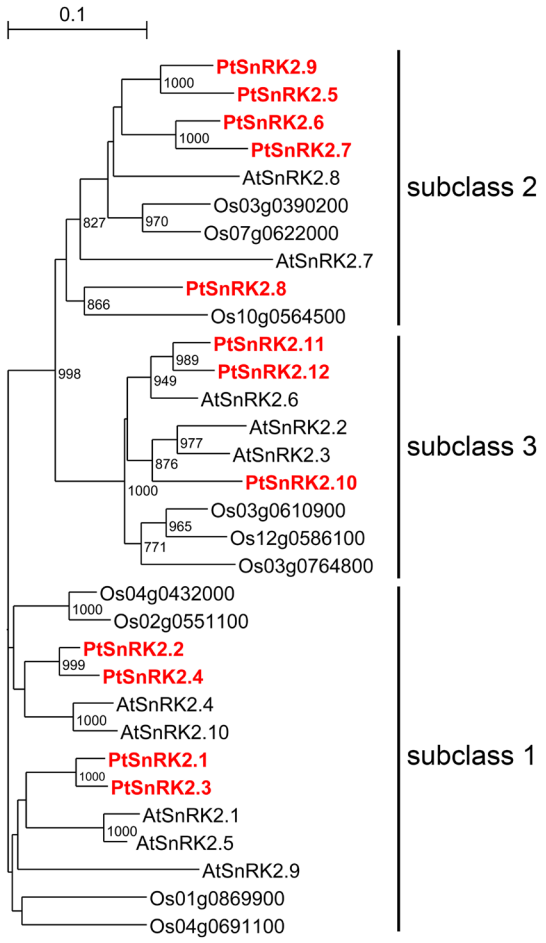

Figure 1. Phylogenetic tree of SnRK2 proteins from *Populus trichocarpa* (red, Pt), *Oryza sativa* (Os) and Arabidopsis (At). The phylogenetic tree was constructed using the neighbor-joining method (for the aligned sequences, please see Supplementary Table 1). Numbers are bootstrap values and are given for clades that received support values of over 70% (1000 resamplings). The scale (0.1) represents a 10% change in sequence.

and pAD-GAL4-GWRFC vectors (Yamaguchi et al. 2008), respectively, by the LR clonase (Invitrogen) reaction. The pAD-GAL4-GWRFC and pBD-GAL4-GWRFC plasmids containing the multi-cloning sequence (Yamaguchi et al. 2008) were used as negative controls. The SRK2E-pGBKT7 and ABI1-pGADT7 plasmids, into which the Arabidopsis *SnRK2.6* and *ABI1* genes were inserted, respectively, were kindly provided by Dr. Umezawa and used as positive controls (Umezawa et al. 2009; R. Yoshida et al. 2006).

Using the plasmids described above, yeast two-hybrid analysis was carried out using the GAL4 Two-Hybrid Phagemid Vector Kit (Agilent Technologies) according to the manufacturer's instructions. The yeast strain AH109 was transformed with the pBD-GAL4-GWRFC harboring PtSnRK2 and with pAD-GAL4-GWRFC harboring PtPP2C in all combinations using the *S.c.* EasyComp™ Transformation Kit (Invitrogen). After the transformed colonies were cultured in SD agar medium

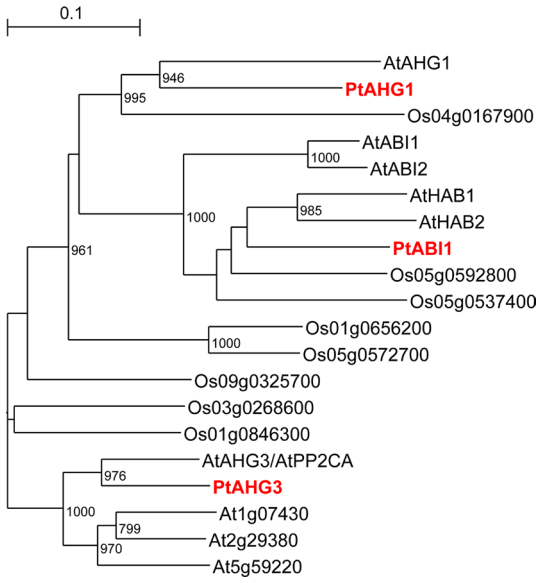

Figure 2. Phylogenetic tree of PP2C proteins from *Populus trichocarpa* (red, Pt), *Oryza sativa* (Os) and Arabidopsis (At). The phylogenetic tree was constructed using the neighbor-joining method (for the aligned sequences, please see Supplementary Table 1). Numbers are bootstrap values and are given for clades that received support values of over 70% (1000 resamplings). The scale (0.1) represents a 10% change in sequence.

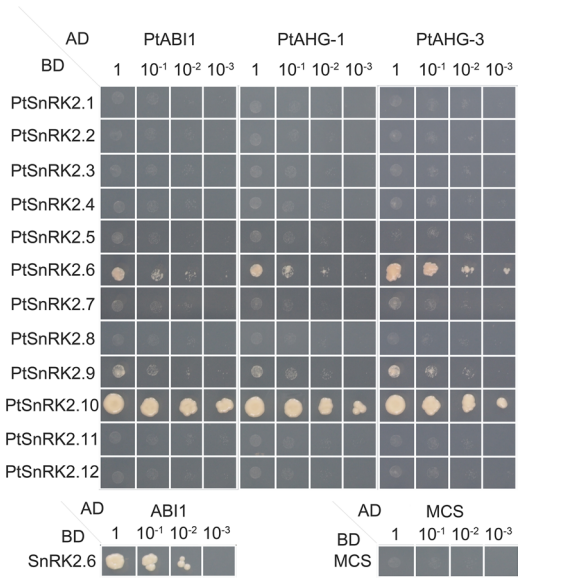

Figure 3. Yeast two-hybrid analysis of PtSnRKs and PtPP2C. Both GAL4AD–PP2Cs and GAL4BD-SnRKs were introduced into yeast cells (strain AH109) as indicated, and grown in liquid SD medium without Leu and Trp. The diluted series of the liquid culture was spotted onto SD agar medium without Leu, Trp or His and grown for 3 days at 28°C. The Arabidopsis ABI1 and SnRK2.6 genes (Umezawa et al. 2009) and the multi-cloning sequence (Yamaguchi et al. 2008) were used as the positive and negative controls, respectively.

(–Leu, –Trp) at 28°C, the well-grown colonies were picked and cultured in liquid SD medium (–Leu, –Trp). The liquid culture was grown to mid-log phase at 28°C and adjusted to an A_{600} of 0.1, after which 2-μl aliquots of serial 10-fold dilutions of the cultures were spotted onto SD agar medium (–Leu, –Trp, –His). The plates were incubated at 28°C for 3 days to check the growth of each yeast strain (Figure 3).

The results showed that three PtSnRK2s, PtSnRK2.6, PtSnRK2.9 and PtSnRK2.10, which are classified into subclass 2 or 3 (Figure 1), can interact with all PtPP2Cs tested (Figure 3). PtSnRK2.10 in subclass 3 seemed to bind especially strongly to all three PP2Cs, because the 10^{-3} diluted culture cells formed well-grown colonies in all combinations. Compared with PtSnRK2.10, PtSnRK2.6 and PtSnRK2.9 possessed relatively weaker, but significant binding activities to PtPP2Cs; moreover, PtSnRK2.6 and PtSnRK2.9 exhibited stronger binding to PtAHG3, suggesting that these two PtSnRKs have preferential binding partners. Among PtSnRKs in the same subclass, we could not find large differences in amino acid sequences contributing to the SnRK–PP2C interaction in Arabidopsis (Soon et al. 2012; Zhou et al. 2012). It was shown using the alfascreen method in Arabidopsis that the ABA box region of all SnRKs, except SnRK2.1 and SnRK2.8, can interact with PP2Cs in vitro (Zhou et al. 2012), although a positive interaction between full-length SnRK protein and PP2Cs was not observed in the case of yeast two-hybrid experiments for SnRK 2.10 (Umezawa et al. 2009). Thus, the difference in interaction activities largely depends on conformational diversity, which is difficult to determine from primary amino acid sequences. In *Arabidopsis*, all three members of subclass 3 SnRK2 can interact with group A PP2Cs. However, only PtSnRK2.10 interacted with PP2C in our study, indicating there might be some SnRK2 functional differences between Arabidopsis and poplars in ABA signaling pathway. Further detailed analyses will clarify these issues.

In summary, our results showed that the SnRK2–PP2C interaction is conserved in the model tree *P. trichocarpa*, and that the interaction with PP2C is basically limited to PtSnRK2s in subclass 2 or 3, as shown for Arabidopsis (Figure 3; Umezawa et al. 2009). Recently, we also revealed that the expression levels of some *PtSnRK* genes are upregulated by ABA treatment (Yu et al. unpublished data). These findings suggest that the key components and basic molecular mechanisms of ABA signaling are conserved between Arabidopsis and poplars. Plant growth and development require the integration of a variety of environmental and endogenous signals. Further intensive research on the function of the SnRK–PP2C complex in poplars will improve our understanding of the ABA signaling pathway in tree species, and also help us further explore the roles of SnRK–PP2C in plant growth and development as well as the response to biotic and abiotic stresses for commercial application of the super trees with high yield and strong resistance characters.

Acknowledgements

We are grateful to Dr. Taishi Umezawa (Tokyo University of Agriculture and Technology) for kindly providing the SRK2E-pGBKT7 and ABI1-pGADT7 vectors. This work was supported by the International Science & Technology Cooperation Program of China (2014DFG32440), the National Science Foundation of China (No. 31170561), the Priority Academic Program Development of Jiangsu Higher Education Institutions, the Program for Innovative Research Team in University of Educational Department and Jiangsu Province, China, and in part by the Biomass Engineering Program Cooperation Division, RIKEN Center for Sustainable Resource Science.

References

Arend M, Schnitzler JP, Ehlting B, Hänsch R, Lange T, Rennenberg H, Himmelbach A, Grill E, Fromm J (2009) Expression of the Arabidopsis mutant *ABI1* gene alters abscisic acid sensitivity, stomatal development, and growth morphology in gray poplars. *Plant Physiol* 151: 2110–2119

Belin C, de Franco PO, Bourbousse C, Chaignepain S, Schmitter JM, Vavasseur A, Giraudat J, Barbier-Brygoo H, Thomine S (2006) Identification of features regulating OST1 kinase activity and OST1 function in guard cells. *Plant Physiol* 141: 1316–1327

Boudsocq M, Barbier-Brygoo H, Lauriere C (2004) Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in *Arabidopsis thaliana*. *J Biol Chem* 279: 41758–41766

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2010) Abscisic acid: Emergence of a core signaling network. *Annu Rev Plant Biol* 61: 651–679

Fujii H, Verslues P, Zhu JK (2007) Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in *Arabidopsis*. *Plant Cell* 19: 484–494

Fujii H, Zhu JK (2009) Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. *Proc Natl Acad Sci USA* 106: 8380–8385

Fujita Y, Nakashima K, Yoshida T, Katagiri T, Kidokoro S, Kanamori N, Umezawa T, Fujita M, Maruyama K, Ishiyama K, et al. (2009) Three SnRK2 protein kinases are the main positive regulators of abscisic acid signaling in response to water stress in Arabidopsis. *Plant Cell Physiol* 50: 2123–2132

Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD, Fazo J, Mitros T, Dirks W, Hellsten U, Putnam N, et al. (2012) Phytozome: A comparative platform for green plant genomics. *Nucleic Acids Res* 40(D1): D1178–D1186

Hirayama T, Umezawa T (2010) The PP2C–SnRK2 comples: The central regulator of an abscisic acid signaling pathway. *Plant Signal Behav* 5: 160–163

Kobayashi Y, Yamamoto S, Minami H, Kagaya Y, Hattori T (2004) Differential activation of the rice sucrose nonfermenting1-related protein kinase2 family by hyperosmotic stress and abscisic acid. *Plant Cell* 16: 1163–1177

Leung J, Merlot S, Giraudat J (1997) The Arabidopsis abscisic acid-insensitive 2 (ABI2) and ABI1 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. *Plant Cell* 9: 758–771

Leung J, Michelle BD, Morris PC, Guerrier D, Chefdor F, Giraudat J (1994) Arabidopsis ABA response gene ABI1: Features of a calcium-modulated protein phosphatase. *Science* 264: 1448–1452

Meyer K, Leube MP, Grill E (1994) A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. *Science* 264: 1452–1455

Nakashima K, Fujita Y, Kanamori N, Katagiri T, Umezawa T, Kidokoro S, Maruyama K, Yoshida T, Ishiyama K, Kobayashi M, et al. (2009) Three Arabidopsis SnRK2 protein kinases, SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, involved in ABA signaling are essential for the control of seed development and dormancy. *Plant Cell Physiol* 50: 1345–1363

Ng LM, Melcher K, Teh BT, Xu HE. (2014) Abscisic acid perception and signaling: structural mechanisms and applications. *Acta Pharmacologica* 35: 567–584

Nishimura N, Yoshida T, Kitahata N, Asami T, Shinozaki K, Hirayama T (2007) *ABA-Hypersensitive Germination1* encodes a protein phosphatase 2C, an essential component of abscisic acid signaling in Arabidopsis seed. *Plant J* 50: 935–949

Ohtani M, Nishikubo N, Xu B, Yamaguchi M, Mitsuda N, Goué N, Shi F, Ohme-Takagi M, Demura T (2011) A NAC domain protein family contributing to the regulation of wood formation in poplar. *Plant J* 67: 499–512

Saez A, Apostolova N, Gonzalez-Guzman M, Gonzalez-Garcia MP, Nicolas C, Lorenzo O, Rodriguez PL (2004) Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C *HAB1* reveal its role as a negative regulator of abscisic acid signalling. *Plant J* 37: 354–369

Soon FF, Ng LM, Zhou XE, West GM, Kovach A, Tan MH, Suino-Powell KM, He Y, Xu Y, Chalmers MJ, et al. (2012) Molecular mimicry regulates ABA signaling by SnRK2 kinases and PP2C phosphatases. *Science* 335: 85–88

Tuskan GA, Di Fazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A, et al. (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* 313: 1596–1604

Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, Shinozaki K (2009) Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. *Proc Natl Acad Sci USA* 106: 17588–17593

Umezawa T, Nakashima K, Miyakawa T, Kuromori T, Tanokura M, Shinozaki K, Yamaguchi-Shinozaki K (2010) Molecular basis of the core regulatory network in ABA responses: Sensing, signaling and transport. *Plant Cell Physiol* 51: 1821–1839

Vlad F, Rubio S, Rodrigues A, Sirichandra C, Belin C, Robert N, Leung J, Rodriguez PL, Laurière C, Merlot S (2009) Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in *Arabidopsis*. *Plant Cell* 21: 3170–3184

Welling A, Palva ET (2006) Molecular control of cold acclimation in trees. *Physiol Plant* 127: 167–181

Yamaguchi M, Kubo M, Fukuda H, Demura T (2008) VASCULAR-RELATED NAC-DOMAIN7 is involved in the differentiation of all types of xylem vessels in Arabidopsis roots and shoots. *Plant J* 55: 652–664

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptionl regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu Rev Plant Biol* 57: 781–803

Yoshida R, Umezawa T, Mizoguchi T, Takahashi S, Takahashi F, Shinozaki K (2006) The regulatory domain of SRK2E/OST1/SnRK2.6 interacts with ABI1 and integrates abscisic acid (ABA) and osmotic stress signals controlling stomatal closure in Arabidopsis. *J Biol Chem* 281: 5310–5318

Yoshida T, Nishimura N, Kitahata N, Kuromori T, Ito T, Asami

T, Shinozaki K, Hirayama T (2006) *ABA-hypersensitive germination3* encodes a protein phosphatase 2C (AtPP2CA) that strongly regulates abscisic acid signaling during germination among Arabidopsis protein phosphatase 2Cs. *Plant Physiol* 140: 115–126

Yunta C, Martínez-Ripoll M, Zhu JK, Albert A (2011) The structure of *Arabidopsis thaliana* OST1 provides insights into the kinase regulation mechanism in response to osmotic stress. *J Mol Biol* 414: 135–144

Zhou XE, Soon FF, Ng LM, Kovach A, Suino-Powell KM, Li J, Yong EL, Zhu JK, Xu HE, Melcher K (2012) Catalytic mechanism and kinase interactions of ABA-signaling PP2C phosphatases. *Plant Signal Behav* 7: 581–588