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Two genes encoding protein phosphatase 2A catalytic subunits are differentially expressed in rice

Richard Man Kit Yu[1], Yan Zhou[1,2], Zeng-Fu Xu[3], Mee-Len Chye[3] and Richard Yuen Chong Kong[1,*]

[1]*Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong Special Administrative Region, People's Republic of China*

[2]*Present address: The State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, People's Republic of China*

[3]*Department of Botany, University of Hong Kong, Pokfulam Road, Hong Hong Special Administrative Region, People's Republic of China*

**(author for correspondence; e-mail: bhrkong@cityu.edu.hk)*

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Abstract

Type 2A serine/threonine protein phosphatase (PP2A) plays a variety of regulatory roles in metabolism and signal transduction. Two closely related PP2A catalytic subunit (PP2Ac) genes, *OsPP2A-1* and *OsPP2A-3*, have been isolated from the monocot *Oryza sativa*. Both genes contain six exons and five introns which intervene at identical locations, suggesting they have descended from a recent duplication event. Their encoded proteins share 97% sequence identity and are highly similar (94–96%) to a PP2Ac subfamily (AtPP2A-1, -2 and -5) identified in *Arabidopsis thaliana*. Both *OsPP2A-1* and *OsPP2A-3* are ubiquitously expressed, with the expression levels high in stems and flowers and low in leaves. *OsPP2A-1*, but not *OsPP2A-3*, is also highly expressed in roots. Transcript levels of *OsPP2A-1* in roots and *OsPP2A-3* in stems are elevated at the maturation and young stages, respectively. Drought and high salinity upregulate both genes in leaves, whereas heat stress represses *OsPP2A-1* in stems and induces *OsPP2A-3* in all organs. These findings indicate that the two PP2Ac genes are subjected to developmental and stress-related regulation. *In situ* hybridization results show that both transcripts exhibit nearly identical cellular distribution, except in leaves, and are abundant in meristematic tissues including the young leaf blade of stems and the root tip.

Abbreviations: cDNA, complementary DNA; ORF, open reading frame; PCR, polymerase chain reaction; PP2A, protein phosphatases 2A; RACE, rapid amplification of cDNA ends; rRNA, ribosomal RNA; UT, untranslated; UTR, untranslated region; SDS, sodium dodecyl sulfate.

Introduction

Reversible protein phosphorylation is a major regulatory mechanism in a variety of cellular processes (Cohen, 1989). In contrast to protein kinases, our understanding of the structure, expression and functional roles of the protein phosphatases has been developed only relatively recently. Protein phosphatases (PPs) are structurally and functionally diverse enzymes that are divided into two major types: serine/threonine PPs and protein tyrosine phosphatases (PTPs). Interestingly, at least in eukaryotes, protein phosphorylation occurs predominantly (97%) at serine and threonine residues (Shenolikar and Nairn, 1991). Based on differences in biochemical and structural characteristics,

The genomic sequences for OsPP2A-1 and OsPP2A-3 have been deposited in GenBank under the accession numbers AF097182 and AF159061, respectively.

the protein Ser/Thr phosphatases are further divided into PP1, PP2A, PP2B, and PP2C. PP2A is recognized as a highly regulated family of Ser/Thr phosphatases, playing important roles in cellular growth and signalling (reviewed by Janssens and Goris, 2001). Native PP2A enzymes are heterotrimers, consisting of a catalytic C subunit, a structural A subunit and a regulatory B subunit (Cohen, 1989; Shenolikar and Nairn, 1991).

The catalytic subunit of PP2A (PP2Ac) has been identified in a variety of eukaryotes by molecular cloning. Two PP2Ac isoforms have also been identified from Mammalia (Ariño *et al.*, 1988), *Xenopus* (van Hoof *et al.*, 1995) and, both budding (Sneddon *et al.*, 1990) and fission (Kinoshita *et al.*, 1990) yeasts. In contrast, only one form of PP2Ac has been reported in either *Drosophila* (Orgad *et al.*, 1990), *Trypanosoma* (Erondu and Donelson, 1991) or *Dictyostelium* (Murphy *et al.*, 1999). Interestingly, a PP2Ac family complex has been described in *Arabidopsis thaliana* where five isoforms can be grouped into two subfamilies based on amino acid sequence identity (Ariño *et al.*, 1993; Casamayor *et al.*, 1994; Stamey and Rundle, 1995).

The plant PP2Ac genes appear to be expressed ubiquitously in various organs, albeit at varying levels. In alfalfa, the *pp2aMs* transcript is present in leaves, stems, roots and bud flowers, but the maximal mRNA level is found in stems (Pirck *et al.*, 1993). In *A. thaliana*, *AtPP2A-1*, *AtPP2A-2*, *AtPP2A-3* and *AtPP2A-4* are expressed in leaves, stems, roots and flowers, and comparatively high expression is found in roots (Pérez-Callejón *et al.*, 1993; Casamayor *et al.*, 1994). *Nicotiana tabacum NPP4* transcript is found in all tissues while, in contrast, significant *NPP5* expression is restricted to only leaves and flowers (Suh *et al.*, 1998). Previously, Chang *et al.* (1999) demonstrated that *OsPP2Ac* is expressed in the shoots and roots of *Oryza sativa* seedlings. The multiplicity of PP2Ac isoforms, in combination with different regulatory subunits, could generate a large array of PP2A heterotrimers. In a given plant cell, the relative abundance of each PP2A subunit might lead to the prevalence of a defined subset of PP2A holoenzymes by affecting their subunit composition. The specificity, activity and subcellular localization of these PP2A holoenzymes will in turn affect the cell functions.

While much is known about the distribution and diverse roles of PP2A in animals, similar information about the functions of PP2A in plants remains limited. PP2A has been shown to dephosphorylate sucrose-phosphate synthase (Siegl *et al.*, 1990) and nitrate reductase (MacKintosh, 1992) in spinach, phosphoenolpyruvate carboxylase in maize (McNaughton *et al.*, 1991) and quinate dehydrogenase in carrot (MacKintosh *et al.*, 1991). PP2A has also been implicated in the regulation of auxin transport in *Arabidopsis* (Garbers *et al.*, 1996) and seed germination in rice (Chang *et al.*, 1999). To elucidate the physiological functions of PP2A in plants, we have isolated and characterized two closely related PP2Ac genes (*OsPP2A-1* and *OsPP2A-3*) from *O. sativa* L. They share an identical genomic organization (6 exons and 5 introns) and are distinct from those reported in *Arabidopsis* (11 exons and 10 introns) (Pérez-Callejón *et al.*, 1998). Northern blot analysis demonstrated differential expression of these rice genes during development and in response to stress. To examine their cellular localization, *in situ* hybridization was performed on tissue sections of developing seedlings, and we found that both genes exhibit a largely overlapping expression pattern.

Materials and methods

Plant material and growth conditions

Rice (*Oryza sativa* L. indica var. IR36) seeds (kind gift from Dr. G. S. Khush, IRRI, Philippines) were surface-sterilized with 70% ethanol and 2% sodium hypochlorite, and then germinated on 0.5× Murashige-Skoog (MS) salts medium (Invitrogen) at 30 °C for 2 days in total darkness. Germinated seeds were grown on a white gauze net (1-mm meshes) on 1× MS salts under continuous light (white fluorescent light, photon flux of 35 μmol m^{-2} s^{-1}) for 7 days in a controlled growth chamber (25 °C and 75% RH). After 7 days, seedlings were subjected to various treatments including desiccation (on paper towels), heat-shock (42 °C) and salt stress (0–300 mM NaCl). Leaves, stems and roots were dissected and snap frozen in liquid nitrogen at −80 °C until used. For longer growth periods, rice plants were grown in soil and maintained in a greenhouse under natural illumination. Plants were harvested at biweekly intervals and different tissues were dissected and processed as described above.

Screening of cDNA and genomic libraries

An *Oryza sativa* (L. indica, var. IR36) cDNA library constructed in λgt10 (Clontech) was screened

with a 1.3-kb barley PP2Ac cDNA labelled with [$\alpha-^{32}$P]-dCTP by random priming. Positive phages were recovered, purified and sequenced. A rice PP2Ac cDNA (PPcDNA-1) was selected and used to screen an *O. sativa* (L. indica var. IR36) genomic library constructed in EMBL3 by *Sau*3A I partial digestion (Clontech). Approximately 10^5 plaques were screened and, after restriction and hybridization analysis of the DNA from positive phages, two genomic clones were selected for further analysis.

DNA sequencing and analysis

Nucleotide sequences were determined by an automated DNA Sequencer (ABI 377, Applied Biosystems) using the dRhodamine terminator cycle sequencing kit. DNA sequencing was carried out with vector-specific or sequence-specific oligonucleotides (Invitrogen). Routine DNA sequence analyses and homology searches were performed using the Wisconsin Package Version 10.0 (GCG) and BLAST (NCBI, USA) programs, respectively. Consensus transcription factor-binding sites and oligonucleotide repeats were analysed using the PLACE (Higo *et al.*, 1999) and OligoRepFinder (Institute of Cytology and Genetics, Novosibirsk, Russia) programs, respectively.

RNA isolation and northern analysis

Total RNA was isolated using TRIZOL Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (15 μg) was electrophoresed on 1.2% agarose/formaldehyde gels and blotted onto nylon membranes (Hybond-XL, Amersham Biosciences). DNA probes were radiolabelled by the random priming method and 2.0×10^6 cpm/ml were used in northern hybridizations, which were carried out at 60 °C for 2 h in ExpressHyb solution (Clontech). Blots were washed thrice with 2× SSC, 0.05% SDS for 10 min at room temperature, and twice with 0.1× SSC, 0.1% SDS for 20 min at 50 °C. Blots were exposed on a phosphor screen (Kodak-K) for 2 days at room temperature, and the signals were captured using the Molecular Imager FX System (Bio-Rad).

Genomic DNA isolation and Southern hybridization

Genomic DNA was prepared from 2-week old leaves using the Plant DNeasy Mini Kit (Qiagen). Total DNA was digested with the appropriate restriction enzymes, separated on 0.8% agarose gels and blotted onto nylon membranes (Hybond N+, Amersham Biosciences). Preparation of radiolabelled DNA probes, Southern hybridization (60 °C), membrane washing and autoradiography were performed as described above for the northern analysis.

Random amplification of cDNA ends (RACE) by PCR

The start and termination sites of gene transcripts were determined by 5′-RACE (Marathon cDNA Amplification kit; Clontech) and 3′-RACE PCR (3′-RACE System; Invitrogen), respectively, according to the instructions provided with the kits. Poly(A)$^+$ RNA (1 μg) from 1-week old leaf fragments was used as a source of template. Gene-specific nested primers for 5′-RACE were: primer C, 5′-GTTCTTGTCCTGGCACCAATTGAAG-3′ and primer E, 5′-ATCCAATGACGGCGAGAGACCG-3′ for *OsPP2A-1*; and primer D, 5′-GATAACAGTAGTT TGGTGCACTGAAG-3′ and primer F, 5′-TGTATAA CCTGCTCCTCTCGGT-3′ for *OsPP2A-3*. Gene-specific nested primers for 3′-RACE were: primer G, 5′-TTCCTCTCTGCTGCGTCTGGT-3′ and primer I, 5′-ATGTAGATCTTCGTCCTTAGAA-3′ for *OsPP2A-1*; and primer H, 5′-GTAGATCTTCTGTCCTTAGAT AC-3′ and primer J, 5′-TTCCACGAGCCCGGCTGT ATG-3′ for *OsPP2A-3*. Thirty-five cycles of PCR were performed according to the manufacturer's recommendations. The amplification products were cloned into pUC18 using the SureClone Ligation kit (Amersham Biosciences) for DNA sequencing.

Primer extension analysis

Primer extension was carried out as described by Sambrook *et al.* (1989) with slight modifications. Two gene-specific primers of OsPP2A-1 and *OsPP2A-3* were used. Primer A, 5′-CCTCCCCACCTCCCCCTCCTCCTCCTCCCTCCT CA-3′ for *OsP2A-1* and primer B, 5′-CGCCGCTCGC CGCCGGCGAGGAGGGTGGGGGAGAG-3′ for *OsPP2A-3*, were 5′ end-labelled with [$\gamma-^{32}$P]ATP using T4 polynucleotide kinase. Total RNA from 1-week old leaves (10 μg) was hybridized overnight at 30 °C with $\sim 5 \times 10^4$ cpm ^{32}P-labelled oligonucleotides. The hybridized probes were extended by Superscript II reverse transcriptase (Invitrogen). Primer extension products were analysed on a 6% acrylamide/7M urea sequencing gel together with a sequencing ladder produced from an appropriate genomic clone.

In situ *hybridization*

Plant organs were fixed in 3.7% formaldehyde, 5% acetic acid, 50% ethanol, dehydrated through an ethanol series and embedded in Paraplast Plus (Oxford, St. MO, USA) according to the method of Cox and Goldberg (1988). Longitudinal and transverse sections of 8 μm were cut and mounted on poly-L-lysine-coated slides (Electron Microscopy Sciences, USA). The 3′-UT sequence (100 bp) of *OsPP2A-1* and *OsPP2A-3* was cloned into pBluescript KS+ (Stratagene) and digoxigenin (DIG)-labelled sense and antisense RNA probes were synthesized *in vitro* using T3 or T7 RNA polymerase (Roche), respectively. Hybridizations were performed overnight at 45 °C with DIG-labelled riboprobes (1.5 ng/μl) in 10 mM Tris (pH 7.5), 1 mM EDTA, 0.3 M NaCl, 50% deionised formamide, 1× Denhardt's solution, 10% dextran sulfate, 500 μg/ml herring sperm DNA and 250 μg/ml yeast tRNA. After hybridization, slides were washed twice at room temperature with 2× SSC for 5 min and treated with RNaseA (10 μg/ml in 10 mM Tris, pH 7.5, 1 mM EDTA, 500 mM NaCl) for 30 min at 37 °C, followed by two 10-min rinses in the same buffer. Slides were washed twice with 2× SSC for 30 min at room temperature and twice with 0.1× SSC for 30 min at 50 °C. Hybridization signals were detected using an alkaline phosphatase-linked immunoassay (DIG Nucleic Acid Detection Kit, Roche) in accordance with the manufacturer's instructions. Sections were examined with an Olympus BH-2 light microscope and photographs were taken with Kodak Gold 100 film using an Olympus C-35AD-4 camera.

Results

Isolation and characterization of OsPP2A-1 *and* OsPP2A-3

A 1.3-kb cDNA fragment encoding a barley PP2Ac protein (Dr P. L. Xu, unpublished) was used to screen a rice cDNA library (Clontech). A rice cDNA clone (PPcDNA-1) was obtained and DNA sequencing confirmed that it contains an incomplete PP2Ac open reading frame (ORF) of 807 bp that lacks the start codon. PPcDNA-1 was used to screen a λEMBL-3 rice genomic library from which seven phage clones were obtained. Restriction enzyme digestion and Southern blot analysis showed that the clones corresponded to two groups of PP2Ac-related genes, each represented by λg7-1 and λgS-5, repectively. A 7.8-kb *Bam*HI fragment of λg7-1 and a 6.6-kb *Hin*dIII fragment of λgS-5, which hybridized strongly to PPcDNA-1, were cloned into the pBluescript II vector to yield plasmids p7-1B and pS-5H, respectively (Figure 1).

Sequence analysis indicated that p7-1B and pS-5H contain two distinct rice PP2Ac genes, *OsPP2A-1* and *OsPP2A-3*, spanning 6.1 kb and 4.9 kb, respectively. The 5′-untranslated (UT), ORF and 3′-UT sequences of *OsPP2A-1* are almost identical to the *OsPP2Ac* cDNA reported by Chang *et al.* (1999). In the 5′-UT region, a sequence identity of 94% over a stretch of 104 bp was observed. In the coding sequence, a 98% identity with several base differences resulting in six amino acid substitutions in the deduced protein was found. Also, a comparison of the 291-bp 3′-UT sequence of *OsPP2A-1* with the 620-bp 3′-UT sequence of *OsPP2Ac* indicated a 98% identity. Curiously, the additional 329-bp sequence at the 3′-end of *OsPP2Ac* was not found in the 3′-flanking sequence of *OsPP2A-1*. To verify the authenticity of this additional 3′-UT stretch, we performed genomic- and RT-PCR amplifications using a pair of specific primers that were targeted at this region; however, no detectable PCR product was obtained. Since the studies by Chang *et al.* (1999) and our group were performed on the same rice variety, the nucleotide and size differences noted above are most likely due to cloning artefacts.

Both *OsPP2A-1* and *OsPP2A-3* share an identical genomic organization, each consisting of six exons and five introns. Homologous exons of the two PP2Ac genes are largely identical in size and show high sequence conservation both at the nucleotide (80–89%) and amino acid (92–100%) levels (Table 1). DNA sequence conservation was also observed in the 5′- (58%) and 3′- (63%) UT regions. The exon/intron boundaries were identified by comparing the genomic sequences of *OsPP2A-1* and *OsPP2A-3* with the corresponding full-length cDNAs (derived by reverse-transcription PCR using gene-specific primers) and conform to the invariant gt/ag sequences at the 5′- and 3′-splice sites, respectively (Table 1). Unlike the coding regions (52%), intronic sequences of the rice PP2Ac genes are AT-rich (56–69%), which is typical of dicot genes (Goodall and Filipowicz, 1989). Interestingly, such sequences have been demonstrated to promote efficient splicing of certain monocot introns (Luehrsen and Walbot, 1994). The ORFs of *OsPP2A-1* (918 nt) and *OsPP2A-3* (921 nt) encode polypeptides of 306 and 307 amino acids, respectively, with predicted molecular masses of ca. 35 kDa. The predicted

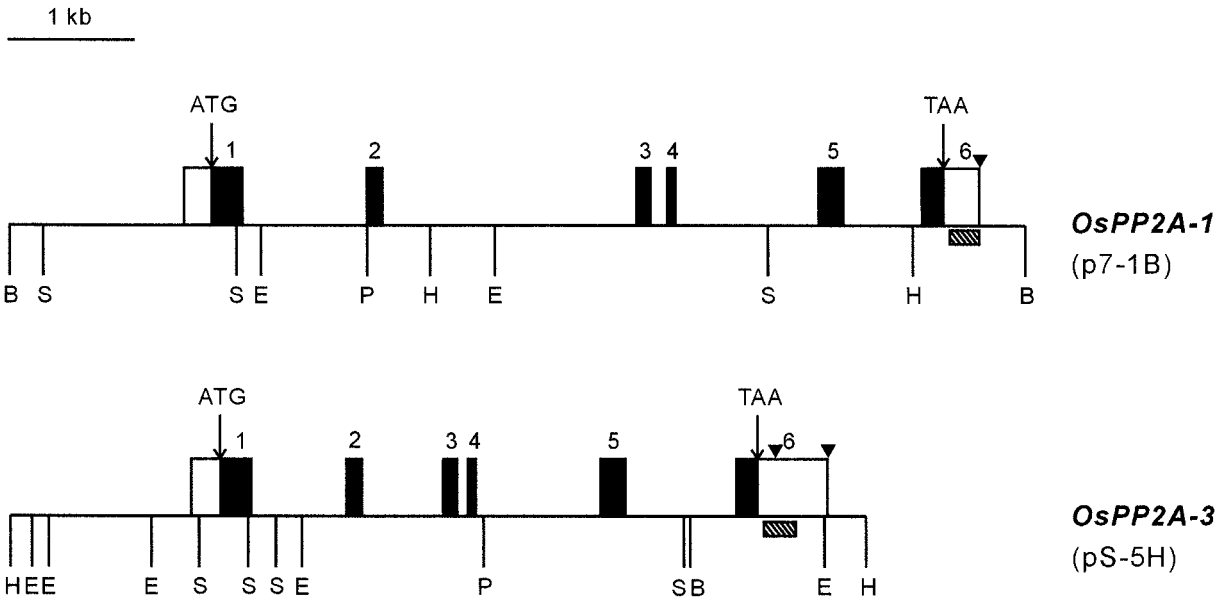

Figure 1. Genomic structure of *OsPP2A-1* and *OsPP2A-3*. The six exons of each gene are shown as boxes above the sequence. The position of the start (ATG) and stop (TAA) codons are indicated by arrows. Filled and open boxes indicate translated and untranslated regions, respectively. The 5′-termini of exons 1 were inferred by primer extension. Inverted triangles (▼) represent the positions of the polyadenylation sites determined by 3′-RACE. Gene-specific probes used for DNA- and RNA-blot hybridization studies are marked by hatched boxes in the 3′-UT regions of the respective genes. The restriction enzymes used to establish the structure of both genes are indicated by: B = *Bam*HI, E = *Eco*RI, H = *Hin*dIII; S = *Sac*I and P = *Pst*I.

pIs for both polypeptides are low: 4.84 for *OsPP2A-1* and 4.91 for *OsPP2A-3*.

Genomic Southern hybridization analysis was performed to estimate the gene copy number of *OsPP2A-1* and *OsPP2A-3*. High molecular weight genomic DNA was digested with *Bam*HI, *Eco*RI, *Hin*dIII and *Sac*I, and hybridized with probes that correspond to the 3′-UT region of each gene. Both gene-specific probes hybridized to only one major genomic fragment in each restriction digest (data not shown), which indicate that *OsPP2A-1* and *OsPP2A-3* are both single-copy genes in the rice genome.

Mapping the 5′- and 3′-ends of OsPP2A-1 *and* OsPP2A-3 *transcripts*

The transcription start sites of *OsPP2A-1* and *OsPP2A-3* were determined using 5′-RACE and primer extension analyses. Single fragments of ca. 700 and 850 bp, respectively, were obtained by 5′-RACE PCR for *OsPP2A-1* and *OsPP2A-3* (data not shown). The two fragments were cloned into the pUC18 vector and five independent clones from each were randomly selected for DNA sequencing. DNA sequencing of the longest 5′-RACE products indicated that the transcription start sites of *OsPP2A-1* and *OsPP2A-3* are located at nucleotide positions −143 and −162, respectively, upstream of the ATG start codon (Figure 2A). The 5′-RACE that we performed in this study could not accurately map the 5′-end of a cDNA because the T4 DNA polymerase that was used for second-strand cDNA synthesis may remove nucleotides from the 5′-end. For this reason, primer extension analysis was performed to more precisely define the transcription start sites of the genes. The primers used were complementary to the 5′-UT sequences that were derived from the above 5′-RACE experiments. Primer extension analysis indicated that the 5′-UT regions of *OsPP2A-1* and *OsPP2A-3* extended 211 and 211/213 bp, respectively, upstream from the ATG start codon (Figure 2A).

The 3′-ends of the PP2Ac genes were determined using 3′-RACE. A single RACE-PCR product of ca. 280 bp was obtained for *OsPP2A-1*, while two major products of ca. 130 and 480 bp were obtained for *OsPP2A-3* (data not shown). DNA sequencing of these products mapped the transcript end of *OsPP2A-1* to nucleotide position +1212, and the 3′-end of *OsPP2A-3* to two sites; one at nucleotide position +1106 and the other at +1452 (Figure 2B). The presence of a poly(A) tail at the 3′-end of both amplified products of *OsPP2A-3* indicated that the two transcripts re-

A.

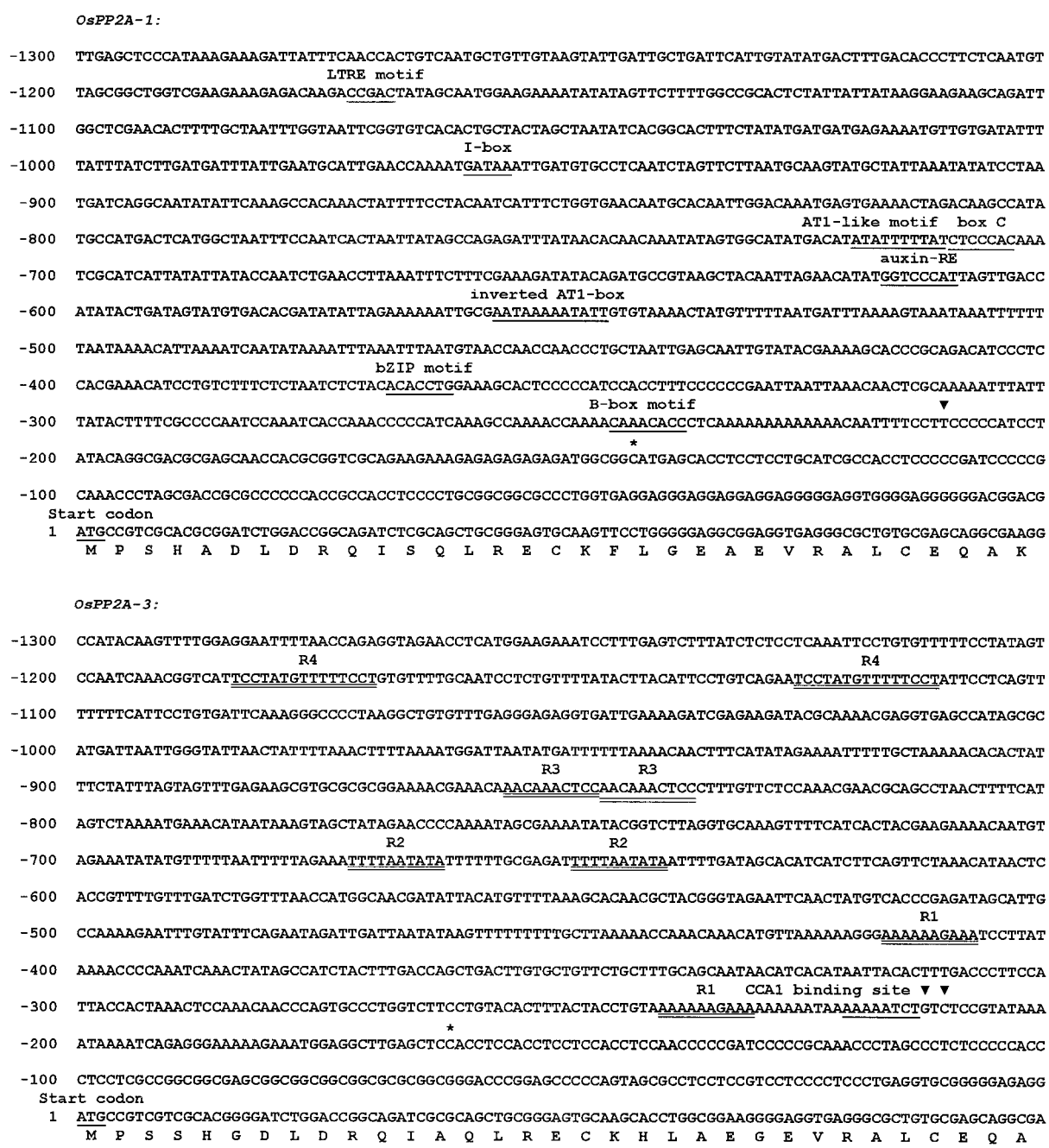

Figure 2. Nucleotide sequences of the 5′- and 3′-flanking regions of *OsPP2A-1* and *OsPP2A-3*. (A) The 5′-flanking sequences of *OsPP2A-1* and *OsPP2A-3* were compared to the nucleotide sequences of the corresponding primer extension and 5′-RACE products to determine the transcription start sites. Inverted triangles (▼) and asterisks (*) indicate transcriptional start sites mapped by primer extension and 5′-RACE, respectively. Position +1 represents the A nucleotide of the start codon, ATG. Negative numbers represent sequences upstream from this position. The putative CANNTG and GATA motifs are shaded. Direct sequence repeats (R1–R4) are double-underlined. The GAGA-repeat in *OsPP2A-1* is indicated in bold. (B) The transcription termination sites in the 3′-flanking sequences of *OsPP2A-1* and *OsPP2A-3* determined by 3′-RACE are indicated by arrowheads (↓). The TAA stop codons are labelled. The putative poly(A) signals are underlined and labelled.

B.

Figure 2. Continued.

sulted from alternative polyadenylation. These analyses resulted in an estimated exon-6 size of 465 bp for *OsPP2A-1* and, 356 and/or 702 bp for *OsPP2A-3* (Table 1). Examination of the 3′-flanking genomic sequences of the two PP2Ac genes revealed that a putative polyadenylation signal (AATAGA) is located 24 bp upstream from the transcript end of *OsPP2A-1*, while a consensus polyadenylation signal (AATAAA) is located 203 bp upstream from the end of the larger *OsPP2A-3* transcript (Figure 2B). Northern analysis using the 3′-UT sequences as probes confirmed that *OsPP2A-1* produces a single transcript of ca. 1.4 kb and *OsPP2A-3* produces two transcripts ca. 1.3 and 1.65 kb in size.

Promoter structure of OsPP2A-1 *and* OsPP2A-3

A comparison of the 5′-flanking sequences (1000 nucleotides upstream of the major transcription start site) of *OsPP2A-1* and *OsPP2A-3* revealed no significant sequence homology. Both promoters lack putative TATA and CAAT boxes and are notably AT-rich (67%). AT-rich sequence motifs are likely to enhance transcription by binding to high mobility group (HMG) proteins which are involved in the formation of active RNA polymerase II initiation complexes (Grasser, 1995). A stretch of GAGA-repeats responsible for transcriptional activation (Granok *et al.*, 1995) is located near the transcription start site of *OsPP2A-1* (Figure 2A), which has also been reported in the 5′-flanking sequences of *AtPP2A-2*, *AtPP2A-3* and *AtPP2A-4* in *Arabidopsis* (Pérez-Callejón *et al.*, 1998; Thakore *et al.*, 1999). In addition, *OsPP2A-1* and *OsPP2A-3* promoters contain two putative regulatory sequence motifs with similarity to those reported in other plant genes, for example *AtPP2A-2* (Thakore *et al.*, 1999), and include the CANNTG motifs (E-boxes) that bind to basic helix-loop-helix (bHLH) transcription factors (Ludwig and Wessler, 1990), and GATA motifs that are responsible for the light-responsiveness of plant promoters (Gilmartin *et al.*, 1990). Furthermore, several direct sequence repeats ($\geqslant$10 bp) that could serve as transcription factor binding sites are found in the *OsPP2A-3* promoter (Figure 2A).

Similarity of OsPP2A-1 *and* OsPP2A-3 *to other plant PP2Ac homologues*

OsPP2A-1 and *OsPP2A-3* share 96% identity with each other and are most closely related (90–93%) to the AtP2A-1, AtPP2A-2 and AtPP2A-5 isoforms (members of the same PP2Ac subfamily) of *Arabidopsis* (Ariño *et al.*, 1993; Stamey and Rundle, 1995). The major sequence variabilities between the two groups of PP2Ac proteins are largely in the N-terminal region (Figure 3). When the rice PP2Ac isoforms are compared to *AtPP2A-3* and *AtPP2A-4* (members of a second PP2Ac subfamily) of *Arabidopsis*, sequence variabilities are apparent at both the N- and C-terminal

Table 1. Exon/intron organization and percentage of identity between equivalent exons and introns of *OsPP2A-1* and *OsPP2A-3.*

EXON	Size (bp)	GC Content (%)	% Nt Identity	% Aa Identity	†5′ Splice site	INTRON	Size (bp)	GC Content (%)	% Nt Identity	†3′ Splice site
1	464	68	80	90	TC**G**g**t**a**a**g**t**tt	1	960	41	42	tgc**tgcag**ACC
	467	68			TC**G**g**t**c**a**g**t**tc		723	44		ta**ttgcag**ACC
2	115	41	85	97	AGT**gt**g**agt**tt	2	1956	35	40	gga**tacagGT**A
	115	43			AGT**gt**g**agt**tt		621	31		ttc**tacagGT**A
3	109	40	89	97	C**AGgt**t**ag**ctc	3	103	35	51	t**tttgtagGT**C
	109	37			C**AGgt**t**tgt**ta		81	35		t**ttt**a**tagGT**G
4	78	53	82	100	G**AGgta**t**g**caa	4	1092	35	39	gg**tt**a**tagGT**C
	78	45			G**AGgta**t**g**aag		945	35		gc**ttgcagGTT**
5	192	49	84	98	C**AGgt**t**ggt**at	5	603	36	37	tta**tgcagG**AC
	192	44			C**AGgta**g**g**cgt		857	33		tcat**ccagG**AC
6	465	45	*75/72	100						
	*356/702	*45/43								

†Nucleotides identical with the consensus are indicated in boldface. Upper lines correspond to *OsPP2A-1* and lower lines to *OsPP2A-3*.
*Alternatively spliced exon 6 of *OsPP2A-3.*

regions which suggest that these variable domains in PP2Ac may have a role in determining its interaction specificity with other PP2A regulatory subunits.

The aligned proteins show that the typical sequence motifs known to be important for catalytic activity of type 2 Ser/Thr phosphatases (Zhuo *et al.*, 1994) are conserved in the rice PP2Ac isoforms and include: the 'phosphoesterase signature' motif (residues 54, 56, 81, 82, 85, 112–116 (Figure 3); the 'YRCG' motif (residues 264-267), purportedly involved in okadaic acid binding; and the 'DYFL' residues at the C-terminus believed to be important in regulating PP2A activity by covalent modification (Mayer-Jaekel and Hemmings, 1994).

Spatio-temporal expression of the PP2Ac genes

To determine the spatial and temporal expression patterns of *OsPP2A-1* and *OsPP2A-3*, northern blot analysis was performed with equal amounts of total RNA isolated from different plant organs at biweekly time intervals after seed germination. The results show that *OsPP2A-1* and *OsPP2A-3* are ubiquitously expressed in all plant organs, albeit at varying levels (Figure 4). *OsPP2A-1* transcript is expressed at a similar level in stems, roots and flowers, with the lowest expression observed in leaves. *OsPP2A-3* is predominantly expressed in stems and flowers, with minimal expression in leaves and roots. Changes in temporal expression of the two PP2Ac genes are also apparent during the life cycle of the rice plant. Expression of *OsPP2A-1* in roots increases at least two-fold by week 8, and remains high until week 14. *OsPP2A-3* is highly expressed in young stems (up to week 8 but rapidly declines from week 10 onwards), and in flowers especially at the heading stage. These observations indicate that expression of *OsPP2A-1* and *OsPP2A-2* is spatially and temporally regulated.

Gene expression in response to environmental stresses

In order to analyse the effects of various environmental stresses on the expression of *OsPP2A-1* and *OsPP2A-3*, 7-day old rice seedlings were subjected to high salinity, drought and heat stress, and the relative mRNA levels in different organs monitored by northern blot analysis. As shown in Figure 5A, drought stress induces expression of *OsPP2A-1* more strongly than *OsPP2A-3* in leaves, while only a marginal increase in transcript levels is observed in stems. Interestingly, whilst *OsPP2A-1* expression is unaffected in roots, *OsPP2A-3* expression is repressed in response to drought stress. In response to heat-shock, *OsPP2A-3* expression is upregulated in all three organs, while *OsPP2A-1* expression is downregulated in stems (Figure 5B). Salt stress at 300 mM NaCl results in increased expression of *OsPP2A-1* and *OsPP2A-3* in leaves (Figure 5C). Overall, our findings indicate that *OsPP2A-1* and *OsPP2A-3* are differentially expressed (especially in the leaves) in response to different environmental stresses.

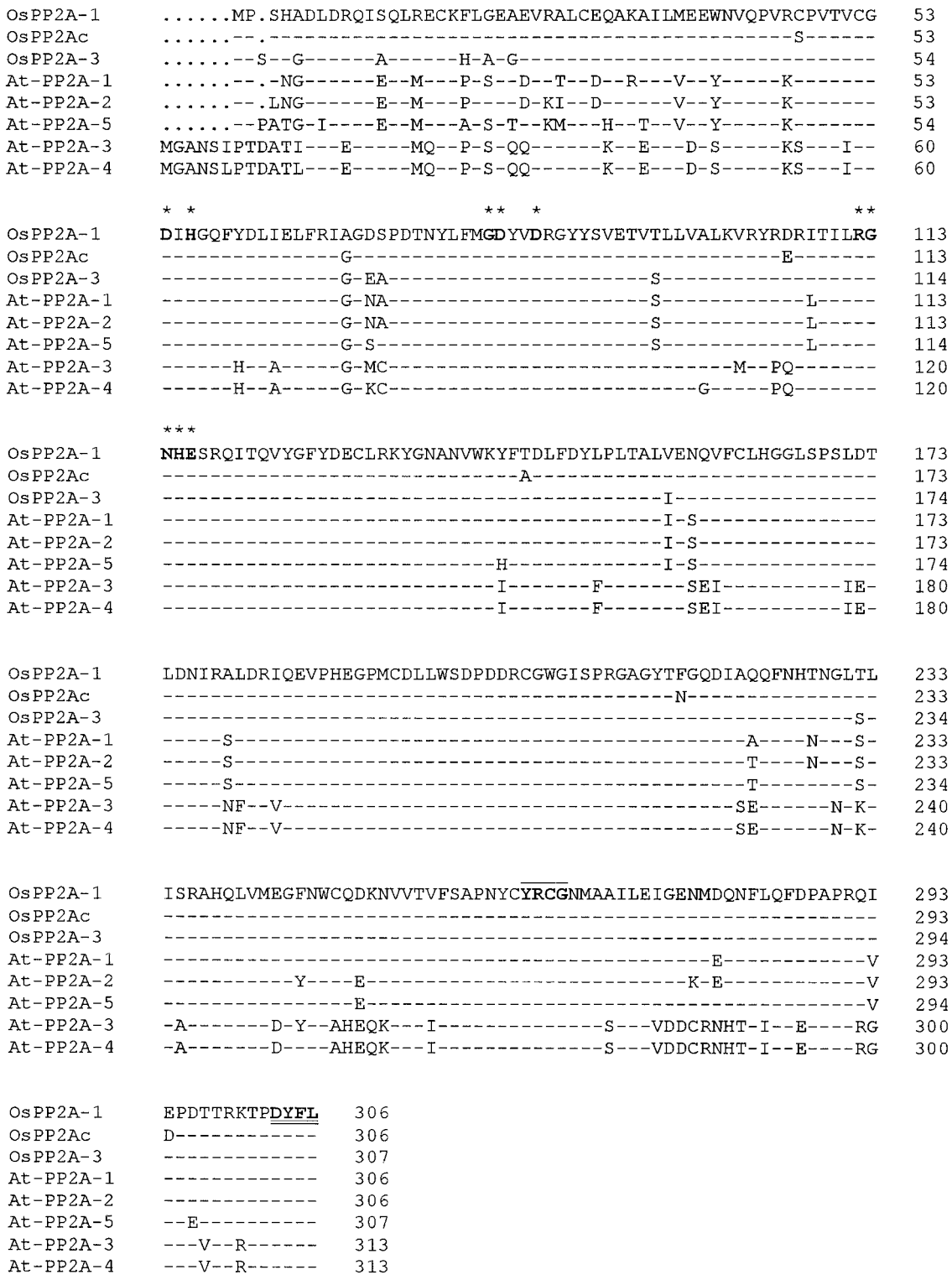

Figure 3. Comparison of the deduced amino acid sequences of *OsPP2A-1* and *OsPP2A-3* with the PP2Ac homologues of *A. thaliana*. From top to bottom: deduced amino acid sequences of rice *OsPP2A-1* and *OsPP2A-3* (AF097182 and AF159061, respectively, this work); *A. thaliana* AtPP2A-1 (M96733), AtPP2A-2 (M96732), AtPP2A-3 (M96841), AtPP2A-4 (U08047) and AtPP2A-5 (U39568). Alignment was created using ClustalW 1.8. Dashes (–) indicate amino acid residues identical to the rice OsPP2A-1 sequence. Dots (..) indicate gaps inserted in the sequences for better alignment. Amino acid numbers are given on the right. Conserved residues of the 'phosphoesterase signature' motif are highlighted in bold and marked with asterisks (*). The 'YRCG' and 'DYFL' residues are in bold type and are overlined and double underlined, respectively.

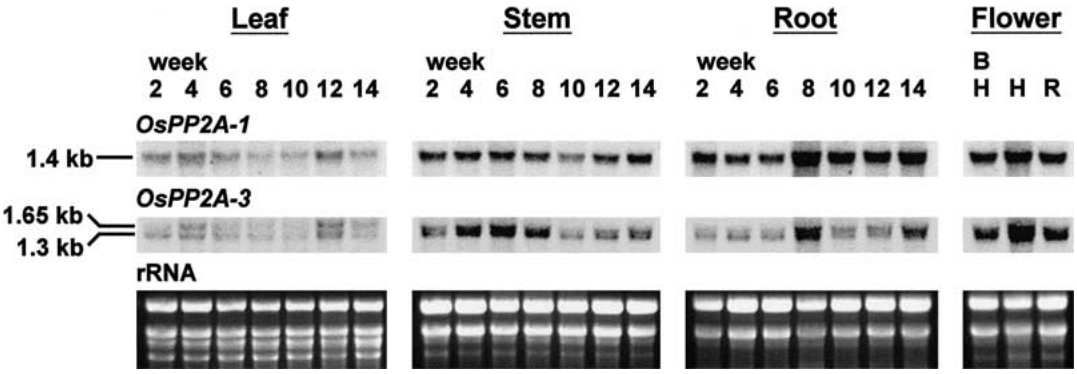

Figure 4. Northern blot analysis of *OsPP2A-1* and *OsPP2A-3* mRNAs in rice plant organs at various stages of development. Total RNAs from leaves, stems and roots at 2, 4, 6, 8, 10, 12 and 14 weeks after seed germination; and flowers (panicles) at three developmental stages: before heading (BH), heading (H) and ripening (R), were used for Northern analysis. Hybridization signals were normalized against rRNAs stained with ethidium bromide.

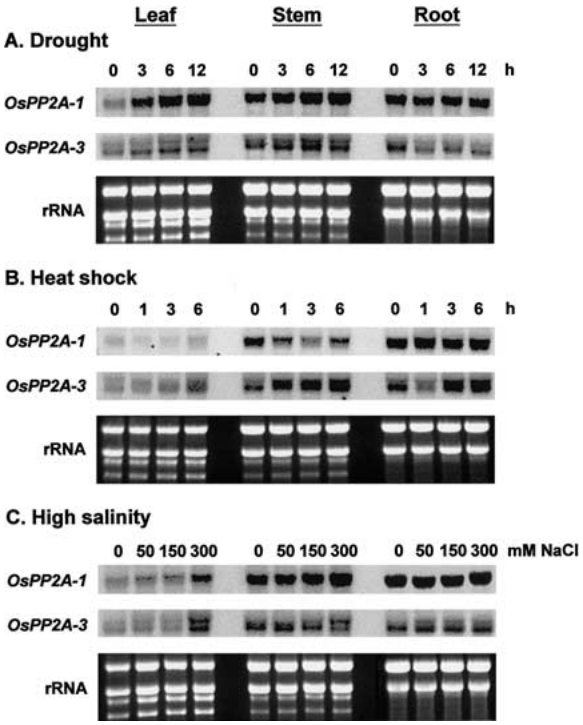

Figure 5. Northern blot analysis of *OsPP2A-1* and *OsPP2A-3* mRNAs under different environmental stresses. Hybridization with RNAs isolated from leaves, stems and roots of 7-day old plantlets in response to: (A) drought stress (0, 3, 6 and 12 h); (B) heat stress at 42 °C (0, 1, 3, and 6 h); and (C) salt stress (8 h in 0, 50, 150 and 300 mM NaCl). Hybridization signals were normalized against rRNAs stained with ethidium bromide.

In situ *localization of PP2Ac mRNAs*

Cellular distribution of *OsPP2A-1* and *OsPP2A-3* mRNAs was examined by *in situ* hybridization on paraffin-embedded sections of different plant organs using DIG-labelled RNA probes. In all tissues examined, with the exception of the leaf, the cellular expression patterns of *OsPP2A-1* and *OsPP2A-3* are identical. Therefore, unless otherwise stated, the *in situ* results described henceforth are representative of both PP2Ac genes.

On transverse section of the third leaf, PP2Ac expression is detected in the mesophyll, bundle sheath cells and cells in the vascular bundle (Figure 6, A1 and B1). In the vasculature, the highest expression is detected in companion cells of the leaf phloem. Interestingly, positive hybridization in the epidermal cell layer is observed only with the *OsPP2A-3* (Figure 6, B1) but not *OsPP2A-1* (Figure 6, A1) antisense probe. In stems, expression is enriched in three specific cell layers: young leaf blade (strongest), inner sheath (moderate) and outer sheath (weakest) (Figure 6, C1). At higher magnification, the *in situ* hybridization results show that PP2Ac expression is most prominent in the mesophyll and phloem companion cells of young leaf blade (Figure 6, D1) and the inner sheath (Figure 6, E1) layers of the stem, while weaker expression is detected in the outer sheath layer in the companion cells of the stem phloem (Figure 6, F1). A transverse section of the root at the elongation zone (Figure 6, G1) shows especially high accumulation of PP2Ac mRNA in a number of cell types in the central stele, in particular cells near the protoxylem and protophloem. On longitudinal section of the root tip, the highest expression is observed in the root apex (Figure 6, H1). In the young panicle of a mature plant, strong hybridization signals are detected in the ovary, style, stigma and filament (Figure 6, I1). Moderate mRNA signals are detected in the lodicule, palea and lemma, however,

no signal is detectable in the anther. From the above observations, only a subtle difference in the cellular expression patterns of *OsPP2A-1* and *OsPP2A-3* is evident.

Discussion

In this study, two PP2Ac isogenes, *OsPP2A-1* and *OsPP2A-3*, have been isolated from *O. sativa*. Several lines of evidence suggest that *OsPP2A-1* and *OsPP2A-3* are derived from a recent duplication of an ancestral gene. First, both genes contain 6 exons and 5 introns. Moreover, homologous exons of *OsPP2A-1* and *OsPP2A-3* are identical in size and share high sequence identity at the nucleotide (> 80%) and amino acid (> 92%) levels. In addition, structures at the exon-intron boundaries of both genes are also highly conserved (Table 1) and, finally, sequence homology was also observed in the 5′- and 3′-UT regions of both genes. This is in agreement with the observations from studies of the corresponding human (Khew-Goodall *et al.*, 1991) and *Arabidopsis* (Pérez-Callejón *et al.*, 1998) PP2Ac genes. Recently, Pérez-Callejón *et al.* (1998) reported on the isolation of two *Arabidopsis* PP2Ac genes, *AtPP2A-3* and *AtPP2A-4*, which consist of 11 exons and 10 introns. We have also isolated and completely sequenced three other PP2Ac isogenes from *O. sativa* (which we have named *OsPP2A-2*, *OsPP2A-4* and *OsPP2A-5*); the genomic structure and nucleotide sequences of which are highly homologous to *AtPP2A-3* and *AtPP2A-4* of *Arabidopsis* (unpublished observations). Taken together, there is strong evidence indicating that in higher plants, there are at least two subfamilies of PP2Ac isogenes with two distinctive genomic structures.

The ubiquitous expression of *OsPP2A-1* and *OsPP2A-3* is consistent with the notion that PP2A plays an essential role in cellular homeostasis. Although a number of PP2Ac cDNAs have been described in several plant species, detailed information on their spatio-temporal expression patterns and/or possible functions is sparse. We have demonstrated that the spatio-temporal expression patterns of the rice PP2Ac genes are differentially regulated in a tissue- and stage-specific manner. For example, O*sPP2A-1* and *OsPP2A-3* mRNAs are abundantly expressed in stems and flowers, but are scarce in leaves; while *OsPP2A-1* is more highly expressed than *OsPP2A-3* in roots at all of the developmental stages examined (Figure 4). Most notably, elevated expression of *OsPP2A-1* in roots and *OsPP2A-3* in stems is most prominent at the maturation and juvenile stages, respectively. In *Arabidopsis*, expression of *AtPP2A-3* (Pérez-Callejón *et al.*, 1993) and *AtPP2A-1* (Ariño *et al.*, 1993) is elevated at the seedling and adult stages, respectively, during plant growth. These results suggest that PP2Ac genes probably play very specialized roles in different organs during plant development.

Thus far, very little is known about the roles of protein phosphatases in environmental signaling in plants. The rice cultivar IR36 is highly tolerant to adverse environmental conditions (Khush, 1987) and in this study, we have demonstrated that *OsPP2A-1* and *OsPP2A-3* expression are upregulated in the aerial organs in response to drought and high salinity stress (Figure 5). This suggests that PP2A may be involved in adaptive responses to counteract osmotic perturbations and ion toxicity in the rice plant. Interestingly, only *OsPP2A-3* expression is upregulated by heat stress in leaves, stems and roots of the rice plant. In *Arabidopsis*, it has been demonstrated that the PP2A B′ regulatory subunit gene, $AtB'\gamma$, is upregulated by heat stress (Latorre *et al.*, 1997), but whether or not any of the *Arabidopsis* PP2Ac genes are also responsive to heat stress is not known.

In situ hybridization of tissue sections from different organs of a 7-day old rice plantlet demonstrated that *OsPP2A-1* and *OsPP2A-3* are expressed in a variety of cell types. In nearly all tissues examined, identical expression patterns were observed. In leaves and stems, prominent expression was detected in the companion cells of the phloem suggesting that PP2A may play a role in regulating the translocation of macromolecules in the sieve tube. In stems, PP2Ac mRNA is localized mainly in the young leaf blade where active cell division and differentiation occurs; and in the root tip, PP2Ac mRNA is predominantly expressed in the apical meristem. In *Arabidopsis*, elevated expression of *AtPP2A-2* in the root tip of young seedlings was also observed using the GUS assay (Thakore *et al.*, 1999), suggesting that PP2Ac expression is also elevated in regions of active growth. Taken together, there is strong evidence that PP2A is a key regulator of mitosis (Janssens and Goris, 2001). In the panicle (heading stage) of 14-week old rice plants, high PP2Ac expression is detected in the ovary and filaments (Figure 6, D1). Curiously, in *Arabidopsis*, *AtPP2A-2* promoter-directed GUS activity was detected only in anthers and in the pollen of mature flowers (Thakore *et al.*, 1999).

Both PP2Ac genes are widely expressed in a variety of rice tissues, and are regulated in a seem-

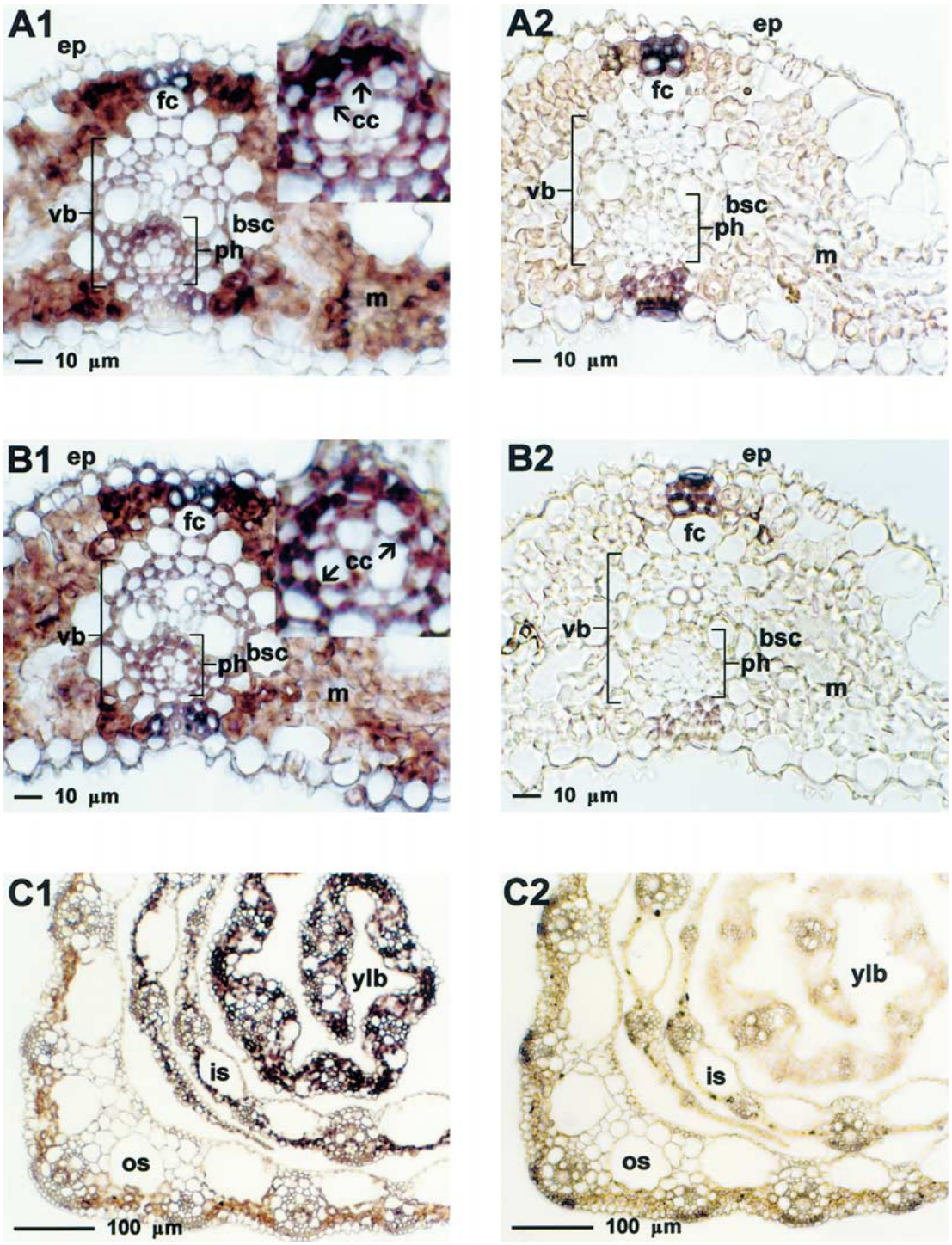

Figure 6a–c. In situ localization of PP2Ac mRNAs. Tissue sections of 7-day-old plantlets were hybridized with *OsPP2A-1* (A1, C1–I1) or *OsPP2A-3* (B1) antisense riboprobes. Control experiments were performed using *OsPP2A-1* (A2, C2–I2) or *OsPP2A-3* (B2) sense riboprobes. Positive hybridization signals appear purple in color. A1, A2, B1 and B2, transverse section (TS) of third leaf (scale bar = 10 μm); C1 and C2, TS of stem (mesocotyl) (scale bar = 100 μm); D1 and D2, TS of young leaf blade of stem (scale bar = 10 μm); E1 and E2, TS of inner sheath of stem (scale bar = 10 μm); F1 and F2, TS of outer sheath of stem (scale bar = 10 μm); G1 and G2, TS of root from the elongation zone (scale bar = 50 μm); H1 and H2, longitudinal section (LS) of root tip (scale bar = 100 μm); I1 and I2, LS of panicle (heading stage) (scale bar = 100 μm). Insets correspond to a higher magnification of the phloem tissue in A1, B1 and D1–F1, and root stele in G1. Abbreviations: a, anther; bsc, bundle sheath cell; cc, companion cell; ep, epidermis; f, filament; fc, fiber cell; is, inner sheath; le, lemma; lo, lodicule; m, mesophyll; o, ovary; os, outer sheath; pa, palea; ph, phloem; pp, protophloem; px, protoxylem; ra, root apex; s, stele; st, style; sti, stigma; vb, vascular bundle; ylb, young leaf blade.

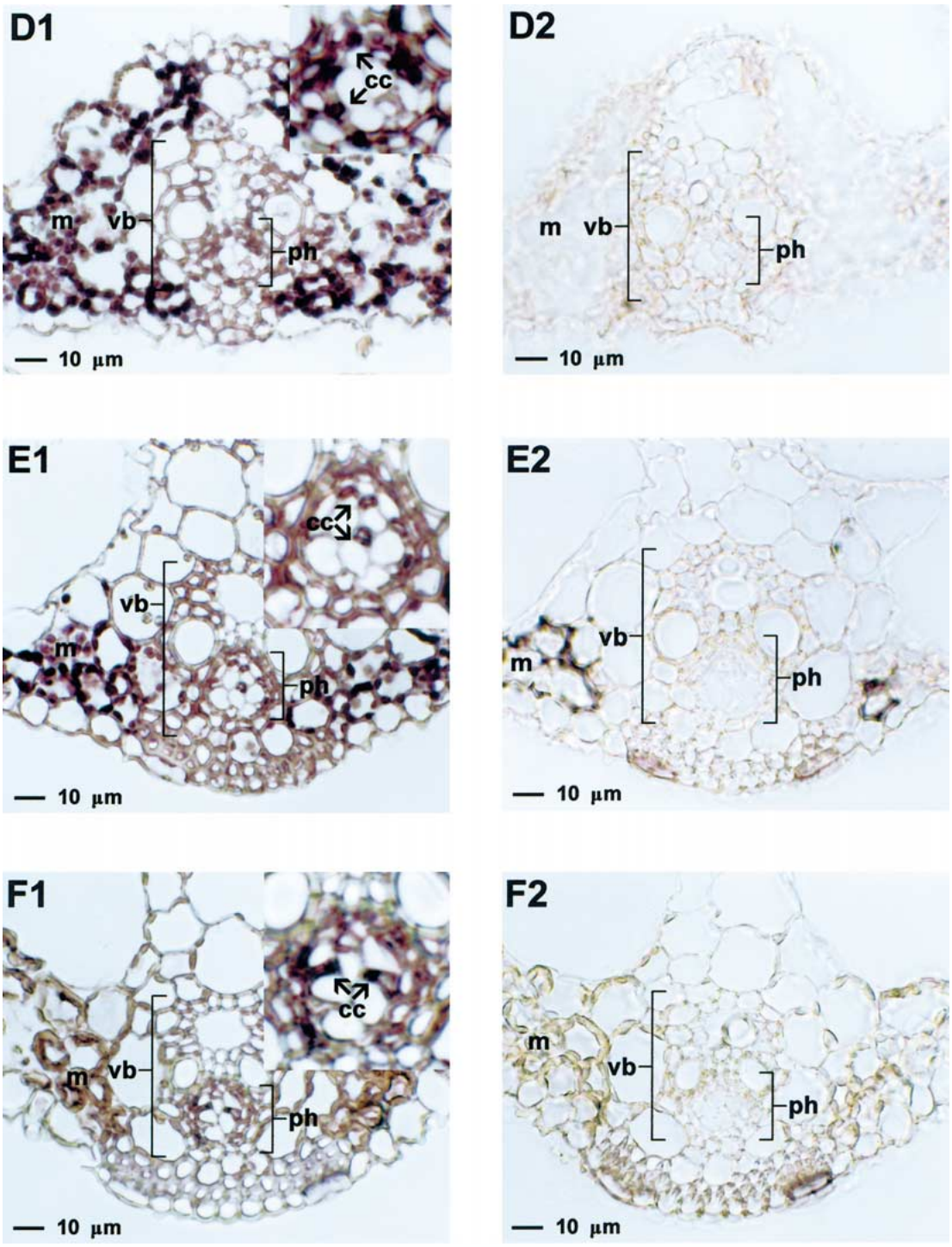
D1
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Figure 6d–f.

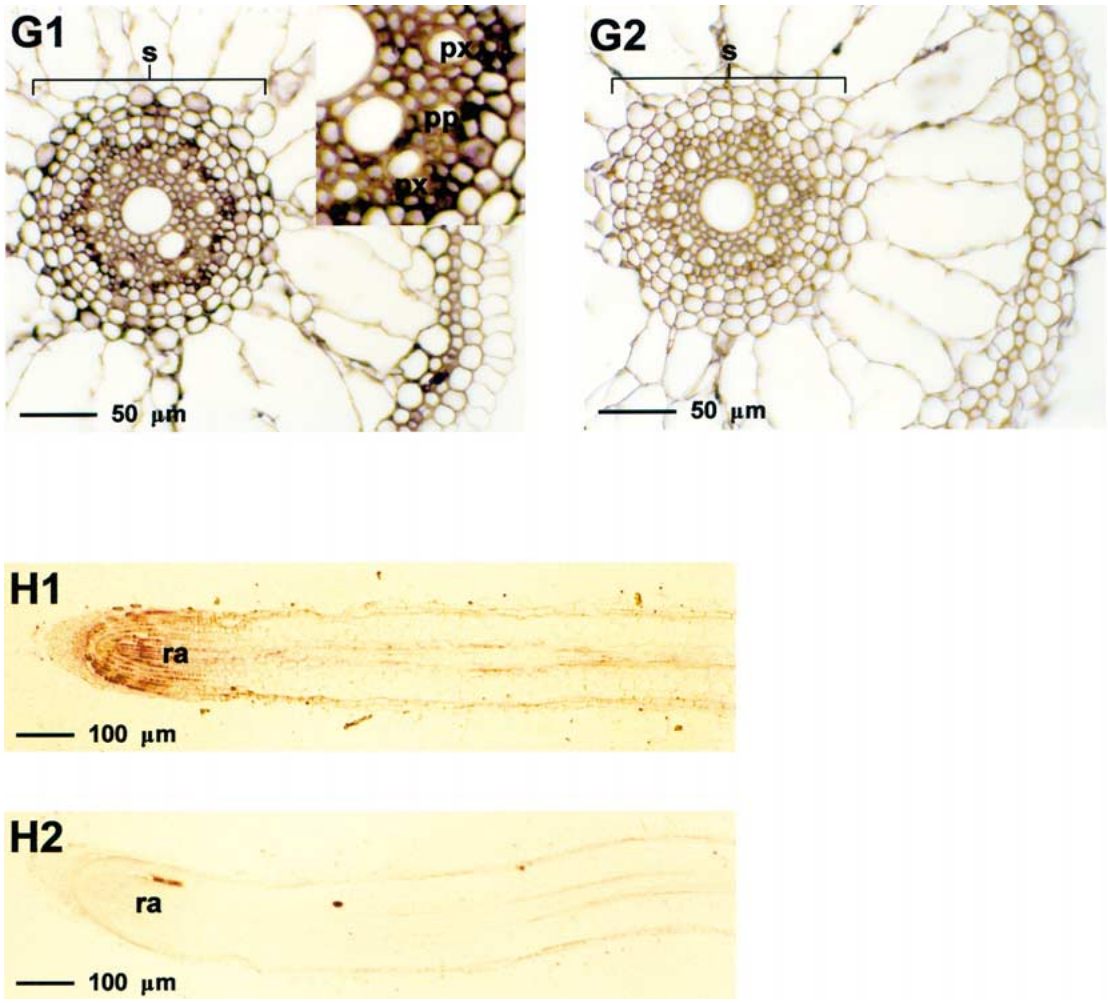

Figure 6g–h.

ingly fine-tuned manner. This is consistent with the housekeeping features and integral roles of PP2A as reported by other investigators. Although the expression patterns of both PP2Ac genes are not completely overlapping, they share many similarities in terms of organ and cellular distributions, and transcriptional response to external stimuli. The question that arises is whether the multiplicity of PP2Ac isoforms in plants is redundant with respect to biological function, and to address this question, deletion mutants in other eukaryotes, for example mice and yeasts, have been produced. Knock-out mice lacking the Cα subunit gene are embryonically lethal, despite the fact that both mutant and wild-type embryos have been shown to express PP2Ac at comparable levels (Götz *et al.*, 1998). This indicates that the roles of Cα and Cβ are not truly overlapping since Cβ is unable to compensate for the absence of Cα. While a disruption in either the *PPH21* or *PPH22* gene of *Saccharomyces cerevisiae* is not deleterious, a defect in both of the genes together was found to be lethal (Sneddon *et al.*, 1990), suggesting that the *PPH* genes in yeast perform basically very similar functions. Similarly, a double defect in the *ppa1* and *ppa2* genes of *Schizosaccharomyces pombe* also resulted in a lethal phenotype (Kinoshita *et al.*, 1990). However, while no abnormality was observed in *ppa1*-defective mutants, mutants defective in the *ppa2* gene exhibited reduced cell size and retarded growth, indicating that the functions of the two PP2Ac genes in fission yeast are not completely overlapping. Presently, there is a paucity of similar information on plant PP2Ac proteins and further experiments are needed to determine the function

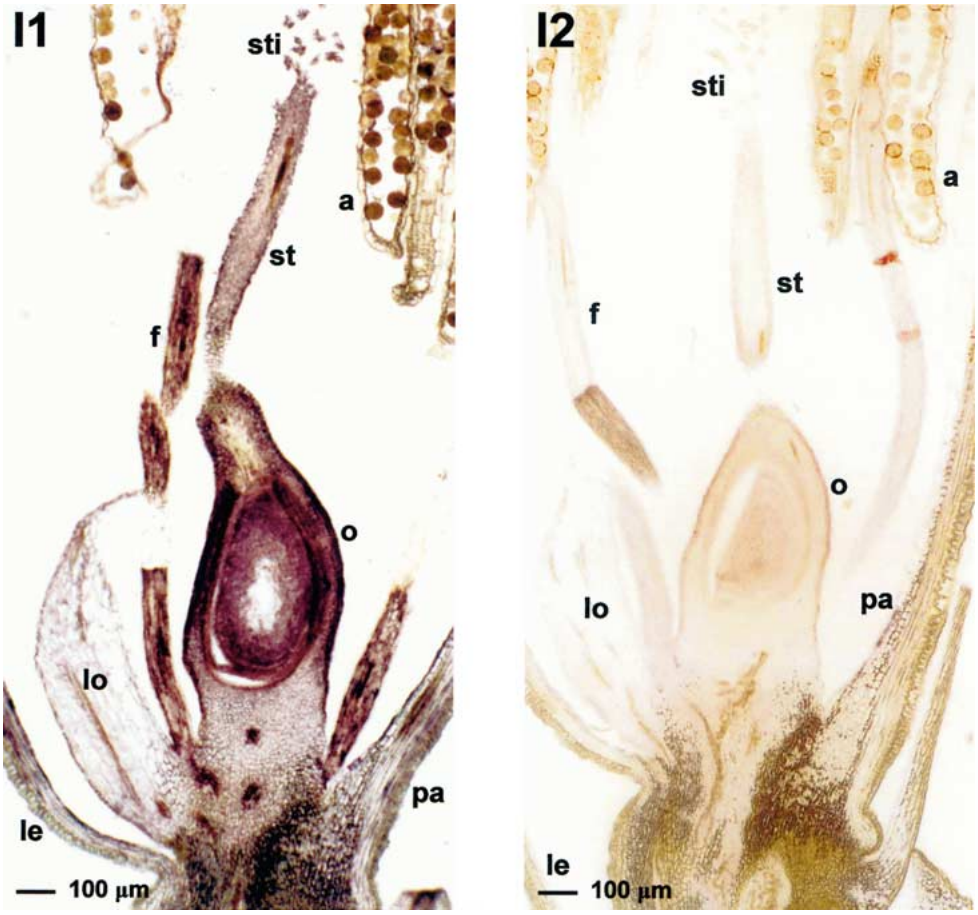

Figure 6i.

and physiological roles of the PP2Ac isoforms in plant growth and development.

Aside from transcriptional regulation, PP2Ac activity may be modulated at the translational (Baharians and Schönthal, 1998) and post-translational levels (Chen *et al.*, 1992). One impact of post-translational modification of PP2Ac is in modulating its association with the regulatory subunits of PP2A. In *Arabidopsis*, it is estimated that the 15 different PP2A subunits - five catalytic subunits (Ariño *et al.*, 1993; Casamayor *et al.*, 1994; Stamey and Rundle, 1995), three structural A subunits (Slabas *et al.*, 1994); two regulatory B subunits (Rundle *et al.*, 1995; Corum *et al.*, 1996), four regulatory B′ subunits (LaTorre *et al.*, 1997; Haynes *et al.*, 1999), and one regulatory B′′ subunit (Hendershot *et al.*, 1999) - could generate up to 105 distinct PP2A heterotrimers in one plant cell (Thakore *et al.*, 1999). Nonetheless, it has been suggested that only a small subset of heterotrimeric PP2A proteins (assembled from different catalytic and regulatory subunits) are produced in any one type of plant cell (Thakore *et al.*, 1999). In *Medicago sativa*, organ-specific expression of regulatory subunit genes has been demonstrated, leading to the speculation that only specific types of PP2A isoforms may be produced (Tóth *et al.*, 2000). The differential accumulation of *OsPP2A-1* and *OsPP2A-3* transcripts in various rice organs during plant growth, and their responses to different external stimuli, suggest that the type of PP2A heterotrimers produced are tightly regulated in the plant organs under different physiological conditions.

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