Isolation and Expression analysis of *OsPME1*, encoding for a putative Pectin Methyl Esterase from *Oryza sativa* (subsp. *indica*)

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ABSTRACT

Pectin Methyl Esterases (PMEs) play an essential role during plant development by affecting the mechanical properties of the plant cell walls. Recent studies indicated that PMEs play important role in pollen tube development. In this study, we isolated a 1.3 kb cDNA clone from rice panicle cDNA library. It contained a 1038 bp of open reading frame (ORF) encoding for a putative pectin methyl esterase of 345 aminoacids with a 20 aminoacid signal peptide and was hence designated as *OsPME1* (*Oryza sativa* Pectin Methyl Esterase 1). It contained the structural arrangement GXYXE and GXXDFIF, found in the active groups of all PMEs. *OsPME1* gene product shared varying identities, ranging from 52 % to 33 % with PMEs from other plant species belonging to *Brassicaceae*, *Fabaceae*, *Amaranthaceae* and *Funariaceae*. Southern blot analysis indicated that PME1 exists as a single copy in the rice genome. Expression pattern analysis revealed that *OsPME1* is expressed only in pollen grains, during the later stages of their development and was also regulated by various abiotic stress treatments and phytohormones. Functional characterization of this pollen specific PME from rice would enable us to understand its role in pollen development. [*Physiol. Mol. Biol. Plants* 2009; 15(2) : 123-131] E-mail : akg54@sify.com

Key words : *Oryza sativa*, Pectin Methyl Esterase, Gene Expression, Cell wall and pollen development

INTRODUCTION

The plant cell wall is a highly complex structure, composed of polysaccharide, structural proteins and various enzymes. Besides cellulose and hemicellulose, pectins constitute a major portion of cell walls from dicot species accounting for ~35 % of the primary cell wall dry weight (Varner and Lin, 1989). Pectins are synthesized in the Golgi, methyl esterified and modified with side chains and subsequently released into the apoplastic space as highly methylesterified polymer (Micheli, 2001). The homogalacturonan (HGA) component of pectin can later be demethylesterified by pectin methyl esterases (PME, EC 3.1.1.11) (Micheli, 2001), which belong to class 8 (CE8) of the carbohydrate esterases (CAZy website, <http://www.cazy.org.org.fam/CE8.html>). The demethylesterified HGA can either form Ca^{2+} bonds, which promote the formation of the so-

called 'egg-box' model structure, thus forming gels, or become a target for pectin-degrading enzymes, such as polygalacturonases, affecting the texture and rigidity of the cell wall. Thus, PMEs play major roles in pectin remodeling *in muro* (Micheli, 2001), thereby affecting shape and growth of the plant cells.

Data acquired from genome and EST sequencing projects have shown that PMEs belong to large multigene families in all plant species examined to date. (CAZy, <http://www.cazy.org/fam/CE8.html>; TIGR <http://www.tigr.org/plantProjects.ahtml>). For instance in *Arabidopsis thaliana*, 66 ORFs have been annotated as putative full-length PMEs, whereas in *Oryza sativa* (<http://www.tigr.org/tbd/e2k1/osa1/>) only 35 putative ORFs were found. The lower number of PME genes in rice is consistent with findings that HGA is less abundant in grasses than in dicotyledenous species. Higher plant PMEs are often pre-pro-proteins, in which the mature, active part of the protein (PME domain, IPR000070) is preceded by an N-terminal extension (PRO region) that varies in length and aminoacid identity between isoforms (Markovic and Janacek, 2004). The PRO region is similar

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Footnotes : 1302 nt *OsPME1* sequence was deposited in the GenBank with the accession number AY343494.

to the PME inhibitor domain (PMEI domain, IPR006501) (Camardella *et al.*, 2000). PMEs are classified based on the presence or absence of the PMEI domain. Group 1 (type II) has no PMEI domain and group 2 (type I) has 1–3 PMEI domains (Micheli, 2001; Tian *et al.*, 2006). Group 2 PMEs constitute 65 % of total PMEs in *Arabidopsis*, 54% in *Oryza sativa* and 57 % in *Populus trichocarpa* (Pelloux *et al.*, 2007).

PMEs are ubiquitous enzymes involved, directly and indirectly, in various physiological processes associated with both vegetative and reproductive plant development. Several studies have shown a strong correlation between PME activity or PME gene expression and physiological processes such as hypocotyls elongation (Bordenave and Goldberg, 1994; Derbyshire *et al.*, 2007)), fruit ripening and maturation (Tieman and Handa, 1994; Prasanna *et al.*, 2007), cellular adhesion (Roberts *et al.*, 2000), seed germination (Ren and Kermode, 2000), cambial cell differentiation (Micheli *et al.*, 2000) and cell growth in developing wood cells (Siedlecka *et al.*, 2008). Other studies have shown that PME acts as methanol source (Frenkel *et al.*, 1998) and also as host-cell receptor for Tobacco Mosaic Virus (TMV) (Dorokhov *et al.*, 1999; Chen *et al.*, 2000). Recent studies demonstrated the role of PMEs in disease resistance and abiotic stress tolerance (An *et al.*, 2008). Furthermore, analysis of pollen-specific transcriptome of *Arabidopsis* indicated that several PMEs are specifically expressed in floral buds, including pollen (Pina *et al.*, 2005). However, despite the apparently major role that PMEs may play in the growth of the pollen tube – the cell wall of which is composed mainly of pectins – the functional studies of pollen-specific PMEs are still in their infancy. Different studies examined the PME function during pollen development and pollen tube growth; specifically, exogenously added tobacco PME has been shown to inhibit pollen tube growth by thickening the apical cell wall (Bosch *et al.*, 2005) whereas the inactivation of VANGUARD1 (VGD1), *Arabidopsis* PME with a demonstrated function in the pollen tube growth, resulted in unstable and poorly growing pollen tubes (Jiang *et al.*, 2005). Similar effects, but weaker, have been observed in *AtPPME1* (*A. thaliana* pollen-specific PME 1) mutants (Tian *et al.*, 2006). Also, QUARTET1 (QRT1), another PME from *Arabidopsis* was shown to have a role in pollen tetrad separation during floral development (Francis *et al.*, 2006). A single PME from rice, Rice Immature Pollen 1 (RIP1) was characterized as regulator of late pollen development (Han *et al.*, 2006).

PME's are characterized mostly from *Arabidopsis*, Tomato, Pea, Tobacco, Petunia, Yellow cedar, *Brassica* and Maize etc. Most of these characterized PMEs belong to group 2, that contain PMEI domain at their N terminal end. Till date only one PME gene belonging to group 1, *AtPPME1* was characterized to play a role in pollen tube growth (Tian *et al.*, 2006). Here, we identified a new pollen-specific group 1 PME from rice, *OsPME1*, and characterized its native expression patterns and also its expression during various stress and phytohormone treatments. Further characterization of this gene may help to understand the role played by group 1 PMEs in pollen development.

MATERIALS AND METHODS

Isolation of *OsPME1*

A small number of random clones from rice (subsp *indica*) pre-pollinated spike cDNA library (cloned in lambda-ZAP express vector, Stratagene) were picked up and single-clone excision was performed to obtain recombinant pBK-CMV phagemid vectors, in which cDNA inserts were directionally cloned in *EcoRI* (5' end) and *XhoI* (3' end) sites. T3 and T7 promoter regions present on either side of MCS were utilized for sequencing of cDNA inserts. One of the phagemid clone designated as pAKV1, containing ~1.3 kb insert was sequenced completely and further the sequence was analyzed by various bioinformatic tools.

Bioinformatic analysis

The BLAST search was performed using the nr database at NCBI (<http://www.ncbi.nlm.nih.gov/Blastp>). Various tools from Expasy (<http://www.expasy.org/tools>) were used to deduce the translated product and compute theoretical pI (Isoelectric point) and Molecular weight. SignalP (<http://www.cbs.dtu.dk/services/signalP>) was used for the prediction of signal peptide. The degree of aminoacid sequence identity was determined by the use of Wu-Blast from EBI (<http://www.ebi.ac.uk/blast2>). The position of a cDNA in the in the rice genome was predicted by blasting the cDNA sequence with the rice genome sequence at GRAMENE (<http://www.gramene.org/db/searches/blast>). Multiple sequence alignments involved use of ClustalW (<http://www.ebi.ac.uk/clustalW>). And the dendrogram file generated through clustalW was used to view the phylogenetic tree using treeview (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>). The cis-acting regulatory elements in the promoter region were predicted using PLACE database (<http://www.dna.affrc.go.jp/PLACE>).

Plant material, plant growth conditions and stress treatments

Indica rice (*Oryza sativa* L. cv IR-50) seeds used in this study were obtained from Tamil Nadu Farm Aid services, Tamil Nadu, INDIA. Healthy seeds were surface sterilized with 70% ethanol, rinsed thoroughly, soaked in double distilled water overnight, and the seedlings were raised on the water saturated cotton in a Biotron PH100 growth chamber (Nippon, Japan) maintained at 28 °C, 80 % relative humidity and 16 h photoperiod ($180 \mu\text{Em}^{-1} \text{s}^{-1}$). After 8 days of growth, they are transferred to pots containing soil and were grown in green house till maturity. The panicles from the 120 day old rice plants were collected at various stages of growth and the stress treatments were given for 24 h by dipping the panicles in the 100 ml beakers containing water (mock solution) and kept at 4°C for cold stress and 42°C for heat stress. The panicles were treated with 250 mM NaCl for salinity stress, 400mM mannitol for drought stress and 100 mM of salicylic acid (SA) to mimic biotic stress. The panicles were treated with 100 mM of various hormones like Indole Acetic Acid (IAA), Indole Butyric Acid (IBA), 2,4, di-chloro phenoxy acetic acid (2,4D), Naphthalene acetic acid (NAA), Benzyl amino purine (BAP) and Gibberellic acid (GA3). Desiccation stress was simulated, by drying the panicles, on tissue paper and keeping them wrapped in dry tissue paper. For submergence stress, the beaker with panicles was submerged under water in a 2 L glass beaker. Absciscic acid (ABA) was dissolved in DMSO to make a stock of 10 mM and was diluted further in water. The panicles were also treated with 50 mM of various sulphates or chlorides of Zinc, Cadmium, Mercury and Calcium for heavy-metal stress.

Southern blot analysis

Genomic DNA was extracted from the leaf materials of *Oryza sativa* by Dellaporta method (Dellaporta *et al.*, 1983). Aliquots of genomic DNA (10 $\mu\text{g/sample}$) were digested to completion at 37°C with *HindIII*, *KpnI* and *SacI* respectively, which did not cut within the probe region. These were then fractionated by 0.8 % agarose gel electrophoresis and transferred on to a positively charged Biobond N⁺ nylon membrane (Sigma Aldrich, India). The 1267 bp cDNA fragment of *OsPME1* obtained by *SacI* digestion of a clone pAKV1, harboring full-length *OsPME1* cDNA was used as probe in Southern blot hybridization. Probe labeling was done using random primer labeling kit from Bangalore genei, India. The hybridization was carried out at 65 °C for 12 h. The membrane was washed under stringent conditions (65

°C) and the hybridized signals were detected by exposure to Kodak X-ray film at –80 °C.

Northern blot analysis

Northern blot analysis was performed to investigate the *OsPME1* expression profiling in various tissues of rice and in response to various abiotic stresses and phytohormones. RNA was extracted from the roots and shoots of 8-day-old rice seedlings and from leaf sheaths and pre-pollinated and post-pollinated panicles from 120-day-old rice plant. Panicles collected from a 120-day old rice plant at various stages of pollen development were subjected to various abiotic stress treatments using TRIreagent (Sigma Aldrich) as per the manufacturers instructions. 5 μg of the total RNA was size fractionated on 1.0 % formaldehyde gel and was transferred onto a positively charged Biobond N⁺ nylon membrane. The probe and the probe labeling are same as described in Southern blot analysis. Hybridization was carried out at 42 °C for 12 h followed by stringent washes at the same temperature. The hybridized signals were detected by exposing the membrane to Kodak X-ray film at -80 °C.

RESULTS AND DISCUSSION

Isolation and sequence analysis of *OsPME1*

A total of 1302 bp sequence was obtained by sequencing the cDNA insert in pAKV1 clone. It contained an open reading frame of 1038 bp. Homology searches run with the full-length nucleotide sequence of cDNA revealed 100 % identity to another cDNA clone (AK100728) from *japonica* rice. *In silico* analysis (GENSCAN) of the genomic sequence corresponding to *OsPME1* revealed that the full-length cDNA is of 1434 bp with 60 bp of 5' untranslated region (UTR), 1038 bp of open reading frame and 336 bp of 3' UTR (Fig. 1).

Characterization of the predicted *OsPME1* protein

Computational translation of the cDNA clone indicated that the 1038 bp ORF of the *OsPME1* encodes for a 345 aminoacid peptide (Fig. 1) with a predicted molecular mass of 37.994 kDa and theoretical pI of 8.99. Homology searches run with this deduced aminoacid sequence revealed significant identity to the pectin methyl Esterases (PMEs) from *Arabidopsis*. The identity values at the aminoacid levels varied in between 52 % to 36.7 % with the *Arabidopsis* PMEs. This result suggests that the isolated gene may be coding for a putative PME from rice and hence designated as *OsPME1* (*Oryza sativa* Pectin Methyl Esterase 1). *OsPME1* did not show


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gcacgagaagccatcttcgatcgacgagaaccccgcggtgcgtagcgatgggcccgccacgc
M A R P R
ctctctctctctctctctctctcgccgctgcgcggcggtgctctctctctctctctctctc
L L L L P L L A V A A A V L S S P L G R
gcgctcgccaagacggcccaagaagacgaacgacatcgtaacggcccaactgctcaactcc
A L A K T A K K S N D I V N G P L L T S
aagatcaacgccaagcgcaacgctgatcgtcgcccgaggacgagttcaagacgctgcaag
K I N A K R T L I V G P E D E F K T V Q
tccgcatcgacgcccgtgcccgtcggaacacccagtggtgctcgtccacccctccgctcc
S A I D A V P V G N T E W V I V H L R S
ggcatctacagggagaaagtattgatcccggaacacacgcttcatctctgtagggggc
G I Y R E K V M I P E T K P F I F V R G
aacggcaagggcggaactcaatcaacacgagtcgcttctctccacacgcccagtgctt
N G K G R T S I N H E S A S S H N A E S
ggcgcttcaacgctgacgagagctgttacatccaggcaacatcgatctctctcagcagc
A A F T V H A D N V I V F G L S I R N S
ggcgcccggggctgcccacgctcccgaggtgcgcacggtggcgccgctgctgtagggcg
A R A G L P N V P E V R T V A A M V G G
gacaagattgcttctaccactgcgcgttctacagccccaccacacctctctcagctc
D K I A F Y H C A F Y S P H H T L F D V
ggcgcccgccactactacgagagctgttacatccaggcaacatcgatctctctcagcag
A G R H Y Y E S C Y I Q G N I D F I F
ggcgcccgagtcctctctccaatcccgagatctctgtagaagcctgacggcgtagggag
G G Q S I F Q C P E I F V K P D R R T E
atcaaggggtccatcagcgcgacgaacgcaagcaagaggacggcagcggtctctctc
I K G S I T A Q N R K Q E D G S G F V F
atcaagggcaaggtgtacgagctgttacatccaggcaacatcgatctctctcagcag
I K G K V Y G V G Q V Y L G R A N E A Y
tcgcgctcatctctcgccgacacctacctctccaagaccatcaacccggcggtggacc
S R V I F A D T Y L S K T I N P A G W T
agctacgctacacgggacacccgacacggtgatgctcggggagttcaactgcacccgg
S Y G Y T G S T D H V M L G E F N C T G
ccgggctccgaagcgacgaagcgagcggtgctcgccgacgctcaccagggaggagcc
P G S E A T K R E P W S R Q L T Q E E A
gacaagttcatcaacatcgacttcatcaacggcaagagtggtgctcccggtactactac
D K F I N I D F I N G K E W L P A Y Y Y
tgatcatatccaacaaaaagagaacgtagtaacatgtatgtcgtgtgcatgcatgct
*
ctgggcccggctagccgtaaacatggtgtcaagagttctaaacttctaactgaaacggaag
cgcaactggaacgattggtgtcttcttcagtaggaagatggcagatttaattgtgtgatcat

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Fig. 1 The nucleotide (1302 bp) and deduced amino acid sequence (345 aa) of *OsPME1*. The start codon (atg) and the stop codon (tga) were underlined in bold. Asterisk mark (*) represents the stop codon that cannot be translated into any amino acid. The conserved domains, GXYXE and GXXDFIF in the active groups of PME were boxed. The putative processing sites for signal peptide sequence (Between S and S) is indicated by arrow.

any significant homology with the RIP1, a previously characterized PME from *japonica* rice.

Apart from *Arabidopsis* PMEs, it also showed identity with the amino acid sequences of PMEs reported from other plant species like *Lupinus* (41.5 %, Q84K37; Fabaceae), *Physcomitrella* (35.4 %, Q4A3V7, Funariaceae (Bryophyte), *Brassica oleracea* (35.4 % Q2A9P0, Brassicaceae), *Medicago truncatula* (34 % Q1RUM0, Fabaceae) and *Salsola* (33 %, Q17ST3, Amaranthaceae).

As mentioned earlier, in higher plants, PMEs can be categorized into two groups, group 2 genes that encode for proteins with a long pro-region characterized as PME inhibitor (PMEI) (Camardella *et al.*, 2000) and group 1 genes, that encode for proteins without any pro-region (Micheli, 2001). Bioinformatic analysis revealed that *OsPME1* belongs to the group 1 PMEs as its encoded protein lacks any pro-region and contains only catalytic PME domain.

Spatial and temporal regulation of PME activity during plant development is based on a large family of isoforms. Biochemical analysis revealed that most of the *Arabidopsis* PME isoforms encode for a PME domain of an alkaline pI. Apart from these, some of the PME domains are also acidic and neutral. The presence of many alkaline isoforms explains their tight association with the slightly acidic cell wall. However, the presence of the acidic PME isoforms indicates that not all PMEs are necessarily tightly associated with the cell wall components. Indeed highly soluble acidic PME isoforms have been detected in hypocotyls of mung bean, across the cambial region of hybrid aspen and in the seeds of yellow cedar (Bordenave and Goldberg, 1994; Micheli *et al.*, 2000; Ren and Kermode, 2000). Bioinformatic analysis revealed that *OsPME1* encodes for an alkaline isoform, thus might be associated with cell wall. This is further substantiated by the prediction of N' signal sequence, that would target the protein to the cell membrane.

Putative structure of *OsPME1* gene product

As mentioned earlier, BlastP analysis revealed that *OsPME1* is homologous to PMEs from various plant species belonging to different families and also to the PMEs from fungi (*Aspergillus*) and bacteria (*Erwinia*), indicating that the PMEs are conserved during evolution. Indeed, multiple alignment of the PME protein sequences from various plant species, fungi and bacteria revealed that the structural arrangement **GXYXE** and **GXXDFIF** found in the active groups of PME's was also conserved in *OsPME1* protein.

PME belongs to the family of parallel beta-helix proteins. The folding of PME protein into parallel beta helix structure is important for carrying out its activity. The crystal structures of *Erwinia chrysanthemi* PME (Jenkins *et al.*, 2001) and carrot (*Daucus carota*) PME (Johanson *et al.*, 2002) shows that the fold of PME is a single stranded right-handed beta-helix. In this study we attempted to analyze the putative 3D structure *OsPME1* using Modbase. The analysis indicated that it showed 28 % identity to Igg84 template (PME from carrot) and 17 % homology to Iru4A (*Erwinia* pectate lyase pel9A). Further structural analysis was performed with Igg84 as template. The predicted 3D structure reveals it to be a single stranded right-handed beta-helix (Fig. 2a) with a central hollow cylindrical structure lined by several aromatic residues, which could be involved in ligand binding. The structure also revealed that the amino acid residues at the active site and those involved in ligand binding are conserved (Fig. 2b). Also,

all the amino acid residues predicted to be involved in ligand binding could be located on the central region of the cleft (Fig. 2c). These results indicate that *OsPME1* might be coding for a functional PME in rice.

Genomic organization of *OsPME1*

To gain insight into the *OsPME1* gene structure, full-length cDNA sequence of *OsPME1* was blasted against the *Oryza sativa* genome sequence database (NCBI).

Fig. 2. Putative Structure of *OsPME1* gene product. (A) Putative 3D structure of *OsPME1* protein showing right handed beta helix fold and a central long cleft. The figures were obtained by using Rasmol. A. side view; B. Upper view. (B) Sequence based comparison of carrot (*Daucus carota*) PME (*DcPME*, Acc.N0. P83218) and *OsPME1*. The Proposed active sites (red), ligand binding sites (blue) and the aromatic amino acids lining the cleft (yellow) are mostly conserved. (C) The ligand binding sites (pink) are located in the central part of the cleft. The putative ligand binding sites in *OsPME1* protein are R157, H179, H180, D201, F204, A232, R260, N262 and Y287.

The sequence showed 100% identity to sequences of single contig NT107192.1 (NCBI) from chromosome 3 of *indica* rice. To localize *OsPME1* gene in rice genome, its cDNA sequence was used as query for BLAST search against nipponbare (*Oryza sativa* ssp. *Japonica*) database at GRAMENE (<http://www.gramene.org/db/searches/blast>). Only a single clone (Acc. No. AC137071) located at the locus LOC_OSO3G19610 was predicted to be 100% identical to the *OsPME1* sequence. According to the physical map of the *Nipponbare*, *OsPME1* locates in the region 11.012355 to 11.014375 MB on the upper arm of the chromosome 3, flanked by genetic markers RM14828 and RM563 (Fig. 3c). Comparison of the full-length cDNA sequence with the corresponding genomic sequence of *OsPME1*, showed the presence of five-exons of 266 bp, 163 bp, 207 bp, 245 bp and 157 bp separated by four-introns of 243 bp, 113 bp, 97 bp and 134 bp respectively. So *OsPME1* may be a single copy gene with five exons and located on chromosome 3 of *indica* rice.

Fig. 3. Organization of *OsPME1* gene in rice genome. Restriction analysis of *OsPME1* genomic DNA. All restriction sites are not represented. (B) Southern Blot analysis of *OsPME1*. 5 mg of total genomic DNA was digested with HindIII (lane 1), KpnI (lane 2) and SacI (lane 3). The digested samples were resolved on 0.8% agarose gel, transblotted onto positively charged nylon membrane and probed with 1.3 kb *OsPME1* cDNA fragment. (C) Physical location of *OsPME1* on *Oryza sativa* genome.

Copy number of *OsPME1* in rice genome

In order to find out the exact copy number of *OsPME1* in the rice genome, genomic southern blot analysis was performed. The autoradiogram revealed that the *OsPME1* probe hybridized with only single 11.0 kb fragment, ~9.0 kb fragment and ~3.5 kb fragment generated by digestion of rice DNA with *HindIII*, *KpnI* and *SacI* respectively (Fig. 3b). Except for *HindIII*, other restriction sites are present in the genomic sequence of *OsPME1* (Fig. 3a). *SacI* digested genomic DNA hybridized with a fragment of 3.5 kb as expected. Thus, these observations confirm that *OsPME1* exist as a single copy in the rice genome.

Tissue specific expression of *OsPME1*

In higher plants, though some PMEases are ubiquitously present (Gaffe *et al.*, 1997), others are specifically expressed during root development (Wen *et al.*, 1999), fruit ripening (Frenkel *et al.*, 1998), or stem elongation (Bordenave, 1996; Pilling *et al.*, 2000). Furthermore, recent analysis of pollen-specific transcriptome of *Arabidopsis* indicated that several PMEs are specifically expressed in floral buds, including pollen (Pina *et al.*, 2005). To determine the organ-specific expression of *OsPME1*, northern blot analysis was performed with total RNA extracted from rice seedlings (8-day old), shoots, roots and panicle (90-day old plant). The probe hybridized only with the RNA from pre-pollinated and post-pollinated panicle indicating that *OsPME1* gene expresses only in the flowers under normal conditions (Fig. 4a). *In silico* analysis predicted the presence of pollen specific elements in the regulatory sequences of *OsPME1*, suggesting it to be a pollen-specific gene. To test this hypothesis, total RNA from lemma & palea and pollen grains from pre-pollinated- (opened flowers with un-dehiscent anthers) and post-pollinated- (closed flowers with dehiscent anthers) flowers was extracted and northern analysis was performed. The probe hybridized only to the RNA from pollen grains of both pre-pollinated and post-pollinated flowers, however, comparatively more with the RNA from pollen grains of post-pollinated flowers. No such hybridization was observed with the RNA from lemma & palea thus indicating its expression to be pollen specific (Fig. 4b). To gain further insight into the expression pattern of the *OsPME1* during pollen development, total RNA was extracted from the anthers at different days of flowering (different stages of pollen development) and northern analysis was performed. Results revealed that *OsPME1* transcript was not expressed at very early stages of pollen development [2 DAF (Days after flowering)] but the transcript could be detected in anthers from flowers

of 4 DAF and increased gradually till the stage (6 DAF) at which mature and viable pollen grains are formed. These results suggested that *OsPME1* expresses specifically in pollen grains during their later stages of development (Fig. 4c). Indeed PME-related genes are expressed in pollen of many diverse plant species, e.g. *Brassica* (Albani *et al.*, 1991), *Alfaalfa* (Qiu and Erickson, 1995), Maize (Wakeley *et al.*, 1998), Tobacco (Bosch *et al.*, 2005; Lacoux *et al.*, 2003; *Salix* (Futamura *et al.*, 2000), *Medicago* (Rodriguez Lorente *et al.*, 2004) and *Arabidopsis* (Jiang *et al.*, 2005; Pina *et al.*, 2005; Tian *et al.*, 2006). Other works have shown that the PME has role either in tetrad dissolution of pollen grains (Albani *et al.*, 1991; Francis *et al.*, 2006), or in pollen ornamentation (Lacoux *et al.*, 2003) or in pollen tube germination or elongation (Jiang *et al.*, 2005; Bosch *et al.*, 2005; Tian *et al.*, 2006).

Fig. 4. Expression patterns of *OsPME1* in different tissues of rice plants. 5mg of the total RNA extracted from Leaves (L), Shoots (S), Roots (R), Leaf sheath (Ls), Pre pollinated panicle (Pr), Post pollinated panicle (Po), Lemma and palea from Pre-pollinated panicle (L1), Lemma and Palea from Post-pollinated panicle (L2), Pollen of Pre-pollinated panicles (P1), pollen of Post-pollinated panicles (P2) and Panicle from 1-days after flowering (DAF) (I1), 2 DAF (I2); 4 DAF (I3); 5 DAF (I4) and 6 DAF (I5) was size fractionated on 1% formaldehyde gel and transblotted onto a positively charged nylon membrane and probed with radio labeled *OsPME1* cDNA fragment. Lower panel depicts the EtBr stained total RNA that served as a control for equal loading.

Effect of various abiotic stress treatments and phytohormones on the expression of *OsPME1*

Apart from the spatial regulation, PME activity is also regulated by phytohormones. Auxin-induced PME activity was shown to increase cell wall extension and, as a result, water absorption by the cell (Micheli, 2001). Some contradictory results have been obtained about the role of abscisic acid (ABA) on PME regulation. For example, although ABA enhanced PME activity in tomato seeds (Downie *et al.*, 1998), it inhibited PME activity during seed germination in yellow cedar (*Chamaecyparis nootkatensis*) (Ren and Kermode, 2000). Moreover, in these cedar seeds, gibberellic acid (GA₃) had a stimulatory effect on PME activity. In the present study,

efforts were made to understand the regulation of *OsPME1* expression. *In silico* analysis of ~1.5 kb genomic sequence upstream to the transcriptional start site of *OsPME1* (retrieved from the sequence of contig NT107192.1 of *indica* rice showing 100% identity to *OsPME1* cDNA) using PLACE database predicted the presence of several *cis*-acting regulatory elements responsive to Absciscic acid (ABREOSRAB21 (Marcotte *et al.*, 1989) at -1435 position), dehydration (DRECRCTCOREAT (Dubouzet *et al.*, 2003) at -172 position; MYCCONSUSAT (Abe *et al.*, 2003) at -402 position), Low temperature (LTRE1HVBLT49 (Dunn *et al.*, 1998) at -122 position), salicylic acid (WBOX (Yamamoto *et al.*, 2004) at -1449 position) and gibberellic acid (PYRAMIDINEBOXORAMY1A at -1136 position). Therefore, in this study, an attempt has been made to validate the functionality of responsive elements experimentally. For this, northern blot analysis was performed with the total RNA extracted from the pre pollinated panicles from 120-day old rice plants treated with various stresses like heat, cold, salt, drought, desiccation, submergence and salicylic acid; heavy metals like mercury, copper, cadmium and zinc; phytohormones like IAA, IBA, 2,4D, NAA, BAP, GA₃ and ABA. The probe hybridized differentially with the RNA from different samples indicating the differential regulation of *OsPME1* by various signals. The results indicated that *OsPME1* mRNA levels were upregulated by Mannitol treatment, downregulated by heat, cold, desiccation, submergence and salicylic acid and

unaltered by NaCl treatment (Fig. 5a). The *OsPME1* mRNA levels were downregulated by Cadmium and Zinc and unaltered by Mercury and Copper (Fig. 5b). *OsPME1* transcript levels were upregulated by various auxins with higher accumulation by IBA and NAA treatments as compared to IAA and 2,4-D treatments, downregulated by GA₃ and unaltered by BAP (Fig. 5c). Stress hormone ABA also upregulated the *OsPME1* expression in dose-dependent manner showing higher accumulation with 100μM of ABA as compared to lower ABA concentration (10μM) (Fig. 5d). These results indicate that *OsPME1* expression is regulated by phytohormones and various stress conditions.

Recently, some of the pollen-specific PMEs were shown to have role in the pollen tube growth and structure. However, the exploration of the specific effects of PMEs on pollen tube growth is just in the beginning. PMEs together with PMEs are thought to regulate this pollen growth. As mentioned earlier, till date only one PME belonging to group 1 from *Arabidopsis* was characterized (Tian *et al.*, 2006). Here we report the isolation and expression analysis of a group 1 PME from rice. Functional characterization of *OsPME1* might provide an insight into further understanding of roles played by it and group 1 PMEs role in the pollen development of rice.

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Fig. 5. Expression patterns of *OsPME1* in response to various abiotic stress treatments, heavy-metals and phytohormones. 5μg of the total RNA extracted from the pre-pollinated panicles of rice treated with water (C), Heat (H), Cold (Co), NaCl (Na), Mannitol (M), Desiccation (De), Submergence (Su), Salicylic acid (SA), HgCl₂ (Hg), CuSO₄ (Cu), CdCl₂ (Cd), ZnSO₄ (Zn), IAA (IA), IBA (IB), 2,4-D (4D), NAA (NA), BAP (BA), GA₃ (GA), 1μM ABA (A1), 100μM ABA (A100) was size fractionated on 1 % formaldehyde gel and transblotted onto a positively charged nylon membrane and probed with radio labeled *OsPME1* cDNA fragment.

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