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Molecular Cloning and Characterization of a Rice PP2C, *OsPP2C4*

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Protein phosphorylation and dephosphorylation are major regulatory mechanisms that cells use to transmit signals from their extracellular environment to the interior. Up to now, two structurally distinct groups of ser/thr phosphatases are known of: the PP1/PP2A family and the PP2C family. Here, we focus our efforts to reveal the functions of the PP2C family in rice. It has been known that PP2C has diverse functions related to developments and stress responses. We have obtained a rice EST clone, OsPP2C4, that contained the highly conserved PP2C motifs. RNA gel-blot analysis showed that *OsPP2C4* was expressed highly in panicles, while it was expressed weakly in seedling leaves, seedling roots, and mature leaves. Assay of the PP2C enzyme activity with a substrate, *para*-nitrophenyl phosphate, showed that *OsPP2C4* encoded an active PP2C. Transgenic plants expressing the antisense construct of this clone were generated to study the functional roles of the PP2C clone in rice.

Keyword : dephosphorylation, phosphatase, phosphorlation, PP2C, rice

Protein phosphorylation and dephosphorylation are major mechanisms that are involved in conveying developmental signals and environmental information to the cell (Luan, 1998). Protein kinases and phosphatases carry out these functions. Protein phosphatases are classified into two major groups based on their substrate specificity: tyrosine and serine/threonine phosphatases. The serine/threonine phosphatases are also classified into two groups: the PP1/PP2A family and the PP2C family. PP2Cs require Mg^{2+} or Mn^{2+} for their activity and are insensitive to okadaic acid, an inhibitor of PP1/PP2A enzymes (Cohen, 1989).

Several functional roles of PP2Cs have been reported. In *Arabidopsis*, genetic studies have shown that ABI1/ABI2 have functions related to the signal transduction cascade of abscisic acid (Leung et al., 1994; Leung et al., 1997; Gosti et al., 1999). Moreover, kinase associated protein phosphatase (KAPP) is known to be an important element in flower meristem development (Stone et al., 1994; Williams et al., 1997; Stone et al., 1998) and an alfalfa PP2C, MP2C, has been found to act as a negative regulator of a stress activated MAPK (Meskiene et al., 1998). In addition, ten PP2C transcripts, *Mpc1-Mpc10*, of ice plant showed tissue- and environmental response-specific expression patterns (Miyazaki et al., 1999). Besides these, it was reported that Ptc2p/Ptc3p of budding yeast dephosphorylated the major cyclin dependent kinase, Cdc28p, and Ptc4 of fission yeast was involved in the regulation of vacuole fusion (Cheng et al., 1999; Gaits and Russel, 1999). Fem-2 of *Caenorhabditis elegans* was demonstrated to be required for male development (Chin-Sang and Spence, 1996). It was also revealed that the human PP2Cs do functions in the monocytic differentiation and cystic fibrosis transmembrane conductance regulation (Nishikawa et al., 1995; Travis et al., 1997). However, the roles of PP2Cs in rice were not reported except OsKAPP, a homologue of KAPP, which interacts with a transmembrane kinase (Knaap et al., 1999). Here, we tried to reveal the functional roles of another rice PP2C, OsPP2C4.

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MATERIAL AND METHODS

Bacterial Strain and Plant Transformation

The *Escherichia coli* strain JM83 was used as the recipient for routine cloning experiments. *Agrobacterium tumefaciens* LBA4404 containing the Ach5 chromosomal background and a disarmed Ti plasmid pAL4404 was used for rice transformation (Hoekema et al., 1983). A japonica rice variety, Dongjin, was used for transformation by the *Agrobacterium*-mediated cocultivation method as described previously (Jeon et al., 1999; Lee et al., 1999).

RNA Gel-Blot Analysis

Total RNA was extracted from various tissues of rice plants using the RNA isolation kit, TRI REAGENT® (Molecular Research Center). Ten microgram RNA was used for electrophoresis in a 1.3% (w/v) formaldehyde-agarose gel as described previously (Jeong et al., 1999; Moon et al., 1999). After RNA transfer onto a nylon membrane, the blot was prehybridized and hybridized in a solution containing 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 7% (w/v) SDS, and 1% (w/v) BSA overnight at 60°C. After hybridization, the blot was washed with a solution containing 0.2X SSC and 0.1% (w/v) SDS for 5 min at 45°C.

Construction of Antisense Expression Binary Vector

A binary vector, pGA1611, was used for the construction of an antisense expression vector (Kim et al., 2000). The binary vector, a derivative of pGA482 (An et al., 1988), contains hygromycin phosphotransferase gene (*hph*) as a selectable marker under the control of cauliflower mosaic virus 35S promoter followed by the termination region of the 7 gene of pTiA6. The vector also contains several unique restriction sites (HindIII, SacI, HpaI, and KpnI) between the maize ubiquitin promoter, including the first intron of the ubiquitin gene, and the nopaline synthase (*nos*) terminator. The 450 bp 3′ fragment of the OsPP2C4 cDNA between the restriction site, SacI, and the end of the clone was inserted into pGA1611 in an antisense orientation.

PP2C Enzyme Assay

Catalytic region of the OsPP2C4 cDNA was inserted into the pMALc2x vector (NEB) and the maltose binding protein (MBP)-OsPP2C4 fusion protein was expressed in *E. coli*, JM83 (Maina et al., 1988). This fusion protein was purified with amylose resin, following the procedure described by a manufacturer (NEB).

PP2C enzyme activity was assayed using *para*-nitrophenyl phosphate (*p*NPP) as a substrate (Takai and Mieskes, 1991). Eighty five microgram of purified MBP-OsPP2C4 was incubated with 10 mM *p*NPP in a solution containing 40 mM Tris·Cl, 20 mM KCl, 30 mM $MgCl_2$, 10 mM $MnCl_2$, and 0.5 mM dTT at 30°C for 5 min (Marley et al., 1996). The absorbance at 405 nm wavelength was measured.

RESULT AND DISCUSSION

Molecular Cloning of a Protein Phosphatase 2C (PP2C) Gene in Rice

Protein phosphatases in the 2C family contain 11 conserved motifs that are scattered along the catalytic sequence (Bork et al., 1996). Therefore, we searched the DDBJ rice EST database using the OsKAPP catalytic domain as a query to isolate rice PP2C clones. Several clones contained some of these PP2C conserved motifs. However, it was revealed that only one clone, D46746, covered all catalytic sequence. We obtained this EST clone, which was isolated from 8 days old rice seedling shoots, from the Ministry of Agriculture, Forestry, and Fisheries (MAFF) DNA bank of Japan. We named this clone OsPP2C4 (*Oryza sativa* protein phosphatase 2C 4). Since the released EST sequence of OsPP2C4 was 450 bp in length, we determined the full-length sequence. The result showed that the clone was 1419 bp and encoded 342 amino acids. Eleven conserved motifs of PP2C were identified through the amino acid sequence analysis (Fig. 1). This suggests that OsPP2C4 is a true PP2C.

Based on the amino acid sequence, OsPP2C4 showed the highest identity (34%) with an alfalfa PP2C clone, MP2C (Figs. 2 and 3). However, the homologous regions were restricted to the PP2C conserved motifs. Additional information is needed to anticipate the functional roles of the rice clone.

RNA Expression Level Analysis of OsPP2C4

The levels of the *OsPP2C4* transcript were analyzed by RNA gel-blot analysis. The 450-bp 3′ fragment downstream of the SacI site was used as a probe. The result showed that *OsPP2C4* was transcribed preferentially in panicles and mature flowers while weakly in seedling shoots, seedling roots, and mature leaves (Fig. 4). It implies that *OsPP2C4* may play important roles during panicle development.

The transcript length was about 1.5 kb, which is similar with that of the cDNA clone. It indicates that the OsPP2C4 clone covered almost all of the *OsPP2C4* gene sequence. In addition, it also indicates that OsPP2C4 may have a distinctive function from KAPP since KAPP is composed of more than 580 amino acids (Stone et al., 1994; Braun et al., 1997; Knaap et al., 1999).

PP2C Enzyme Activity Assay

We have examined whether OsPP2C4 has phosphatase enzyme activity. First, we cloned the catalytic

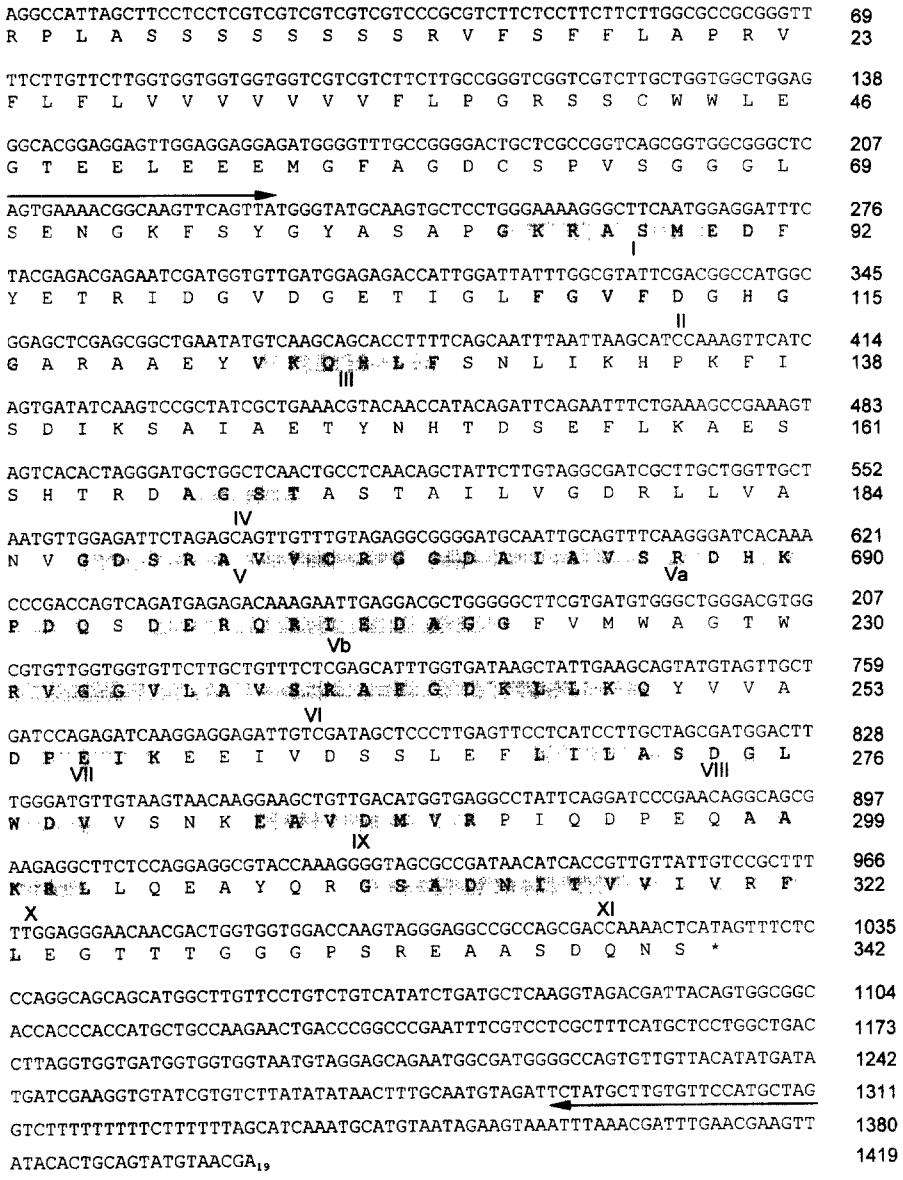

Figure 1. Nucleotide sequence of *OsPP2C4*. The shaded sequences are the conserved motifs in the PP2C phosphatases. The arrow-lined sequences are the primers that were used to generate the fusion protein MBP-OsPP2C4.

domain of OsPP2C4 into the expression vector, pMALc2x, making a fusion between the maltose binding protein (MBP) and OsPP2C4. The MBP fusion protein, MBP-OsPP2C4, was expressed in *E. coli* and purified with amylose resin. The purified MBP-OsPP2C4 was used for the phosphatase activity assay with a substrate, *para*-nitrophenyl phosphate (*p*NPP). The assay uses the principle that the absorbance of 405 nm wavelength should change when the phosphate of *p*NPP is released by a phosphatase. The difference of the absorbance between purified MBP and MBP-OsPP2C4 proteins was 0.144. It can be calculated that MBP-OsPP2C4 has the phosphatase activity at the value of 19.4 μmole min^{-1} per mg (Fig. 5). This value is higher than that of human PP2Cα which was previously reported to have an activity of 3 μmole min^{-1} per mg (Marley et al., 1996). The N-terminal domain of PP2C that is thought to have an inhibitory role may be a reason to create the difference (Rodriguez, 1998; Sheen, 1998) because we deleted the N-terminal domain of OsPP2C4 while the other group used a full length PP2Cα in the phosphatase activity assay (Fig. 1).

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OsPP2C4   LS----ENGKFSYGYASAPGKRASMEDFYETRIDGVDGET------------IGLFGVF
MP2C      VARDVVEAEGDGYSVYCKRGRREYMEDRYTAGVNLRGENN------------LAFFGVF
Mpc5      RS-------GS----FADIGPRKYMEDEHIRIDDLSVQLG-----SLFRCPKPSAFYGVF
ABI1      RS-LFEFKSVPLYGFTSICGRRPEMEDAVSTIPRFLQSSSGSMLDGRFDPQSAAHFFGVY
KAPP      FQIPFKIGVASDPMAMRRGGRKLPMEDVCHYKWPLPGANK------------FGLFCVC
                                    I

OsPP2C4   DGHGGARAAEYVKQ-------HLFSNLIKHPKFIS-------DIKSAIAETYNHTDSEFL
MP2C      DGHGGAKAAEFARN-------NLEKNILDEVIMTDE-----DDVEEAVKRGYLNTDSEFM
Mpc5      DGHGGSEAAAYVREN----VMRFFFEDVSFPEASELDEIFLEGVENCLRRAFFLADLALA
ABI1      DGHGGSQVANYCRERM---HLALAEEIAKEKPMLCDGDTWLEKWKKALFNSFLRVDSEIE
KAPP      DGHGGSGAAQSAIKIIPEVLANILSDSLRKEKVLSK-----RDASDVLRDMFAKTEARLE
            II          III

OsPP2C4   KAESSHTRDAGSTASTAILVGDRLLVANVGDSRAVVCRGGDAIAVSRDHKPDQSDERQRI
MP2C      KKD----LHGGSCCVTAFIRNGNLVVSNAGDCRAVISRGGVAEALTSDHRPSREDEKDRI
Mpc5      DDCS-ISTSSGTTALTALVLGRLLLVANAGDCRAVLCRKGEAIDMSQDHRPTYPSEKRRV
ABI1      SVAP---ETVGSTSVVAVVFPSHIFVANCGDSRAVLCRGKTALPLSVDHKPDREDEAARI
KAPP      EHQYEGCTATVLLVWKDNEENFFAQCANLGDSACVIHLGGRYIQMTEDHRVVSLSERKRF
                      IV                V                    Va

OsPP2C4   EDAGGFVMWAG-TWRVGGVLAVSRAFGDKLLKQ------YVVADPEIKEEIVDSSLEFLI
MP2C      ETLGGYVDLCRGVWRIQGSLAVSRGIGDRHLKQ------WVTAEPETKVIRIEPEHDLLI
Mpc5      EELGGYVDDGY---LNG-VLSVSRALGDWDMKLPKGSASPLISEPELRQIILTEDDEFLI
ABI1      EAAGGKVIQWN-GARVFGVLAMSRSIGDRYLKP------SIIPDPEVTAVKRVKEDDCLI
KAPP      QEAGLALRDGE---TRLFGINLARMLGDKFPKQQDSRFSAEPYISEPLRIDQSSKDVFAV
            Vb                      VI                    VII

OsPP2C4   LASDGLWDVVSNKEAVDMVR--------------------------PIQDPEQAAKRLL
MP2C      LASDGLWDKVSNQEAVDIARQ---------------------FCVGNNNQQPLMACKKLA
Mpc5      IGCDGIWDVISSQQAVSIVRWG-----------------------LKRHDDPEQSAKDLV
ABI1      LASDGVWDVMTDEEACEMARKRILLWHKKNAVAGDASLLADERRKEGKDPAAMSAAEYLS
KAPP      LASDGLWDVVSPKKAVQLVLQMR-------------------DKERGRESSAEKIANGLL
              VIII        IX                                    X

OsPP2C4   QEAYQRGSADNITVVIVRFLEGTTTGGGPSREAASDQNS----------------
MP2C      QLSVSRGSLDDTSVMIIKFKHYV-----------------------------------
Mpc5      NEALRRHTIDNLTVIIVCFSSIDHQREQTGPRPRRFRCSLSAEALSSLRSLLEGNESH
ABI1      KLAIQRGSKDNISVVVVDLKPRRKLKSKPLN---------------------------
KAPP      NEARAMRTKDNTSIIYLDFDTSL-----------------------------------
                  XI
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Figure 2. Multiple alignment of PP2C catalytic domain obtained with the CLUSTALW program. Alignment of several plant PP2C phosphatases: OsPP2C4 (rice), MP2C (alfalfa), Mpc5 (ice plant), ABI1 (*Arabidopsis*), and KAPP (*Arabidopsis*). The 11 conserved motifs are indicated as Roman numerals. Dashes indicate gaps to allow maximum alignment.

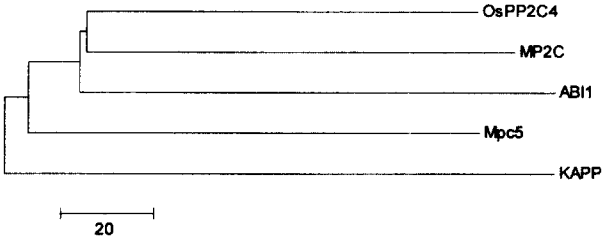

Figure 3. Phylogenetic tree showing the relationship of PP2Cs. The neighbor-joining analysis was used. The bar means the number of different amino acids.

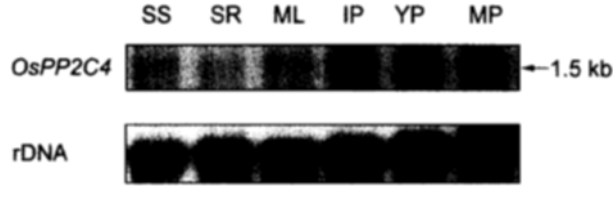

Figure 4. The *OsPP2C4* transcripts level in different organs. Ten microgram of total RNA was used for the RNA gel-blot analysis. The 3′ fragment from SacI site was used as a probe. **SS**, seedling shoots; **SR**, seedling roots; **ML**, mature leaves; **IP**, immature panicles smaller than 5 cm; **YP**, panicles at a 5 - 10 cm stage; **MP**, mature panicles at a heading stage. rDNA indicates 18S ribosomal DNA.

OsPP2C4 Antisense Transgenic Rice Plants

In order to study the functional roles of OsPP2C4, we have generated antisense transgenic rice plants by agrobacterium-mediated transformation using a binary vector, pGA1611. About thirty rice plants have been generated and analyzed for developmental abnormalities. However, we have not noticed any abnor-

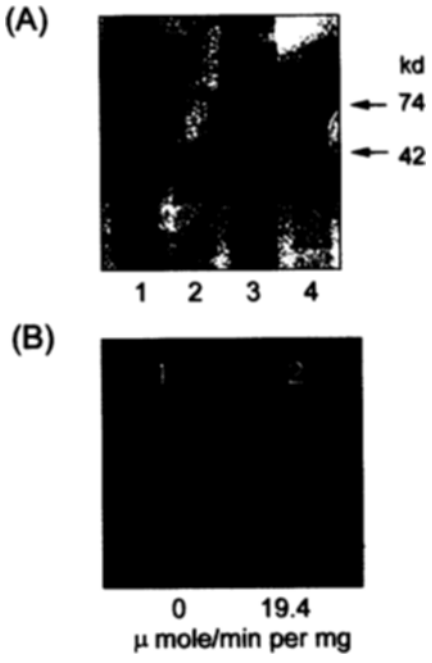

Figure 5. Enzyme activity of OsPP2C4. (**A**) Total proteins extracted from *E. coli* expressing MBP (lane 1) and MBP-OsPP2C4 (lane 3) were subjected to 10% SDS-PAGE. Lanes 2 and 4 show purified MBP and MBP-OsPP2C4 proteins. (**B**) Phosphatase activity of the pruified MBP-OsPP2C4 protein was measured in a solution containing 30 mM $MgCl_2$, 10 mM $MnCl_2$, and 10 mM *para*-nitrophenyl phosphate at 30°C for five minutes. 1, purified MBP; 2, purified MBP-OsPP2C4. The solution was changed from colorless liguids to yellow.

mal phenotypes from the primary transgenic plants.

It is possible that the OsPP2C4 is a member of a multiple gene family that has redundant functional roles. Alternatively, phenotypic alterations will not be visible until the plants are faced to specific conditions. Transgenic plants over-expressing this clone may provide some clue for the function. OsPP2C4 have some homology with MP2C that is up regulated by ABA treatment. Therefore, it may be worth to examine the effects of ABA, cold, drought, and some other stresses to the antisense transgenic plants.

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