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## *OsPM1* is a positive regulator of rice tolerance to drought stress but a negative regulator of rice tolerance to salt stress

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### ABSTRACT

To understand stress response mechanisms, a multiple stress-responsive gene *OsPM1* with a disordered region containing about 30 amino acid residues was screened by microarray. Promoter region analysis using PlantCARE showed this gene contains multiple cis-regulatory elements responding to abiotic stresses. Quantitative real-time PCR and promoter-GUS transgenic plant analysis verified that *OsPM1* was induced by multiple abiotic stresses. *OsPM1*-GFP fusion protein expression showed that *OsPM1* was localized at the cell membrane. The rice plants overexpressing *OsPM1* exhibited hypersensitivity to salt stress but enhanced drought tolerance compared to the wild-type plants; the rice plants expressing antisense of *OsPM1* were hyposensitive to salt stress but with weak drought tolerance compared to the wild-type plants. These results showed *OsPM1* is a positive regulator of rice tolerance to drought, but it's also a negative regulator of rice tolerance to salt.

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Rice; intrinsically disordered; salt; abscisic acid; cis-acting element

## 1. Introduction

Abiotic stresses, such as drought, high-salinity, low-temperature and oxidative stresses, are principal causes limiting crop productivity throughout the world (Mahajan and Tuteja 2005; Tan et al. 2013). Compared with crops such as wheat and corn, rice is more sensitive to salt stress, and approximately 30% of rice-growing areas in the world are suffering from salt persecution (Takehisa et al. 2004). Rice (*Oryza sativa* L.) is a staple crop for more than half of the world's population (Todaka et al. 2012). Salinity stress affects growth at various developmental stages, leading to reduced crop yield (Munns et al. 1995; Ahmad et al. 2014; Roy et al. 2014). With the rapidly increasing human population, enhancing crop salinity tolerance is a key solution to increase agricultural productivity (Qin et al. 2020). Therefore, it is necessary to vigorously excavate some genes related to abiotic stress in rice, and use genetic engineering to study and modify them, so that rice has corresponding resistance under different stresses (Filiz and Akbudak 2020). This is of great significance for ensuring the normal growth and development of rice in harsh environments and maintaining stable and considerable yields (Cao et al. 2016).

Phytohormones are synthesized in certain cell types but mediate physiological responses throughout the plant (Weyers et al. 2002; Boursiac et al. 2013). The phytohormone abscisic acid (ABA) controls a variety of aspects in plant growth and development (Himmelbach et al. 2003; Boursiac et al. 2013). During vegetative growth, ABA plays an important role in regulating adaptive responses to various environmental stresses, such as high salinity, drought, low temperature, oxidative

stress, and mechanical wounding (Chan 2012). The pathway of stress response is categorized as ABA-dependent and ABA-independent (Jakoby et al. 2002). Previous study have shown that *OsPM1* as an ABA influx carrier that plays an important role in drought responses (Yao et al. 2018).

We found that the rice plants overexpressing *OsPM1* exhibited hypersensitivity to salt stress but enhanced drought tolerance compared to the wild-type plants; the rice plants expressing antisense of *OsPM1* were hyposensitive to salt stress and with weak drought tolerance compared to the wild-type plants. *OsPM1*, therefore, is a positive regulator of rice tolerance to drought but a negative regulator of rice tolerance to salt.



## 2. Materials and method

### 2.1. Plant materials

Rice (*Oryza sativa* L. subsp. *japonica* cv. *Nipponbare*) plants was cultured in a climate incubator at 22°C to 28°C, 16 h light / 8 h dark conditions for 14 days (Campo et al. 2014). Then the seedlings were treated under different stresses (ABA, NaCl, drought, 4°C, 42°C). The leaves were collected according to different time periods of the stress treatment to extract RNA (Figure 3b–e).

### 2.2. Generation of transgenic lines

To produce overexpression and antisense expression vectors of *OsPM1*, the open reading frame (ORF) of *OsPM1* was cloned into D-163 + 1300 vector (derived from *pJIT163*

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and *pCambia1300*). The overexpression primers were F: 5'-tgg aga gga cag ccc aag ctt ATG GCC GGA GTA GGG AGG A-3' and R: 5'-gta ccg aat tcc cgg gga tcc TTA GAC CCT GGC CGC GGC-3'. The antisense expression primers were F: 5'-tgg aga gga cag ccc aag ctt TTA GAC CCT GGC CGC GGC-3' and R: 5'-gta ccg aat tcc cgg gga tcc ATG GCC GGA GTA GGG AGG A-3'. In order to obtain *OsPM1pro: GUS* vector, *OsPM1* promoter sequence was cloned into *pCambia3301* vector and the primer were 5'-gag ctc ggt acc cgg gga tcc GGT TAA ATC TGC ATC CAC-3' and 5'-tta ccc tca gat cta cca tgg TCA CAA ATC AAC ACA AAT TAG C-3'. Then the constructed vector is infected with wild-type plant callus by *Agrobacterium*-mediated transformation to obtain the corresponding transgenic plant (Hiei and Komari 2008).

### 2.3. RNA extraction and cDNA synthesis

Total RNA was extracted by using trizol reagent (Solarbio, China) according to the manufacturer's protocol (Guan et al. 2010). cDNA synthesis was completed using PrimeScript RT reagent Kit (TaKaRa, Japan).

### 2.4. RT-PCR

The primers for *OsPM1* and the reference gene Ubiquitin were designed by using primer premier 5.0. The primers for *OsPM1* gene were F: 5'-CGC TGC TGG TGC TGA ATC TGA T-3' and R: 5'-AGG ATG GCG AAG ACG AGG AAG T-3', the primer for UBQ were F: 5'-AAC CAG CTG AGG CCC AAG A-3' and R: 5'-ACG ATT GAT TTA ACC AGT CCA TGA-3'.

### 2.5. Sequence analysis

Obtained about 1.5 kb sequence upstream of the translation start site of rice *OsPM1* gene from Gramene database (<http://www.gramene.org/>). The promoter of *OsPM1* was analyzed by Plant-CARE database (<http://bioinformatics.psb.ugent.be/we-btools/plantcare/html/>) (Liao et al. 2017).

### 2.6. Subcellular localization of *OsPM1*

*OsPM1* gene is connected with Hind III and BamH I of *pAcGFP* vector, and its primers were 5'-tgg aga gga cag ccc aag ctt ATG GCC GGA GTA GGG AGG A-3' and 5'-ctc acc atg acc ggt gga tcc CTG GCC GCG GCG GCG GGG G-3'. The successfully ligated vector was transformed into *Agrobacterium* EHA105 and stored at -80°C. Take 35:GFP as the control group, observe by laser confocal microscope 48 h after tobacco injection (Waadt et al. 2008).

### 2.7. Transgenic plants treated with abiotic stress

To better study the sensitivity of transgenic rice to exogenous hormone ABA, the wild-type plants and transgenic plants that had grown for 4 days were treated with 10  $\mu$ M ABA solution for 12 days. In order to study the tolerance of overexpression and antisense expression to salt, three-week-old wild-type plants and transgenic rice seedlings were treated with 150 mM NaCl for 6 days, and the culture was resumed for 7 days, and the survival rate was calculated. Finally, a field experiment was simulated by sand culture, and rice seedlings

cultivated in sand for 30 days were subjected to 150 mM NaCl stress treatment to observe survival.

### 2.8. Histochemical localization of *GUS* activity in plants

Using the method described by Jefferson to histochemically detect *GUS* activity, different tissues of *OsPM1pro: GUS* transgenic rice were placed in a buffer containing 50 mM NaPO<sub>4</sub> buffer (pH 7.2), 5 mM K<sub>3</sub>Fe (CN)<sub>6</sub>, 5 mM K<sub>4</sub>Fe (CN)<sub>6</sub>, 0.1% Triton-100 and 1 mM X-Gluc, incubated overnight at 37°C (Jefferson 1989). Take out the material and soak in 70% ethanol for 5 min to stop the dyeing reaction, then add 95% alcohol and boil until the chlorophyll is completely removed, and finally take a photo with a ZEISS stereo microscope.

### 2.9. Statistical analysis

The data were analyzed by analysis of variance using SPSS statistics program. The statistical difference was clarified through analysis of variance by t-test, with  $P < 0.05$  (\*) and  $P < 0.01$  (\*\*) to be significantly different.

## 3. Result

### 3.1. *OsPM1* sequence analysis

In order to understand the organization of the regulatory region of *OsPM1*, we analyzed the upstream 1500 bp promoter sequence of *OsPM1* through plantCARE. We found some cis-acting elements related to stress response, such as ABRE (ABA response element), ATCT-motif (Participating in light response elements), TC-rich repeats (defense and adversity response cis elements), CGTCA-motif (jasmonic acid response element), ERE (ethylene response element), MRE (MYB binding site), GC-motif (participate in anaerobic specific induction), circadian (cis-acting element involved in physiological regulation) (Figure 1(a)).

RiceGE (Gene Expression Atlas) website analysis found that the expression level of *OsPM1* was higher in rice seeds, but lower in other organs (Figure 1(b)).

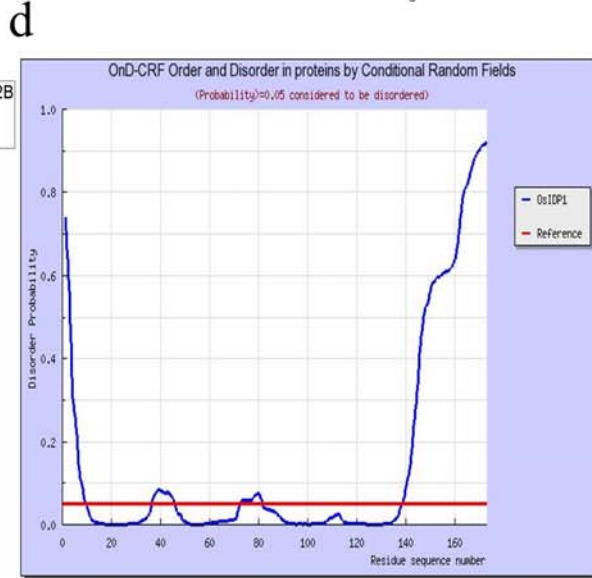
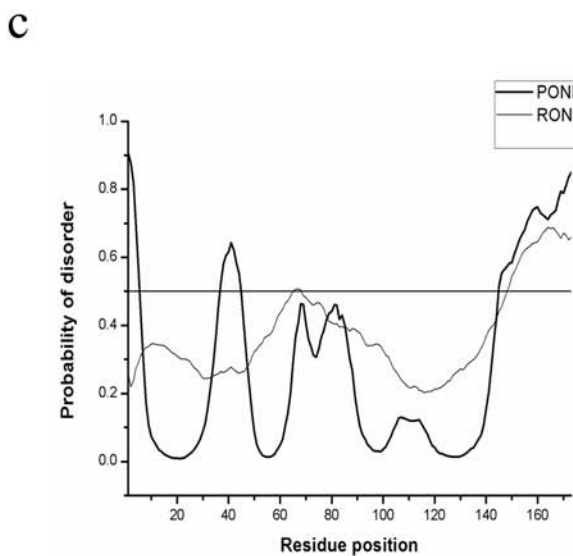
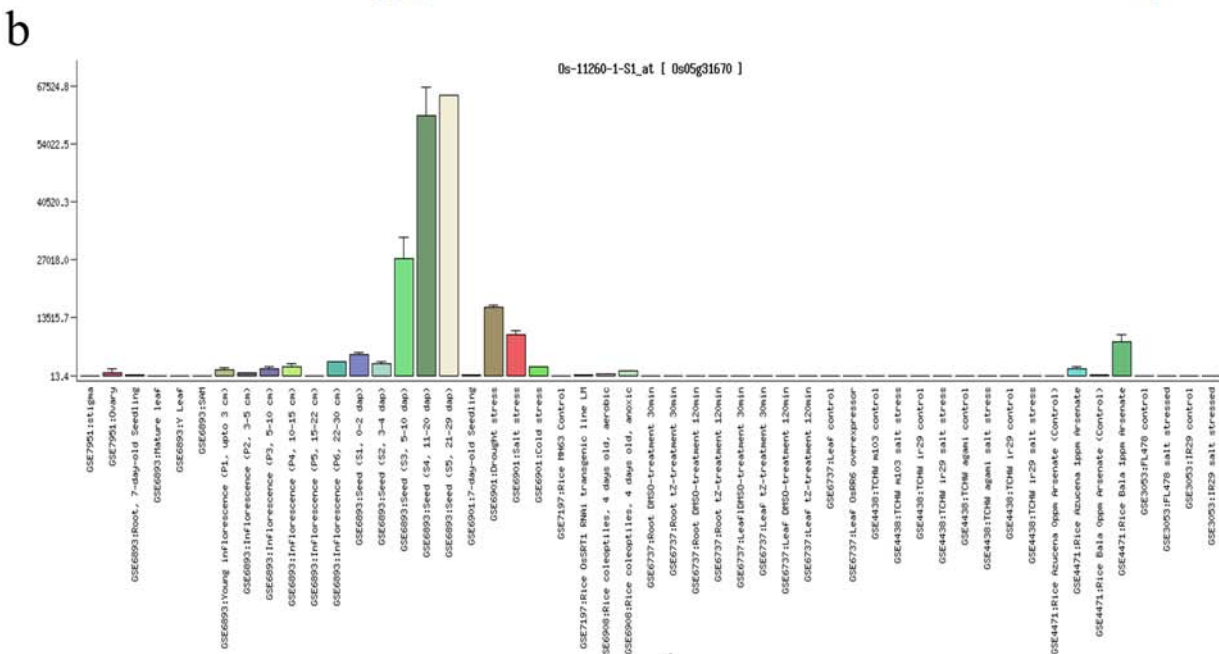
PONDR136 VSL2B, RONN and OnD-CRF were used to analyze the protein sequence of *OsPM1*. The results showed that a disordered region was predicted at the c-terminal, and the predicted fragment sizes were 29, 25, and 35 amino acid residues, respectively. (Figure 1(c,d)).

### 3.2. *OsPM1* is mainly expressed in cell membrane system

Tobacco subcellular localization results show that *OsPM1-GFP* localizes to cell membranes system (Figure 2(a)). This result is consistent with existing research conclusions on the positioning of *OsPM1* (Chen et al. 2015; Yao et al. 2018).

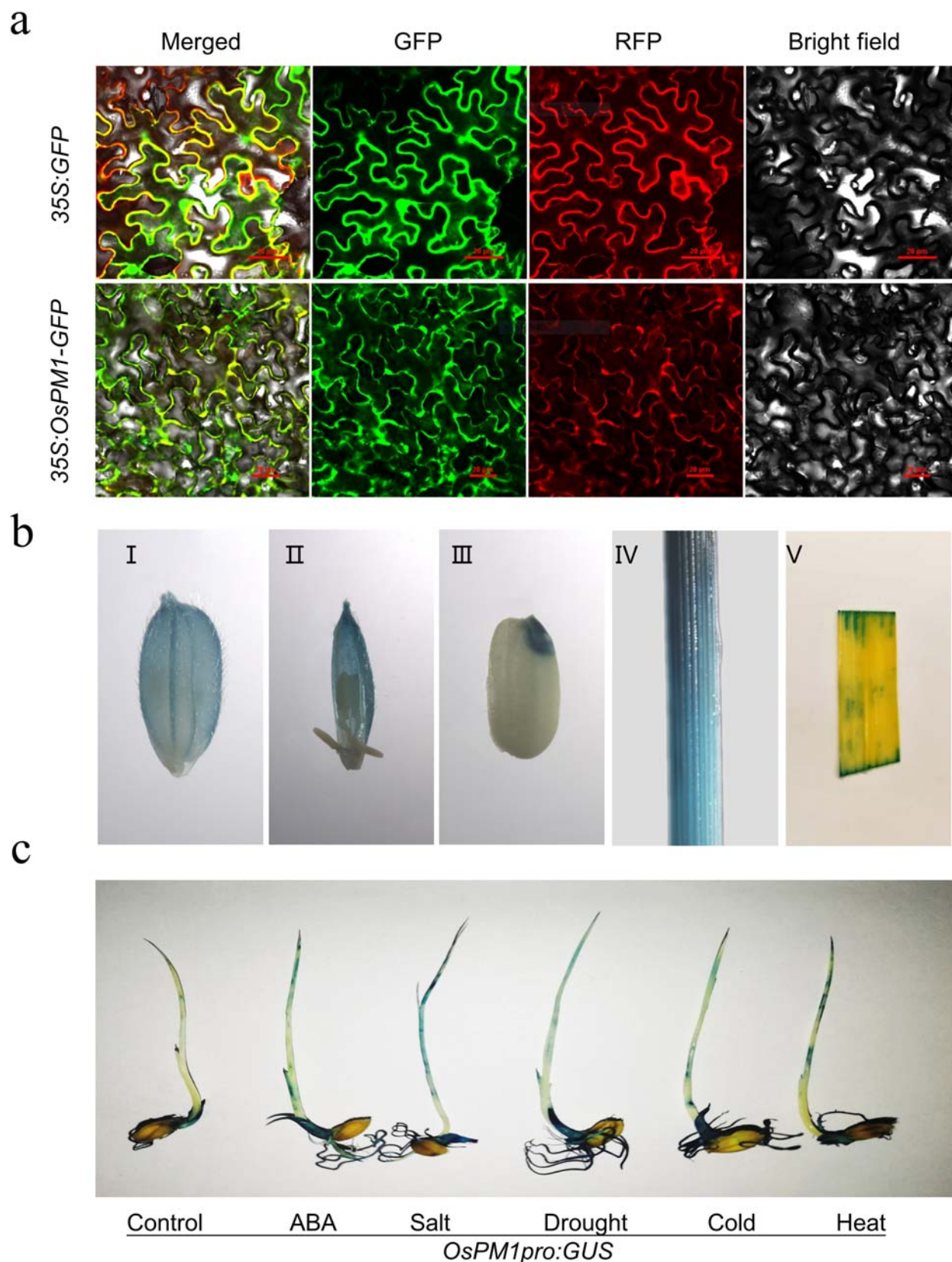
### 3.3. *OsPM1* is highly induced by multiple stresses

In order to screen out more rice stress response genes, we used the Affymetrix gene chip array to study the overall genome expression profile of rice under cold, heat and drought stress (Xu et al. 2011; Chen et al. 2012; Xu et al. 2013; Zhang et al. 2016). Microarray data analysis showed that *OsPM1*



**Figure 1.** Bioinformatics analysis of the *OsPM1* gene. (a) The analysis of cis-acting elements among the putative promoter Region of *OsPM1*. (b) The expression level of *OsPM1* in different time and organs in rice. (c) and (d) The prediction of intrinsically disordered regions in *OsPM1*.



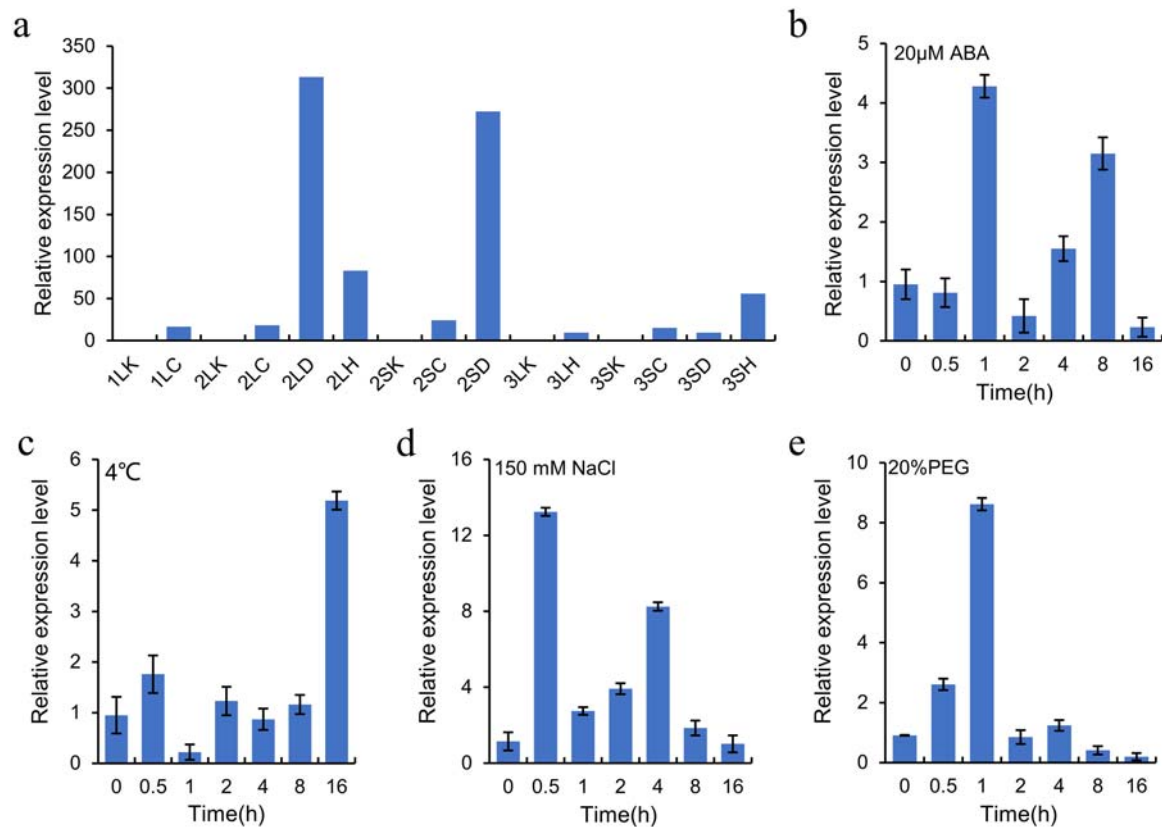


**Figure 2.** Analysis of the expression pattern of *OsPM1*. (a) Subcellular localization of *OsPM1*-GFP protein in tobacco cells. (b) Expression of *OsPM1* in different tissues. (Note: I: glumes; II: inflorescences; III: seed embryos; IV: stems; V: mature leaves.) (c) GUS activity of rice seedlings under different stress conditions.

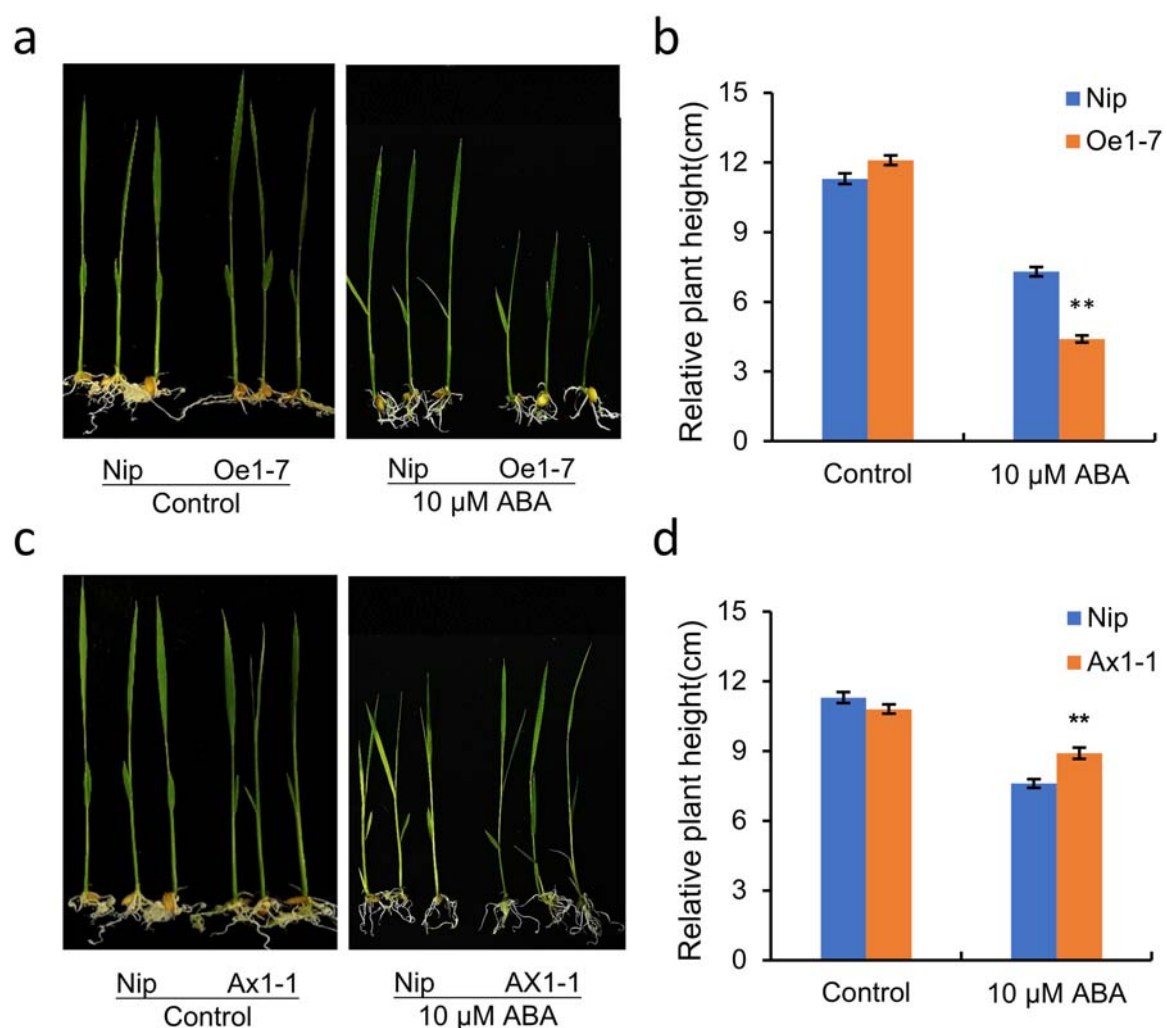
was highly responsive (>10 times) to three stresses on rice leaves and panicles at the seedling, booting and heading stages of rice. (Figure 3(a)). In addition, the gene was significantly up-regulated in the seedling and booting stages, and the number of leaves and ears increased by 313.32 times, especially under drought treatment (Figure 3(a)). A search of gene expression data on the RiceGE (Gene Expression

Atlas) website shows that drought and salinity stress treatments also induce *OsPM1* expression (Figure 1(b)).

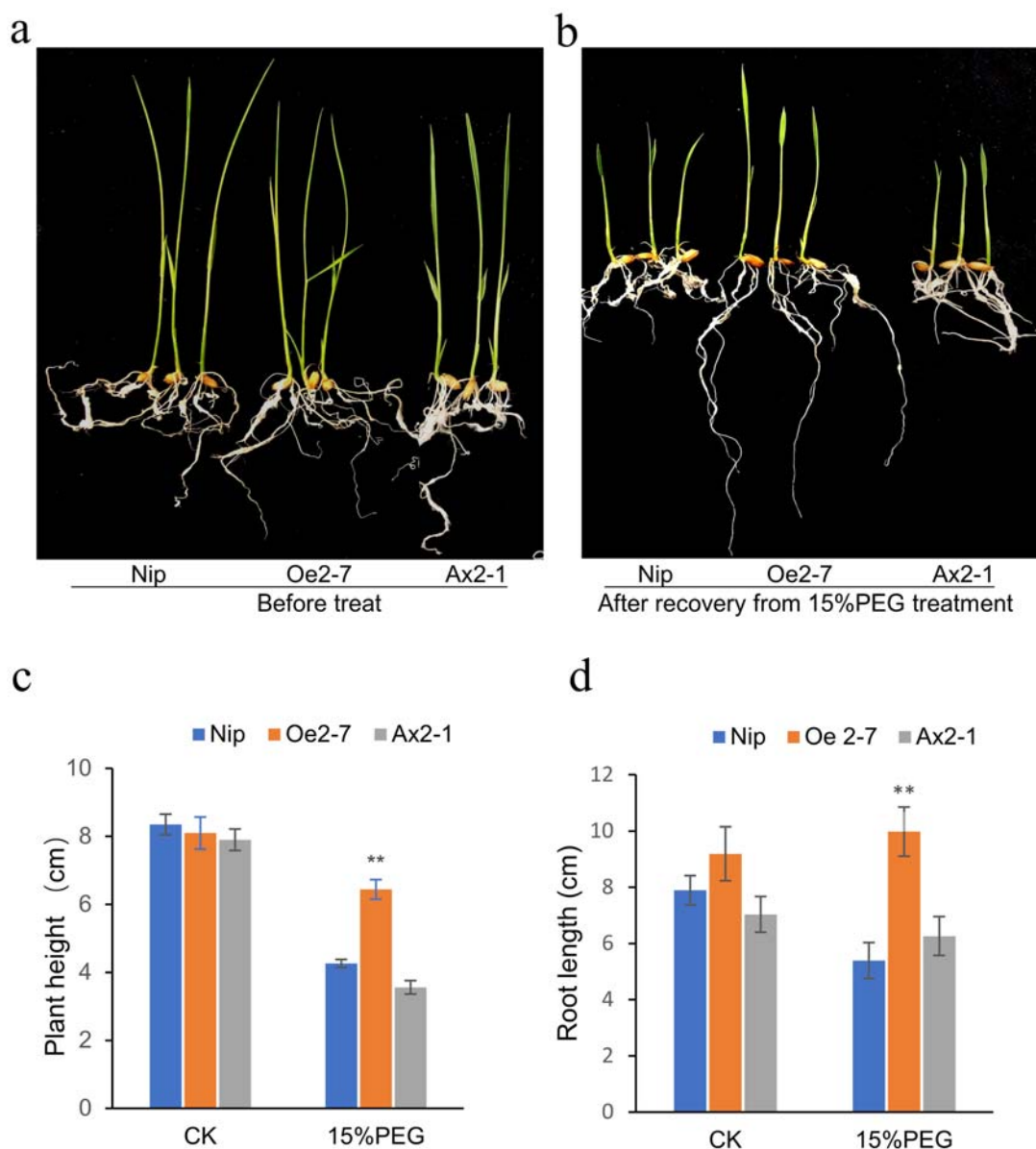
Further analysis of the expression of *OsPM1* by RT-PCR revealed that the expression level of this gene under stress is generally 4.24–13.24 times higher than that of control plants, and the expression level is the highest under salt stress and drought stress (Figure 3(d,e)). The differential expression



**Figure 3.** Expression analysis of *OsPM1* under multiple pressures. (a) Microarray analysis for *OsPM1*. (Note: 1: seedling stage; 2: booting stage; 3: heading and flowering stage; L: leaf; S: spike K: control; C: cold; H: heat; D: drought.) (b) and (d) RT-PCR analysis for *OsPM1* (Note: b: 20  $\mu$ M ABA; c: 4; d: 150 mM NaCl; e: 20 % PEG.)



**Figure 4.** ABA treatment of *OsPM1* transgenic plants. (a) and (c) *OsPM1* transgenic plants with 10  $\mu$ M ABA for 8 days. (b) and (d) Relative plant height. Values are means  $\pm$  SE. \*\* indicate significant difference  $P \leq 0.01$  probability.



**Figure 5.** Drought stress treatment of *OsPM1* transgenic lines. (a) and (b) Oe2-7 and Ax2-1 were treated with 15% PEG for 16 days. (c) Relative plant height. (d) Root length. The value is the mean  $\pm$  SE ( $n = 8$ ). \*\* indicate significant difference  $P < 0.01$  probability.

patterns are in good agreement with the obtained microarray data, and this also confirmed that under abiotic stress, *OsPM1* was strongly induced in different tissues at different developmental stages of rice.

To confirm the expression pattern of *OsPM1*, we analyzed the activity of  $\beta$ -glucuronidase (GUS) in transgenic plants controlled by the *OsPM1* promoter (*OsPM1pro::GUS*). Consistent with RT-PCR results, GUS staining results showed that *OsPM1* was expressed in most tissues, including mature leaves, stems, glumes and seed embryos, but was not expressed in anthers (Figure 2(b)). In addition, after salt stress and high temperature stress, the expression of *OsPM1* in roots was significantly increased. This result is consistent with the results of Rerksiri et al. (Figure 2(c)).

The above results indicate that *OsPM1* participate in multi-stress responses.

### 3.4. *OsPM1* overexpression is more sensitive to ABA

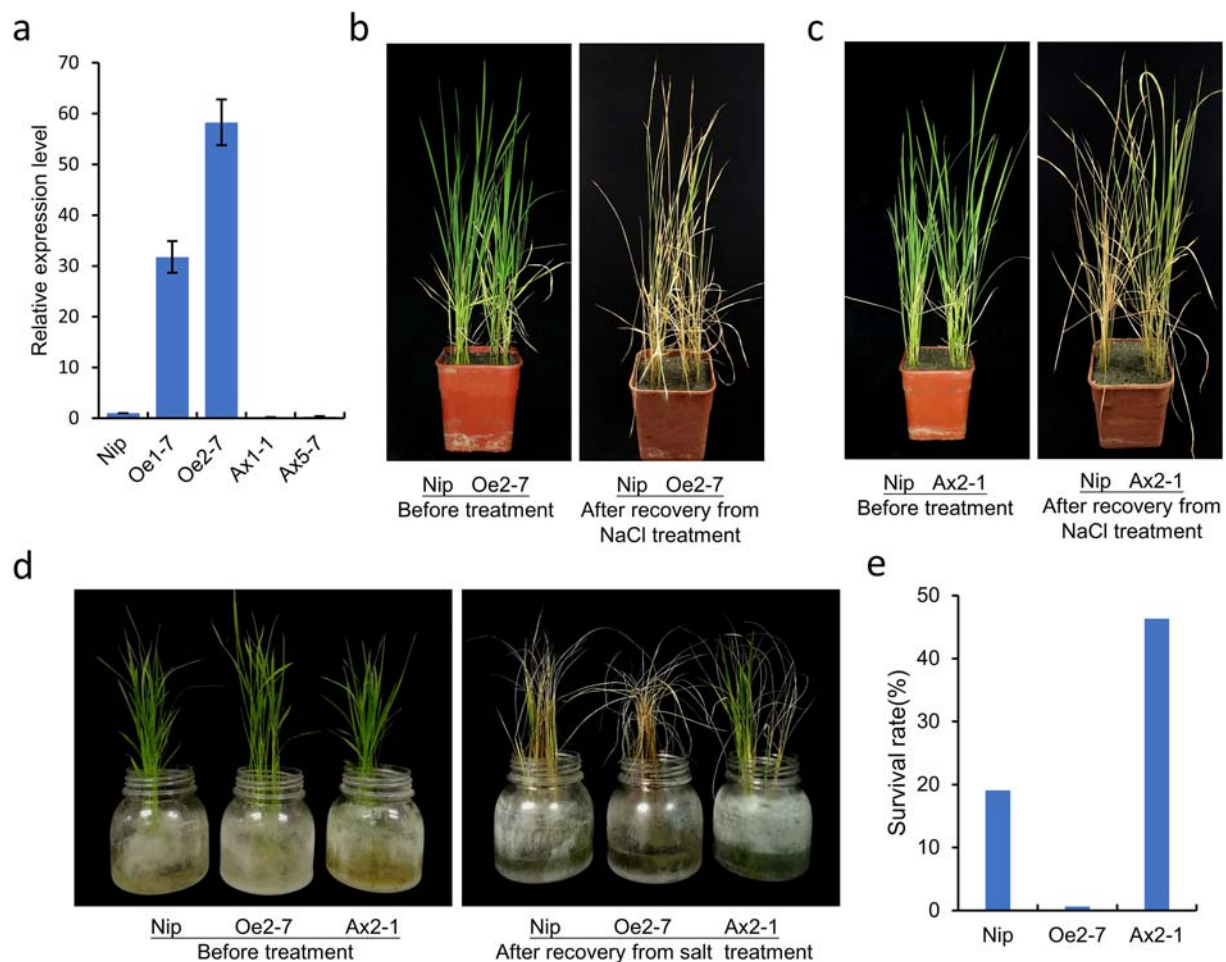
In order to further verify that *OsPM1* is involved in the stress response of ABA-dependent pathways, *OsPM1* overexpression and antisense overexpression rice were treated with

ABA. Under normal hydroponic conditions, the average plant height of overexpression lines and antisense expression lines were not different from that of the wild-type plants (Figure 4(a,c)). After exogenous ABA treatment, the height of the overexpression line was significantly shortened, and the antisense expression line was significantly higher than that of the wild-type plants (Figure 4(a,c)). This result confirms the conclusions of existing studies that overexpression of *OsPM1* can enhance the sensitivity to ABA and promote the introduction of exogenous ABA into cells.

### 3.5. Overexpression of *OsPM1* increases tolerance to drought

Based on the experimental results of Yao L et al, we conducted 15% PEG simulation drought treatment experiments on the overexpression and antisense expression of *OsPM1*, which further confirmed that the overexpression of *OsPM1* increased the tolerance to drought. The overexpression and antisense expression plants of *OsPM1* were treated with 15% PEG for 16 days from germination. There was basically no big difference in germination rate, but after 16 days of





**Figure 6.** Salt stress treatment of *OsPM1* transgenic lines. (a) The expression level of *OsPM1* in overexpression and antisense expression transgenic rice. (b) and (c) Oe2-7 and Ax2-1 treated with 150 mM NaCl for 7 days. Values are means  $\pm$  SE (n=15). (d) Oe2-7 and Ax2-1 treated with 180 mM NaCl. (e) Survival rate. Values are means  $\pm$  SE (n=20).

treatment, Oe2-7 plants were not only significantly higher than the wild-type plants, but also had a very significant difference in root length from the wild-type plants (Figure 5(b,d)). Under the same treatment, there was no significant difference in plant height and root length between Ax2-1 plants and the wild-type plants (Figure 5(b,d)).

### 3.6. *OsPM1* antisense overexpression leads to salt tolerance faster recovery

The rice grown for one month in sandy soil was watered with 150 mM NaCl. After 7 days of treatment, the Oe2-7 plants basically withered and died, while some of the wild-type plants still had green leaves and were still alive, but under the same treatment, most of the Ax2-1 plants survived and the leaves were stretched (Figure 6(b,c)). Moreover, three-week-old rice seedlings were treated in a hydroponic solution containing 180 mM NaCl for 12 days (Oe2-7 was only treated for 6 days), and hydroponics were resumed for 8 days. The survival rate of Ax2-1 was 45%, and the survival rate of the wild-type plants was 20%. None of the plants in Oe2-7 survived (Figure 6(d,e)).

## 4. Discussion

In this study, by predicting the disorder and transmembrane region of the amino acid sequence of *OsPM1*, it was found that it has 4 transmembrane helices and a

disordered structure of about 30 amino acid residues at the c-terminal. Intrinsic disordered protein is a kind of protein with no specific three-dimensional structure and was considered as a kind of junk protein in the early stages of discovery. However, with the deepening of research, it has been found that inherent disordered proteins or regions of inherent disorder (IDP/IDR) are involved in numerous cellular processes, including transcriptional regulation, protein interaction and signal linking (Kragelund et al. 2012; Pazos et al. 2013; Jensen et al. 2014). In recent years, with people's recognition, intrinsic disorder proteins have gradually attracted the attention of researchers, and certain progress has been made in plant stress resistance and signal response. At present, the most researched are NAC transcription factor family genes and LEA genes (Taoka et al. 2004; Yamaguchi et al. 2010; Jensen et al. 2014). Most LEA (late embryogenesis abundant) are inherently disordered proteins, and it has been reported that disordered LEA proteins are closely related to abiotic stress tolerance (Garayarroyo et al. 2000; Mouillon et al. 2006). In addition, disordered LEA proteins also play an important role in maintaining membrane stability under stress, such as *COR15A* and *LEA18* of *Arabidopsis* (Hundertmark et al. 2011; Bremer et al. 2017). The subcellular localization results show that *OsPM1* is located in on the cell membrane. Therefore, *OsPM1* may be a functional protein that exerts certain functions or participates in certain signaling pathways under stress conditions. As shown in the present



study, we found that compared with the control group, *OsPM1* overexpression of transgenic rice gave rise to increased sensitivity to ABA and salt treatments, whereas the expression of antisense transgenic rice exhibit hyposensitivity to ABA and salt treatments. Studies have shown that *OsPM1*, as an ABA influx carrier, promotes the entry of ABA into cells, and plays an important role in plant response to drought stress, and many ABA response genes are significantly up-regulated in *OsPM1* overexpression lines (Yao et al. 2018). Salt stress is mainly caused by the absorption of a large amount of  $\text{Na}^+$  in the soil by plants, which disrupts the cytoplasmic  $\text{Na}^+/\text{K}^+$  balance and causes cell membrane damage. (Saqib et al. 2005; Zhao et al. 2006). In addition, studies have shown that the microbiome can affect the ability of root diffusion barrier function and reduce the deposition of cork by inhibiting the ABA signal transduction pathway in the local endoderm of plants, which can enable plants to resist nutrient stress and salt stress (Salas-González et al. 2021). SODIUM ion transport protein HKT plays an important role in  $\text{Na}^+$  absorption. For example, rice *OsHKT2;1* can mediate a large amount of  $\text{Na}^+$  into the  $\text{K}^+$  starved rice roots. Tomoaki et al. also found that *OsHKT2;1* is localized in the cell membrane and its root expression is also high (Horie et al. 2007). In this experiment, we also found that *OsPM1* is located in the membrane system, and the GUS test results also show that its expression in roots is higher, similar to *OsHKT2;1*, so it is speculated that *OsPM1* may be involved in the transport of  $\text{Na}^+$ .

According to previous research results, the mechanisms of plants adapting to drought and salt stress are often interrelated and affect each other. Yao L et al. reported that the overexpression of *OsPM1* can increase the drought resistance of rice and the sensitivity of rice to ABA. Our results also showed that *OsPM1* is a positive regulator of rice drought tolerance, which is consistent with results of Yao L et al. Although a recent study showed that *OsPM1* expression in *OsNAC45* knockout mutant rice with a weak salt tolerance is significantly reduced, (Zhang et al. 2020) our results of transgenic plants showed *OsPM1* is a negative regulator of rice to salt stress. The salt sensitivity of *OsPM1* overexpression lines may be due to the fact that *OsPM1* overexpression promotes the influx of  $\text{Na}^+$ , which is more likely to cause ion poisoning in salt stress and ultimately lead to plant death (Ashraf et al. 2020; Liu et al. 2020).

## 5. Conclusion

In summary, the gene *OsPM1* is mainly located on the cell membrane, and its expression increases under various abiotic stresses such as salt, cold, and drought. In addition, further phenotypic treatment experiments confirmed that the antisense overexpressed *OsPM1* plants are hyposensitive tolerance to salt stress, but are not sensitive to ABA. These results lay a foundation for the potential use of *OsPM1* to improve rice abiotic stress.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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## Author contributions

Rongjun Chen conceived and designed the experiments; Wei Wang and Changqian Quan performed the experiments, and wrote the article; Shiwei Zheng, Yuwei Wang, Yuhuan Mo, Chuan Ma analyzed the data, produce the figures; Zhengjun Xu, Lihua Li, Zhengjian Huang, Xiaomei Jia, Xiaoying Ye, Jianqing Zhu, Huainian Liu provided support and experimental guidance for this study. All authors read and approved the final manuscript.

## Availability of data and material

All the data supporting the findings of this study are available within the article.

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