



Characterization of *OsPM19L1* encoding an AWPM-19-like family protein that is dramatically induced by osmotic stress in rice

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ABSTRACT. The plant-specific AWPM-19-domain proteins play important roles in plant development and stress responses. In the current study, *OsPM19L1* encoding *Oryza sativa* AWPM-19-like protein 1 was isolated from rice. Tissue-specific gene expression analysis revealed that *OsPM19L1* was highly expressed in the leaf sheath of rice. Interestingly, expression of *OsPM19L1* was high at the early stage of panicle development and decreased thereafter. qRT-PCR analysis indicated that *OsPM19L1* was dramatically induced by 20% PEG stress (>600-fold), exogenous abscisic acid (>350-fold), salt and cold stress. Subcellular localization assay suggested that the *OsPM19L1*-GFP (green fluorescent protein) fusion protein was localized in the membrane system in rice cells. Moreover, under stress conditions, *OsPM19L1* expression was enhanced in an *ABI5-Like1* (*ABL1*) deficiency rice mutant, *abl1*, suggesting that *ABL1* negatively regulates *OsPM19L1* gene expression. Thus, *OsPM19L1*

appears to be closely associated with stress tolerance through ABA-dependent pathway in rice.

Key words: Rice; Membrane protein; AWPM-19-like; Abiotic stress; Panicle

INTRODUCTION

Drought, cold, and high salinity are major abiotic stresses that affect plant growth and crop production. In order to respond and adapt to these adverse factors, plants can initiate a number of molecular, cellular, and physiological changes. The stress-inducible genes play significant roles in response to these stresses (Kreps et al., 2002; Seki et al., 2002; Xiong et al., 2002; Zhu, 2002; Shinozaki et al., 2003; Lee et al., 2005). The phytohormone ABA plays a central role in the regulation of plant growth and developmental processes, as well as in the abiotic stress response, especially the response to drought or salt stress. ABA serves as an endogenous signal to initiate the adaptive responses when plants are challenged by abiotic or biotic stress (Zhu, 2002; Adie et al., 2007; Bari and Jones, 2009; Huang et al., 2012a; Ye et al., 2012; Nakashima and Yamaguchi-Shinozaki, 2013; Danquah et al., 2014; Mehrotra et al., 2014). It has been established that two distinct pathways (ABA dependent and ABA independent) are involved in stress-responsive gene expression (Huang et al., 2012b; Yoshida et al., 2014). Previous research has helped to identify the key components of ABA signaling. ABA signaling involves an ABA receptor complex, consisting of an ABA receptor family, phosphatases and kinases. These proteins play a central role in regulating a variety of responses to diverse stresses, including the activities of membrane-localized factors (Osakabe et al., 2014). However, most studies at present focus on ABA functions in stress responses in *Arabidopsis*. Since there is limited insight into the specificity of rice signaling, studies on rice ABA signal transduction will be very valuable to elucidate the functional mechanism of ABA. Rice ABI5-Like1 (ABL1), a basic region/leucine zipper motif transcription factor, modulates ABA responses by directly regulating the ABA-responsive element (ABRE)-containing genes (Zou et al., 2007, 2008; Yoshida et al., 2010; Yang et al., 2011).

Proteins with the AWPM-19 domain are classified into the AWPM-19-like family of proteins. The AWPM-19-like family proteins are widely distributed in various plant species. The roles for these proteins in seed development, dormancy and stress responses have been studied. GmPM3, the first identified AWPM-19-like family protein, was isolated from developing soybean seeds at the late embryogenesis stage, also classified as late embryogenesis-abundant (LEA) protein (Hsing et al., 1995). *WPM-1*, isolated by PCR from the library prepared from the ABA-treated cultured winter wheat (*Triticum aestivum* L. cv. Chihoku) cells, encodes a putative plasma membrane localized protein, WPM-1. Studies suggest that the WPM-1 promotes freezing tolerance of the wheat suspension-cultured cells by ABA treatment (Koike et al., 1997). The expression pattern of the *PM19* gene, encoding a putative barley (*Hordeum vulgare* L.) plasma membrane protein, indicates that it is associated with embryo development, dormancy as well as with environmental stress during germination (Ranford et al., 2002). Besides soybean, wheat and barley, several AWPM-19 genes have been isolated from other plant species, such as *Populus trichocarpa*, *Arabidopsis* and rice, but the functional mechanisms for these proteins in plant development or stress tolerance are not clear.

In the present study, a novel AWPM-19-like family protein gene, *OsPM19L1* was identified through microarray-based gene expression analysis in rice under ABA stress. Gene expression

analysis suggested that *OsPM19L1* was largely induced upon ABA stress and also induced by simulated drought, salt and cold stresses. Interestingly, *OsPM19L1* expression was negatively regulated by an ABI5-like transcription factor, ABL1 under stress conditions, implying that *OsPM19L1* might play a crucial role in osmotic stress response through the ABA-dependent pathway.

MATERIAL AND METHODS

Plant materials and stress treatments

The rice (*Oryza sativa* L. ssp *japonica*) cultivars Jiucaiqing, Zhonghua11 (ZH11), and *abl1* mutant of Zhonghua11 (Yang et al., 2011) were used in this study. The seeds were sterilized in 0.1% HgCl₂ and germinated at 30°C. The seedlings were cultured with Yoshida's solution in a growth chamber as previously described (Sun et al., 2012). All seedlings were kept under continuous light for 48 h before treatment and continuous light was used during the treatments. For stress and ABA treatments, the seedlings were cultured in Yoshida's solution supplemented with 100 mM NaCl, 20% PEG6000 (w/v), and 50 mM ABA, respectively. For the cold treatment, seedlings were transferred to a growth chamber maintained at 4°C. The seedling samples were collected at different time points after each treatment, immediately frozen in liquid nitrogen, and stored at -80°C. For tissue-specific gene expression analysis, the roots, stems, leaf blades, leaf sheathes, and panicles at different stages from adult plants were harvested and immediately frozen in liquid nitrogen.

RNA isolation and first strand cDNA synthesis

Total RNA was extracted from rice seedlings and different tissues treated with stress and ABA, using the Trizol reagent (Invitrogen, USA), as per manufacturer protocols. The RNA was subsequently treated with DNase I (Promega, USA) to remove the remaining genomic DNA. The first strand cDNA was synthesized with 2 µg purified total RNA using the reverse transcription system (Promega).

Cloning of *OsPM19L1*

OSPM19L1 was cloned by PCR approach with a pair of primers (sense: 5'-GATTTGTGATTTTGATACGAGATGG-3' and antisense: 5'-TGCACGAGCGGAAACAC-3'). The PCR conditions for amplifying *OSPM19L1* were as follows: 5-min pre-denaturation at 98°C, 28 cycles of 10 s at 98°C, 15 s at 56°C, 50 s at 72°C, and a final extension for 10 min at 72°C. The PCR product was purified and cloned into pEASY-T vector (TransGen, China) for sequencing.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was carried out on an Applied Biosystem 7500 Fast Real Time PCR System (Applied Biosystems). Quantification of gene expression was done using the comparative CT method. The *18S rRNA* gene was used as an internal control (Jain et al., 2006). The primers of *18S rRNA* were: sense primer 5'-ATGGTGGTGACGGGTGAC-3' and antisense primer 5'-CAGACACTAAAGCGCCCGGTA-3'.

Multiple sequence alignment and phylogenetic analysis

Alignment of *OsPM19L1* and other plant AWPM-19-domain protein sequences were performed with the ClustalX program (ver 2.1). The phylogenetic tree was constructed with the MEGA program (ver 4.0) by neighbor-joining (NJ) method. The parameters pairwise deletion and *p*-distance model were used. Bootstrap test of phylogeny was performed with 1000 replicates.

Subcellular localization

A green fluorescent protein (GFP) fusion protein was constructed using the full-length *OSPM19L1* cDNA clone, with an N-terminal fusion of the GFP clone under the control of CaMV 35S promoter. *Agrobacterium tumefaciens* strain EHA105, containing an *OSPM19L1::GFP* fusion and a GFP alone was separately inoculated through the stomata using a 1-mL plastic syringe and by applying gentle pressure on the lower epidermal surface of tobacco leaves (Maselli et al., 2014). The subcellular distribution of the GFP fusion protein was examined by confocal laser scanning microscopy (Zeiss, LSM780).

In silico sequence analysis

For promoter sequence analysis, the 2000-bp upstream sequence of *OSPM19L1* was analyzed by MatInspector program at Genomatix website (<http://www.genomatix.de/>). The co-expression analysis was performed both with Rice Oligo Array Database (<http://ricearray.org/>) based on 1081 rice Affymetrix microarray data (NCBI GEO AC: GPL2025) and by genevestigator (<https://www.genevestigator.com/gv/>). The genes coexpressed with *OSPM19L1* were selected with a Pearson's correlation coefficient (PCC) cut-off of 0.6 in both results. The changes in expression of co-expressed genes under drought, salt, and cold stresses were investigated using GEO (Gene Expression Omnibus) datasets (<http://www.ncbi.nlm.nih.gov>) under accession No. GSE6901.

RESULTS

Sequence analysis of *OsPM19L1*

From microarray data on ABA-responsive genes in rice seedlings (Huang J and Zhang H, unpublished data), an EST probe (probe ID: Os.11260.1.S1_at) showing ~1220-fold induction upon exogenous ABA treatment was chosen for further analysis. Database search indicated that this EST encodes a putative AWPM-19-like family protein containing the AWPM-19 domain (Gene locus name: Os05g0381400). We designated this gene *OsPM19L1* as it encodes an AWPM-19-like family protein in rice. The full-length *OsPM19L1* gene, containing a complete ORF of 522 bp, was cloned from total RNA prepared from rice seedlings by RT-PCR. The predicted protein product of *OsPM19L1* comprised of 173 amino acids, with a calculated molecular mass of 18.06 kDa and isoelectric point of 9.68. Multiple sequences alignment showed that *OsPM19L1* was similar to other plant AWPM-19-domain-containing proteins (Koike et al., 1997; Ranford et al., 2002) (Figure 1). In particular, the N-terminal AWPM-19 domain is well conserved among *OsPM19L1* and other AWPM-19-like proteins, whereas the C-terminal domain exhibits diversities. To investigate the evolutionary relationship among plant AWPM-19-like family proteins, a phylogenetic tree was

constructed using NJ method with the full-length amino acid sequences of several AWPM-19-like family proteins - four members in rice (OsPM19L1, OsPM19L2, OsPM19L3, OsPM19L4), one in wheat (WPM-1), one in barley (pm19), one in sorghum (SbPM19L), one in *Arabidopsis* (AtPM19L), one in soybean (GmPM3), one in *P. trichocarpa* (PthPM19L), and one in peach (PpaPM19L). Phylogenetic analysis revealed that OsPM19L1 was clustered with WPM-1, PM19, and other AWPM-19-like proteins existing in rice and *Sorghum bicolor*, whereas others were categorized into another branch (Figure 2).

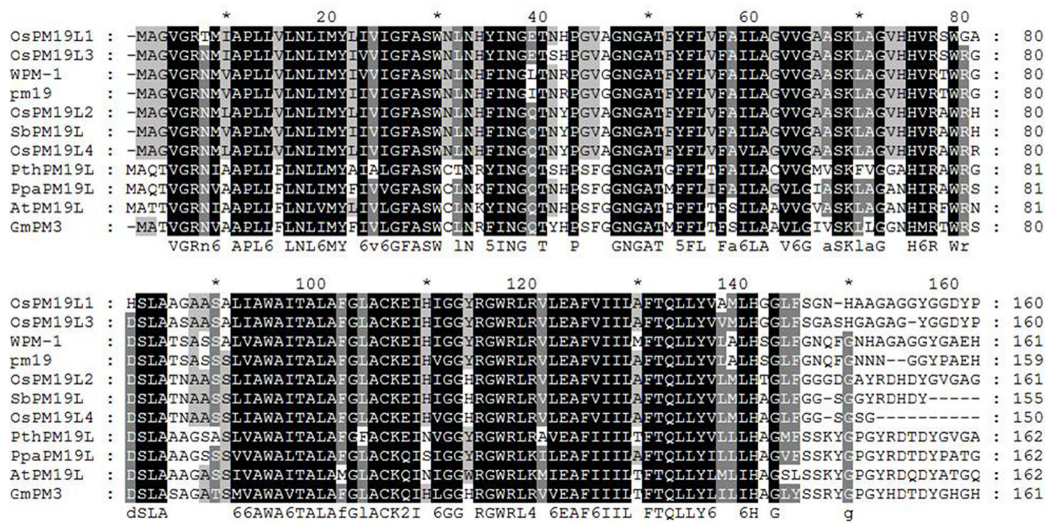


Figure 1. Multiple sequence alignment of AWPM-19-like family proteins. The amino acid sequences marked in black belong to conserved AWPM-19-domain. GenBank accession Nos. for the sequences: OsPM19L1 (NP_001055398.1), OsPM19L2 (NP_001059485.1), OsPM19L3 (XP_006654338.1), OsPM19L4 (XP_006658505.1), AWPM-19 (AAB38504.1), pm19 (AAF29532.1), SbPM19L (XP_002459766.1), AtPM19L (NP_563710.1), GmPM3 (NP_001238105.1), PthPM19L (XP_002314514.1), PpaPM19L (XP_007223551.1).

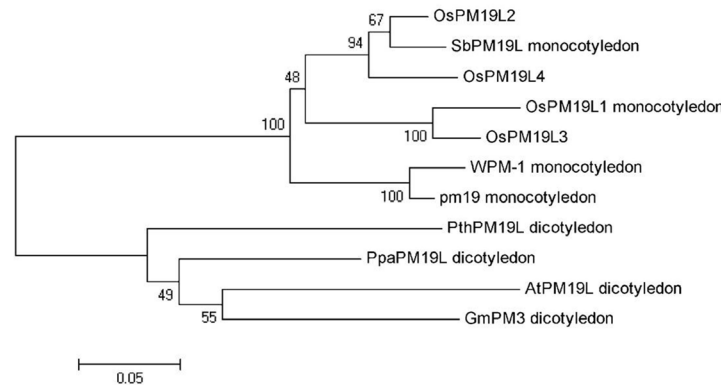


Figure 2. Phylogenetic analysis of AWPM-19-like family proteins. The neighbor-joining tree was constructed with MEGA 4.0. Branch numbers represent a percentage of the bootstrap values in 1000 sampling replicates and the scale bar indicates branch length.

The 2000-bp promoter sequence of *OsPM19L1* was cloned and analyzed through the MatInspector program (<http://www.genomatix.de/>). The promoter sequence contained some putative stress-related cis-acting elements, such as GTBX (GT-box elements) (Hamann et al., 1994), ABRE (Uno et al., 2000), DREB (dehydration responsive element binding factors) (Agarwal et al., 2006), MYBL (MYB-like proteins) (Abe et al., 2003), MYCL (MYC-like binding factors), G-BOX (plant G-box/C-box bZIP proteins) (Figueroa and Browse, 2012), CGCG (calmodulin-binding/CGCG box binding proteins) (Yang and Poovaiah, 2002), NACF (plant specific NAC transcription factors) (Takasaki et al., 2010), and WBXF (W Box family) (Figure 3). These stress-related cis-acting elements may be responsive for stress-regulated expression of *OsPM19L1*.



Figure 3. Distribution of stress-related cis-acting elements in the 2000 bp promoter region of *OsPM19L1*. Different cis-elements for TFs were labeled with different colors. Right end 2000 bp is the transcriptional start site (TSS) of *OsPM19L1*.

Expression analysis of *OsPM19L1* in different rice tissues

For tissue specific expression analysis, roots, stems, leaf blades, leaf sheathes, and panicles with lengths of 3 cm (P1), 8 cm (P2), and 12 cm (P3) were collected from adult rice plants. qRT-PCR results indicated that *OsPM19L1* was constitutively expressed in various rice tissues, and predominately in leaf sheathes. We also investigated *OsPM19L1* expression in panicles at different developmental stages, and found that the highest expression levels were in 3-cm young panicles, followed by a 4- and 5-fold decrease in expression levels in 8- and 12-cm panicles, respectively (Figure 4).

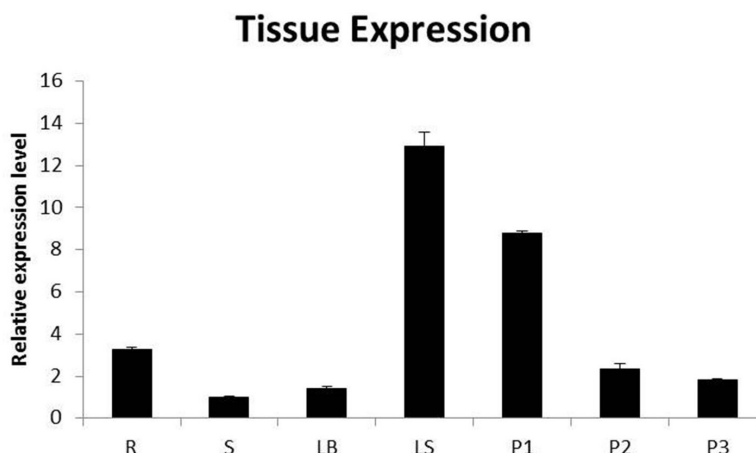


Figure 4. Expression pattern of *OsPM19L1* in various tissues by real-time qPCR. Roots (R), stems (S), leaf blades (LB), leaf sheathes (LS), and panicles with the length of 3 cm (P1), 8 cm (P2), and 12 cm (P3) in rice (*Oryza sativa* L. ssp. *japonica* cultivar JiUCAIQING). 18S-rRNA was used as an internal control. Data are reported as means and standard errors of three replicates.

Induction of expression of *OsPM19L1* in rice under abiotic stress

Since the microarray data showed that *OsPM19L1* was largely induced by ABA treatment, we investigated the expression pattern of the gene in response to various abiotic stresses. qRT-PCR assay showed that *OsPM19L1* expression was highly induced by salt, PEG, low temperature, and ABA treatment. For the 100-mM NaCl treatment, *OsPM19L1* transcript started responding to the treatment after 3 h, and showed two-peak induction pattern at 6- and 24-h treatment with a decrease in expression at 12 h (Figure 5D). The transcript levels of *OSPM19L1* increased remarkably after 3 h of 20% PEG treatment, and compared to control, a 660-fold increase at 12 h was observed (Figure 5B). After 50- μ M exogenous ABA treatment, *OSPM19L1* expression in seedlings showed a 360-fold increase at 12 h, which remained at a similar level till 24 h, while a 95-fold increase was observed at 48 h (Figure 5A). For the low temperature treatment, *OsPM19L1* expression changes were not as dramatic as those observed for other stresses, and the *OsPM19L1* transcript accumulated slowly, starting to respond to the stress after 24 h (Figure 5C).

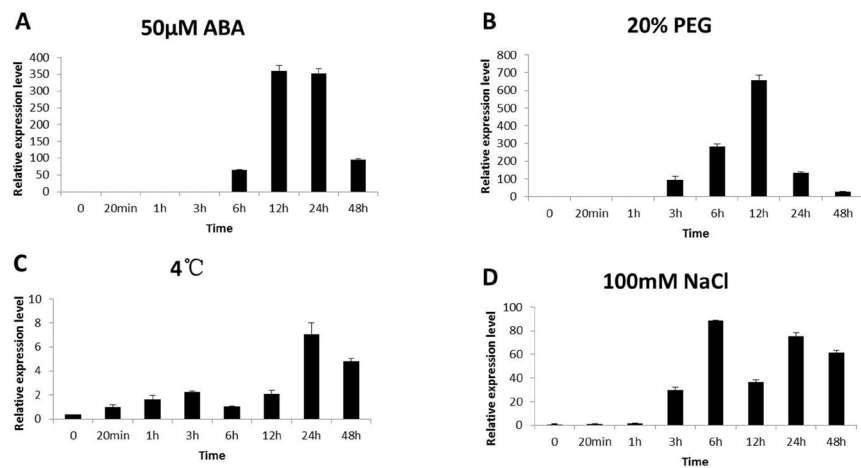


Figure 5. Expression patterns of *OsPM19L1* in rice seedlings treated with various stresses and ABA by real-time qPCR. **A.** 50 μ M ABA, **B.** cold stress (4°C), **C.** 100 mM NaCl, **D.** stimulated drought stress (20% PEG). Three to four leaf rice seedlings (Jiucaiqing) were used. 18S-rRNA was used as an internal control. Data are reported as means and standard errors of three replicates.

Expression of *OsPM19L1* in rice *abl1* mutant under PEG and NaCl stress

The rice ABI5-like1 (ABL1) deficiency mutant, *abl1*, shows suppressed ABA responses (Yang et al., 2011). Since previous data suggest that *OsPM19L1* expression is regulated by ABA treatment, it was of interest to analyze whether *OsPM19L1* was involved in ABL1 signaling pathway. *OsPM19L1* gene expression patterns in *abl1* and wild-type rice ZH11 under NaCl and PEG stresses were examined. Compared to untreated samples, a 2140-fold increase in *OsPM19L1* expression was observed in *abl1* after 12 h of 20% PEG6000 treatment, while in WT a 276-fold increase was observed. A similar trend was also observed for NaCl treatment, where after 12 h of treatment, *OsPM19L1* expression in *abl1* was 25-fold higher than in untreated samples. *OsPM19L1* expression was up-regulated greatly in *abl1* mutant under PEG and NaCl treatment (Figure 6A-C).

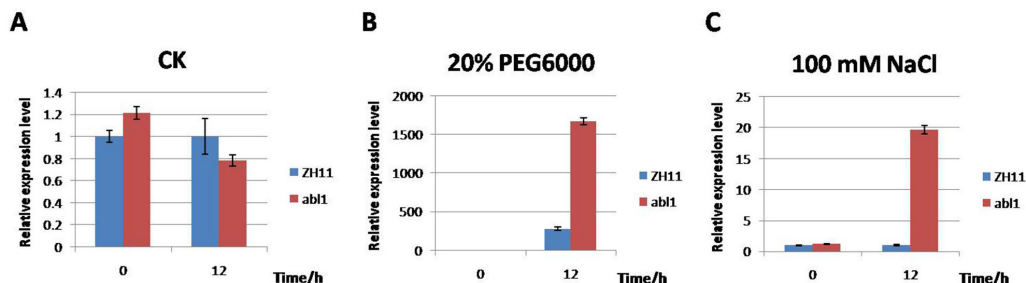


Figure 6. Expression change of *OsPM19L1* in rice *abl1* mutant and WT (ZH11) seedlings under different treatments. **A.-C.** Expression of *OsPM19L1* in the seedlings exposed to water (CK), stimulated drought stress (20% PEG6000) and 100 mM NaCl, respectively. 18S-rRNA was used as an internal control. Data are reported as means and standard errors of three replicates.

Co-expression analysis of *OsPM19L1*

The identities of seven genes, that were selected from the *OsPM19L1* co-expression gene data as having PCC higher than 0.6, provide clues about the functions of *OsPM19L1* in plant stress responses (Table 1). These genes might function coordinately in response to abiotic stress. Six of the seven genes (*RAB16D*, *RAB16B*, *RAB21*, Os05g0349800, Os06g0246500, and Os03g0322900) showed highly up-regulated response to all three abiotic stresses. Interestingly, only Os03g0111300, a lipid-transfer protein, was induced by drought and salt treatments and inhibited by cold stress.

Table 1. List of genes co-expressed with *OsPM19L1*.

Gene ID	Annotation	PCC	Drought	Salt	Cold
Os11g0453900	Dehydrin Rab16D	0.7765	5.81 ± 0.43	5.18 ± 0.77	0.07 ± 1.21
Os11g0454200	Dehydrin Rab16B	0.7304	9.54 ± 0.20	7.34 ± 0.33	0.06 ± 0.76
Os11g0454300	Water stress-inducible protein Rab21	0.6882	8.04 ± 0.35	7.19 ± 0.35	0.20 ± 0.36
Os05g0349800	Late embryogenesis abundant protein	0.6736	6.68 ± 0.20	3.35 ± 0.60	1.83 ± 1.88
Os03g0111300	Nonspecific lipid-transfer protein 2 (nsLTP2)	0.6467	3.35 ± 0.36	3.59 ± 0.52	-2.04 ± 2.66
Os06g0246500	Pyruvate dehydrogenase E1 alpha subunit	0.643	4.23 ± 0.34	3.60 ± 0.77	0.85 ± 1.13
Os03g0322900	Late embryogenesis abundant protein	0.636	7.41 ± 0.10	6.33 ± 0.41	2.52 ± 1.99

PCC = Pearson's correlation coefficient. The data in the table indicate the log₂ fold of the microarray data in rice seedlings under cold, drought, or salt stress relative to that under control condition. The normalized microarray data were generated from GEO database under accession No. GSE6901. Standard error was based on three biological repeats.

OsPM19L1 locates in the membrane system

As *OsPM19L1* encodes a putative AWPM-19-domain protein, it was of interest to investigate whether *OsPM19L1* was a membrane protein. We analyzed the subcellular localization of *OsPM19L1* using the transient expression system in tobacco leaves. *OsPM19L1*-GFP fusion protein was localized in the organelle membranes, while the control GFP protein was detected throughout the cell, indicating that the GFP protein remained in the cytosol (Figure 7).

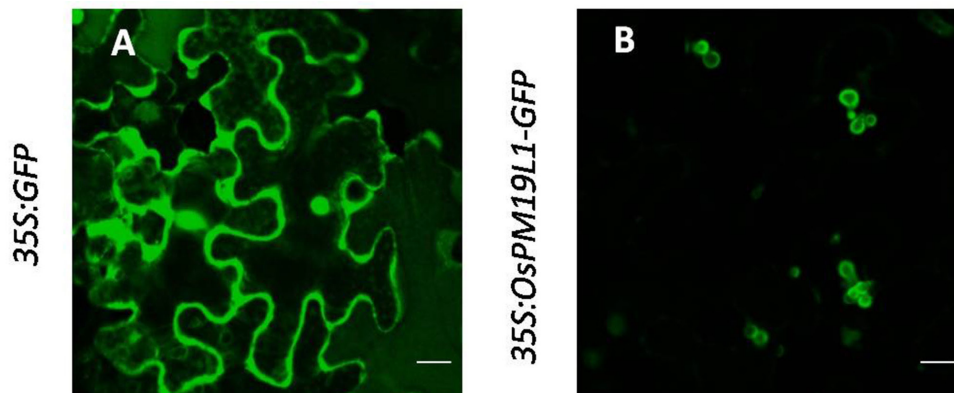


Figure 7. Subcellular localization of OsPM19L1 in tobacco epidermal cells. Each *Agrobacterium tumefaciens* strain EHA105 containing the 35S::GFP (control empty vector) (A), 35S::OsPM19L1-GFP (B) proteins was transiently expressed in *Nicotiana benthamiana* leaves. (bar = 20 μ m).

DISCUSSION

In present study, we report a novel AWPM-19-like family protein gene *OsPM19L1*, whose expression was dramatically induced by simulated drought, osmotic stress and exogenous ABA. Through multiple sequence alignment and phylogenetic analysis, it was found that OsPM19L1 is mostly related to wheat protein WPM-1 and barley protein pm19. These two plants both belong to monocotyledons. The phylogenetic analysis indicated that the AWPM-19 gene family might be formed by the species-specific gene expansion. The results of tissue specific expression analysis indicate that *OsMP19L1* is highly expressed at the early stage of panicle development and probably plays an essential role in immature panicles. However, this hypothesis needs further validation using *OsMP19L1* knock-out mutant. MYB transcription factors are important for normal reproductive development of plants. Overexpression of cotton *GhMYB24* in *Arabidopsis* led to flower malformation, shorter filaments, non-dehiscent anthers and fewer viable pollen grains (Li et al., 2013), while, in transgenic rice, supra-optimal expression of *OsMyb4* transcription factor caused mis-expression of alternative targets with costly trade-offs to panicle development (Park et al., 2010). Since there is a MYB-like protein-binding site in the promoter region of *OsMP19L1*, it will be of interest to investigate the functions of OsMP19L1 in plant reproductive development.

Co-expression analysis shows a number of genes involved in abiotic stress coexpressed with *OsPM19L1*. The top three genes (*RAB16D*, *RAB16B*, *RAB21*) and Os06g0246500, encoding pyruvate dehydrogenase E1 alpha subunit, are well-documented stress-induced ABA late responsive genes (Wang et al., 2011). Two genes (Os05g0349800 and Os03g0322900) encoding LEA proteins are highly induced by drought, salt and cold stresses. Many LEA proteins are known to be involved in abiotic stress tolerance in plant (Dalal et al., 2009). Collectively, these coexpression results suggest that *OsPM19L1* plays a positive role in drought salinity and cold tolerance by regulating stress-induced ABA response in rice.

Gene expression analysis indicated that *OsPM19L1* was significantly induced by salt and PEG stresses but not markedly by cold, implying that *OsPM19L1* is mainly involved in signal transduction induced by osmotic stress. Based on the observation that *OsPM19L1* was dramatically induced by ABA treatment, it may play an important role in ABA signaling. The ABL1

protein is localized to the nucleus and modulates the plant stress and ABA responses by directly regulating the ABRE-containing genes (Yang et al., 2011). An ABRE protein binding site in the promoter region of *OsPM19L1* suggests that OsPM19L1 may participate in plant stress and ABA responses under the regulation of ABL1 transcription factor. Although the role of OsPM19L1 in ABA signal transduction is not yet understood, it provides an interesting avenue for further study of rice OsPM19L1 functions in stress response via the ABA dependent pathway.

The subcellular localization results suggest that OsPM19L1 is a membrane protein. The features of WPM-1 amino acid composition indicate that WPM-1 is a membrane protein (Koike et al., 1997). Membrane-delimited events play a crucial role in ABA signaling, and ABA receptors (PYR/PYL/RCAR) modulate the activity of different plasma membrane components involved in ABA action (Bueso et al., 2014).

In conclusion, a new AWPM-19-like family protein gene *OsPM19L1* was identified and characterized in present study. OsPM19L1 was localized in plasma membrane and endomembrane system, implying that OsPM19L1 functions as a membrane-localized protein in ABA signaling pathway. Interestingly, we found that expression of *OsPM19L1* was regulated by ABL1. As *OsPM19L1* expression was largely induced upon drought, osmotic stress and exogenous ABA application, we hypothesize that OsPM19L1 is involved in stress response via an ABA-dependent pathway in rice. However, this hypothesis needs further experimental validation by use of *OsPM19L1* knock-out mutant or transgenic approach.

Conflicts of interests

The authors declare no conflicts of interests.

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