Chapter III
Isolation and characterization of a Pectin Methyl Esterase (OsiPME1) gene from rice.
Introduction

Pectin methyl esterases (PMEs) are ubiquitous enzymes involved in various physiological processes in plants. 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), fruit ripening and maturation (Tieman and Handa, 1994), cellular adhesion (Robert et al., 2000), seed germination (Ren and Kermode, 2000) and cambial cell differentiation (Micheli et al., 2000), microsporogenesis. 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). PMEs have been shown to play a central role in growth of pollen tube wall, which is composed mainly of pectin (Ferguson et al., 1998; Li et al., 1994). 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). Many reports have shown that the PME has role in tetrad dissolution of pollen grains (Albani et al., 1991), in pollen ornamentation (Lacoux et al., 2003) and in pollen tube germination or elongation.

PME’s are characterized mostly from Arabidopsis, Tomato, Pea, Tobacco, Petunia, Yellow cedar, Brassica, Maize etc. To the best of our knowledge, there is no report on PME’s from rice in the Literature.

In this study, genome wide analysis of pectin methyl esterases from rice was performed. Also a pollen specific putative pectin methyl esterase gene lacking a PMEI domain was isolated and it’s detailed sequence analysis and expression analysis was performed.
Review of Literature

The most distinguishing feature of the plant cell is the presence of cell wall. The Plant Cell Wall is a complex and intricate structure involved in the determination of cell size and shape, growth and development, intercellular communication and interaction with the environment (Carpita and Gilbeaut, 1993). The cell wall is largely composed of polysaccharides, structural proteins and various enzymes. Besides cellulose and hemicellulose, pectins constitute a major portion of cell wall dry weight accounting for ~35% of the dry weight of the dicot cell wall (Fig.1). Pectins are also abundant in gymnosperms cell walls, but less so in the walls of grasses (Varner and Lin, 1989). The pectin matrix is structurally complex and heterogenous and it contributes to both the physical integrity and physiological status of cell walls.

Typically heterogenous population of pectic polymers are present in the primary cell walls and essentially consists of galacturonan backbones with or with out various side chain additions (O’Neil et al, 1990). The back bone domains consists of either contiguous 1,4 linked α-D-galacturonic acid (homogalacturonan, HG also referred to as smooth pectin) or repeats of the disaccharide (-4)-α-D-GalA-(1-2)-α-L-Rha-(1-) (rhamnogalacturonan, RG also referred to as hairy pectin). GalA residues in HG may

Figure 1: Diagrammatic representation of plant cell wall components
be methyl esterified, acetylated and/or substituted with xylose or apiose (Schols, 1995). Oligosaccharide side chains may be attached to both RG and HG domains to form the branched domains known as RG-I and RG-II, respectively (O’Neil et al., 1996).

It is widely accepted that the pectins are polymerized in the cis Golgi, methyl-esterified in the medial Golgi and substituted with side chains in the trans Golgi cisternae (Micheli, 2001). Subsequently pectins are released into the apoplastic space as highly methyl esterified polymers. Those can later be de-esterified by Pectin Methyl Esterases (PMEs) present in the cell wall, converting the methoxy groups into carboxyl groups on the polygalacturonic acid chain and releasing both methanol and proton (Fig.2).

![Image](image.png)

**Figure.2:** Demethylesterification of pectins by pectin methyl esterase (PME).

**Biochemical study of Pectin methyl-esterases**

Historically, the first PME analyses were made on PME isoforms purified from plant cell walls using standard conditions of extraction, assay and electrophoretic migration from large amounts of plant tissues. Most of these purified PME isoforms have neutral or alkaline isoelectric points (pIs), and only a few studies have revealed the presence of acidic pectin methyl-esterases (Bordenave, 1996). The existence of acidic isoforms suggests that not all PMEs are necessarily tightly associated with the cell wall components. By using newer methods such as microanalysis on 25μM cryosections or a gel diffusion assay, PMEs analyzed at the tissue level shown the importance of acidic PMEs in plant physiology. Indeed, highly soluble acidic PME isoforms have been detected in hypocotyls of mungbean (*Vigna radiata*), across the cambial region of hybrid aspen (*Populus tremula*×*Populus tremuloides*) and in the seeds of yellow cedar (Bordenave and Goldberg, 1994; Micheli et al., 2000; Ren and Kermode, 2000). Because acidic isoforms are probably only weakly adsorbed onto the cell wall components, soluble-protein extraction procedures increase their recovery.
dramatically. These observations suggest that some of the failures to detect acidic isoforms in higher plants might be related to the experimental conditions used for protein extraction (Micheli, 2001).

**Pectin Methyl Esterases: a multiple gene family**

Various molecular analyses in *Arabidopsis* have shown that the several PME isoforms detected in the cell walls are encoded by multigene family (Richard *et al*., 1996; Micheli *et al*., 1998). The systemic sequencing of the *Arabidopsis* genome has greatly contributed to the identification of 67 PME-related genes in this species (Kaul *et al*., 2000).

The PME genes were shown to encode pre-pro proteins that have peptide motifs considered to be signatures of PMEs. The pre region or signal peptide is required for protein targeting to the endoplasmic reticulum. The pro-PME is secreted to the apoplasm via the cis, medial and trans-Golgi cisternae and the trans Golgi network, and only the mature part of the PME (without the pro region) is found in the cell wall (Micheli, 2001).

According to the data from the systemic sequencing (Kaul *et al*., 2000), PME genes can be classified into two classes namely type I and type II. The type I genes consist of only two to three introns and a long pro region. The genes in the type II contain five to six introns with either small or nonexistent pro region. The type II sequences have a structural similarity to the phytopathogenic (bacterial and fungal) PMEs that are involved in cell wall soaking during plant infection (Micheli, 2001). This data forces us to consider the role and development of the PME pro region.

**The role of pro region**

As noted earlier only the mature PME, without the pro-region can be detected with PME antibodies or activity-based staining in the cell wall extracts, indicating that the pro-region is cleaved off either during the secretion (Fig.3a) or at some point after secretion (Fig.3b). In the first case, the pro region might be degraded (Fig.3d) or play a role either inside the cell (Fig.3e) or in the apoplasm (Fig.3f) (Micheli, 2001).

Several functions for the pro region have been suggested including, the role in biological function of PME, targeting of PME to the cell wall, correct folding of PME (intramolecular chaperon) and inhibition of PME enzyme activity (PMEI). Recent studies with pollen specific PME in *Nicotiana tabaccum* showed that the pro region may help in targeting the PME and may act as inhibitor of enzyme activity and its regulation is governed by various factors like pH, ionic balance etc.. Although
experimental evidence to support the view of the pro region of PMEs as intramolecular chaperons is lacking, the functions of chaperons can be applied to PMEs, supporting the hypothetical role of the PME protein in inhibiting the mature part of the protein and protein folding. These supporting similarities include high specificity of chaperons for mediating folding (pro regions of PMEs do not have high homologies and are variable in size and sequence according to the isoform, suggesting the specificity of the pro region with respect to the corresponding mature part) and folding mechanism in which the chaperons are covalently attached to the N-terminus of the protein and gets released via a proteolytic cleavage (The mature part of the PMEs is covalently attached to the pro region and are cleaved by a proteolytic cleavage at a motif RR(K)LL that is highly conserved with the cleavage site (RRKRR) found in some intramolecular chaperones from animals (Micheli, 2001).

Figure 3: Hypothesis for pectin methylesterase (PME) excretion into the apoplasm and maturation of the protein. Pro region and mature PME are depicted in red and green respectively.
It is hypothesized that the pectins and PMEs are co-secreted into the apoplasm via Golgi vesicles and pro region could inhibit the mature part during secretion to the apoplasm to prevent premature demethyleneesterification of pectins before their insertion into the cell wall (Fig. 4). This point is particularly interesting because the first two PME isoforms identified to be involved in microsporogogenesis and pollen tube growth are type II PMEs, related to bacterial PMEs. The PME activity in this type II PME should be inhibited by other proteins apart from the pro region. This can be explained by the regulation of PME with PMEinhibitors (PMEIs) (Giovane et al., 2004).

**Figure 4:** Hypothesis of co-secretion of the pectins and the pectin methylesterases (PME) into the apoplasm. In the scheme, the pro region (red) could inhibit the mature part (green) during its secretion to the apoplasm to prevent the premature demethyleneesterification of pectins before their insertion into the cell wall. Methylesterified galacturonic acids are represented in blue and demethylesterified galacturonic acids in yellow.
Activity of PME enzyme

It is generally believed that most plant PMEs remove methyl esters in a block-wise fashion (linearly), creating long contiguous stretches of deesterified pectins (Limberg et al., 2000). The negatively charged carboxyl groups from neighboring chains can be co-operatively cross-linked by Ca$^{2+}$, which results in the stiff threedimensional pectate network (Grant et al., 1973; Catoire et al., 1998). The strength of the interaction between Ca$^{2+}$ and pectin increases with decreasing average degree of pectin methylesterification and increasing length of the unsubstituted galacturonan backbone. Therefore, the affinity of highly methylesterified pectins for Ca$^{2+}$ is generally not enough to induce the formation of a pectate gel (Tibbits et al., 1998).

The observation that HGs with a more random distribution of methyl esters are also abundant in primary cell walls suggests that at least some PMEs are able to deesterify pectins in a non-block wise fashion, similar to the action pattern of bacterial and fungal PMEs (Williats et al., 2001). These differences in mode of action, block-wise versus non-block wise, as well as degree of methyl esterification, determine the mechanical and porosity properties of the cell wall and influence its pH and ion balance (Goldberg et al., 2001). It is important to note that cell wall properties are also affected by the hydrodynamics of the pectin network. The hydration of the network is influenced by the balance between the osmotic stress exerted on the cell wall by the cell contents and cross-linking of the network, which tend to restrict swelling, and the affinity of the network for water, which drives swelling (Zsivanovits et al., 2004).

In addition to exposing carboxyl residues, the changes brought about by PME activity have further important consequences. For example, the localized reduction in pH, generated by the protons released during the deesterification process, can inhibit some PME isoforms (Moustacas et al., 1991) and at the same time stimulate the activity of cell wall hydrolases, such as polygalacturonases and pectate lyases (Nari et al., 1986). The combined effect can result in cell wall loosening (Wen et al., 1999), which will enhance wall yielding and cell expansion. Yet another factor to consider is the possibility that demethylation mediated by PMEs renders pectins susceptible for degradation by pectinolytic enzymes (Koch and Nevins, 1989). The released oligo galacturonides, which consists of 2 to ~20 GalA residues, can act as signaling molecules in plant defense responses and plant growth and development (Cote and Hahn, 1994; Ridley et al., 2001).

Analysis of the Arabidopsis thaliana transcriptome reveals that the plants invest extensively in the biosynthesis and modification of pectins. An analysis of the
putative PME isoforms encoded in the Arabidopsis genome is presented in Fig.5 (redrawn from Bosch and Hepler, 2005 data according to Pina et al., 2005). Most Arabidopsis isoforms encode a PME domain with a predicted molecular mass between 33 and 43 kDa and an alkaline pI. The latter feature explains their tight association with the slightly acidic cell wall. However, acidic isoforms were also found mostly in the tissues and was discussed in the biochemical study of PMEs in this chapter.

While it is clear that PME activity must be tightly regulated to fine-tune properties of the pectin network in specific region of the cell wall, the precise nature of the control mechanism in vivo is poorly understood. One level by which PME activity is regulated is the differential expression, both spatially and temporally of PME isoforms. However multiple PME isoforms have been shown to be expressed in certain tissues at the same time. From the action pattern of the few PME isoforms studied so far, it appears that the intrinsic activity of different isoforms is the same but that certain substrate specificities and reaction mechanisms require different environmental conditions (Denes et al., 2000; Limberg et al., 2000; Goldberg et al., 2001). It has been shown that the distribution of carboxyl units along the pectin backbone, and to a lesser extent the methylation degree; are important in controlling PME activity. Free carboxyl groups in the vicinity of the ester linkage increase the affinity of the enzyme for its substrate (Catoire et al., 1998; Golberg et al., 2001). The immobilization of PMEs at these anionic sites has been reported to either increase or decrease their deesterification rate, possibly due to confirmation changes induced by the microenvironment (Bordenave and Goldberg, 1994).
Previously it was hypothesized that random demethylesterification depended on acidic PMEs where as linear demethylesterification depended on alkaline PMEs. However later it was shown that PME activity also depends on pH and the initial degree of methylesterification of pectins. Some isoforms can act randomly at acidic pH but linearly at alkaline pH. And at a given pH some isoforms are effective than others on highly methylesterified pectins (Catoire et al., 1998). Moreover, PME activity is enhanced by cations: trivalent cations are more effective than bivalent cations, which are themselves more effective than monovalent cations (Schmohl et al., 2000). Depending on their concentration, cations might also modify the affinity of PMEs for their substrate. In conclusion, the action pattern of mature PMEs is regulated in the cell wall by numerous factors that both reveal the complexity of the enzymes and challenge the simplistic hypothesis that divides the PMEs into two groups: ‘alkaline, linear demethylesterification’ and ‘acidic, random demethylesterification’ (Fig.6).

![Figure 6: Modes of action of the Pectin methylesterases (PMEs). Colour codes as described in previous figures.](image)

**Regulation of PME activity**

Post-translational regulations of PMEs by proteinaceous inhibitor PMEIs represent another important control mechanism (PMEIs; Giovane et al., 2004). PMEIs
are small proteins that inhibit PME activity through the formation of a reversible 1:1 complex of which the stability is pH dependent, being higher in acidic conditions, typical of the apoplastic environment (D’Avino et al., 2003). Although we know that PMEIs inhibit plant PMEs in vitro and the crystal structure of the complex between a plant PME and PMEI has recently been resolved (DiMatteo et al., 2005), little is known about how PMEIs regulate PME activity in planta. The first functionally and structurally characterized PMEI from kiwi fruit shows significant sequence homology to the well-characterized group of plant invertase inhibitors (Greiner et al., 1998, 1999) and to the pro-region of type I PMEs (Scognamiglio et al., 2003). Homology search revealed that in Arabidopsis, this newly discovered inhibitor protein family includes at least 14 genes, which may encode either PMEIs or invertase inhibitors (Rausch and Greiner, 2004). However some inhibitors might also have other target proteins. Both invertase inhibitors and PMEIs contain four cysteins at the same conserved positions, which form two intra molecular disulphide bridges critical for protein folding (Greiner et al., 2000; Camardella et al., 2000). The members of this family are either inhibitors of PME or invertase but never both (Scognamiglio et al., 2003). Assigning function to any of the protein family cannot be done by comparison of aminoacid sequences, because of the common structural features between both PMEIs and invertase inhibitors. Two proteins at1g47960 and at3g17130 were shown to work as invertase inhibitors and they group with similar functionally characterized tobacco invertase inhibitors (Greiner et al., 1998; 1999) and at1948020 and at3g17220 termed as AtPMEI1 and AtPMEI2 were shown to function as PMEIs and they show 22% identity with other pro regions Arabidopsis PMEs, with the four cysteins present in the conserved positions, and were found to be predominantly expressed in pollen (Sebastian Wolf et al., 2003). This gives a speculation that pro-region could function as an auto-inhibitory domain. And as pro region is usually removed during PME maturation, it remains unclear whether AtPMEI1 and 2 interact with type I and/or type II PME enzymes. Further work on PMEI knockouts mutants can explain the possible roles of PMEI proteins during plant development.

PME activity is also regulated by hormones. Auxin-induced PME activity increases 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.

Recently it is thought that PME is subjected to intra molecular regulation by pro region. As noted earlier, the pro region shares some homology to PMEIs. Recent localization studies on tobacco (Nicotiana tabaccum) pollen-specific PME, \( \text{NtPPME1} \) reveal that the full-length product fused to green fluorescent protein is secreted, where as \( \text{NtPPME1} \) lacking the pro region is not, suggesting that the pro region participates in the correct targeting of the mature PME (Bosch et al., 2005). In addition, where as over-expression of the whole \( \text{NtPPME1} \) protein, including its pro-region, does not affect \textit{in vitro} pollen tube growth, transient expression of only the PME domain reduces the growth dramatically. Expression of the PME domain also leads to the accumulation of de-esterified pectins in the apical pollen tube wall, which appears to alter the rheological properties of the apical wall concomitant with the cessation of growth (Bosch et al., 2005). Importantly, the inhibitory effect caused by the PME domain can be partly rescued by co-expressing the pro-region. These experiments not only show that the PME domain alone is sufficient for exerting its enzymatic activity but also support the idea that the pro region acts as an intramolecular inhibitor of PME activity, thereby preventing the premature de-esterification of pectins prior to secretion. The predicted structural similarity between the pro-region and PMEIs (Pfam 4043; Bateman et al., 2004) substantiates this idea.

**Structure of pectin methyl esterases**

A better understanding of the pectin-PME interaction could be obtained by over-expression followed by analysis of the structure of PMEs using X-ray crystallography. The PMEs from phytopathogenic fungi and bacteria are expressed in heterogeneous lower eukaryotic (christgau et al., 1996) and prokaryotic systems (Laurent et al., 2000). Two PME three dimensional (3D) structures have been determined to date, the bacterial PME from \( \text{Erwinia chrysanthemi} \) pemA (Jenkins et al., 2001) (Fig.7a) and the plant PME from carrot (Johansson et al., 2003) (Fig.7b). In the frame of \textit{CAZy} classification system (http://afmb.cnrs-mrs.fr/~cazy/CAZY/CE_8.html), it belongs to the carbohydrate esterase family CE_8. Based on the proposed active sites it was possible to characterize the enzyme PME as aspartyl esterase. Both structures adopt the right-handed \( \beta \)-helix structural domain (In \textit{Erwinia} it is organized as a \( \beta \)-cylinder and in carrot PME it is organized as a \( \beta \)-prism) first found in pectin lyase (Yodar et al., 1993). The parallel \( \beta \)-helix has been divided
into seven classes consisting of all four pectinolytic enzymes. It was suggested by comparing their substrate binding sites that the right-handed parallel $\beta$-helix enzymes might have evolved from a common ancestor (Jenkins and Pickersgill, 2001).

The general structure of carrot PME and the bacterial enzyme from *Erwinia chrysanthemi* is very similar except for the presence of significantly longer loops in *Erwinia* and some differences in structure at termini. The plant PME at N-terminus has a short antiparallel $\beta$-strand corresponding to a loop in the *Erwinia* PME enzyme. The C-terminus in the carrot enzyme contains an additional helix and has a longer extended chain at the end than *Erwinia chrysanthemi* (Johansson et al., 2003).

![Crystal structures of (a) *Erwinia chrysanthemi* (bacterial) and (b) carrot (plant) Pectin methylesterase (PME).](image)

*Figure 7:* Crystal structures of (a) *Erwinia chrysanthemi* (bacterial) and (b) carrot (plant) Pectin methylesterase (PME).

The lack of data showing higher plant PME over-expression shows that, during export towards the apoplasm, plant isoenzymes probably undergo organism-specific post-translational processing that is necessary for their structural and functional integrity. As a consequence, the functional characterization of the PME-related genes identified to date is generally difficult and requires alternative methods based on the over-expression of the genes in heterologous plant systems (Gaffe et al., 1997). Since the sequence similarities among the plant PMEs is high and the residues at the active site are conserved, the structure of carrot PME can be considered a prototype for the whole family of plant PMEs and serve as a basis for future structure-function studies.

**PMEs are ubiquitous enzymes involved in growth and developmental processes in plants**

Several studies have shown a strong correlation between PME activity or PME gene expression and physiological processes such as hypocotyls elongation, fruit
ripening and maturation, cellular adhesion, seed germination, cambial cell differentiation, microsporogenesis and pollen tube growth. Other studies have shown that PME acts as methanol source and also as host-cell receptor for Tobacco Mosaic Virus (TMV). It was also shown to have a role in pollen ornamentation.

1. Seed germination and dormancy

PME was shown to break the dormancy and induce the seed germination in yellow cedar. In these seeds, GA3 was shown to have stimulatory effect on the activities of the PME isoform in embryo and megagametophyte (Ren and Kermode, 2000). It is hypothesized that PME plays a role in weakening of the megagametophyte allowing radicle emergence and the completion of germination.

2. Hypocotyl elongation

Purified PMEs from mung bean (*Vigna radiata*) hypocotyls cell walls were shown to have a role in hypocotyls elongation (Bordenave and Goldberg, 1993).

3. Stem elongation

Transgenic potato plants were transformed with a *Petunia inflata* derived cDNA encoding a PME in sense orientation under a constitutive promoter. This showed that the apex of the stem contained less PME activity than the wild type. Furthermore, during the early stages of development, stems of transgenic plants elongated more rapidly than those of the wild type (Pilling et al., 2000).

4. Stem nodule development

A differential display was applied to the early stages of the interaction between the tropical legume *Sesbania rostrata* and its symbiont *Azorhizobium caulinodans* ORS571 and a clone similar to PME encoding gene Srpme1 was isolated (Lievens et al., 2002), that was shown to have a role in vascular development and cell division or cell expression during stem nodule development.

5. Fruit ripening or maturation

Transgenic tomato fruits exhibiting over 90% inhibition of PME activity develop and ripen normally, yet show alterations in the cation binding capacity of cell walls, regulation of cation levels and maintenance of tissue integrity in ripening fruit, suggesting a significant role for PME in tomato fruit cell wall maturation (Tieman and Handa, 1994).
6. Cellular adhesion

During separation of the border cells of the root cap of pea, PME activity increases and is correlated with an increase in the amount of acidic pectin and a decrease in cell wall pH (Stephenson and Hawes, 1994). This study was performed using an antisense transgenic plant transformed with a PME gene (rcpme1) obtained by screening a root cDNA library (Wen et al., 1999). Analysis of transgenic plants showed that rcpme1 expression is required for the maintenance of extracellular pH, elongation of the cells within the root tip and for cell wall degradation leading to border cell separation. Further more PMEs were shown to help in cell separation during leaf and fruit abscission and pod dehiscence (Robert et al., 2000).

7. Cambial cell wall differentiation

Distribution of pectin methylesterases (PMEs) across the cambial region in active and dormant hybrid aspen (Populus tremula L. × Populus tremuloides Michx) was studied (Micheli et al., 2000). Isoelectrofocusing analysis revealed numerous isoforms that were differentially distributed according to the tissue-type and to the cambial stage. A neutral isoform was found to be a housekeeping isoform involved in the maintenance of the cell wall integrity throughout the stem. A basic isoform appeared to be a physiological marker of the dormant stage involved in the cessation of meristematic radial growth, whereas an acidic isoform was found to be functionally related to the immediate expansion of the cambial daughter cells that occurs bilaterally on each side of the cambium at the active stage.

8. Pollen and pollen tube elongation

PMEs must play a central role on growth of pollen tube wall which is composed mainly of pectin (Ferguson et al., 1998; Li et al., 1994). 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; Rogers et al., 2001, 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). Many works have shown that the PME has role either in tetrad dissolution of pollen grains (Albani et al., 1991), or in pollen ornamentation (Lacoux et al., 2003) or in pollen tube germination or elongation. Exploration of the specific effects of PMEs on the pollen tube growth, however is just the beginning, with one study showing the requirement for the endogenous VGD1
PME for the growth of Arabidopsis pollen tubes (Jiang et al., 2005) and another reporting inhibition of growth of pollen tubes of Lilium formosanum by exogenously added tobacco PME NtPPME1 (Bosch et al., 2005). In both the above studies, VGD1 and NtPPME1 contains a pro region that is similar to PMEI domain, and their disruption effects pollen tube stability, growth, silique morphology and plant fertility. But the recent work with AtPPME1 that contains no pro region was also shown to affect the pollen tube by reducing the growth and leading to irregularity in tube shape suggesting functional differences between different pollen-specific PMEs.

**Apical Wall Dynamics in Pollen Tubes**

Gene expression data from from a comparative Arabidopsis transcriptome study using the affymetrix genome array, which contains 60 of the 66 open reading frames that have been annotated as PMEs, show that at least 18 PME isoforms are expressed in Arabidopsis pollen (Pina et al., 2005) (Fig.8). From a total of 1584 expressed genes in Arabidopsis pollen, 8 PME isoforms are among the 50 highest expressed transcripts, underlining the importance of PMEs for pollen germination and/or pollen tube growth.

Pollen tube growth, which is fast and restricted to the extreme apex of the tube, involves a massive secretion and assembly of new plasma membrane and cell wall. Throughout this process, a balance point must be achieved in which the apical cell wall is plastic enough to allow wall stretching and the incorporation of these new wall components, while at the same time rigid enough to withstand the high internal turgor pressure (e.g., 0.2Mpa in Lilium longiflorum pollen tubes; Benkert et al., 1997) and prevent bursting. The apical tube wall of pollen is exclusively composed of pectic network (Ferguson et al., 1998). The traditional load-bearing components, consisting of a cellulose-hemicellulose framework (O’Neill and York, 2003), can only be found in the subapical and more distal parts of the tube wall (Taylor and Hepler, 1997). Consequently, the pectic network represents the load-bearing component at the pollen tube apex. Not surprisingly, PMEs emerge as potential key regulators of pollen tube growth since these enzymes determine the rheological properties of the apical pollen tube wall.

Evidence for a differential distribution of PME activity can be obtained from examining the localization of methylesterifies pectins along the pollen tube wall. Two monoclonal antibodies, JIM5 and JIM7 (Knox et al., 1990), have been particularly useful in resolving the patterns of esterification. JIM5 binds preferably to at least four contiguous unesterified GalA residues and labels the relatively de-esterified pectin
epitopes. By contrast, JIM7 binds to methylesterified residues with adjacent or flanking unesterified GalA residues and therefore indicates the presence of relatively high methylesterified pectin epitopes (Clausen et al., 2003). In general esterified pectins are more abundant in the apical region, while deesterified pectins are more concentrated in the areas away from the tip (Li et al., 1994; Bosch et al., 2005; Parre and Geitmann, 2005).

Further studies supporting a role of PME in pollen tube growth involved the application of pollen tube growth media supplemented with orange peel PME. The results reveal that exogenous PME inhibits in vitro growth (Bosch et al., 2005; Parre and Girtmann, 2005) and cause a significant thickening of the apical cell wall and dissipation of the intracellular tip-focused Ca\(^{2+}\) gradient (Bosch et al., 2005). As can be expected, exposing pollen tubes to such enhanced extracellular PME activities converts apical methylesterified pectins into deesterified pectins (Parre and Geitmann, 2005), which will readily form a stiff calcium-mediated pectate gel at the tip, preventing turgor driven elongation and instead leading to cell wall thickening. Support for this comes from microindendation experiments showing that treatment with exogenous PME leads to a dramatic increase in the apical cellular stiffness and a decrease in the visco-elastic behavior (Parre and Geitmann, 2005). These data show that the PME-mediated configuration of pectin at apex is an important component in controlling pollen tube growth.

**Figure 8:** Analysis of PME isoforms encoded in the Arabidopsis genome. (a) The total relative expression level of the PME isoform in a certain organ or cell type. (b) Organ and cell-type specificity of the PME isoforms.

Further studies supporting a role of PME in pollen tube growth involved the application of pollen tube growth media supplemented with orange peel PME. The results reveal that exogenous PME inhibits in vitro growth (Bosch et al., 2005; Parre and Girtmann, 2005) and cause a significant thickening of the apical cell wall and dissipation of the intracellular tip-focused Ca\(^{2+}\) gradient (Bosch et al., 2005). As can be expected, exposing pollen tubes to such enhanced extracellular PME activities converts apical methylesterified pectins into deesterified pectins (Parre and Geitmann, 2005), which will readily form a stiff calcium-mediated pectate gel at the tip, preventing turgor driven elongation and instead leading to cell wall thickening. Support for this comes from microindendation experiments showing that treatment with exogenous PME leads to a dramatic increase in the apical cellular stiffness and a decrease in the visco-elastic behavior (Parre and Geitmann, 2005). These data show that the PME-mediated configuration of pectin at apex is an important component in controlling pollen tube growth.
Genetic proof for the critical role of PME activity in regulation of pollen tube growth came from a study in which an Arabidopsis pollen specific PME, VGD1 (Jiang et al., 2005) when disrupted lead to burst of mutant pollen invitro suggesting that the decrease in the over all PME activity leads to an imbalance of the apical wall dynamics, resulting in failure to resist the internal turgor pressure. Initial in vivo growth of the mutant tubes on the surface of stigmatic cells is not affected, however, growth is greatly retarded in the style and transmitting tract. Despite this retardation, a few tubes manage to fertilize ovules in the upper part of the silique (Jiang et al., 2005). Mutant tubes may perceive enough structural support from the surrounding female sporophytic tissues to partially overcome the severe phenotype observed in vitro. Also the presence of endogenous PME activity in the transmitting tract might contribute to less severe phenotype observed in vivo. This indicates that the existing equilibrium of the apical cell wall dynamics in providing both support and plasticity is very delicate and that a minor disturbance of this equilibrium compromises pollen tube growth (Bosch and Hepler, 2005).

Although it is apparent that PMEs are important for controlling pollen tube growth, there are still many open questions. For instance, are all the pollen-expressed PME isoforms redundant or do different isoforms employ different functions? Experiments in which two Arabidopsis pollen-expressed PME isoforms (AT2g47030 and At 3g62170) with high aminoacid identity to vgd1 (85 and 69% respectively) were placed under the control of the VGD1 promoter showed that only At2g4703 was able to complement the VGD1 mutant phenotype, while no complementation was observed for At3g62170 (Jiang et al., 2005). These data, together with the notion that sequence identities between many different isoforms are likely to be lower than in the above example, suggest that many isoforms are not merely redundant. Rather, the differences in pIs, presence of a pro-region, different pH optima, and different substrate specificities and action mechanism at a certain pH among the different isoforms are likely to contribute to certain isoform specificities.

A role for pectin/PME in oscillatory pollen tube growth

An interesting property of pollen tube growth is that the rate oscillates both in vivo (Iwano et al., 2004) and in vitro (Holdaway-Clarke and Hepler, 2003). Since changes in the yielding properties of the cell wall could underline these changes in rate, it is reasonable to imagine that modifications of pectin are a controlling factor (Holdaway-Clarke et al., 1997). One potential regulation of PME activity at the tip involves a negative feedback, in which the local decrease in pH generated by protons
released during the de-esterification reduces PME activity (Moustacas et al., 1991). A
decrease in pH would also be expected to activate enzymes such as
polygalacturonases and pectate lyases. The combined effect of inactivating
polygalacturonase and pectate lyase would loosen the cell wall and facilitate a growth
pulse. However the subsequent dilution of negative charges would increase the pH and
cause a reactivation of PME and inactivation of polygalacturonase and pectate lyase,
leading toward an apical wall stiffening and a decrease in growth rate. Thus, PME,
together with other pectin-associated enzymes, could play a key role in controlling
oscillations in pollen tube growth (Fig. 9).

There are different lines of evidence that support the above scenario. First the
apical wall thickness in lily pollen tubes, which has been shown to oscillate with the
same frequency as growth, becomes thickened in anticipation of the next growth rate
increase (Holdaway-Clarke and Hepler, 2003). These changes might represent an
oscillatory behavior of the apical wall dynamics mediated by PME activity. Second,
polygalacturonases and pectate lyase-related transcripts are highly expressed in
Arabidopsis pollen (Pina et al., 2005), suggesting a function in pollen germination and
tube growth.

![Potential feedback mechanism for the regulation of apical PME activity in
pollen tubes that could contribute to an oscillatory growth pattern. PG-
polygalacturonase; PL-pectate lyase](image-url)

Fig. 9: Potential feedback mechanism for the regulation of apical PME activity in
pollen tubes that could contribute to an oscillatory growth pattern. PG-
polygalacturonase; PL-pectate lyase
Figure 10: Regulation of PME activity in pollen tubes.
Regulation of PME by its pro-region, PMEIs and oscillating ion fluxes. (1) The pro-region functions as an intramolecular inhibitor of PME activity, preventing the premature deesterification of pectins prior to secretion. (2) Expression of only the PME domain can lead to premature, intravesicular, demethylesterification of pectins, causing inhibition of tube growth. (3) It remains to be seen if PMEIs can inhibit premature deesterification of PME isoforms lacking a pro-region. (4) Pectins are secreted in a highly methylesterified state. (5) Removal of pro-region by cleavage of pro-PMEs is necessary for the PME domain to become enzymatically active. (6) The active PME domain catalyses the demethylesterification of the pectins. (7) Ca\(^{2+}\) binds co-operatively to the free carboxyl groups which causes rigidification of the cell walls. (8) PMEIs are able to inhibit the PME activity in the cell wall by forming 1:1 reversible complex. (9) The oscillatory influx of H\(^+\) and Ca\(^{2+}\) ions, which maintain the apical acidic domain and the tip-focused Ca\(^{2+}\) gradient, respectively through the apical cell wall might directly influence the PME activity, the conditions necessary for cleavage of pro-PME and the formation of the pH-dependent PMEI-PME complex. (10) The same is true for the oscillatory efflux of H\(^+\) ions maintaining the alkaline band at the base of the clear zone.
Other factors might include the endogenous oscillations of intracellular H+, which could participate in the regulation of PME activity or vice versa. Pollen tubes possess two domains that oscillate in pH intensity: an acidic domain at the extreme tip and an alkaline band at the base of the clear zone (Feijo et al., 1999; Cardenas et al., 2005), an apical area in which the golgi derived secretory vesicles accumulate and to which larger organelles are excluded. The acidic tip is thought to be maintained by a localized proton influx at the apex, while the alkaline band is maintained by a localized proton efflux, presumably under the control of plasma membrane proton ATPase. These proton fluxes are likely to affect the pH in specific cell wall region, which in turn might influence the activity of PMEs in these regions. As mentioned before the stability of the interaction between PME and PMEIs is pH dependent, as might also be the interaction of the pro region with the PME domain. A local change in pH may even activate specific proteases that process the pro-region and release the active PME domain. It will be interesting to see if the pectin methyl esterase pattern, such as seen in the figure.10, can be attribute to specific pH conditions in the cell wall that are generated by the proton fluxes. However, the rapid turnover of the cell wall material at the apex in oscillating tubes makes pH measurements in this region a particular challenge that still needs to be resolved.

Pollen tubes also display an intracellular tip-focused Ca2+ gradient whose magnitude oscillates markedly. This gradient is thought to be maintained by the influx of the extracellular Ca2+ across the plasma membrane. It has been suggested that both Ca2+ and H+ enter at tip through the same stretch-activated cation channels (Holdaway-Clarke and Hepler, 2003). This might set the stage for a cyclic event in which PME-mediated oscillations in apical wall yielding determine the status of stretch-activated channels. The resulting ion fluxes generated by these channels might in turn affect the activities of PMEs and, thus, wall yielding.

9. Methanol source

In 1998, a close correlation was reported between PME activity and levels of methanol in fruit tissues from both wild-type tomato and PME antisense mutant, indicating that PME is on the primary biosynthetic pathway for methanol production in tomato fruit (Frenkel et al., 1998). Because methanol oxidation to CO2 could result in the incorporation of methanol carbon into metabolites via the Calvin-Benson cycle, PMEs could play an appreciable, albeit indirect, role in the photosynthetic metabolism of the plant.
10. Laticifer development

The poppy laticifer system develops through the gradual disappearance of the common wall between differentiating laticifer element throughout the plant. Gene homologous for cell wall degrading enzymes was found during random sequencing of an opium poppy latex cDNA library (Pilatzke-Wunderlich and Nessler, 2001). PME, Pectin acetyesterase (PAE) and pectin lyase (PL) were highly expressed and latex specific. Enzyme assay confirmed abundance of this transcript encoding pectin-degrading enzyme in latex and suggests that these enzymes may play an important role in laticifer development.

11. Cell to cell systemic movement of Plant virus

PME was shown to be host-cell receptor for the Tobacco Mosaic Virus (TMV) movement protein (Dorokhov et al., 1999; Chen et al., 2000). Thus, the interaction between the virus movement protein and the PME is required for the viral cell-to-cell movement through plasmodesmata. One hypothesis proposed is that binding of the TMV movement protein interferes with PME activity, altering the cell wall ion balance and consequently inducing changes in the permeability of the plasmodesmata (Chen et al., 2000).

Future prospectives

It is apparent that PMEs are essential enzymes involved in many plant physiological processes, including, and maybe in particular, pollen tube growth. Many of the players involved in the regulation of PME activity have been identified. However, resolving the full impact of PME activities in planta requires much more attention. It will be important to unravel the respective functions and action patterns of the array of PME isoforms. The local cell wall properties constitute a major determinant for the PME activity profile. Therefore, advanced probing techniques need to be implemented to obtain information about local wall conditions and link them to the corresponding methyl-esterification patterns observed in these wall areas.

Probably one of the most potent regulators of PME activity is the PMEIs, yet very little is known about the spatial and temporal regulation of these proteins and their interaction with PMEs in vivo. It will be interesting to see if PMEIs, analogous to the action of the pro-region, can prevent premature deesterification of pectins before these are secreted or if complex formation between PMEs and PMEIs is limited to the apoplastic space. A recent model proposed for the interaction between PME and PMEI also allowed convenient intramolecular binding of PMEI homologous pro-region to the
PME domain (Hothorn et al., 2004). Although the importance of the pro-region has been recently established, future studies need to explore their mode of action in more detail. The inhibitory potential of pro-regions needs to be tested \textit{in vitro} with PME activity assays, while co-localization studies with antibodies against the pro-region and the PME domain, as well as fluorescence resonance energy transfer analysis of pro-PMEs, will provide crucial spatial and temporal information (Bosch and Hepler, 2005).

Although many questions still need clarification, it is evident that the enzymatic activity of PME plays a central role in the control of cell wall properties and, thus, in cell growth and development. The pollen tube given its extensive investments in the pectins and its rapid growth rates constitutes a favorable system to examine the role of PME in wall dynamics and cell elongation. While we must not ignore the contribution of other factors in determining the cell wall properties, advances made in current research define PMEs as critical enzymes in the control of pectin dynamics. Thus, it is not surprising that a complex regulatory network emerges that carefully orchestrates PME activity (Bosch and Hepler, 2005).
Materials and Methods

I. Materials

Chemicals enzymes and Instruments

The instruments, chemicals and enzymes used in this study were same as mentioned in chapter 1.

Library used

Rice (indica cv pusa basmati) pre-pollinated spike cDNA library cloned in the lambda-ZAP express vector was obtained as a gift from Prof. Akhilesh K. Tyagi, Delhi University South Campus (DUSC).

Plasmid constructs and vectors used

1. The Plasmid pUCNos harboring NOS terminator in pUC19 vector constructed earlier in the lab was used for cloning OsiPME1 in plant expression vector. (Fig.11)

2. The plasmid pSK50 is a binary vector (10 kb). The 2.0 kb Ubiquitin promoter obtained by digesting this construct with HindIII fragment was used to drive the expression of OsiPME1 in either sense or antisense orientation.

Figure.11. Linear map of pUCnos (3.0 Kb)
II. Methods

1. Bacterial culture and plasmid purification

Bacteria \textit{E.coli} containing the plasmids was grown in Luria Broth, and the plasmid was extracted by alkali lysis method and the pure plasmid was prepared by PEG precipitation according to the methods described in chapter I and used for bombardment.

2. Molecular methods

Cloning and sequencing

General restriction analysis, agarose gel electrophoresis, electro-elution, ligation, transformation cloning and sequencing were done according to the methods given in the Chapter I.

Extraction and quantification of genomic DNA and RNA

Total RNA and DNA were extracted from rice explants as per the methods of Dellaporta \textit{et al.}, (1983) and with TRIreagent respectively. Details of the procedures are given in the chapter 1.

Southern analysis

Southern analysis was performed following the procedure given in the chapter I. Total DNA from the rice leaves was extracted and digested with appropriate enzymes. The digested samples were resolved on 0.8% agarose gel and blotted onto positively charged nylon membrane by downward capillary blotting. The blots were probed with appropriate radiolabelled DNA probes. After post hybridization washes, the blots were exposed to X-ray film for autoradiography.

Northern analysis

Northern analysis was performed according to the procedure given in chapter I using NorthernMax kit (Ambion, U.S.A) as per the manufacturers instructions. The total RNA was size fractionated on 1% formaldehyde gel and transblotted onto positively charged nylon membrane using downward capillary method and probed with the radiolabelled cDNA fragments. After post hybridization washes, the membrane was exposed to X ray film for autoradiography.
Phage titrations and isolation of single plaque

Isolation of single isolated plaques and preparation of phage lysate from pre-pollinated spike cDNA library of rce was performed according to the method described in chapter II.

Phage excision

Phage excision was performed using Rapid excision kit (Stratagene) as per manufacturer's instructions. (Courtesy Dr. Akhilesh Tyagi's lab, DUSC, India).

3. Plant growth conditions and stress treatments

Rice seedlings were grown as described in chapter 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 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 100µM of salicylic acid (SA) to mimic biotic stress. The panicles were treated with 100 µM of various hormones like IAA, IBA, 2,4D, NAA, BAP and 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. Abscisic 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 50mM of various sulphates or chlorides of Zinc, Cadmium, Mercury and Calcium.

4. Bioinformatic analysis

The BLASTN search was performed using the nr database at NCBI (http://www.ncbi.nlm.nih.gov/Blast/). Various tools from Expasy (http://www.expasy.org/tools) were used to deduce the translated product and compute theoretical pI and Molecular weight. The putative domains were identified using the interproscan search (http://www.ebi.ac.uk/interproscan/) and SMART tool search (http://smart.embl-heidelberg.de/). 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(s) in the in the rice
genome were predicted at GRAMENE (http://www.gramene.org/db/searches/blast). Multiple sequence alignments involved use of ClustalW (http://www.ebi.ac.uk/clustalw). The cis-acting regulatory elements in the promoter region were predicted using PLACE database (http://www.dna.affrc.go.jp/PLACE).
Results

In this section, the isolation and expression analysis of a PME from rice, designated as OsiPME1 (*Oryza sativa* Pectin Methyl Esterase 1) is described along with the gene structure and the phylogenetic analysis of Rice Pectin Methyl Esterases (OsPME’s) in general.

1. Isolation of OsiPME1

A small number of random clones from rice pre-pollinated spike cDNA library 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 XhOl (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 containing 1.6 kb insert was end sequenced using T3 and T7 primers, which yielded 628 bp (5' end sequence) and 637 bp (3' end sequence) sequences respectively, leaving an intermediate region of ~370 bp unsequenced. *In silico* analysis of these sequences predicted the presence of start codon in the 5' end sequence and stop codon in the 3' end sequence, thus indicating that the cDNA insert may be of full length. This cDNA clone was designated as pAKV1 (Fig. 12).

Detailed restriction analysis of pAKV1 with the enzymes present in the multiple cloning site (MCS) of PBK-CMV phagemid, revealed the presence of BamHI (~900 bp) and Sacl (~1260 bp) sites in the cDNA (Fig. 13). pAKV1 when digested with BamHI released a 900 bp fragment, containing ~280 bp unsequenced region from a 5.1 kb vector backbone with 700 bp of cDNA insert containing ~80 bp of unsequenced region. Therefore, to complete the total sequence, the 900 bp fragment obtained by digesting pAKV1 with BamHI was subcloned into pBKS+ to obtained pPE1 construct (Fig. 14) and the 5.1 kb vector backbone was self ligated to obtain pPEself (Fig. 15). The cloning of the 900 bp fragment in pPE1 was confirmed by restriction analysis with BamHI (Fig. 16) and the pPEself clone was confirmed by restriction analysis with BamHI/XhOl (Fig. 17). Both the clones were sequenced using T3 primer, to obtain the sequences of unsequenced region. All the sequences obtained were overlapped to complete the
whole 1.6 kb sequence. BlastP analysis of this 1.67 bp region indicated that it contained a 1302 bp of full length gene (with Poly A tail) coding for putative pectin methyl esterase (PME), followed by a 368 bp of 3’ end sequence of chlorophyll a/b binding protein (Fig.18). This might have resulted because of abberation during cDNA library construction. For further analysis, the 368 bp region coding for chlorophyll a/b binding protein has to be excluded. For this, the pAKV1 plasmid was digested with Sacl, that released a 1.267 kb fragment containing the cDNA region encoding for putative PME (with missing 25 bp 3’ UTR). The released 1.267 kb fragment was electro-eluted and cloned into the corresponding Sacl site of pOK12 vector to obtain pOKpec’S’ (Fig.19) and pOKpec’A’ (Fig.20) in which the cDNA fragment was cloned in either sense or antisense orientation respectively. The orientation of the clones was confirmed by restriction analysis with EcoRI that linearised pOKpec’S’ plasmid and released 1.267 kb fragment from pOKpec’A’ plasmid (Fig.21).

2. Bioinformatic analysis

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) 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.12).

Computational translation of the cDNA clone performed by using expasy TRANSLATE tool, indicated that the 1038 bp ORF of the OsiPME1 encodes for a 345 aminoacid peptide with a predicted molecular mass of 37.994 kDa and theoretical pl 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 (Table.I). This result suggests that the isolated gene may be coding for a putative Pectin methyl esterase from rice and hence designated as OsiPME1 (Oryza sativa Pectin Methyl Esterase 1).

Apart from Arabidopsis PMEs, it also showed some identity with the aminoacid 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), Medigaco truncatula (34% Q1RUM0, Fabaceae) and Salsola (33%, Q17ST3, Amaranthaceae) (Table.II).
Table I: Blastp results for Os/PME1 (uniprot, www.ebi.ac.uk).

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<th>Length (aa)</th>
<th>Identity %</th>
<th>Similarity %</th>
<th>Overlap (aa)</th>
<th>E-value</th>
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Table II. Homology of Os/PME1 to other plant PME’s.

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Phylogenetic analysis was performed using the PME sequences from different species like *Medicago, Brassica, Physcomitrella, Salsola, Lupinus, Tomato, Carrot, Tobacco, Arabidopsis, Maize, Aspergillus Neurospora* and *Erwinia* along with Os/PME1 polypeptide sequence. For this, the PME sequence from all the species listed above were retrieved from the GenBank and multiple alignment was performed using ClustalW (www.ebi.ac.uk/clustalw), which revealed that the structural arrangement GXYXE and GXXDFIF found in the active groups of all PME’s is also conserved in Os/PME1 (Fig.23). The phylogenetic tree obtained from this alignment (Fig.24) revealed the conserved nature of the PME’s during evolution. In addition, Os/PME1 gene product showed a 20 aminoacid residues long N’ signal peptide (signalP).

The folding of PME protein into parallel beta helix structure is important for carrying out its activity. In this study we attempted to analyze the putative 3D structure Os/PME1 using Modbase. The analysis indicated that it showed 28% identity to Igg84 template (PME from carrot) and 17.00% 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. 25. A, B, C, D and E) with a central hollow cylindrical structure lined by several aromatic residues. The structure also revealed that the aminoacid residues at the active site and those involved in ligand binding are conserved (Fig.26). All the aminoacid residues predicted to be involved in ligand binding could be located on the central region of the cleft (Fig.27).

To gain insight into the Os/PME1 gene structure, full-length cDNA sequence of Os/PME1 was blasted against the *Oryza sativa* genome sequence database (NCBI). The sequence showed 100% identity to sequences of single contig NT107192.1 (NCBI) from chromosome 3 of *indica* rice representing essentially the same gene. To localize Os/PME1 gene in rice genome, its cDNA sequence was used as query for BLAST search against nipponbare (*Oryza sativa* L, *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 Os/PME1 sequence. According to the physical map of the Nipponbare, Os/PME1 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.29.C). Comparison of the full-length cDNA sequence with the corresponding genomic sequence of Os/PME1, showed the presence of four-intron/five-exon gene structure
with intron sizes ranging from 97-243 bp and exon sizes ranging from 157 bp to 266 bp. These results indicate that OsiPME1 may exist as a single copy on the upper arm of chromosome 3.

3. Molecular analysis of OsiPME1

Copy number in genome

In order to find out the copy number of OsiPME1 in the rice genome, genomic southern blot analysis was performed. For this, 5 µg of total genomic DNA, extracted from the rice leaves was digested with HindIII (no restriction site in introns or exon region), KpnI (no restriction site in introns or exons region) and SacI (single site in the cDNA region) to completion (Fig.29.A). Digested DNA was resolved on a 0.8% agarose gel, transblotted onto a positively charged nylon membrane and probed with radiolabelled 1302 bp fragment obtained by digesting pOKpec’S’ with SacI. The probe hybridized with an 11.0 kb fragment, ~9.0 kb fragment and ~3.5 kb fragment generated by digestion with HindIII, KpnI and SacI respectively (Fig.29.B). These observations suggest that OsiPME1 exist as a single copy in the rice genome.

Expression profile

In silico analysis of ~1.5 kb genomic sequence upstream to the transcriptional start site of OsiPME1 (retrieved from the sequence of contig NT107192.1 of indica rice showing 100% identity to OsiPME1 cDNA) using PLACE database predicted the presence of several cis-acting regulatory elements like ABRE (Abscisic acid responsive element), DRE (Drought responsive element), ERE (Ethylene responsive element), LTRE (Low temperature responsive element), W-box, WRKY, GT-motif, Pollen 1 motif (Summarised in Table.III). Therefore, in this study, an attempt has been made to validate the functionality of responsive elements experimentally.

Table III. Putative cis-acting regulatory elements predicted by PLACE database in OsiPME1 5’ upstream regulatory sequences.

<table>
<thead>
<tr>
<th>S.N o</th>
<th>Element</th>
<th>Sequence</th>
<th>Response element for</th>
<th>Position</th>
<th>Reference</th>
</tr>
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<tr>
<td>1</td>
<td>ABREOSRAB21</td>
<td>ACGTSSSC</td>
<td>Abscisic acid</td>
<td>-1435</td>
<td>Marcotte et al., 1989</td>
</tr>
<tr>
<td>2</td>
<td>DRECRTCOREAT</td>
<td>RCCGAC</td>
<td>Dehydration</td>
<td>-172</td>
<td>Dubouzet et al., 2003</td>
</tr>
<tr>
<td>3</td>
<td>ERELEE4</td>
<td>AWTTCAA A</td>
<td>Ethylene</td>
<td>-890, -859, -726</td>
<td>Itzhaki et al., 1994</td>
</tr>
</tbody>
</table>
Since, in the 5’ upstream regulatory region, pollen specific response elements like GT-motif and Pollen-1 motif were predicted, northern blot analysis was performed to determine the organ-specific expression of Osi\textit{PME1}. For this, total RNA was extracted from seedling (8-day old), shoots, roots and inflorescence (90-day old plant). 5\(\mu\)g of total RNA was size fractionated on 1% formaldehyde gel, transblotted onto a positively charged nylon membrane and probed with radiolabelled 1267 bp of Osi\textit{PME1} cDNA obtained by digesting pOKpec’s’ with SacI. The probe hybridized only with the RNA from pre-pollinated and post-pollinated Inflorescence indicating that Osi\textit{PME1} gene expresses only in the flowers under normal conditions (Fig. 30.A).

\textit{In silico} analysis predicts Osi\textit{PME1} to be pollen-specific. To test this hypothesis, total RNA from lemma & palea and pollen grains from pre-pollinated- (opened flowers with un-dehisced anthers) and post-pollinated- (closed flowers with dehisced anthers) flowers was extracted and northern analysis was performed as described above. The probe hybridized only to the RNA from pollen grains of both pre-pollinated and post-pollinated Inflorescence indicating that Osi\textit{PME1} gene expresses only in the flowers under normal conditions (Fig. 30.A).
stage (6 DAF) at which mature and viable pollen grains are formed (Fig.30.C). These results suggest that OsIPME1 expresses specifically in pollen grains during their later stages of development.

**Effect of Heat, Cold, NaCl, Mannitol, Dehydration, Submergence and Salicylic acid on the expression of OsIPME1**

An attempt has been made to determine the effect Heat, Cold, NaCl, Mannitol, Dehydration, Submergence and Salicylic acid on the expression of OsIPME1. For this, total RNA was extracted from the pre pollinated panicles from 120-day old rice plants were treated with various stresses like salt, drought, desiccation, submergence, heat, cold and salicylic acid. 5 µg of total RNA from all the samples and resolved on a 1% formaldehyde gel, transblotted onto a positively charged nylon membrane and probed with 1267 bp OsIPME1 cDNA obtained by digesting pOKpec’S’ with Sacl. The probe hybridized differentially with the RNA from different samples indicating the differential regulation of OsIPME1 by various signals. The results indicated that OsIPME1 mRNA levels were upregulated by Mannitol treatment, downregulated by heat, cold, desiccation, submergence and salicylic acid and unaltered by NaCl treatment (Fig.31.A).

**Effect of heavy metals on OsIPME1 expression**

An attempt has been made to determine the effect various heavy metals on the expression of OsIPME1. For this, total RNA was extracted from the pre pollinated panicles from 120-day old rice plants were treated with 50 mM mercury, copper, cadmium and Zinc salts. 5 µg of total RNA from all the samples and resolved on a 1% formaldehyde gel, transblotted onto a positively charged nylon membrane and probed as described above. The results indicated that OsIPME1 mRNA levels were downregulated by Cadmium and Zinc and unaltered by Mercury and Copper (Fig.31.B).

**Effect of phytohormones on OsIPME1 expression**

An attempt has been made to determine the effect various phytohormones on the expression of OsIPME1. For this, total RNA was extracted from the pre pollinated panicles from 120-day old rice plants were treated with IAA, IBA, 2,4-D, NAA, BAP and GA3. 5 µg of total RNA from all the samples and resolved on a 1% formaldehyde gel, transblotted onto a positively charged nylon membrane and probed as described above. The results indicated that OsIPME1 mRNA levels were upregulated by all
auxins (IAA, IBA, 2,4-D and NAA), downregulated by GA₃ and unaltered by BAP (Fig.31.C). OsiPME1 transcript accumulation was high with IBA and NAA treatments than with IAA and 2,4-D treatments.

**Effect of ABA on OsiPME1 expression**

An attempt has been made to determine the effect stress hormone, Abscisic acid (ABA) on the expression of OsiPME1. For this, total RNA was extracted from the pre pollinated panicles from 120-day old rice plants were treated 10 µM and 100 µM ABA. 5 µg of total RNA from all the samples and resolved on a 1% formaldehyde gel, transblotted onto a positively charged nylon membrane and probed as described above. The results indicated that OsiPME1 mRNA levels were upregulated with ABA treatment. However, the transcript accumulation was more with 100µM ABA. Therefore, OsiPME1 may be regulated by ABA in dose dependent manner (Fig.31.D).

4. **Construction of plant expression vector with OsiPME1**

Con*struction of plant expression vector with OsiPME1 in sense orientation*

Towards understanding the functional role of OsiPME1 in planta, plant expression vectors expressing the gene constitutively in sense under Ubiquitin promoter and NOS Terminator was constructed in pUC19 based vector. For this, the 1.267 kb fragment obtained by KpnI/XbaI digestion of pOKpec’S’ was cloned into the corresponding sites of pUCNOS (Fig.11) to obtain pUCNOSpec’S’ (Fig.32). The cloning was confirmed by restriction analysis with KpnI (linearize) and EcoRI (releases 1.5 kb fragment) (Fig.33, lane 1, 2). Later a 2.0 kb ubiquitin promoter obtained as a HindIII digested product from a binary vector pSK50 was cloned into the HindIII site of pUCNOSpec’S’ obtain pUbpec’S’ (Fig.34) (expressing OsiPME1 constitutively in sense orientation). The clone with proper orientation of the ubiquitin promoter was selected after restriction analysis with KpnI (linearize) and EcoRI (releases 1.5 kb fragment (Fig.35. lane 3, 4).

**Construction of plant expression vector with OsiPME1 in antisense orientation**

Towards understanding the functional role of OsiPME1 in planta, plant expression vectors expressing the gene constitutively in antisense under Ubiquitin promoter and NOS terminator was constructed in pUC19 based vector. For this, The 1.267 kb fragment obtained by KpnI/XbaI digestion of pOKpec’A’ was cloned into the corresponding sites of pUCNOS (Fig.11) to obtain pUCNOSpec’A’ (Fig.36). The
cloning was confirmed by restriction analysis with KpnI (linearize) and EcoRI (releases 1.2 kb and 0.3 kb fragments) (Fig.33, lane 3, 4). Later a 2.0 kb ubiquitin promoter obtained as a HindIII digested product from a binary vector pSK50 was cloned into the HindIII site of pUCNOSpec'A' to obtain pUbpec'A' (Fig.37) (expressing OsiPME1 constitutively in antisense orientation). The clone with proper orientation of the ubiquitin promoter was selected after restriction analysis with KpnI (linearize) and EcoRI (releases 1.2 kb and 0.3 kb fragments) (Fig.35, lane 1,2). These clones would be used further for Plant transformation studies to gain insight into the in vivo functions of OsiPME1.

II. Genome wide analysis of Pectin Methyl Esterase (PME) gene family from Oryza sativa (OsPME).

In this study, genome-wide survey of genes encoding proteins possessing PME catalytic domain and/or PMEI domain, named PME gene family, has been carried out in rice. The domain structure, genomic distribution and gene architecture of the encoded PME proteins in rice were studied along with their phylogenetic relationship with Arabidopsis PMEs.

In silico analysis

Putative PME domain containing proteins were extracted from the NCBI protein database using ‘Pectin Methyl Esterase’ as keyword. Apart from this, all the genes annotated as putative PME were retrieved from different databases like SMART, pfam and Interproscan. In total, 132 proteins annotated as PME were obtained (SMART search-29; pfam search-47 and Interproscan search 56). Pairwise alignment of all the retrieved sequences were done and the identical genes from the same contig but annotated with different putative names in the various databases were identified and removed. After filtering out, a total of 51 putative OsPME’s were identified in Oryza sativa subsps japonica. Only one putative OsPME was identified in Oryza sativa subsps indica (OsiPME1, discussed in the earlier sections), identical to one of the annotated putative OsPME from japonica. The nucleotide length, number of introns, predicted amino acid length and chromosomal location of each rice gene were obtained from GRAMENE database (http://www.tigr.org/). The presence PME and/or PMEI domain, their length and organization in each sequence was characterized by
domain search in the SMART (http://www.smart.embl-heidelberg.de/) and InterPro database (http://www.ebi.ac.uk/interpro/).

**Classification of OsPME gene family**

Aminoacid sequence analysis of these proteins using the SMART tool (Letunic *et al.*, 2004, Schultz *et al.*, 1998) showed that, 31 of them contain only the catalytic PME domain (represented as Ospro # in phylogenetic analysis for convenience), while 20 of them also contain a PME inhibitor (PMEI) domain (represented as OsPMEI #). Thus, *Oryza sativa* PME’s can be subdivided into two general groups based on their domain structure (Table.IV). Group I includes smaller protein with 189-540 aminoacid residues (with exception like 71 aa residues and 867 aa residues) and a predicted molecular mass of 17-57 kDa that contain a PME domain (exceptions include 7.97 and 96.67 kDa proteins), and group II comprises larger proteins with 336-971 aminoacid residues and a molecular mass of 35-105 kDa, that include in addition to a catalytic PME domain, an extra PMEI domain also. Only one protein (Q01UA3) is of 2446 aa residues long and with a molecular mass of 265.3. This protein contains PMEI and catalytic PME domains at its N’ end, followed by many leucine rich repeats and a C’ protein kinase domain.

**Genomic structure of OsPMEs**

0-2 intron gene structure patterns was generally conserved in the group II OsPME’s with an exception of Q01UA3 with 10 introns. Whereas, the intron structure is not conserved in Group I. 0-6 introns were found in the genes belonging to Group I (Table.IV).

**Biochemical features of PMEI and PME domains of OsPME’s**

An attempt was made to understand the biochemical nature of the predicted PMEs. For this the molecular weight and theoretical pl of all the catalytic PME domains of all the 51 OsPMEs and molecular weight and pl of PMEI domain of all the 20 group II OsPMEs was predictected using expasy tools. It was observed that most of the OsPME isoforms encode a PME domain with predicted molecular mass between 10-37 kDa and alkaline pl (28/51). Neutral (15/51) and acidic (8/31) isoforms were also observed. Whereas, the Group II OsPME’s encode PMEI domain of molecular mass ranging from 15-20 kDa and acidic pl (14/20). Neutral (4/20) and acidic (2/20) PMEI domains are also seen (Fig.38).
### Table IV. Classification and characterization of OsPMEs

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<th>Group</th>
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<th>Uniprot ID</th>
<th>PME domain present</th>
<th>Peptide length (aa)</th>
<th>Estimated molecular mass (kDa)</th>
<th>Estimated theoretical pl</th>
<th>Located on the chromosome</th>
<th>Position on the chromosome</th>
<th>Introns (#)</th>
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Physical Locations of OsPME’s on Rice Chromosomes

Since all rice clones used for genomic sequencing in the International Rice Genome Sequencing (IRGS) project can be physically anchored on the 12 rice chromosomes, To physically link all the OsPMEs to the chromosomes of rice, sequence of each OsPME was used as a query in the BLAST analysis against the mapped *Oryza sativa* genome in GRAMENE database. As a result, all the 51 OsPME’s encoded on individual clones could be correspondingly mapped on japonica chromosome (Fig.39). This analysis showed that OsPME’s are not clustered on chromosomes and always only a single gene is present at a given chromosomal location. The OsPME’s with PMEI or pro region (OsPMEI’s) and that with out pro or PMEI domain (Ospro−’s) also does not cluster but are uniformly distributed on all the 12 chromosomes. The majority of OsPME’s are located on chromosomes 1 (10/51), 4 (10/51) and 11 (11/51), accounting for 60.1% of total OsPME’s. The remaining genes are distributed on other chromosome (4 genes on chromosome 3, 3 genes each on chromosomes 2 and 7, 2 genes each on chromosomes 5and 9 and single genes are located on chromosomes 6, 10 and 12). Majority of the OsPro− proteins are also present on chromosome 1 (5/31), 4 (8/31), and 11 (9/31). Whereas, ~25% (5/20) of OsPMEI proteins are located on chromosome 1.

Phylogenetic analysis of OsPME’s and Arabidopsis PME’s

In this study, an attempt was made to perform Phylogenetic analysis of OsPME’s to understand its evolution. For the phylogenetic analysis, typically conserved regions of proteins would be used. Since the PME domains are distributed along the protein, full-length protein sequences were used for phylogenetic analysis in this study. For this, all the 51 OsPME aminoacid sequences were retrieved from the GenBank and multiple alignment was performed using clustalW (www.ebi.ac.uk.clustalW). The distance file obtained from this clustalW analysis was viewed using Tree view. The rice PME family members formed four distinct groups (Fig. 40). This included two specific groups, group I (only group I proteins i.e, OsPro− ) and group II (only group II members i.e, OsPMEI) and two other group with both group I and Group II rice PMEs together, group III (OsPMEI 13 is grouped with other Ospro− proteins) and group VI (OsPMEI 10, 11, 15, 17, 18, 19, 23 and 24 are grouped with other Ospro− 11, 15,19,22,24 and 25). This analysis revealed that Ospro- and OsPMEI i.e, Group I and Group II OsPMEs form separate clades (Fig.40, 41).
Later, all these 51 OsWAKs were analyzed phylogenetically together with the 66 Arabidopsis PME's (Fig.42). All the sequences were retrieved from the database and clustalW was performed as described above and the phylogenetic tree was viewed in the tree view. This analysis revealed that OsPME’s and Arabidopsis PME’s do not cluster into species-specific clades. All the Arabidopsis PME’s cluster together with OsPME’s rather than diverging into a group unique for each species, suggesting that PMEs are conserved during the evolution. Analysis also revealed that Group I and Group II PMEs, from both Rice and Arabidopsis fall into two separate clades suggesting their parallel evolution (Fig.40).
Discussion

Pectins are polymerized in the Golgi, methylesterified and modified with side chains, and subsequently released into the apoplastic space as highly methylesterified polymers. The homogalacturonan component of pectin can later be demethylesterified by pectin methylesterases (PMEs) (Micheli, 2001). This enzymatic activity of PMEs can lead either to cell wall loosening or to cell wall stiffening, depending on the apoplastic pH (Catigne et al., 1998; Denés et al., 2000; Micheli, 2001). In higher plants, pectin demethylesterification is catalyzed by a number of PME isoenzymes and are ubiquitous enzymes involved in various physiological processes in plants. Several studies have shown a strong correlation between PME activity or PME gene expression and physiological processes such as hypocotyls elongation (Bordenave and Goldberg, 1993), fruit ripening and maturation (Tieman and Handa, 1994), cellular adhesion (Robert et al., 2000), seed germination (Ren and Kermode, 2000), cambial cell differentiation (Micheli et al., 2000), microsporogenesis. 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). PMEs have been shown to play a central role in growth of pollen tube wall, which is composed mainly of pectin (Ferguson et al., 1998; Li et al., 1994).

PME's are characterized mostly from Arabidopsis, Tomato, Pea, Tobacco, Petunia, Yellow cedar, Brassica, Maize etc. No report is available on the rice PME’s in the literature. Here we report a genome wide analysis of Pectin methyl esterases (PME) from rice and isolation of OsPME1 from rice along with its bioinformatic analysis and expression profile.

The Arabidopsis genome encodes 66 PME-related genes (CAZY database, http://afmb.cnrs-mrs.fr/CAZY/), that can be classified into two groups based upon the presence and absence of PMEI domain in their putative encoded proteins. Group I contain 22 members of smaller proteins with molecular mass of 30-45 kDa and contain only PME catalytic domain. Whereas Group II includes remaining 44 with larger molecular mass of 55-105 kDa and contains 1-3 PMEI domains along with catalytic PME domain (Tian et al., 2006). A genome wide analysis in rice using SMART domain
search, pfam and interproscan search programmes revealed the presence of 51 members of OsPMEs. By employing the criterion used to classify PMEs from *Arabidopsis*. OsPMEs could also be divided into two groups based on presence or absence of PMEI domain. (a) Group I containing 31 members of smaller proteins with molecular mass of 17-57 kDa and containing only PME catalytic domain and (b) Group II containing remaining 20 members of larger proteins with molecular mass of 35-105 kDa containing a single PMEI domain along with a catalytic PME domain (Table. IV).

In *Arabidopsis* group I PMEs show a conserved gene structure pattern with 5-6 introns as against 1-2 intron patterns in group II PMEs (Micheli, 2001). Whereas, in rice number of introns in group I PMEs varied from 0-6. However, group II PMEs show a conserved gene structure with 0-2 intron pattern (Table. IV). These results indicate that the gene structure of PMEs is not conserved across the species.

Biochemical analysis revealed that most of the *Arabidopsis* PME isoforms encode for a PME domain of 33-43 kDa and an alkaline pl (44/66). Apart from these, some of the PME domains are also acidic (9/66) and neutral (13/66). In contrast, the PMEI domain is mostly acidic (24/44), followed by neutral (13/44) and alkaline (7/44) pl (Bosch and Hepler, 2005). In rice too, it was observed that most of OsPME isoforms encode for a PME domain of 10-37 kDa and an alkaline pl (28/51). 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). As in case of *Arabidopsis* the pl of PMEI domain is mostly acidic (Fig.38).

In *Arabidopsis* the genes encoding PME are not clustered. An attempt was made to map all the 51 PMEs on *japonica* chromosome. The analysis showed that the Group I and group II PMEs did not cluster separately on a single chromosome but were distributed randomly on all the 12 chromosomes with majority (~60%) of the OsPMEs located on chromosome 1, 4 and 11 (Fig.39).

Phylogenetic analysis of PMEs from rice and *Arabidopsis* indicated that the PMEs are conserved during evolution since *Arabidopsis* and rice PMEs were grouped into a single-clade without diverging into species-specific clades. Further, the Group I and Group II PMEs of *Arabidopsis* and rice fall into two separate clades suggesting their parallel evolution (Fig.40, 41 and 42).
In this study, an attempt was also made to characterize a PME isolated from pre-pollinated spike cDNA library of *indica* rice (designated as OsiPME1). Bioinformatic analysis revealed that OsiPME1 belongs to group I OsPMEs since, it contains only catalytic PME domain. Biochemical analysis revealed it to encode for an alkaline isoform, thus might be associated with cell wall. This is further substantiated by the prediction of N\\textsuperscript{\prime} signal sequence, that would target the protein to the cell membrane.

BlastP analysis revealed that OsiPME1 is homologous to PMEs from various plant species belonging to different families and also to the PMEs from fungi (*Aspergillus* and *Neurospora*) 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 GXKFIF found in the active groups of PME’s was conserved (Fig.23).

PME belongs to the family of parallel beta-helix proteins. The crystal structures of *Erwinia chrysanthemi* PME (Jenkins et al., 1997) and carrot (*Daucus carota*) PME (Johanson et al., 2002) shows that the fold of PME is a single stranded right-handed beta-helix. The putative 3D structure of OsiPME1 protein also shows single stranded right-handed beta-helix (Fig.25.A, B, C). A central hollow cylindrical structure was also noticed and the central part of this long shallow cleft is lined by several aromatic residues, that could be involved in ligand binding (Fig.25.D, E). The aminoacid residues at the active site are also conserved and many aminoacids involved in ligand binding are also conserved (Fig.26). These results indicate that OsiPME1 might be coding for a functional PME in rice.

Southern blot analysis indicated that OsiPME1 exists as a single copy in the rice genome (Fig.19) and was further substantiated by identification of only single 100\% identical genomic sequences in the rice genome.

In higher plants, a number of PME isoenzymes were shown to express in response to certain developmental or environmental cues and/or in a tissue-specific fashion. For example, while some PMEs are ubiquitously present (Gaffe et al., 1997), others are specifically expressed during root development (Wen et al., 1999), fruit ripening (Brummell and Harpster, 2001; Frenkel et al., 1998), or stem elongation (Bordenave et al., 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). Indeed PME-related genes are expressed in pollen of many diverse plant species, e.g *Brassica* (Albani et
Many works have shown that the PME has role either in tetrad dissolution of pollen grains (Albani et al., 1991), or in pollen ornamentation (Lacox et al., 2003) or in pollen tube germination or elongation (Jiang et al., 2005; Tian et al., 2006). In this study, OsPME1 was found to express in organ specific manner in the pollen grains with increasing levels of expression during later stages of pollen development (Fig.30).

In addition, PME activity was also shown to be regulated by hormones. Auxin-induced PME activity increases 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 this study, OsPME1 expression was found to be regulated by heat, cold, drought, mannitol, submergence, desiccation, heavy metals, phytohormones and ABA. OsPME1 mRNA levels were found to be upregulated by Mannitol, down-regulated by heat, cold, desiccation, submergence, salicylic acid, Cadmium and zinc and unaltered with NaCL, Mercury and copper salts (Fig. 31.A, B). OsPME1 mRNA levels were regulated by various plant hormones like auxins, cytokinins and Giberellic acid. The OsPME1 transcript level was downregulated by Giberellic acid (GA3) and was not affected by cytokinins (Fig.31.C). However had differing effect by various auxins. A higher level of OsPME1 transcript accumulation was observed with IBA and NAA treatments as compared to that of 2,4-D and IAA (Fig.31.C). ABA, a stress hormone affected expression of OsPME1 in a dose dependent manner. Therefore, it may be suggested that the expression of OsPME1 gene is regulated by these stimuli, confirming the roles of various regulating elements of ABA, GA3, drought, stress, temperature responsive elements existing at 5' upstream of OsPME1 gene. In conclusion, since OsPME1 expresses in pollen specific manner, it may be speculated to have a role in pollen development.
Summary

a. Isolation of OsiPME1 and its Bioinformatic analysis

The 1302 bp OsiPME1 (Acc No. AY343494) was isolated from pre-pollinated stage rice panicle cDNA library cloned in Lambda ZAP express vector. The *in silico* analysis of OsiPME1 revealed that its total size is 1434 bp and consists of 60 bp of 5' UTR, 1038 bp of open reading frame and 336 bp of 3' UTR. The ORF of the OsiPME1 encoded a 345 aminoacid peptide with a predicted molecular mass of 37.994 kDa and theoretical pl of 8.99. The deduced aminoacid sequence showed high homology (52%) to *Arabidopsis* PME (Q9LSP1). It also showed similarity to other plant encoded Pectin methyl esterases. SignalP analysis predicted the presence of 20 bp N' signal peptide. The conserved aminoacids GXYXE and GXXDFIF found in the active groups of all PME's has also been identified in rice OsiPME1. The putative 3D structure of OsiPME1 protein showed it to be a single stranded right-handed beta-helix, similar to the crystal structures of carrot and *Erwinia* PMEs. A central hollow cylindrical structure was also noticed and is lined by several aromatic residues thought to be involved in ligand binding.

Comparison of the full-length cDNA sequence with the corresponding genomic sequence of OsiPME1, showed the presence of four introns ranging from 97-243 bp and five exons ranging from 157 bp to 266 bp.

b. Molecular characterization

Genomic Southern analysis revealed the presence of single copy of OsiPME1, which is substantiated by *in silico* analysis of OsiPME1 gene sequence showing 100% similarity only with sequences located on the upper arm of chromosome 3 at 11.012355 to 11.014375 MB position in rice genome (*Japonica*).

Northern analysis revealed that the gene expressed in pollen specific manner during later stages of development. Northern blot analysis was performed in order to understand the expression patterns of OsiPME1 upon treatments with various stresses, and phytohormones. The results showed that OsiPME1 mRNA levels were upregulated by Mannitol, Auxin and Abscisic acid treatments and downregulated by abiotic stresses like heat, cold, desiccation, submergence and salicylic acid, heavy metals like Cadmium.
and zinc and with phytohormone Giberellic acid (GA3). The transcript levels of OsPME1 were found to be unaltered with Nacl treatment and also with heavy metals like Hg and Cu. These observations were validated by In silico analysis (PLACE search) of 1.2kb 5’ upstream promoter region of OsPME1 which predicted the presence of various cis-acting regulatory elements like ABA, GA3, drought, stress and temperature responsive elements.

c. Functional characterization

Towards understanding the function of OsPME1 in vivo, Plant expression constructs harboring OsPME1 gene in sense and antisense orientation under ubiquitin promoter and NOS terminator were constructed in pUC19 vector backbone. These constructs would be used in the future to raise homologous and heterologous transgenic plants either overexpressing and/or underexpressing OsPME1 gene product to gain insight into the function of OsPME1 in pollen development.

d. Genome wide analysis of OsPME gene family

Different bioinformatic tools like SMART, pfam and Interproscan searches were used to identify and classify OsPME’s from Oryza sativa. Based on these, a total of 51 putative OsPME’s were identified in Oryza sativa subsps japonica. One of them had been found to be 100% identical to OsiPME1.

Aminoacid sequence analysis of these proteins using the SMART tool showed that 31 of them contain only the catalytic PME domain, while 20 of them also contain a PME inhibitor (PMEI) domain. Thus, like Arabidopsis PME’s, Oryza sativa PME’s also could be subdivided into two general groups based on their domain structure. Group I includes smaller protein with 189-540 aminoacid residues and a predicted molecular mass of 17-57 kDa and has only catalytic PME domain, and group II comprises larger proteins with 336-971 aminoacid residues and a molecular mass of 35-105 kDa, that include in addition to a catalytic PME domain, an extra PMEI domain at the N’ end. 0-6 introns were found in the genes belonging to Group I as against 0-2 intron pattern in group II PME’s.

The 51 OsPME’s could be correspondingly mapped on japonica chromosome. This analysis showed that OsPME’s are not clustered on chromosomes and always only a single gene is present at a given chromosomal location. The majority of OsPME’s are located on chromosomes 1 (10/51), 4 (10/51) and 11 (11/51), accounting for 60.1% of total OsPME’s. Remaining PMEs are distributed on other chromosomes (2 (3/51), 3 (4/51), 5 (2/51), 6 (1/51), 7 (3/51), 8 (3/51), 9 (2/51), 10 (1/51), 12 (1/51).
Phylogenetic analysis of all 51 OsPMEs revealed that Group I and Group II OsPMEs form separate clades. Phylogenetic analysis along with 66 Arabidopsis PMEs revealed that OsPME’s and Arabidopsis PME’s do not cluster into species-specific clades. All the Ara PME’s cluster together with OsPME’s rather than diverging into a group unique for each species, suggesting that PMEs are conserved during the evolution. But the analysis also revealed that all the Group I and Group II PME’s from Arabidopsis and Oryza sativa cluster in different clades, hence suggesting their parallel evolution.


plant cell walls. Implications for pectin methyl esterase action, matrix properties, and cell adhesion. J.Biol. Chem. 276, 19404-19413.


500 ng of pAKV1 plasmid was digested with SacI (lane 1), PstI (lane 2), BamHI (lane 3), EcoRI (lane 4), KpnI (lane 5), ClaI (lane 6), ApaI (lane 7), XbaI (lane 8) and Xhol (lane 1). The digested samples were resolved on 1% agarose gel along with 1 kb ladder (lane M).

Figure 13. Restriction analysis of pAKV1 construct.

Figure 12. Linear map of pAKV1 (6.1 Kb)

Figure 14. Linear map of pPE1 (3.9 Kb)
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Figure 15. Linear map of pPEself (5.2 Kb)

Figure 16. Restriction analysis of pPE1 construct.
500 ng of pPE1 plasmid was digested with BamHI (lane 2) and HindIII (lane 3). The digested samples were resolved on 1% agarose gel along with a 100 bp marker (lane 1).

Figure 17. Restriction analysis of pPEself construct.
500 ng of pPEself plasmid was digested with BamHI/XhoI (lane 2) and BamHI (lane 3). The digested samples were resolved on 1% agarose gel along with a 1 kb marker (lane 1).

Figure 18. Total sequence of pAKV1 insert (1670 bp). The 1302 bp sequence (small letters) code for a putative pectin methyl esterase and the region coding for a part of chlorophyll a/b binding site is indicated in capital letters.

 kakagacagaa gtcacattgag atcgcaagag ccctggcttg gttgaagcag cctctctctc
ccccggccag cctcctccac

Total sequence of pAKV1 insert (1670 bp). The 1302 bp sequence (small letters) code for a putative pectin methyl esterase and the region coding for a part of chlorophyll a/b binding site is indicated in capital letters.
**Figure 19.** Linear map of pOKpec’S’ (3.25 Kb)

**Figure 20.** Linear map of pOKpec’A’ (3.25 Kb)

**Figure 21.** Restriction analysis of pOKpec’S’ and pOKpec’A’ constructs.
500 ng of pOKpec’S’ and pOKpec’A’ plasmids was digested with Kpn1 (lane 1, 2) and EcoRI (lane 3, 4) respectively. The digested samples were resolved on 1% agarose gel along with a 100 bp marker (lane M).
Figure 2. The nucleotide (1302 bp) and deduced amino sequence (345 aa) of OsPME1. The N’ signal peptide is marked with blue colour and the red colour indicated the position of start and stop codons. The Pectin methyl esterase domains are marked with yellow colour and the restriction sites BamHI and SacI present in the cDNA sequence are marked with green colour.
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Figure 23. ClustalW of the PME’s sequences from different plant species, bacteria and fungi, used in phylogenetic analysis. OsiPME1 peptide sequence is marked with yellow color. The conserved functional domains of PME’s across all the kingdoms are marked with blue. Any variation from the consensus in conserved domains are represented with orange color letters.

Figure 24. Phylogenetic analysis using full-length peptide of PME’s from different plant species, bacteria and fungi.
Figure 25. Putative 3D structure of OsIPME1 protein showing right-handed beta helix fold and a central long cleft. The figures were obtained by using Rasmol. A. Front view; B. View from N’ end; C. View from C’ end; D&E: Side views.

Figure 26. Sequence based comparison of carrot PME (CARPME, Acc.N0. P83218) and OsIPME1 (RICEPME). The Proposed active sites (red), ligand binding sites (blue) and the aromatic amino acids lining the cleft (yellow) are mostly conserved.
Figure.27. The ligand binding sites (pink) are located in the cleft. The putative ligand binding sites in OsiPME1 protein are R157, H179, H180, D201, F204, A232, R260, N262 and Y287.

Figure.28. Genome organization of OsiPME1 gene.
Figure 29. Organization of *OsiPME1* gene in rice genome

(a) Restriction analysis of *OsiPME1* genomic DNA. All restrictions are not represented.

(b) Southern Blot analysis of *OsiPME1*. 5 µg of total genomic DNA was digested with HindIII (lane 1); KpnI (lane 2) and ScaI (lane 3). The digested samples were resolved on 0.8% agarose gel along with 5 µg of Undigested genomic DNA (U), transblotted onto positively charged nylon membrane and probed with 1.3 kb *OsiPME1* cDNA obtained by digesting pOKpec’S’ plasmid with ScaI.

(c) Physical location of *OsiPME1* on *Oryza sativa* genome.
Figure 30. Tissue specific expression of *OsiPME1* in rice plants,
5μg of the total RNA extracted from Leaves (L), Shoots (S), Roots (R), Leaf sheath (Ls), Pre pollinated panicle (P1), Post pollinated panicle (P2), 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 *OsiPME1* cDNA fragment.
Figure 3.1. Northern analysis of OsPME1 expression in response to environmental factors.

5µg of the total RNA extracted from the pre-pollinaed panicles of rice treated with water (C), Heat (He), Cold (Co), NaCl (Na), Manittol (Ma), De-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.
Figure 32. Linear map of pUCnospec’S’ (4.26 Kb)

Figure 33. Restriction analysis of pUCnospec’S’ and pUCnospec’A’ constructs. 500 ng of pUCnospec’S’ cut with KpnI (lane 1) and EcoRI (lane 2) and 500 ng of pUCnospec’A’ plasmids cut with KpnI (lane 3) and EcoRI (lane 4) were resolved on 1% agarose gel along with 100 bp ladder (lane M).

Figure 34. Linear map of pUbpec’S’ (6.26 Kb)
Figure 3.5. Restriction analysis of pUbpec’S’ and pUbpec’A’ constructs.
500 ng of pUbpec’A’ cut with KpnI (lane 1) and EcoRI (lane 2) and 500 ng of pUbpec’S’ plamids cut with KpnI (lane 3) and EcoRI (lane 4) were resolved on 1% agarose gel along with 1 kb ladder (lane M).

Figure 3.6. Linear map of pUCnospec’A’ (4.26 Kb)

Figure 3.7. Linear map of pUbpec’A’ (6.26 Kb)
Figure 38. Biochemical analysis of PME isoforms encoded in *Oryza sativa* genome. The number of isoforms without a pro-region as well as the number of isoforms of which pI of the predicted PME domain and pro-region falls within a certain pH interval are depicted.

Figure 39. Physical location of 51 OsPME’s on the 12 japonica rice chromosomes according to GRAMENE database and/or NCBI Blast analysis.
**Figure 40.** Phylogenetic analysis of *OsPMEs.*

The composite tree of the *OsPME*’s protein family (Drawn using treeview). Branch positions were determined by neighbor-joining analysis of the 51 *OsPME* sequences compiled in the table IV. Two major groups of *Ospro* and one group of *OsPMEI* and one group consisting of both *Ospro* and *OsPMEI* are indicated.
Figure 41. Phylogenetic analysis of OsPMEs.

Phylogenetic analysis using full-length sequences of all 51 OsPME's. The Ospro' and OsPMEI form distinct clades. Any deviations from this are circled. Rectangle highlights the group of Ospro' proteins located on rice chromosome 11, forming a distinct clade along with OsPMEI proteins.
Figure 42. Phylogenetic analysis of 51 OsPMEs and 66 Arabidopsis PMEs. No distinct species specific clades are seen.