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# Identification and characterization of profilin gene family in rice

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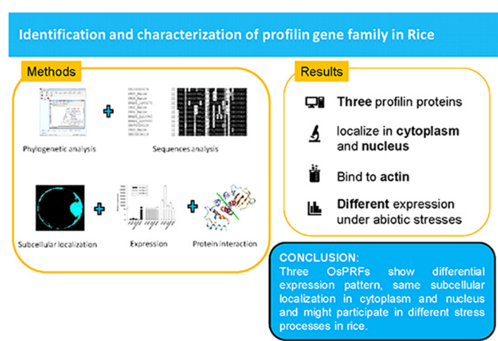
## GRAPHICAL ABSTRACT

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## ABSTRACT

*Background:* Profilin proteins (PRFs) are small (12–15 kD) actin-binding protein, which play a significant role in cytoskeleton dynamics and plant development via regulating actin polymerization. Profilins have been well documented in *Arabidopsis*, *Zea mays L.* as well as *Phaseolus vulgaris*, however no such fully characterization of rice (*Oryza sativa L.*) profilin gene family has been reported thus far.
*Result:* In the present study, a comprehensive genome-wide analysis of rice *PRF* genes was completed and three members were identified. *OsPRF1* and *OsPRF2* shared 98.5% similarity (6 nucleotide divergence), but the deduced amino acid sequences of OsPRF1 and OsPRF2 are fully identical. In contrast, the OsPRF3 presents relatively lower similarity with OsPRF1 and OsPRF2. Phylogenetic analysis also support that OsPRF1 has a closer relationship with OsPRF2. Expression pattern analysis revealed the differential expression of *OsPRFs* in tissues of mature plant, which suggested the potential spatial functional specificity for rice profilin genes. Subcellular localization analysis revealed the OsPRFs were localized in cytoplasm and nucleus and all of them could bind actin monomers. Furthermore, abiotic stresses and hormones treatments assay indicated that the three *OsPRF* genes could be differentially regulated, suggesting that *OsPRF* genes might participate in different stress processes in rice.
*Conclusions:* Taken together, our study provides a comprehensive analysis of the *OsPRF* gene family and will provide a basis for further studies on their roles in rice development and in response to abiotic stresses.
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## 1. Introduction

The actin cytoskeleton plays an important role throughout the whole plant life cycle, such as cell expansion and division [1,2,3], vesicle trafficking [4], organelle movement [5], as well as the response to abiotic and biotic stress signals [6,7,8,9,10]. Originally, there are a lot of proteins and factors taking part in these cellular processes by regulating the formation of microfilaments and microtubules, actin nucleation and maintain the homeostasis of G-actin [11,12]. ABPs (actin binding proteins) is a very important class of proteins participating in the regulation of this dynamic rearrangement of actin filaments, of which, profilin is thought to be one of the major modulators [13].

Profilin was discovered firstly as a small molecular mass (12–15 kD), abundant protein which could form a 1:1 complex with actin [14]. It specifically binds to G-actin, thus preventing the incorporation of G-actin and actin filaments as well as its nucleate [15]. Also, it is found that profilin is a dual-function actin regulatory protein that has been implicated in both actin polymerization and depolymerization in vivo [16]. Under low concentrations (1–5 μM), Human profilin interacts with the barbed ends of actin filaments and facilitates actin polymerization. On the contrary, higher concentrations (10–100 μM) could augment actin filament depolymerization and uncap actin filaments [17]. Besides binding to actin, profilins also can interact with poly-L-proline (PLP) and proline-rich proteins (such as formin), membrane polyphosphoinositides, phosphatidylinositol-3-kinase, annexin, and several multiprotein complexes (Arp2/3) that are implicated in the regulation of actin nucleation and endocytosis [18,19,20]. Additionally, there is growing evidence that, profilins function as hubs in mammalian cells that control a complex network of molecular interactions [21]. For plant cells, profilin expressed and accumulated at site of infection on the plasma membrane during defending exogenous fungi [22], it also accumulated in tip-growing plant cells [22,23,24], thus playing a part in both signal transduction and linkages between the plasma membrane and actin cytoskeleton. Furthermore, profilin has been reported to regulate the movement of chloroplast and membrane trafficking by enhancing the connection between chloroplast and actin filaments and its interaction with Class III PI3Ks [25,26].

Plant profilin was discovered by Valenta et al. [27] as an allergen source in birch pollen and confirmed as a plant actin binding protein. Since then, the structure, physical and chemical properties and functions of plant profilins have been widely and deeply studied and important progress has been made. Multiple profilin proteins are encoded in higher plants and are divided into two different classes mainly based on their distinct tissue expression patterns (vegetative profilins and reproductive profilins) [28]. Currently, about more than 400 profilin proteins from plants are available at NCBI (National Center for Biotechnology Information) gene database [29].

In *Arabidopsis thaliana* L., there are five profilin proteins (AtPRF1-AtPRF5). Three profilins (AtPRF1-AtPRF3) are vegetative (also constitutive) that are predominantly expressed in all vegetative tissues and ovules. By contrast, AtPRF4-AtPRF5 are selectively expressed in pollen [28,30,31]. Even for the three vegetative profilins, their expression patterns in young leaf tissue are not fully consistent [32]. AtPRF1 has much higher affinities for both poly-L-proline and G-actin compared with AtPRF2 [33]. Additionally, AtPRF1 contributes to stochastic actin dynamics by modulating formin-mediated actin nucleation and filament elongation during axial cell expansion [34]. Of note, AtPRF1 and AtPRF2 showed distinct distributions with observation of the GFP signals in living cells in stable transgenic lines [33]. AtPRF3 has notable biochemical features that differ from the other four AtPRFs, owing to an N-terminal extension, which was previously overlooked but is widely present in multiple species of eukaryotes. AtPRF3 had a stronger binding affinity to the AtFH1 polyP region, which subsequently induces the oligomerization of AtPRF3, leading to a negative regulation of actin polymerization. In addition, AtPRF3 plays specific roles in the growth-defense trade-off phenomenon as well as the plant's defensive responses during pathogen-plant interactions [8]. For *Maize*, there are two classes profilin proteins including five members. Class II profilins, including ZmPRO5 and ZmPRO4, have higher affinity for PLP and sequestered more monomeric actin than class I profilins. However, class I profilin (ZmPRO1) inhibited hydrolysis of membrane phosphatidylinositol-4,5-bisphosphate ($PIP_2$) by phospholipase C much more strongly than class II profilins [18]. Cotton profilin (*GhPFN1*) expresses closely related to the elongation of cotton fibers and plays a critical role in the elongation of cotton fibers by promoting actin polymerization [35]. In wheat, three isoforms of profilin were identified by polymerase chain reaction with profilin-specific primers [36]. In addition, a complete cDNA clone coding for timothy grass pollen profilin had been isolated and the predictive encoding protein has highest similarity with birch profiling [37]. Beyond that, profilin proteins were also studied in tobacco and tomato. Pronp1 (tobacco profilin gene) prominently functioned in two kinds of tip-growing cells and promoted the organization of actin cytoskeleton [38]. By contrast, tomato profilin LePRO1 was found only in pollen grains [39]. Common bean profilin protein was purified by poly-L-pro affinity chromatography and gel filtration, and it showed a diffuse distribution in the cytoplasm of hypocotyls and nodules but enhanced staining signal at the vascular bundles [40]. Furthermore, *Ricinus communis* phloem profilin (RcPRO1) showed its expression in epidermal, cortex, pith, and xylem tissue [41].

It is reported that even small changes in amino acid sequence can alter the biochemical properties of profilin substantially [42]. Therefore, characterizing the biochemical properties of individual profilin isoforms is important for gaining insight about its potential cellular function. In rice, two pollen-specific cDNA clones, *RproA* and *RproB*, were isolated from a cDNA library of rice mature pollen. Further southern and northern blot confirmed the existence of two rice profilin gene [43]. However, the specific biological role of profilins in rice still remains a mystery. Especially, whether there is any rice PRF proteins responding to abiotic stresses like AtPRF3. In the present study, we characterized the *PRF* genes in rice genome and analyzed the evolutionary relatedness of these rice PRFs with other species as well as the expression patterns. We also examined the subcellular localization of the OsPRFs and demonstrated the binding activity of OsPRFs to actin with different affinity. More importantly, we clarified the expression patterns of the *OsPRF* genes in response to abiotic stresses and exogenous hormones and found the unique features of OsPRFs upon hormones and abiotic treatment. All these results will provide a biological reference for further elucidating the role of *PRF* genes in rice.

## 2. Materials and methods

### 2.1. Sequences analysis

All the three rice profilin proteins sequences were obtained from the Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/). For Arabidopsis, data were collected from TAIR (https://www.arabidopsis.org/index.jsp). Others (*Sorghum bicoior, Maize, Brachypodium distachyon, Chlamydomonas reinhardtii, Yeast*) were obtained from UniProt (https://www.uniprot.org/). All the sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and then the

Multiple-Alignment file was shaded by ExPASy (https://embnet.vital-it.ch/software/BOX_form.html). The physicochemical properties and post-translational modifications such as sumoylation sites and phosphorylation sites were predicted at ExPASy Protein (www.expasy.org). The actin interaction sites and ploy-proline binding sites among the three rice profilin proteins were predicted at NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi).

### 2.2. Phylogenetic analysis

All the profilin proteins sequences of various rice varieties and others profilin proteins sequences (*Sorghum bicoior, Maize, Brachypodium distachyon, Triticum aestivum*) were obtained from UniProt (https://www.uniprot.org/). The 36 profilin proteins from different rice subspecies were aligned using Clustal alignment and the evolutionary history was inferred using the Neighbor-Joining method [44]. The optimal tree with the sum of branch length = 127.25170898 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches [45]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method [46] and were in the units of the number of amino acid differences per sequence. The analysis involved 36 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 115 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [47].

### 2.3. Plant material and growth conditions

Rice seeds (Nipponbare) were sterilized with 95% ethyl alcohol and washed with sterile water five times. The sterilized seeds were soaked in water for 2 d, and the uniformly germinated seeds were sown in bottomless 96-well PCR plates with appropriate spacing in nutrient solution. Then they were grown at 30°C under white light at approximately 20 μmol $m^{-2}$ $s^{-1}$ (14 h) and 25°C in dark (10 h) cycle in a growth chamber before experiments. Plants were cultured for one or four weeks then treated with different hormones and abiotic stresses.

### 2.4. RNA isolation and quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were treated with DNase I (TaKaRa) and then subjected to Prime-ScriptTMRT reagent Kit (TaKaRa), and qRT-PCR was performed using SYBR Premix (TaKaRa). The relative expression level of a target gene was normalized to that of rice ACTIN1 (LOC_Os03g50885). All primers used in qRT-PCR are listed in Table S1

### 2.5. Subcellular localization

To investigate the subcellular localization of rice profilin genes (*OsPRF1*, *OsPRF2*, *OsPRF3*), the coding sequences were amplified by PCR and separately inserted into Gateway expression vector pGWB444 [48] using the pENTR/D-Topo donor vector. The constructs were transiently expressed in protoplasts from wild type Nipponbare cell suspensions by polyethylene glycol-mediated transformation. Fluorescence signals were visualized with confocal fluorescence scanning microscope (LSM710, Carl Zeiss, Germany) [49].

### 2.6. Expression and purification of OsPRF1, OsPRF2 and OsPRF3 in *Escherichia coli*

The GFP fragment was amplified from pGA3427 [50] and digested with EcoRV and BglII, then inserted into pET30a to generate pET30a-GFP vector. The *OsPRF1, OsPRF2* and *OsPRF3* fragments were amplified from wild type Nipponbare cDNA. *EcoR* I and *BamH* I were used to digest the fragments, which were then inserted individually into pET30a-GFP for GFP-HIS-tagged protein expression in the *E. coli* transetta (DE3) strain. Cells containing these vectors separately were grown to an OD600 of 0.6 at 37°C and were induced with 0.6 mmol/L Isopropyl β-D-Thiogalactoside (IPTG) at 18°C overnight. Cultures were collected by centrifugation and resuspended in binding buffer (40 mM PBS, pH 7.4). GFP-HIS-tagged proteins were purified using a Ni-NTA His bind resin. Protein concentrations were determined using the Bradford reagent (Bio-Rad) and BSA as a standard [51].

### 2.7. Actin binding assay

The in vitro actin binding assay was performed according to the previously described methods with minor modifications [52]. OsPRF1-GFP-HIS, OsPRF2-GFP-HIS and OsPRF3-GFP-HIS obtained before were mixed with rabbit monomeric actin (Sigma) followed by absorption using Ni-NTA His bind beads. After several washes of beads, the eluted protein samples were subjected to the protein gel blot analysis described below.

### 2.8. Immunoblotting experiments

Proteins were separated by 10% SDS-PAGE and then transferred to nitrocellulose for immunoblotting experiments. Nitrocellulose membrane was blocked with 3% milk in TBST (0.8% NaCl, 0.02% KCl, 0.3% Tris, pH 7.4, 0.05% Tween20) for 60 min at room temperature. Excess milk was washed off with TBST. Blots were incubated in primary antibodies overnight at 4°C. Secondary antibodies were then applied for 1 h at room temperature. Blots were developed using the ECL solutions (Invitrogen) and detected by ChemiDoc Touch (BioRad).

### 2.9. Ultraviolet absorption spectra of actin polymerization

The effects of profilins on rabbit muscle actin polymerization in vitro were analyzed by using ultraviolet absorption spectrum measurement according to Ren et al. [53,54]. G-actin from rabbit was added to the incubation buffer (PBS, pH = 7.5, 0.2 mmol/L ATP, 5 mmol/L $MgCl_2$, 0.1 mol/L KCl) to a concentration of 4 μmol/L and then incubated at 4°C for 2 h. After incubation, added GFP-HIS, OsPRF1-GFP-HIS, OsPRF2-GFP-HIS and OsPRF3-GFP-HIS at the ratio of 1:8 to G-actin separately. The absorption of G-actin solution at 232 nm was measured every 10 s by spectrophotometer for 30 min.

### 2.10. Abiotic stress treatments

For abiotic stress treatments, seedlings were grown at 30°C in nutrients solutions under white light at approximately 20 μmol $m^{-2}$ $s^{-1}$ (14 h)/dark (10 h) cycle in a growth chamber for four weeks. Then the seedlings were transferred to nutrients solutions containing 20% PEG6000, and 1% NaCl, respectively [55]. For low and high temperature stresses, the seedlings were grown inside a cooler at 4°C or incubator at 45°C [56,57]. UV-B and white light treatments were induced by placing them under UV-B (supplemented with Philips UV-B tubes, TL20W/01RS; 1.5 μmol $m^{-2}$ $s^{-1}$) [58] and white light (20 μmol $m^{-2}$ $s^{-1}$) (darkness treatment for 24 h before treatment). Leaf samples were

collected at indicated time points after treatment for further analysis.

### 2.11. Hormones treatment

For hormone treatment and gene expression analysis, 7-d-old plants were immersed in nutrients solutions containing different kinds of phytohormones for 2 h, including BR (brassinolide, 10 μM), $GA_3$ (gibberellin, 10 μM), IAA (10 μM), Me-JA (jasmonic acid, 10 μM), ABA (abscisic acid, 10 μM), SA (salicylic acid, 10 μM), and GR-24 (strigolactone, 10 μM). Twenty plants for each treatment were collected for RNA isolation [59].

## 3. Results

### 3.1. Identification of OsPRF genes in the rice genome

To better understand the role of profilins in rice, we did a BLAST search using all Arabidopsis profilin protein sequences as query in Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu/) and three profilin protein (OsPRF1, LOC_Os10g17660; OsPRF2, LOC_Os10g17680; OsPRF3, LOC_Os06g05880) were identified in the rice genome. Furthermore, we used the RAP-DB (https://rapdb.dna.affrc.go.jp/) and SMART (http://smart.embl-heidelberg.de/smart/) database to find if there are any potential profilin proteins and to prevent the loss of some atypical profilin genes. All the analysis confirmed that there are three profilin genes in rice. Subsequently, they were used for further study (Table 1).

*OsPRF1* and *OsPRF2* are both located on chromosome 10, whereas *OsPRF3* is distributed on chromosome 6. At the nucleotide level, three genes shared 79.3–98.5% similarity. Gene structure analysis revealed that each *OsPRFs* has two introns, whereas the length of each intron was different. *OsPRF3* has larger intron than *OsPRF1* and *OsPRF2* (Fig. S1). Moreover, the 5′UTR and 3′UTR sequences of the three genes are diverse from each other, suggesting that they might perform various functions (Fig. S2). When we zoomed in the coding region, six nucleotide differences between *OsPRF1* and *OsPRF2* were discovered, whereas their corresponding deduced protein sequences were fully identical. In contrast, OsPRF3 has 23 amino acids variation compared with the other two (Fig. 1, Fig. S3). It is interesting to point out that all the OsPRFs consisted of 131 amino acid residues and shared nearly the same molecular weight, isolectric point value, instability index, aliphatic index and hydropathicity. Also, they might keep the same phosphorylation sites and sumoylation sites, excepting that OsPRF3 missed one sumoylation site (K43) predicted by ExPASy website (https://www.expasy.org/) (Table 1, Fig. S1).

Alignment of the profilin amino acid sequences among different eukaryotic kingdoms revealed relatively low levels of sequence identity. However, among plant kingdoms, such as *Oryza sativa* L., *Arabidopsis, Maize, Brachypodium distachyon* and *Sorghum bicoior*, the proteins sequence of profilins are highly conserved. For example, only single residue was inconsistent in comparison of profilin sequence pair between rice OsPRF1 and *Sorghum* SbPRF1. Previous studies have shown that profilin can bind to monomeric actin and poly-proline protein [60,61]. As shown in Fig. 1, it predicted at NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi) that there are five sites marked with red star have high affinity to monomeric actin and eleven sites marked with green triangle have high affinity to poly-proline proteins (like formins) in the three rice profilins. Thus, the OsPRFs, characterized here, are appropriated for functional exploring.

### 3.2. Phylogenetic analysis of OsPRF proteins

Previously, analyses of phylogeny and expression patterns of mRNA and promoter-reporter gene fusions in transgenic plants have suggested that there are two distinct classes of profilin proteins in higher plants: constitutive (vegetative) and pollen-specific (reproductive). These proteins in the two classes differ by 27% in amino acid sequence [28,62]. To analyze the evolutionary relationships of the profilin gene family in different rice varieties or other monocots, an unrooted phylogenetic tree was drawn by the neighbor-joining (N-J) method using MEGA 5 Software using 36 full-length amino acid sequences (Fig. 2). Based on the statistical analysis and high bootstrap support value, monocotyledonous plant profilins were classified into two clades: vegetative and reproductive, as expected. Both different rice varieties and other monocots as shown in Fig. 2 all possess the above two types of profilin proteins basically. Furthermore, the amount of reproductive protein was more than that of vegetative protein in most species. OsPRF1 and OsPRF2 have very high sequence homology in both *japonica* and *indica* and make a big clade with *Maize* (PRO1, PRO2, PRO3), which were suggested as reproductive protein [18]. In contrast, OsPRF3 falls apart and forms a branch with *Maize* (PRO4 and PRO5), which belong to vegetative protein. These analyses indicated a low sequence homology, different ancestors and individual evolutionary path for their own group. Moreover, OsPRF1 and OsPRF2 made a phylogenetic clade with *Sorghum bicoior* profilins (SB03G034110) and *Maize* profilins (PRO3) with relatively low bootstrap support value suggested their diversification during the course of evolution even after being originated from common ancestor. Followed by *Brachypodium distachyon* and *Triticum aestivum*, rice profilin proteins and the other monocots profilin proteins have a high degree of homology in generally and they may play similar roles in the reproductive growth process of plants (Fig. 2). The phylogenetic analysis helped in the nomenclature and classification of rice profilin genes.

### 3.3. Expression patterns of OsPRFs

To further study tissue-specific expression of the three profilin genes, multiple different rice tissues were used for qRT-PCR and semi-quantitative RT-PCR analyses including roots, stems, leaves, leaf sheath, spikes. The relative transcript levels of these three *OsPRFs* were examined by gene-specific PCR primers that amplified portions of the coding region and UTR of the transcripts from these profilin genes. *ACTIN* was collected as an internal reference gene. Generally, the transcription of *OsPRF3* is the highest and *OsPRF1* is the lowest, according to the qRT-PCR analysis. In addition, the expression of *OsPRF2*, even it encoded identical amino acid sequence with *OsPRF1*, is significantly higher than *OsPRF1*. It is

**Table 1**
Predicted rice profilin protein sequence features.

| Protein | LOC | AA | MW (KD) | pI | instability index | Aliphatic index | hydropathicity | sumoylation sites | phosphorylation sites |
|---|---|---|---|---|---|---|---|---|---|
| OsPRF1 | LOC_Os10g17660 | 131 | 14.24 | 4.85 | 28.91 | 78.09 | −0.108 | 4 (K43, K87, K52, K97) | 17 |
| OsPRF2 | LOC_Os10g17680 | 131 | 14.24 | 4.85 | 28.91 | 78.09 | −0.108 | 4 (K43, K87, K52, K97) | 17 |
| OsPRF3 | LOC_Os06g05880 | 131 | 14.13 | 4.62 | 31.57 | 84.81 | −0.071 | 3 (K87, K52, K97) | 17 |

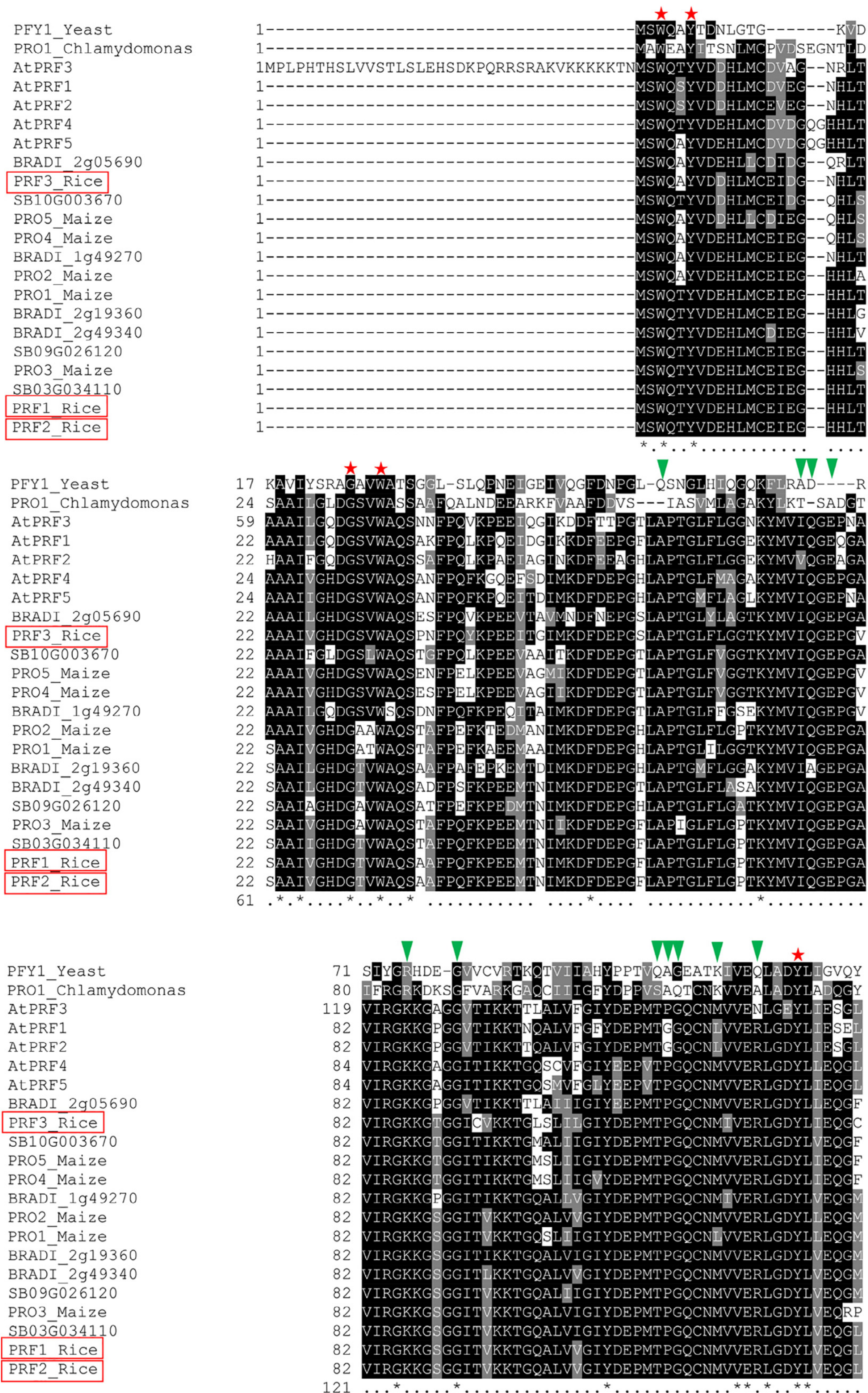


**Fig. 1.** Multiple sequence alignment of amino acid sequences of 22 profilin proteins by ClustalW. The multiple alignment results clearly showed the high conservation among these species. The actin interaction sites and ploy-proline binding sites were marked with red star and green triangle, respectively. AtPRF1/2/3/4/5, Arabidopsis; BRADI_2g05690, BRADI_1g49270, BRADI_2g19360, BRADI_2g49340, Brachypodium distachyon; SB_10G003670, SB_06G026120, SB_03G034110, Sorghum bicoior.

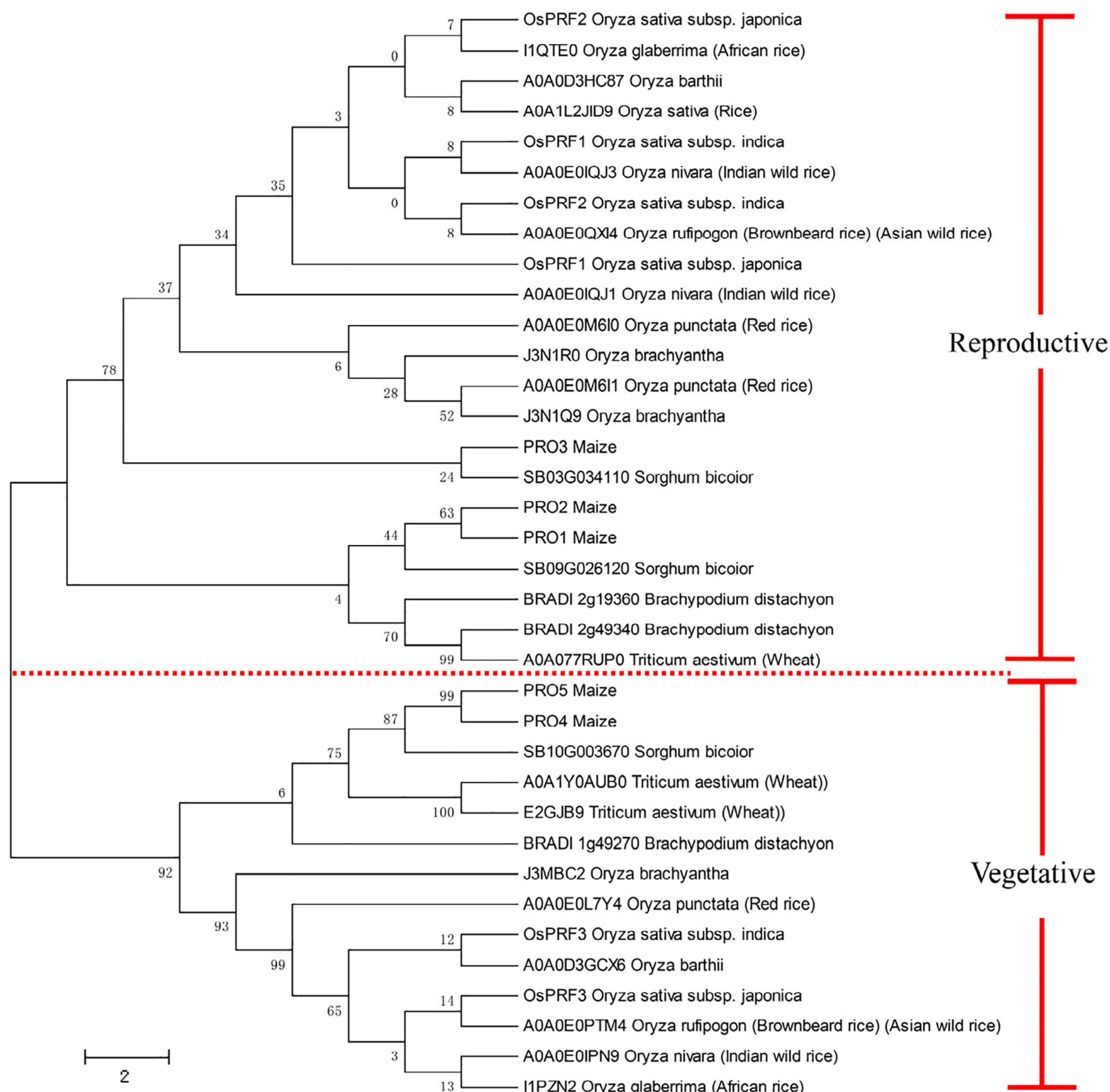


**Fig. 2.** Phylogenetic tree of profilin proteins. Protein sequences were aligned and a phylogenetic tree was constructed by the neighbor-joining (N-J) method using MEGA5.1 software with 1000 bootstrap replicates using 36 full-length amino acid sequences from 5 species. The numbers above the branches indicate the bootstrap value.

noteworthy that, both OsPRF1 and OsPRF2 were predominantly accumulated in the spikes. That phenomenon is consistent with findings in *AtPRF4* and *AtPRF5* [28]. The difference is that, OsPRF2 was relatively high in all collected tissues, while OsPRF1 was hardly detected in root, stem and leaf tissues. Unlike the *OsPRF1* and *OsPRF2*, *OsPRF3* showed significantly higher expression in various plant tissues with highest expression in stems and lowest in spikes (Fig. 3a).

Semi quantitative RT-PCR analysis was conducted to confirm the expression patterns of the three profilin genes. The results suggested that *OsPRF3* was constitutively expressed in all the tested tissues, including root, sheath, leaf, spike, relatively higher levels detected in stems. However, *OsPRF1* and *OsPRF2* were expressed mainly in the spikes (Fig. 3b). All these investigations were consistent with those of qRT-PCR result. Our data indicate that rice profilin genes are expressed differently in different tissues of mature plant. The differences in individual profilin expression patterns suggest the potential spatial functional specificity during rice domestication.

### 3.4. Subcellular localization of OsPRF proteins

Because the protein's function is usually related to its subcellular localization, the ability to predict subcellular localization directly from protein sequences and validation by substantial experiments had been broadly applied for inferring protein functions [63]. In order to determine the localization of OsPRFs in rice cells, we analyzed its signal peptide using TargetP [64], but no clear clues were obtained. Therefore, we used PRF-CFP fusion protein to assess the subcellular localization of OsPRFs in rice protoplasts. Meanwhile, the blue fluorescence signal from DAPI staining and red fluorescence signal from chlorophyll were used to indicate the position of nucleus and chloroplast. By the confocal laser scanning microscope analysis, we found both OsPRF1-CFP and OsPRF2-

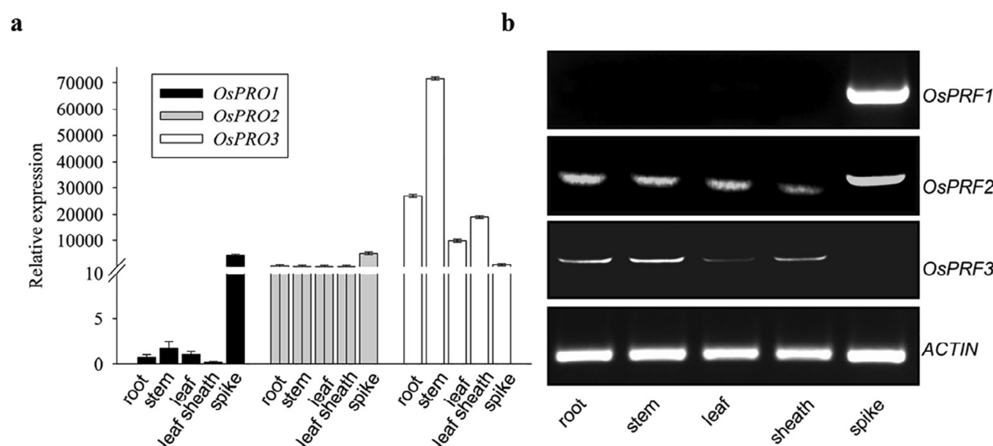


**Fig. 3.** Expression patterns of *OsPRF1*, *OsPRF2* and *OsPRF3*. (a) qRT-PCR analysis of *OsPRF1/2/3* expression patterns in various tissues. (b) Semi quantitative RT-PCR analysis showed differential expression levels of *OsPRF1/2/3* in various tissues of mature seedlings. Error bars represent the standard error of the mean. The qRT-PCR experiments represent three independent experiments (biological replicates). *ACTIN* was used as an internal standard.

CFP signals were co-localised with 35S-GFP, which had been revealed previously as markers of cytoplasm and nucleus [65,66], suggesting that OsPRF1 and OsPRF2 were cytoplasm and nucleus-localized (Fig. 4a–l).

Similar to the OsPRF1 and OsPRF2, OsPRF3 was suggested to localize in cytoplasm and nucleus as well (Fig. 4m–r). However, it is notable that its distribution in the nucleus and cytoplasm is not the same. It is clearly found from the cyan fluorescent signal that it is more distributed in the nucleus. This may be a way of PRF protein especially OsPRF3 participating in actin arrangement linked with various biological and abiotic signals.

### 3.5. OsPRF proteins can bind to actin in vitro

Profilin proteins had been reported to perform actin binding activity in several organisms [67]. To test whether OsPRFs also present the similar actin binding activity, we inserted GFP tag into the protein expression vector pET30a, and *OsPRF1*, *OsPRF2* or *OsPRF3* fragments were inserted into the modified vector individually. Finally, the resulting fusion proteins (OsPRF1-GFP-HIS, OsPRF2-GFP-HIS and OsPRF3-GFP-HIS) with correct size were purified (Fig. 5a). Then, HIS antibody was used to detect the quality of the purified proteins (Fig. 5b) and the results indicated that the quantity and quality of purified proteins are competent for further investigation. We then mixed the GFP-HIS, OsPRF1-GFP-HIS, OsPRF2-GFP-HIS and OsPRF3-GFP-HIS proteins with rabbit muscle actin to do in vitro binding assay followed by protein gel blot analysis. As shown in Fig. 5c, the OsPRF1-GFP-HIS, OsPRF2-GFP-HIS and OsPRF3-GFP-HIS bound to actin in vitro (lane 2, 3, 4), whereas the control GFP-HIS failed (lane 1). It should be noted that the actin-binding capacity of OsPRF1 and OsPRF2 is greater than that of OsPRF3.

Previously, profilin had been suggested as a dual-function actin regulatory protein that function in both actin polymerization and depolymerization in vivo [16]. In order to examine whether OsPRFs also present actin polymerization ability and any difference between OsPRF1, OsPRF2 and OsPRF3, we performed the ultraviolet spectrophotometer experiment following the instruction by Ren et al. [53] with the ultraviolet spectrophotometer. As shown in Fig. 5d, after 2 h of polymerization, the microfilaments and actin monomers in the system are basically stable, that is, the polymerization and depolymerization reached a dynamic balance (yellow). However, when OsPRF1-GFP-HIS and OsPRF2-GFP-HIS were added, the polymerization was greatly promoted (red and black). In contrast, the addition of OsPRF3-GFP-HIS also promoted the polymerization (green), whereas the polymerization capacity of OsPRF3, compared with OsPRF1 and OsPRF2, was slightly weak. Together, these results suggested that the OsPRFs are involved in actin binding and polymerization reaction.

### 3.6. Expression regulation of OsPRF genes under abiotic stresses and hormones

Many profilin genes play important roles in the growth and development of plants, but less study has focused on the expression pattern of profilin genes under abiotic stresses and hormones. Especially for rice profilins, such kinds of investigation are still missing. In this study, we investigated the expression patterns of rice *PRF* genes in the aerial tissues of four-week-old rice seedlings, in response to various hormones and abiotic stressors including UV-B irradiation, salinity (NaCl), drought (PEG), cold (4°C) and heat (42°C).

As shown in Fig. 6, the *OsPRF* gene family was differentially regulated upon varied treatments. In general, the expression of *OsPRF1* and *OsPRF2* were more active than *OsPRF3*. However, for particular treatment (such as salt), *OsPRF3* was up-regulated greatly than *OsPRF1* and *OsPRF2,* OsPRF3 reached its maximal expression at 12 h under salt stress (Fig. 6a). Upon PEG treatment, the expression of all three *OsPRFs* decreased gradually within 2 h compared with normal conditions (Fig. 6b). Similarly, the decreased expressions were detected on both *OsPRF1* and *OsPRF2* with light treatment (Fig. 6c). Under UV-B conditions, both *OsPRF1* and *OsPRF2* showed quick induction within 15mins and subsequently returned back to normal level for 1 h (Fig. 6d). In addition, the expression of three *OsPRFs* is relatively stable under cold stresses (Fig. 6e). Under heat stress condition, *OsPRF1* and *OsPRF2* were slightly up-regulated, but they present different up-regulation speeds (highest expression of *OsPRF1* was appeared at 30 min and *OsPRF2* was at 1 h) (Fig. 6f). In contrast, the expression of *OsPRF3* was less affected by heat stress. Previous studies have found that ACTIN expression is in co-ordination with profilin transcriptional level and modulation in profilin also affects ACTIN transcription [68]. Therefore, we used another internal reference gene (histone H3) to revalidate the results. As shown in Fig. S4, the expression patterns of profilin genes under NaCl and cold treatments are similar with the results in Fig. 6, suggesting that ACTIN transcription is relatively stable in our analysis. All these results indicated that the *OsPRF* genes might participate in different stress responses in rice.

To investigate whether phytohormones affect the transcription of the *OsPRF* genes, we analyzed the expression levels of *OsPRFs* in

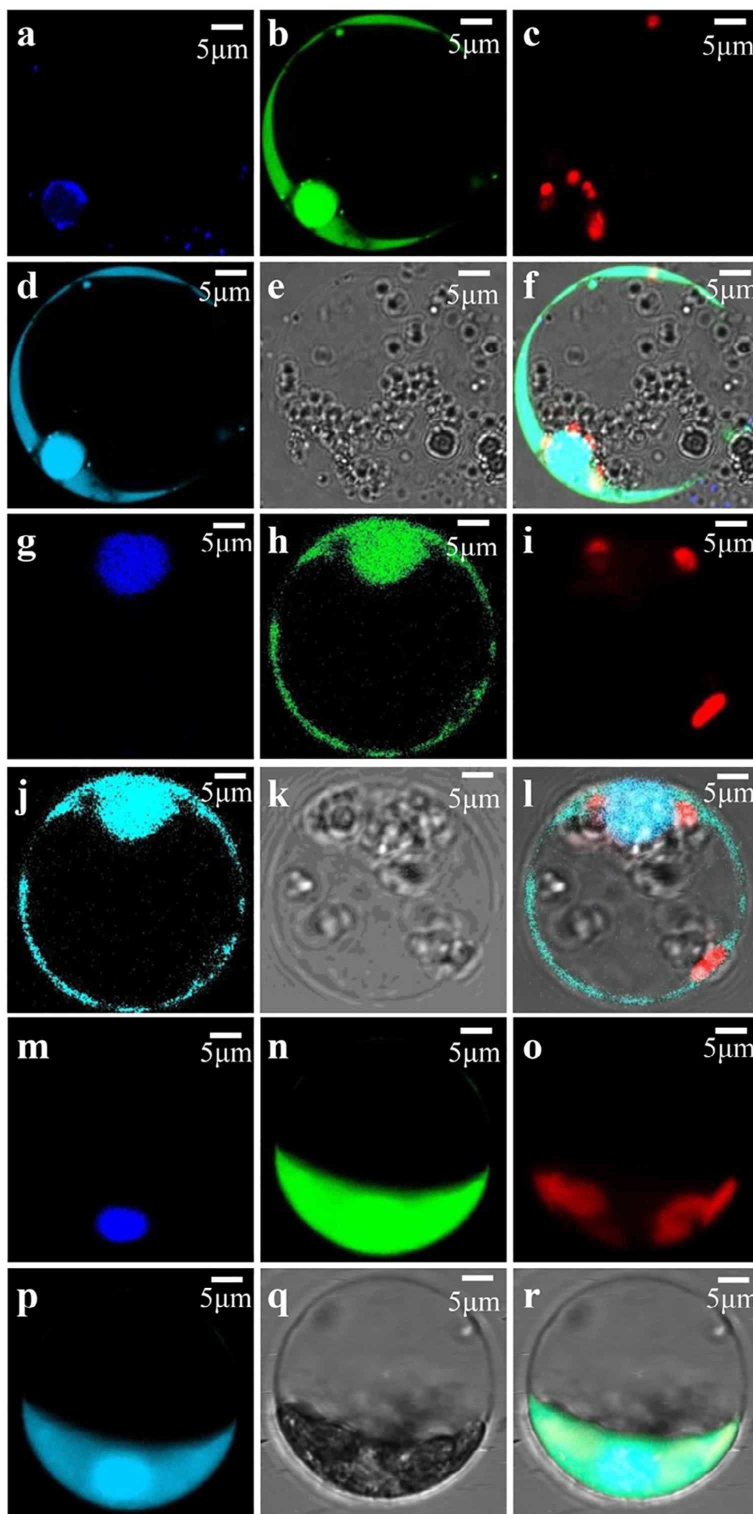


**Fig. 4.** Subcellular localization of OsPRF1/2/3 proteins in rice protoplast. (a-f) Subcellular localization of OsPRF1-CFP. Bar = 5 μm; Images in bright field (e), DAPI (a), 35S-GFP (b), Chloroplast auto-fluorescence signal (c), CFP signal (d), Merge (f). (g-l) Subcellular localization of OsPRF2-CFP. Bar = 5 μm. Images in bright field (k), DAPI (g), 35S-GFP (h), Chloroplast auto-fluorescence signal (i), CFP signal (j), Merge (l). (m-r) Subcellular localization of OsPRF3-CFP. Bar = 5 μm. Images in bright field (q), DAPI (m), 35S-GFP (n), Chloroplast auto-fluorescence signal (o), CFP signal (p), Merge (r).

WT seedlings under the treatment of various hormones. After 2 h application of hormones, we found that the three profilin genes showed different patterns. Generally, the expression of OsPRF1 and OsPRF3 are less changed and the biggest change of *OsPRF1* happened on GA with 1.5-fold increase (Fig. 7a). Meanwhile, *OsPRF3* showed similar expression pattern with *OsPRF1* (Fig. 7c). On the contrary, *OsPRF2* were obviously down-regulated under multiple hormone treatments and the maximal change was

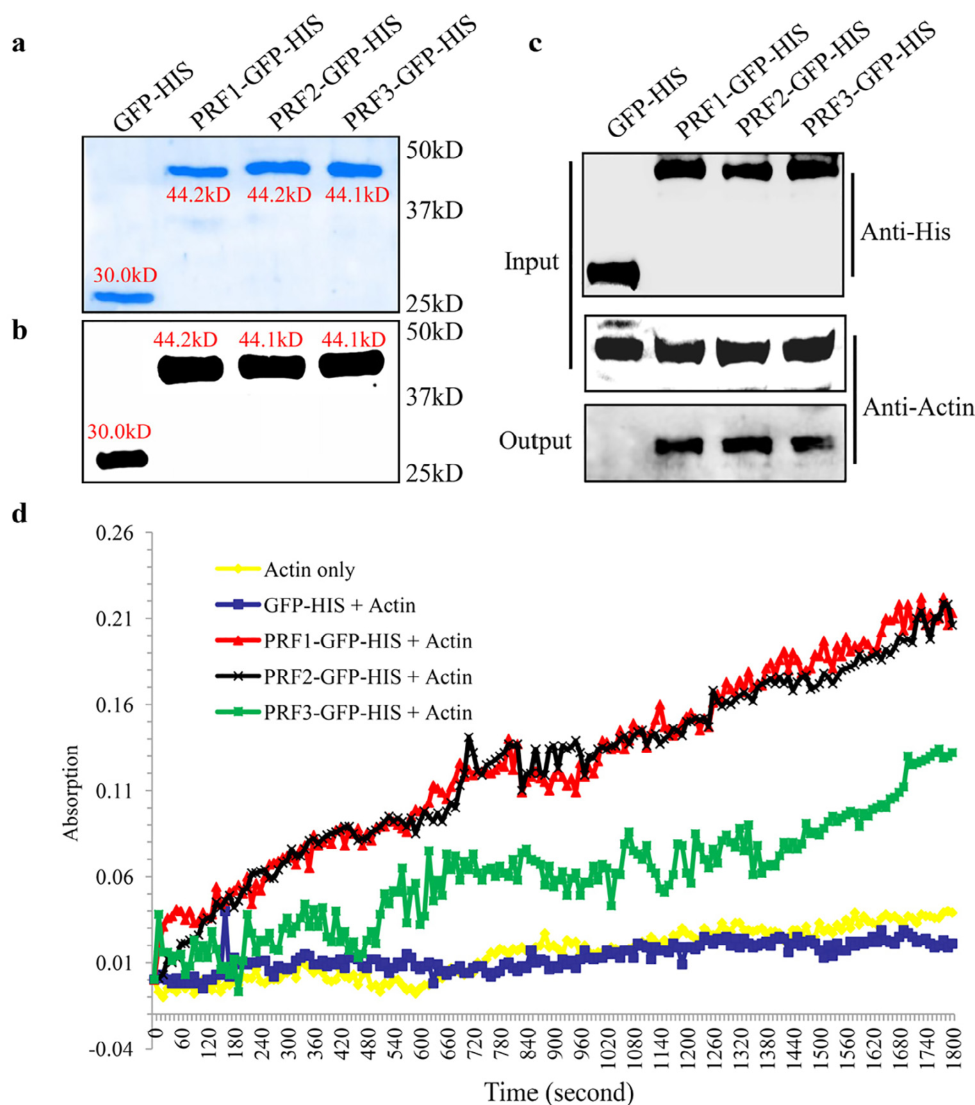


**Fig. 5.** In vitro binding and actin polymerization effect of OsPRF1/2/3-GFP-HIS. (a) Coomassie blue staining of GFP-HIS (lane 1), OsPRF1-GFP-HIS (lane 2), OsPRF2-GFP-HIS (lane 3) and OsPRF3-GFP-HIS (lane 4) proteins on a gel before binding assay to actin. (b) Protein gel blot of GFP-HIS (lane 1), OsPRF1-GFP-HIS (lane 2), OsPRF2-GFP-HIS (lane 3) and OsPRF3-GFP-HIS (lane 4) treated with Anti-His antibody. (c) In vitro binding of OsPRF1/2/3-GFP-HIS to actin. GFP-HIS (lane 1), OsPRF1-GFP-HIS (lane 2), OsPRF1-GFP-HIS (lane 3) and OsPRF3-GFP-HIS (lane 4) were subjected to in vitro binding assay to actin and then analyzed by protein gel blotting using anti-Actin antibody. (d) Ultraviolet absorption spectra of actin polymerization. 4 μmol/L actin only (yellow); 4 μmol/L actin + 0.5 μmol/L GFP-HIS (blue); 4 μmol/L actin + 0.5 μmol/L OsPRF1-GFP-HIS (red); 4 μmol/L actin + 0.5 μmol/L OsPRF2-GFP-HIS (black); 4 μmol/L actin + 0.5 μmol/L OsPRF3-GFP-HIS (green).

observed on SA treatment, followed by GR-24 and BR (Fig. 7b). These data suggested that *OsPRF1* and *OsPRF3* are presumably not involving in hormone signaling pathway.

## 4. Discussion

In plant cells, profilin proteins are encoded by polygenic families. In present study, we identified 3 profilin genes in rice genome. Furthermore, to understand the potential function and evolution of these genes, we analyzed the distribution in the genome, expression patterns and the subcellular localizations, constructed a phylogenetic tree, and performed analysis as actin binding proteins and G-actin polymerization factors. Conclusively, we analyzed the expression patterns of *OsPRFs* under different stresses and hormones. This study is the first comparative analysis of the rice profilin gene family, and we believe that our results showed here should benefit to explore the function of *PRF* genes varied during the development and stresses response in rice.

At present, PRF proteins in plants has been broadly characterized from physiological characters to specific functions, especially in model plants such as *Arabidopsis thaliana*. However, in the pivotal food crop rice, OsPRFs has not been studied in a systematic and in-depth way. Previous study has shown that there are two profilin proteins in rice (named OsPRO1 (LOC_Os10g17680) and OsPRO2 (LOC_Os06g05880)) [69]. However, through exhaustive analysis, we identified one more profilin beside of *OsPRO1* and *OsPRO2* in rice genome. Therefore, we redefined the *OsPRF* genes in rice: Os*PRF1* (LOC_Os10g17660), *OsPRF2* (LOC_Os10g17680) and *OsPRF3* (LOC_Os06g05880). It is noteworthy that even the protein sequences of OsPRF1 and OsPRF2 are identical, but there are six nucleotides difference on coding sequences (Fig. S1). Moreover, the sequences of 5′ and 3′UTR (untranslated region), the important regulatory region for RNA transcription, RNA translation and RNA stability [70], showed obvious difference between Os*PRF1* and Os*PRF2* (Fig. S1), which is consistent with different expression patterns of *OsPRF1* and *OsPRF2* in multiple rice tissues (Fig. 3), abiotic

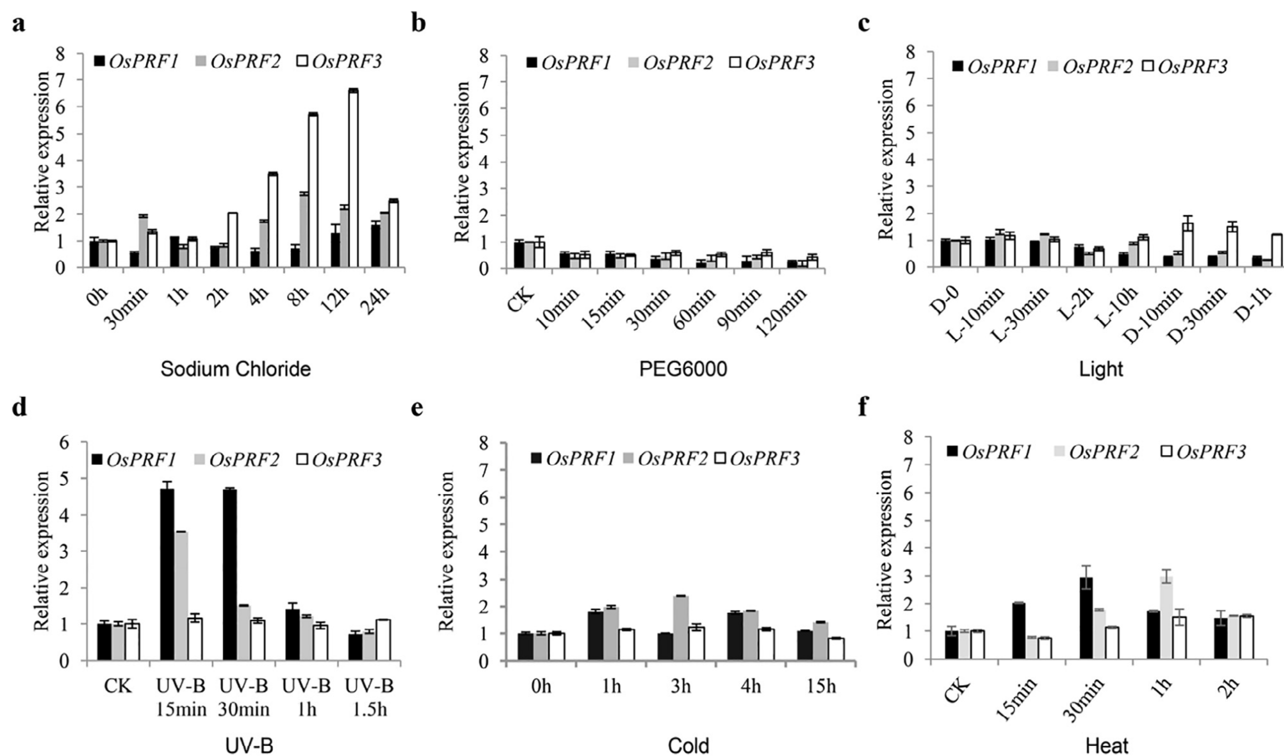


**Fig. 6.** Expression patterns of *OsPRF1/2/3* in response to abiotic stressors. Rice seedlings grown for 30 days in nutrients solutions were treated with (a) NaCl (1% w/v), (b) PEG 6000 (20% w/v), (c) light, (d) UV-B, (e) cold (4°C) and (f) heat (45°C). Total RNA was extracted from shoot parts and used for real-time quantitative PCR. The expression in the untreated control samples was set as 1. Error bars represent the standard error of the mean. The qRT-PCR experiments represent three independent experiments (biological replicates). *ACTIN* was used as an internal standard.

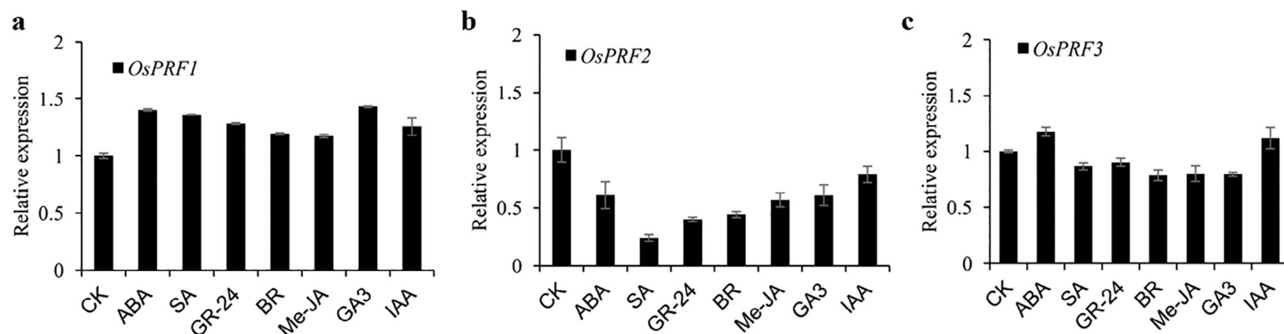


**Fig. 7.** Expression patterns of *OsPRF1/2/3* in response to different phytohormone. Rice seedlings grown for 7 days in nutrients solutions were treated with different hormones. (a) Relative expression level of *OsPRF1* under different phytohormone treatments. (b) Relative expression level of *OsPRF2* under different phytohormone treatments. (c) Relative expression level of *OsPRF3* under different phytohormone treatments. ABA, abscisic acid; SA, salicylic acid; rac-GR-24, Strigolactone; BR, brassinolide; Me-JA, methyl jasmonic acid; GA3, gibberellic acid; IAA, indole-3-acetic acid. Ethanol treatment was used as the standard "1" and *ACTIN 1* as the internal standard.

stresses (Fig. 6) and hormones (Fig. 7). Above all, the identification and characterization of three *OsPRFs* will be helpful to explore the unique functions of the OsPRF proteins.

In the present study, gene structure analysis showed that each of *OsPRFs* contain three exons, and be separated by introns of different sizes. This is similar to the structures of profilin genes found in several species [62]. Comparing the amino acid sequences of different profilins found so far reveal that profilins have less than 25% identity across different kingdoms [71]. Interestingly, they are highly conserved within various plant species with at least 70% identity [72]. This is consistent with the findings by the sequence alignment and phylogenetic tree analysis (Fig. 1 and Fig. 2). As we know, profilin proteins can bind to actin and regulate the cytoskeleton [71]. Our search in NCBI database showed OsPRFs hold several possible actin interaction sites as well as the poly-proline binding sites (Fig. 1) and these sites are highly conserved within the plant kingdom, which indicated the conservation of PRF proteins during the evolution among plants.

PRF proteins are divided into two classes according to their expression patterns [30]. In *Arabidopsis*, *PRF1*, *PRF2*, and *PRF3* are constitutively and strongly expressed in all vegetative tissues at various stages of plant development. In contrast, *PRF4* and *PRF5* are late pollen-specific and are not detectable in other cell types of the plant body [28]. In rice, *OsPRF3* expressed constitutively in all vegetative tissues with a higher level compared with *OsPRF1* and *OsPRF2*. It may be involved in plant vegetative development. *OsPRF1* is similar to *AtPRF4* and *AtPRF5*, which expressed mainly in pollen and little in other tissues. However, *OsPRF2* is unique, which expressed in all tissues with a more similar level (Fig. 3).

Three distinct expression patterns suggested that they had their own responsibilities at different stages of plant growth.

To understand the physiological role of proteins, it would be of great benefit to reveal its localization in living cells. Previous studies have shown that *Arabidopsis* AtPRF2 localized in both nuclear and cytosolic in root cells, and BFA (Brefeldin A) treatment caused enrichment of AtPRF2 [73]. Haiyun Ren and colleagues reported in 2009 that GFP-AtPRF1 fusion protein exhibited filamentous distribution but GFP-AtPRF2 formed polygonal meshes in leaf epidermal cells, trichomes and stem epidermal cells respectively. Moreover, both fusion proteins were associated with moving organelles resembling vesicles [33]. *Maize* profilin is associated with the plasma membrane in microspores and pollen [23]. But for most plant profilins, the precise localization in cells is still unclear, especially rice PRFs. To further address this question, we analyzed the subcellular localization of OsPRFs in rice protoplasts. Similar to *Arabidopsis* AtPRF2 [73], OsPRFs are all localized in nuclear and cytoplasm (Fig. 4). Previously, the investigations suggested that cytoplasmic PRF proteins may involve in the establishment of cytoskeleton in cytoplasm, and directly participate in the transport of organelles and intracellular substances [25,26]. Therefore, the potential role of OsPRFs in cytoplasm is probably to polymerize and depolymerize of actin. Substantially, our data support the mind that OsPRFs did bind to actin in vitro (Fig. 5), which further support the actin binding feature of OsPRFs in cytoplasm. In addition, the investigation also revealed the presence of profilin proteins in the nucleus, probably function in RNA splicing, chromatin remodeling and transcriptional regulation [74,75,76]. For example, in fibroblasts and HeLa cells, profilin I co-localized with small nuclear ribonucleoprotein particle -core proteins and p80 coilin-containing Cajal bodies, indicating a role for profilin during pre-mRNA processing [77]. A Myb-related transcription factor, p42POP, as a new ligand for profilin was regulated by profilin in the nucleus [78]. Besides a general cytoplasmic distribution, profilin specifically accumulates in the nucleus in *Tradescantia virginiana* stamen hair cells [79]. Here, our results showed that rice PRF3 also displayed aggregation in the nucleus (Fig. 4j). We speculate that it may be involved in the transcriptional regulation of some genes or the process of protein nuclear localization. Further study is needed to determine specific function and mechanism of profilin in nucleus.

Previous studies have found that different PRF proteins have different affinity for G-actin [18,33,80]. In our study, we fused GFP to rice profilin isoforms OsPRF1 or OsPRF3 and characterized the fusion proteins in vitro. Our results showed that GFP-OsPRF1 has about 3-fold higher affinity for rabbit actin than GFP-OsPRF3 (Fig. 5c), suggesting that the biochemical properties of GFP-OsPRF1 and GFP-OsPRF3 are substantially different and this may suggest a functional difference between these two profilin isoforms in living cells. For animal actin monomer, as we all know, profilin proteins have two opposite functions: preventing actin polymerization by sequestering actin and promoting actin polymerization by promoting the exchange of ADP with the ATP in solution [54,81,82]. Moreover, the effect of profilin on the polymerization and depolymerization of animal actin depends on the ratio of profilin to actin [54]. For example, profilins purified from *zea mays* increased the polymerization at molar ratios less than or equal to 1 [83]. In our study, we compared the polymerization capacity on rabbit muscle actin of OsPRF1 and OsPRF3 and the results indicated that at ratios (profilin: actin) = 1:2, OsPRF1 is more active than OsPRF3 in promoting actin monomer polymerization (Fig. 5d). This difference in activity may be the reason why there are so many highly conserved PRF proteins in plants.

In *Arabidopsis*, AtPRF3 regulates the PAMP-triggered immune responses by inhibiting the formin-mediated actin assembly [8] suggesting that PRF protein is involving in the process of plants responding to biotic stresses. However, few reports have been published on their expression patterns in different tissues and under abiotic stresses. In this study, we analyzed the expression patterns of *OsPRF* genes under different abiotic stresses (Fig. 6). Under some abiotic stresses, the expression level of rice *PRF* genes were obviously induced or inhibited (Fig. 6), which indicates that *PRF* genes may participate in the regulation of plant tolerance to these stresses. Especially, each of three OsPRF genes show their unique expression pattern even under same treatment, which indicates that different *OsPRF* genes have specific effects under certain stresses. Most interestingly, *OsPRF1* and *OsPRF2* were significantly induced by UV-B treatment, while *OsPRF3* was induced by salt treatment in a slightly lagging manner (Fig. 6c, f). Although the underlying mechanisms should be further explored, the elementary analyses would be helpful for understanding the plant signal transduction pathway responding to stresses. Other plant-specific signal transduction pathways with links to the actin cytoskeleton are now being elucidated, such as Abscisic acid [84,85,86]. Here, the response of three *OsPRF* genes in rice to different plant hormones was studied. It was found that only *OsPRF2* could be significantly inhibited in response to SA treatment (Fig. 7b), which laid the foundation for constructing the connection between cytoskeleton and hormone signal.

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## Conflict of interest

The authors declare that they have no conflict of interest.

## Supplementary material

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