

Protein phosphatase 2A: identification in *Oryza sativa* of the gene encoding the regulatory A subunit

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Abstract

A 2225 bp cDNA, designated RPA1, was isolated from an *Oryza sativa* cDNA library. Analysis revealed a 1761 bp coding sequence with 15 non-identical repeat units. The ORF encoded the A regulatory subunit of protein phosphatase 2A (PP2A-A) as ascertained by complementation of the yeast *tpd3* mutant defective in this gene. The corresponding genomic DNA from a rice genome BAC library revealed that the gene contains eleven introns. The rice genome contains only a single copy of this gene as judged by Southern blot analysis. The PP2A protein is highly conserved in nature; the rice protein shows 88% amino acid identity with its counterparts in *Arabidopsis* or *Nicotiana tabacum*.

Introduction

Reversible phosphorylation of proteins is one of the major general mechanisms for the control of cellular functions in eukaryotes (Edelman *et al.*, 1987; Cohen, 1988). However, most of the information available at present comes from the study of these processes in mammals, whereas the importance of protein phosphorylation as a relevant regulatory mechanism in higher plants has been recognized only more recently (Smith and Walker, 1996). Much work has been devoted to the role of protein kinases and their modulation by hormones, growth factors and neurotransmitters, but it is now appreciated that protein phosphatases are also key players in actively regulating many cellular processes. Protein phosphatases can be divided into three groups: tyrosine phosphatases (Hunter, 1989), serine/threonine phosphatases (Mumby and Walter, 1993) and dual-specificity phosphatases (Haked *et al.*, 1993). Biochemical studies have identified four major types of serine/threonine-specific protein phosphatases in eukaryotic cells: PP1, PP2A, PP2B and PP2C. This

classification was based on substrate specificity, divalent cation requirement and sensitivity to inhibitors (Cohen, 1989). PP1 is inhibited by the thermostable inhibitors 1 and 2, while PP2A and PP2C are unaffected by these inhibitors. In addition, PP2A has no divalent ion requirement for activity, while PP2C activity is Mg²⁺-dependent. Indirect evidence suggests that plant cells also contain PP2B, which is Ca²⁺/calmodulin-dependent (Luan *et al.*, 1993). PP2A is involved in many different cellular processes by regulating protein kinase cascades (Millward *et al.*, 1999).

Plant protein phosphatases are very similar to the enzymes characterized from animals and yeast. Like the animal enzymes, plant PP2A is probably a trimeric holoenzyme; it consists of a 36 kDa catalytic subunit (C subunit) tightly complexed with a 65 kDa regulatory subunit (A subunit). The core dimer complexes with a third, variable subunit (B subunit), which confers distinct properties on the holoenzyme. The activity of the catalytic subunit of PP2A is modulated by A and B regulatory subunits (Imaoka *et al.*, 1983; Mumby *et al.*, 1987; Kamibayashi *et al.*, 1991);

they modulate the specificity and activity of PP2A. Although much is known about the structure and function of PP2A enzymes (Wera and Hemmings, 1995), their regulatory features remain to be elucidated. It was reported that a mutant B subunit of PP2A alters peripheral nervous cell fate choice (Shiomi *et al.*, 1994) and regulates development of *Drosophila* (Wera and Hemmings, 1995). The PP2A C subunit appears to be involved in the expression of the rice gibberellic acid-dependent *Osamy-c* gene (Chang *et al.*, 1999). The regulatory A subunit of PP2A shows a high sequence conservation throughout evolution (ca. 60% amino acid identity between the mammalian and plant proteins), and is reported to be involved in auxin-mediated signal transduction in plants (Garbers *et al.*, 1996). As there was no knowledge of the rice enzyme, we decided to search for the corresponding gene in rice.

Materials and methods

Materials

Seedlings of rice (*Oryza sativa*) cv. Guang Lu Ai 4 (provided by the China National Rice Research Institute, Hangzhou) were grown in the greenhouse at 28 °C for two weeks. *Taq* polymerase, restriction enzymes and ligase were from Boehringer Mannheim or Promega. The rediprime DNA labeling system (Amersham) was used for labeling probes.

Preparation of genomic fragment

Genomic DNA was prepared from two-week old rice seedlings (*indica* variety Guang Lu Ai 4) following the protocol of Zhang *et al.* (1994). Oligo primers P1 (TGATGATCTAACCCAAGATG) and P2 (CCAA-GACCGGGCCATCCCCA) were synthesized according to the sequence of the rice EST (D21972). The genomic fragment was obtained by PCR with the above pair of primers and the genomic DNA as template. PCR conditions were as follows: 50 mM KCl, 10 mM Tris-Cl pH 8.3, 1.5 mM MgCl₂, 200 μM of each dNTP, 1 μM of each primer and 2.5 units of *Taq* polymerase. The samples were heated to 95 °C for 6 min, followed by 30 cycles of amplification at 94 °C for 1 min, 55 °C for 1.5 min, and 72 °C for 3 min. After the last amplification cycle the samples were incubated at 72 °C for 10 min.

Isolation of cDNA encoding PP2A-A

The rice variety Guang Lu Ai 4 ZAP Express cDNA library was obtained from Congron Sun of Fudan University, Shanghai. This library was constructed from mRNA isolated from 8-day old rice shoots. A cDNA cloned into the *EcoRI/XhoI* site of plasmid pBK-CMV (Stratagene) was isolated by hybridization with the above genomic fragment as probe, which was labeled with ³²P-dCTP using the random prime DNA labeling system. Hybridization conditions were according to the ZAP Express cDNA Synthesis Kit (Stratagene).

Screening of BAC library

A representative and genetically stable BAC library of the genome of rice cv. Guang Lu Ai 4 was constructed in this center (Tao *et al.*, 1994), which contained 21 600 BAC clones. BAC clones stored in 96-well plates were taken from the freezer, and were gridded on 12 cm × 8 cm sterile Hybond-N+ filters (Amersham) using a 96-pin gridding tool (Beckman) (Hong *et al.*, 1997). There were 2400 (25 × 96) BAC clones on one filter and therefore nine filters covered the whole library. The BAC clones containing the desired gene were obtained through filter hybridization with the rice cDNA as probe. Hybridization was according to the protocol of Hybond-N+ version 2.0.

Southern blot analysis

Genomic DNA (10 μg) and the BAC clones identified were digested with two restriction enzymes, and the digests were separated by electrophoresis in a 0.8% agarose gel at 1.5 V/cm for 8–10 h. The digests were then transferred onto a Hybond-N+ filter (Amersham) and hybridized as described above with the full-length cDNA as probe.

DNA sequence and analysis

The inserts of positive BAC clones were digested with *Bam*HI, and the fragment containing the PP2A-A gene was subcloned into plasmid pKS. Both cDNA and the genomic DNA of the PP2A-A gene were sequenced on PE Applied Biosystems 377 automatic DNA sequencer. Sequence analysis was done with the Staden package.

Complementation of yeast tpd3 mutant

The rice cDNA for the A regulatory subunit of PP2A was cloned into expression vector pVT102 (Vernet

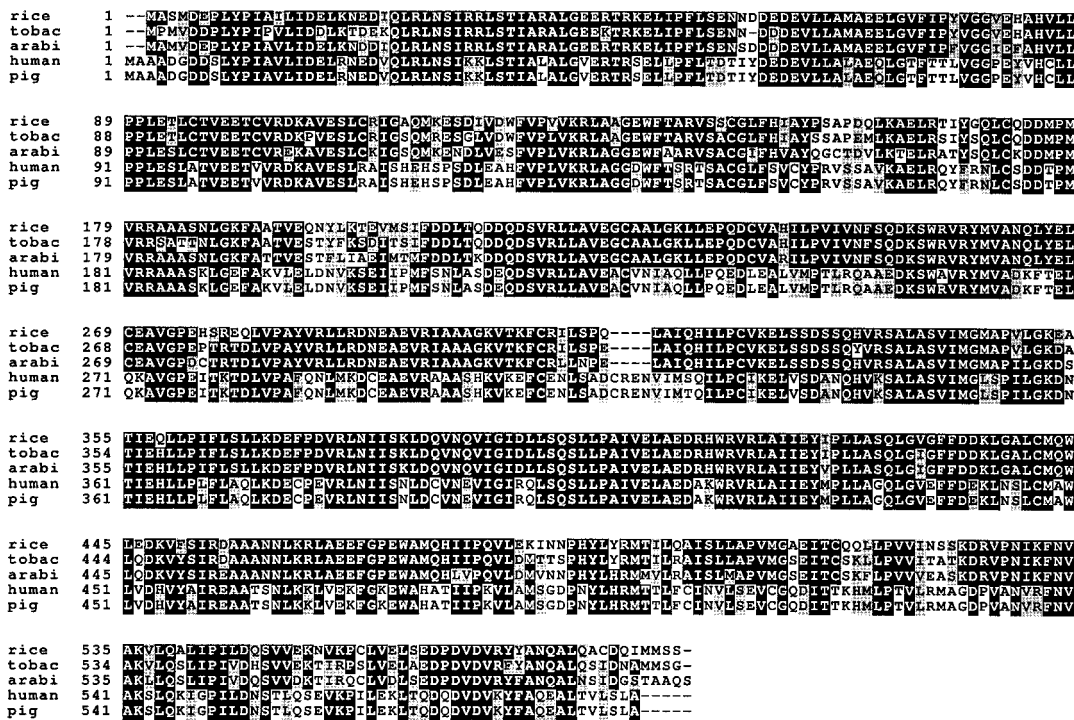


Figure 1. Boxshade presentation of a Clustal X alignment of representative PP2A-A subunits from *Oryza sativa* (this paper), *Arabidopsis* (accession number S51808), *Nicotiana tabacum* (X97913), *Homo sapiens* (P30153), and *Sus scrofa* (P54612). Positions of highest similarity are shaded in black.

et al., 1987). Recombinants were transformed into Y1459 *TPD3* (this yeast strain is *ura*⁻, so the transformants can be selected on a SD/*ura*⁻ plate) and streaked on the SD/*ura*⁻ plate, with the empty plasmid as control. Transformants were again streaked onto YPD (10 g/l yeast extract, 20 g/l peptone, 2% glucose) and YPG (10 g/l yeast extract, 20 g/l peptone, 2% glycerol) respectively. One set of YPD plates was incubated at 37 °C, the other one as well as YPG plates at 30 °C for 4 days.

Results

Isolation and characterization of the rice cDNA encoding the PP2A-A protein

A rice cDNA library was screened with a PCR-derived DNA fragment (based on the sequence of a rice EST, accession number D21972) for the full-length cDNA for PP2A-A. Two positive clones were obtained. The cDNA thus identified was named RPA1. Sequence analysis indicated that RPA1 was 2225 bp long with a unique polyadenylation site and poly(A)⁺ tail of 40 nucleotides. It encoded a 587 amino acid protein.

There was no 9 bp consensus sequence surrounding the ATG initiation codon as found in many other plants (Lütcke *et al.*, 1987). The 3'-untranslated region of RPA1 was 295 bp long. The sequence was deposited in EMBL (accession number AJ243828).

Isolation and analysis of genomic clones containing PP2A-A

The full-length RPA1 cDNA was hybridized to a rice BAC library to generate twelve BAC-positives, seven of which were found to be well filled into BAC contig 354 (Hong *et al.*, 1997). They were digested with *Bam*HI and *Eco*RV, and the digest patterns hybridized with the rice cDNA. One positive band (ca. 16 kb) was found in the *Bam*HI digest and two positives (ca. 3.6 kb and 16 kb) for *Eco*RV. The rice genomic DNA was also digested with both *Bam*HI and *Eco*RV, and the digest patterns produced were the same as those obtained with BAC-positives.

The positive fragment from the BAC *Bam*HI digestion was subcloned into the sequencing vector pBluescript SK⁺. The region starting from 571 bp upstream of the ATG codon down to the site 602 bp

	10	20	30	40	
					MASMDEP 7
1.	LYPIAILIDELKNEDIQLRLNSIRRLSTIARALGEEERTRKE				48
2.	LIPFLSENNDDDEDE.VLLAMAEELGVFIPYVGGVEHAHV				86
3.	LLPPELTCTVEETCVRDKAVESLCRIGAQMKESDIVDW				125
4.	FVVPVKRLAAGEWFTARVSSCGLFHIAYPSPADQLKAE				163
5.	LRTIYGQLCQDDMPMVRRAAASNLGKFAATVEQNYLKTE				202
6.	VMSIFDDLTQDDQDSVRLLAVEGCAALGKLLPEQDCVAH				241
7.	ILPVIIVNFSQDKSWRVRYMVAQLYELCEAVGPEHSREQ				280
8.	LVPAYVRLLRDNEAEVRIAAAGKVKFCRILSPQLAIQH				319
9.	ILPCVKELSSDSSQHVRSALASVIMGMAPVLGKEATIEQ				358
10.	LLPIFLSLKDEFDPVRLNII SKLDQVNQVIGIDLLSQS				397
11.	LLPAIVELAEDRHWRVRLAIEYIPLLASQLGVGFDDK				436
12.	LGALCMQWLEDKVFSTRDAANNLKRLEAEFGPEWAMQH				475
13.	IIPQVLEKINNPHYLYRMTLLQAI SLLAPVMGAEITCQQ				514
14.	LLPVVINSKDRVPNIKFNVAKVLQALIPILDQSVVEKN				553
15.	VKPCLVLELSEDPDQVRYRANQALQACDQIMMSS				587

Figure 2. Internal homology of the rice PP2A A subunit (PP2A-A). The amino acid sequence of RPA1 was manually aligned to show 15 imperfect repeat units, mainly of 39 amino acid residues in length. Conserved residues (bold) delineate strong internal homology with human α and β isotypes and *Arabidopsis* PP2A-A (Hemmings *et al.*, 1990; Slabas *et al.*, 1994). There is a one amino acid gap in unit 2 to allow for better alignment. The numbers at the left side indicate the repeat unit and those on the right side indicate the position of the last amino acid of each repeat in the protein.

downstream of the terminator codon was sequenced using the primer walking approach. Comparison between the genomic sequence obtained and the cDNA showed that the PP2A-A gene contains eleven introns. No TATA box and CAAT box were found in the region upstream of the initiation codon. A simple single polyadenylation signal (AATAAA) was located in the 3'-untranslated region at 201 bp downstream of the terminator codon. The sequence was deposited in EMBL (accession number AJ243829).

Characteristics of the deduced rice PP2A-A protein

The RPA1 coding sequence showed a high degree of conservation with those of regulatory subunits of the PP2A of other organisms. As an example, one gene each from *Arabidopsis*, *Nicotiana tabacum*, *Homo sapiens* and *Sus scrofa* are compared; the Boxshade diagram (Figure 1) makes the high similarity apparent. The rice protein has 88.6% identity with *Arabidopsis*, 88.0% with *N. tabacum*, 59.6% with man, and 59.7% with pig.

As with the PP2A A subunit of other organisms, the amino acid sequence encoded by RPA1 was composed of 15 imperfect leucine-rich repeating structures (Hemmings *et al.*, 1990; Slabas *et al.*, 1994). In the alignment shown in Figure 2, the consensus amino acids are highlighted. With the exception of the first repeat (composed of 41 amino acids) and the fourth

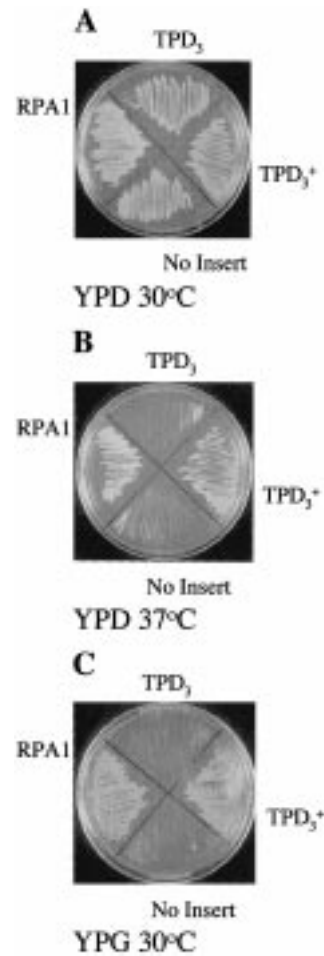


Figure 3. Complementation of the *S. cerevisiae tpd3* mutant strain with rice and yeast PP2A-A genes. The strain and transformants were grown under three conditions. A. YPD medium at 30 °C. B. YPD medium at 37 °C. C. YPG medium at 30 °C. TPD3, yeast mutant gene; TPD3⁺, yeast wild-type gene; RPA1, rice PP2A-A gene; no insert, empty pVT102 plasmid.

repeat (consisting of 38 amino acids), the repeat length is 39 amino acid residues. A gap was observed in the second repeat. The most highly conserved residues are Pro at position 3, Asp at position 11, Val at position 16, and Arg at position 17.

Oryza sativa PP2A-A is functional in *Saccharomyces cerevisiae*

Although the sequence features made it obvious that the rice gene identified encoded the PP2A A subunit, we wished to demonstrate its function *in vivo*. As the *S. cerevisiae* TPD3 gene encodes the PP2A A subunit, and *tpd3* mutants were cold- and heat-sensitive (Van Zyl *et al.*, 1992), we tested the ability of the RPA1

to complement the yeast *tpd3* mutation. All strains grew on YPD plates at 30 °C, while the *tpd3* strain did not grow on YPG plates at 30 °C and YPD at 37 °C. However, transformation of this strain with the yeast wild-type TPD3 gene or the rice RPA1 clone led to growth under the conditions described above. The control strains transformed with empty plasmid vectors behaved like the mutant strain (Figure 3).

Discussion

RPA1, a full-length cDNA isolated from an *Oryza sativa* library, was shown to encode the A regulatory subunit of protein phosphatase 2A. To our knowledge, the gene identified here is the first of its kind characterized in rice. High conservation was observed at the amino acid sequence level between the rice gene and those from animals and plants. PP2A is known to be a key enzyme in living cells. As reported, the mutant of PP2A-A in *Arabidopsis* could result in altered responses to naphthylphthalamic acid (NPA) in root curling, hypocotyl elongation and auxin efflux (Garbers *et al.*, 1996). PP2A has been shown to be involved in signal transduction (Schönthal, 1998; Millward *et al.*, 1999). There are indications that PP2A controls potassium channel activity in guard cells (Li *et al.*, 1994; Thiel and Blatt, 1994) and regulates maturation-promoting factor (MPF) activity (Minshull *et al.*, 1996). *In vitro*, it regulates the activities of metabolic enzymes including phosphoenolpyruvate carboxylase (Carte *et al.*, 1990) and nitrate reductase (Mackintosh, 1992). In rice, okadaic acid-dependent α Amy3 induction is regulated transcriptionally by a signal transduction pathway involving protein phosphatases 2A (Luan *et al.*, 1993) and the transcription of rice chitinase, Rcht2, is also regulated by PP2A (Kim *et al.*, 1998).

In *Arabidopsis* five isoforms of the catalytic subunit of PP2A have been identified (Casamayor *et al.*, 1994; Corum *et al.*, 1996; Perez-Callejon *et al.*, 1998) and the A subunit is likely to be encoded by three genes (Slabas *et al.*, 1994; Rundle *et al.*, 1995). Interestingly, our Southern blotting data showed that rice contains only a single copy for the gene encoding PP2A-A, which was mapped either on chromosome 3 or 7, based on the assignment of contig 354, where the seven positive BACs lodged (Hong *et al.*, 1997).

The A regulatory subunit of PP2A has a proposed rod-like structure consisting of 15 leucine-rich imperfect repeating units of about 39 amino acid residues each (Hemmings *et al.* 1990). The rice gene identi-

fied here was found to share the same structure. Based on a hypothesis that the 15 non-identical repeats are important in the maintenance of the rod-like structure of A regulatory subunit, Ruediger *et al.* (1992) developed a model in which the four C-terminal repeats are proposed to be essential for their interaction with C subunit, whereas N-terminal repeats appeared to interact with B-type subunits. Human and porcine cDNA clones encoding A subunit of PP2A were isolated (Hemmings *et al.*, 1990). Their amino acid sequence alignment showed that they all contained 15 imperfect repeating units of 39 amino acids. The repeating structures were also shown for *Arabidopsis* (Slabas *et al.*, 1994). RPA1 not only shares the considerable internal sequence homology, but also had 15 imperfect repeat units of predominantly 39 amino acid residues each. A comparison of the rice repeat unit sequences with the consensus sequence of the human, porcine, *N. tabacum* and *Arabidopsis* PP2A-A showed a good agreement. Of all 15 units, two showed the highest level of conservation. Unit 10 contained 30 identical amino acids (ca. 77%), while unit 11 contained 32 identical ones (ca. 82%). The predicted protein also has a high leucine/isoleucine ratio (over 20% mol/mol) and these hydrophobic residues are thought to be important for association of the A subunit with C subunit.

Complementation of the *tpd3* mutation by RPA1 demonstrated that the rice gene has the same biological function as the yeast PP2A A subunit. This showed that some essential cellular substrates in yeast may be recognized by rice PP2A-A *in vivo*.

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