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Recognition of a PP2C Interaction Motif in Several Plant Protein Kinases

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Summary

Protein phosphatase 2Cs (PP2Cs) constitute a major class of phosphatases in plants. PP2Cs play important roles in many signaling pathways by countering the action of specific protein kinases. In addition to their role in several environmental stress-related signal transduction pathways, they are also involved in plant metabolism. Protein phosphatases often physically associate with their protein kinase counterparts. One approach to understanding PP2C function is to identify their interacting protein kinases. We describe a yeast two-hybrid assay system used in our lab to determine the interaction between members of the PP2C family and protein kinases in the SOS2 family. This chapter and the cited articles describing related work might be of help in discovering interactions between other protein phosphatases and kinases.

Key Words: PP2C; protein kinase; SOS2; ABI2; yeast two-hybrid

1. Introduction

1.1. Plant PP2C: A Brief Overview

Reversible protein phosphorylation is a fundamental mechanism by which living organisms regulate cellular processes in response to developmental, hormonal, and environmental cues. The phosphorylation status of a protein is determined by the balance between the activities of protein kinases and protein phosphatases. Although the catalytic cores of all eukaryotic protein kinases share extensive similarities in both primary and three-dimensional structures, protein phosphatases display much more diversity. Protein phosphatases can be divided into two major classes: protein tyrosine phosphatases (PTPs) and protein serine/threonine phosphatases. Protein tyrosine phosphatases include PTPs and dual-specificity phosphatases (DSPTPs). The protein serine/

From: *Methods in Molecular Biology, Volume 365: Protein Phosphatase Protocols*
Edited by: G. Moorhead © Humana Press Inc., Totowa, NJ

threonine phosphatases are further classified into the PPP and PPM gene families based on their amino acid sequences. The PPP family includes so-called "signature phosphatases" (types 1 [PP1], 2A [PP2A], and 2B [PP2B]), whereas the PPM family includes type 2C (PP2C) and pyruvate dehydrogenase phosphatase *(1)*.

1.2. Function of PP2C in Plants

The PP2Cs constitute the largest family of protein phosphatases in plants, with 76 members in *Arabidopsis* genome. With merely 15 PP2Cs found in the human genome, 8 in worm and 10 in fly *(2)*, the multiplicity of PP2C in plants suggests broader functional diversity than in other eukaryotes. Members of the PP2C family dephosphorylate the α-subunit of phosphorylase kinase. They require magnesium or manganese for activities and are insensitive to okadaic acids, microcystin, and calyculin A, which are inhibitors of PP1 and PP2A *(3,4)*. Unlike other families of protein phoshatases, members of the PP2C family are monomeric, lacking regulatory subunits *(3,4)*. Most of *Arabidopsis* PP2Cs (44 out of 76) have catalytic domains at their C-terminus with different N-terminal extensions *(2)*.

The PP2Cs are known to reverse stress-induced protein kinase cascades in eukaryotes. For example, PP2C negatively regulate the high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway by derectly dephosphorylating Hog1 MAPK in yeast *(5)*. In plants, alfalfa MP2C can dephosphorylate and inactivate the wound-induced SIMK (stress-induced MAPK), suggesting that MP2C is a part of a negative feedback loop for the SIMK signaling pathway *(6)*.

1.3. PP2Cs Are Negative Regulators of ABA Signaling

The *Arabidopsis* PP2Cs fall into 10 groups (A–J), and ABI1 and ABI2 belong to the group A of PP2C *(2)*. Genetic analysis of *abi1* and *abi2* mutants, their revertants, transient expression studies, and analysis of transgenic antisense plants have revealed that PP2Cs function as negative regulators of ABA signaling *(4,7–9)*. Transient expression assays have demonstrated that ABA signaling could be repressed by ABI-type PP2C, but not by KAPP or other protein phosphatase such as PP1, PP2A, or PP2B, indicating that this function is specific to ABI-type PP2C phosphatases *(7)*. There are as many as nine group-A PP2C members in *Arabidopsis*, which might have redundant functions. *(10)*. ABI1 and ABI2 contribute nearly 50% of the ABA-induced PP2C activity, indicating that other PP2Cs are also involved in ABA signaling *(8)*.

ABI1 and ABI2 encode homologous proteins and are transcriptionally upregulated by ABA *(5)*. In *abi1* and *abi2* mutants, the same Gly to Asp amino acid substitution has occurred at equialent position in ABI1 and ABI2 proteins,

which causes a significant reduction of the ABI1 and ABI2 phosphatase activity *(7)*. The *abi1-1* and *abi2-1* mutations are dominant and lead to largely overlapping sets of phenotypic alterations, including ABA-resistant seed germination and seedling growth, reduced seed dormancy, abnormal stomatal regulation, and defects in various responses to drought stress. Owing to the dominant nature of these mutations, it is still uncertain whether ABI1 and ABI2 were involved in ABA signaling or if the dominant mutations create unspecific phenotypes that are not related to the original function of the wild-type protein.

Several studies have shown that ABI1 and ABI2 have distinct functions in ABA signaling. Guard cells of *abi1-1* and *abi2-1* plants are disrupted in ABA activation of hyperpolarization-activated Ca^{2+} (ICa) channels *(11,12)*. However, the *abi1-1* mutant treated with ABA did not induce production of reactive oxygen species (ROS) but H_2O_2 activation of ICa channels and H_2O_2-induced stomatal closing was not disrupted. This suggested that *abi1-1* impairs ABA signaling between ABA perception and ROS production. Conversely, *abi2-1* impaired H_2O_2 activation of ICa, H_2O_2-induced stomatal closing, and ABA-elicited ROS production. These results suggested that both PP2Cs function at different levels of the same pathway: *abi1-1* acts upstream and *abi2-1* acts downstream of ABA-induced ROS production in guard cells *(12)*. ABA-induction of the alcohol dehydrogenase gene was reduced in *abi2* plants, but not in *abi1* plants *(13)*. *abi1* but not *abi2* mutation abolished induction of cold-regulated (COR) genes by ABA *(14)*. It was found that ABI1 and ABI2 could interact with proteins that are involved in several signaling pathways. SOS2 (Salt Overly Sensitive 2) and some of the SOS2-related protein kinases, PKSs (protein kinase S), interact with ABI1 and ABI2 *(15,16)*. ABI1 also interacts with the ABA-inducible transcription factor ATHB6. ATHB6 promoter–reporter expression was abrogated in *abi1-1* mutant plants, indicating that ABI1 acts upstream of the transcription factor *(17)*.

1.4. Interaction of ABI2 and SOS2

The *Arabidopsis* SOS2 is a serine/threonine kinase that is necessary for sodium and potassium ion homeostasis and salt tolerance *(18)*. SOS2 kinase is activated by the calcium-binding protein SOS3, together with calcium elicited by salt stress *(19)*. The SOS3–SOS2 kinase complex is required for the phosphorylation and activation of the plasma membrane Na^+/H^+ antiporter encoded by the *SOS1* gene *(20)*. *Arabidopsis* SOS2 is a member of a family of 25 protein kinases that are known as protein kinase S (PKS) *(21)*.

We have isolated proteins that interact with SOS2 kinase by yeast two-hybrid screening. Seven of the 101 putative interacting clones encode ABI2 *(16)*. The interaction of SOS2 with ABI2 is mediated through a novel protein domain of 37 amino acid residues, designated as the protein phosphatase interaction (PPI)

motif of the SOS2 that is necessary and sufficient for interaction with ABI2 *(16)*. The PPI motif is conserved in *Arabidopsis* PKS proteins and in the DNA damage repair and replication block checkpoint kinase (Chk1) in various organisms including human. Mutations in the conserved amino acid residues in the PPI motif abolish the interaction of SOS2 with ABI2, indicating that these amino acid residues are important for the interaction with ABI2. Further, a protein kinase interaction (PKI) domain in ABI2 was identified and the interaction specificity between PKS and the ABI phosphatases was investigated. The interaction between SOS2 and ABI2 was disrupted by the *abi2-1* mutation, which causes increased tolerance to salt shock and ABA insensitivity in plants *(16)*.

Some PKSs interact strongly with ABI2, whereas others interact preferentially with ABI1. For example, SOS2, PKS3, PKS11, and PKS24 preferentially interact with ABI2, whereas PKS18 strongly interact with ABI1 *(16)*. PKS3 and its interacting calcium sensor ScaBP5 are regulators of ABA signaling *(15)*. Transgenic plants expressing a constitutive active form of *PKS11* were more resistant to a higher concentration of glucose, suggesting a role of PKS11 in sugar signaling *(22)*. PKS18 is also involved in ABA signaling because RNA interference (RNAi) of *PKS18* conferred ABA insensitivity and transgenic plants expressing an active form of PKS18 were hypersensitive to ABA *(23)*. *sos2* and *sos3* mutants are specific defective in salt tolerance but not in ABA responses *(18,24)*. PKS3 and PKS18 are involved in ABA signaling, but not in salt tolerance *(15,23)*. These studies suggest that different combinations of PKS/SOS2 kinase–ABI1/2 phosphatase complexes might regulate specific signaling pathways.

2. Materials

2.1. Preparation of Test Bait and Interacting Prey Plasmids

We use pAS2 *(25)* and pACT2 *(26)* plasmid vectors to make bait and prey constructs, respectively. The polymerase chain reaction (PCR) is the most preferred experiment for construct preparation, as it greatly helps to make the insert fragments of genes of interest in frame with the vectors. Refer to standard protocols such as "Molecular Cloning" for PCR. However, verify the constructs by restriction digestion/sequencing before proceeding to the next step.

2.2. Yeast Transformation

Transformation of yeast cells can be carried out by the methods developed by Ito et al. *(27)* and modified by Schiestl and Gietz *(28)*, Hill et al. *(29)*, and Gietz et al. *(30)*.

1. YPD medium (1 L): 20 g Difco peptone, 10 g Yeast extract, 18 g Bactoagar (for plates only). Dissolve in 950 mL of H_2O and adjust pH to 5.8, autoclave, and cool to approx 55°C. Add glucose to 2% (50 mL of sterile 40% stock solution).

2. SD medium (1 L): 717 mg dropout, 20 g glucose, 1 mL of 1 *N* NaOH, 10 mL each of 100X amino acid, Bactoagar 20 g (if necessary). Fill up to 900 mL.

Synthetic dropout (SD) is a minimal medium that includes a yeast nitrogen base, a carbon source (generally 2% dextrose), and "dropout" solution that contains essential nutrients (such as amino acids and nucleotides). After autoclaving, wait until the solution is cooled down to 55°C and add 100 mL of 10X YNB (yeast nitrogen base without amino acids; Difco cat. no. 0919-15-3). It is convenient to put a magnetic stirrer bar into the bottle when you autoclave the medium. This helps to mix the medium well after autoclaving.

3. 10X YNB (1 L): dissolve 67 g of the powder in distilled water and sterilize by filtration (0.45-μm filter) and store at 4°C (*see* **Note 1**). We usually make the dropout containing the following amino acids and nucleotides. Take 0.15 g Ile, 0.1 g Ura, 0.75 g Val, 0.1 g Ade, 0.1 g Arg, 0.15 g Lys, 0.1 g Met, 0.25 g Phe, 1 g Thr, and 0.15 g Tyr. Mix and grind these nutrients well with motar and pestle. You can stock the dropout at room temperature. In order to select transformants, you should supplement the following amino acids to the SD medium:

Vector	Amino acid
pAS2	Leu, His
pACT2	Trp, His
pAS2 + pACT2	His

4. 100X amino acid solution: 0.2 g His, 0.2 g Trp, 1 g Leu and dissolve each of them in 100 mL of distilled water and autoclave. 100X amino acids should be kept at 4°C in the dark (Trp is susceptible to photodegradation).
5. 1X TE/LiAc buffer (LATE buffer): 100 m*M* lithium acetate, 10 m*M* Tris-HCl, pH 7.5, and 1 m*M* EDTA. Sterilize by autoclaving.
6. 10 mg/mL salmon sperm carrier DNA. Sonicated. (Salmon sperm carrier DNA in solution can be purchased from commercial sources or can be prepared using a standard method.)
7. PLATE buffer: 40% PEG 4000 in LATE buffer. Dissolve 40 g of PEG 4000 in 100 mL of the LATE buffer. Sterilize by autoclaving; avoid repeated autoclaving.

2.3. Colony-Lift β-Galactosidase Filter Assays

1. Z buffer (1 L): 16.1 g $Na_2HPO_4 \cdot 7H_2O$, 5.50 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, $MgSO_4 \cdot 7H_2O$ 0.246 g. Adjust to pH 7.0 and autoclave.
2. X-gal stock solution: Dissolve 5-bromo-4-chloro-3-idolyl-β-D-galactopyranoside (X-gal) in *N*,*N*-dimethylformamide (DMF) at a concentration of 20 mg/mL. Store in the dark at –20°C.
3. Z buffer/X-gal solution: 100 mL Z buffer, 0.27 mL β-mercaptoethanol (β-ME), 1.67 mL X-gal stock solution. Mix these reagents and use immediately.

4. Whatman #5 or VWR grade 410 paper filters: 75-mm filters (e.g., VWR #28321-055) for use with 100-mm plates or 125-mm filters (e.g., VWR #28321-113) for use with 150-mm plates.

2.4. In vitro Protein-Binding Assay

1. Lysis buffer: 20 m*M* Tris-HCl, pH 8.0, 200 m*M* NaCl, 1 m*M* EDTA, pH 8.0, 0.5% Nonidet P40 (just before use, add protease inhibitors to the following final concentrations: 2 μg/μL aprotinin, 1 μg/μL leupeptin, 0.7 μg/mL peptatin, and 25 μg/mL phenylmethylsulfonyl fluoride).
2. Binding buffer: 20 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 1 m*M* $CaCl_2$, 0.1% Nonidet P-40.

3. Methods

3.1. Yeast Transformation

1. For inoculation, take a single colony 2–3 mm in diameter and scrape the entire colony into 10 mL of YPD. Grow the preculture at 30°C for overnight with shaking at 200 rpm.
2. Add the preculture to produce optical density (OD_{600}) of 0.2–0.3 into 50 mL of main culture and grow further at 30°C untill it reaches mid log phase.
3. Pellet the cells by centrifuging at 1000*g* at 25°C for 5 min. Resuspend the pellet in 35 mL of distilled water and collect the cells again as same as above. Resuspend the cells in 1.5 mL of LATE buffer.
4. Combine the following components in a 1.5-mL tube:

Competent cells	100 μL (*see* **Note 2**)
PLATE buffer	600 μL
10 mg/mL Salmon sperm DNA	5 μL
Plasmid DNA	0.1 μg

5. Incubate the transformation mixtures at 30°C for 30 min with gentle shaking.
6. Add 70 μL of dimethyl sulfoxide (DMSO) and incubate at 42°C for 15 min. Pellet the cells by the centrifugation and resuspend in 100 μL of TE.
7. Plate the cells onto an appropriate SD agar plate (*see* above).
8. Incubate the plates at 30°C for 1 or 2 d until colonies become 2–3 mm in diameter.
9. Streak several colonies to new plates and grow them at 30°C for 1 or 2 d.

3.2. Colony-Lift β-galactosidase Filter Assays

1. In the case that a few colonies are to be assayed, streak them directly onto SD agar plates. Incubate the plates at 30°C for 1–2 days (*see* **Note 3**) and then proceed with the β-galactosidase assay as described in the following steps.
2. For each plate, presoak a sterile Whatman #5 or VWR grade 410 filter by placing it in 2.5–5 mL of Z buffer/X-gal solution in a clean 100- or 150-mm plate.
3. Place a clean, dry filter over the surface of the plate of colonies to be assayed (*see* **Note 4**).

4. Carefully lift the filter off the agar plate with forceps and transfer it (colonies facing up) to a pool of liquid nitrogen (*see* **Note 5**) and submerge the filters for 10 s.
5. Remove the filter from the liquid nitrogen and allow it to thaw at room temperature (this treatment permeabilizes the cells).
6. Carefully place the filter, colony side up, on the presoaked filter (from **step 2**). Avoid trapping air bubbles under or between the filters.
7. Incubate the filters at 30°C and check them periodically for the appearance of a blue color on the colonies (*see* **Note 6**).
8. If you are conducting a screening, identify the β-galactosidase-producing colonies by aligning the filter to the agar plate and pick the corresponding positive colonies from the original plates to fresh medium.

3.3. Yeast Two-Hybrid Screen and Interaction Assay

Colony-lift β-galactosidase filter assays can be used for both screening of interacting proteins and confirmation of the interaction between proteins of interest. When you carry out the two-hybrid experiments for the confirmation of the interactions, you do not have to handle plates and filters aseptically. To do the colony-lift β-galactosidase filter assays semiquantitatively, you must be careful to keep equal amount of colonies among samples. We usually prepare the plates for the colony-lift β-galactosidase filter assays as follows. Inoculate colonies into SD liquid medium and grow them for overnight at 30°C. Check their OD_{600}. Harvest the cells by centrifugation and resuspend them in D.W. so as to adjust their OD_{600} values to 0.1. Drop 20 μL of the cell suspensions (4×10^4 cells) onto SD agar plates and incubate at 30°C for 2 d. In the author's laboratory, the gene for the test protein, SOS2, is fused to the GAL4 DNA-BD in the pAS2 vector and the gene for the target protein ABI2 is fused to the GAL4 AD in the pACT2 vector. To evaluate a positive interaction between test and target proteins, you must make combinations of bait and prey for control experiments. If a combination of your bait construct with pACT2 empty vector or a combination of pAS2 empty vector with your prey construct does not give any background level of positive signal, then the positive signal observed on the colonies harboring your test and target constructs can be considered as a positive interaction.

Using a yeast two-hybrid approach, we screened for proteins that interact with the protein kinase SOS2 from a λ-ACT cDNA library prepared from mRNA isolated from young *Arabidopsis* seedlings ***(31)***. This library can be converted to a pACT plasmid library by infecting *Escherichia coli* BNN132 cells. You can construct a GAL4 AD fusion expression library in pACT2 using either intronless genomic DNA or cDNA such that at least 10^6 different hybrid proteins will be expressed (*see* **Note 7**). (Commercial cDNA libraries from a variety of species and tissues are also available.)

3.4. Identification of Interaction Motif in Test Proteins

Once an interaction between the test and target proteins is confirmed, it might be possible to identify the motif of the test protein that mediates the interaction with the target protein. Usually, deletion clones are made in prey, when a minimal interacting domain is identified. In the authors' laboratory, serial deletions of SOS2 in the bait vector pAS2 were made in order to identify a minimal region of SOS2 that is sufficient and/or necessary for interaction with ABI2. However, *ABI2* could not be used as bait, because pAS-ABI2 activates the *lacZ* reporter gene in yeast. It is important that every deletion construct in bait should be checked for their background transcriptional activation in yeast cells by setting a negative control with an empty prey vector (pACT2) for every bait construct. Some proteins acquire transcriptional activation by removing a portion of the protein, even though the full length does not show transcriptional activation at all. We made two bait constructs, each containing the C-terminal regulatory or N-terminal catalytic domain of SOS2. Because the ABI2 prey interacted with the C-terminal regulatory but not with the N-terminal catalytic domain in bait, we further determined that the minimal C-terminal regulatory sequence of SOS2 interacts with ABI2 using the yeast two-hybrid assay and then made serial deletions in the C-terminal regulatory region. The deletion constructs can be made by PCR with pairs of forward and reverse primers containing suitable restriction sites for cloning. The resulting PCR products can then be digested and inserted between the corresponding sites of pAS2. We found that the SOS2 sequence between amino acid 333 and 369, designated as PPI motif, is necessary and sufficient for interaction with ABI2.

In the next step, we tested the highly conserved amino acid residues in the PPI motif of SOS2. The experiment was to further elucidate the amino acids important for protein interaction. This was done by mutating the conserved amino acid residues of the test protein and then studying the impact of the mutations on the protein interaction. Mutations can be easily introduced by an inverse PCR-based site-directed mutagenesis with double-stranded plasmid DNA as templates. First, you have to phosphorylate one of the PCR primers at the 5' end, followed by PCR with high-fidelity *Taq* DNA polymerase. The resulting PCR products should be digested with *Dpn*I to remove the template plasmid DNA and select for the synthesized DNA-containing mutations. Because DNA isolated from most *E. coli* strains is dam methylated, it is susceptible to *Dpn*I digestion, which is specific for methylated DNA. After digestion, the PCR products are purified by gel electrophoresis, followed by self-ligation. Finally, the circular PCR products are transformed into *E. coli*. All plasmid constructs are completely sequenced to ensure that there is no PCR or cloning errors.

3.5. In Vitro Protein-Binding Assay

It is necessary that the interaction of a test protein with a target protein in yeast two-hybrid assay be confirmed by in vitro protein-binding assays such as a pull-down assay. For the pull-down assay, you have to make two kinds of plasmid construct to express a GST-fusion protein and an in vitro translated protein. We used pGEX-2TK (Pharmacia) and pET146 (Novagen) for the GST fusion and the in vitro translated proteins, respectively *(16)*.

3.5.1. Preparation of GST-Fusion Proteins

1. Make the plasmid construct in an appropriate vector for the GST-fusion protein (e.g., pGEX-2TK) and transform it into the BL21 DE3 *E. coli* strain (Novagen).
2. Inoculate a single colony into 1 L of Luria-Bertani medium containing 100 μg/mL ampicillin and incubate the medium at 30°C until the OD_{600} reaches 0.5–0.7 (freshly transformed colonies give better results of protein expression). You can reduce the size of the culture medium if your protein is well produced in *E. coli* cells.
3. Induce the recombinant protein expression with 0.5 m*M* isopropyl-thio-β-galactoside (IPTG) and incubate 30°C for another 6 h.
4. Harvest the cells by centrifugation at 5000*g* for 15 min at 4°C and resuspend the cells in 20 mL of the lysis buffer.
5. Purify the recombinant fusion proteins from bacterial lysates with glutathione–Sepharose (Pharmacia) described in the manufacturer's protocol.

3.5.2. In Vitro Pull-Down Assay

1. Insert the coding region of the test or interacting protein in a vector for the in vitro translation system such as pET146 (Novagen). Level the expressed proteins by in vitro translation using ^{35}S methionine.
2. Incubate aliquots of the ^{35}S-labeled protein with 20 μg of GST-fusion proteins on Sepharose beads in 150 μL of the binding buffer under constant rocking for 1 h at 4°C. For negative controls, incubate the ^{35}S-labeled protein with 20 μg of GST-Rb and GST beads as same as above.
3. Centrifuge the mixture at 1000 rpm for 2 min at 4°C.
4. Wash the beads extensively with the ice-cold binding buffer. Repeat the washes three times.
5. Elute the bound proteins with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (*see* **Note 8**).
6. Resolve the bound protein on 7.5% SDS-PAGE and detect the bound protein by fluorography.

4. Notes

1. Warm up the 10X YNB before you mix it with the autoclaved agar medium. Otherwise, the agar starts to solidify immediately after you add the chilled 10X YNB.

2. When screening a library, competent cells should be used immediately. However, for small-scale (routine) transformations, the competent cells can be stored at room temperature for several hours without a significant reduction in competency.
3. For best results, use fresh colonies (i.e., grown at 30°C for 2–4 d), 1–3 mm in diameter. If the entire colony was lifted onto the filter, pick it from the filter or incubate the original plate for 1–2 d to regrow the colony.
4. Nitrocellulose filters also can be used, but they are prone to crack when frozen.
5. Liquid nitrogen should be handled with care. Always wear thick gloves and goggles when handling liquid nitrogen.
6. The time that it takes colonies producing β-galactosidase to turn blue varies, typically from 30 min to 8 h in a library screening. Prolonged incubation (>8 h) tends to give false positives.
7. If you construct your own library, you will obtain enough material to perform a library-scale transformation without an extra amplification step. However, be sure to reserve a 1.0-mL aliquot of your library, frozen in 25% glycerol, so that you can go back and amplify it at a later time if necessary.
8. If you get only a low signal, you should change the ion concentration in the binding buffer or incubate target proteins with GST-fusion proteins longer than previously.

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