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Proteins that interact with rice pumilio 1

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ABSTRACT: The N-terminal region of rice pumilio 1 fused with the binding domain (BD-OsPUM1) was used as a bait construct in yeast two-hybrid screening with a rice cDNA library as prey. Several interacting proteins were screened in a stringent media and tested with beta-galactose filter assay. The nucleotide sequences encoding interacting proteins were determined and annotated according to the rice genome database. These proteins are sigma factor F inhibitor, RPL18C, RUBQ2, RPL24A, RCY1, small nuclear ribonucleoprotein G, transferase hexapeptide repeat-containing protein, dormancy-associated protein, and putative expressed proteins with the accessions NP_001067038 and EEE55952. This study suggested that OsPUM1 protein is associated with several biological processes involved in morphology determination, protein folding and plant immunity.

KEYWORDS: interacting proteins, OsPUM1, yeast two-hybrid assay

INTRODUCTION

Pumilio (PUM) proteins are a class of RNA-binding proteins harbouring specific repeated motifs called the Puf domains. These proteins have been reported to play essential roles in several biological processes. For example, Puf3p is involved in mitochondrial biogenesis in yeast[1]. In budding yeast, Puf4p and Puf5p control post translational processes by binding and removing the poly(A) tail of the target mRNAs[2]. Further studies in higher organisms, such as *Drosophila*, *Caenorhabditis elegans*, and humans, indicated that PUM proteins are indispensable for maintaining the germ-line stem cell identity[3–5]. In contrast to abundant information about PUM proteins obtained in yeast and animals, functional studies in plants are lacking.

Recently, by taking advantages of the completed genome and availability of T-DNA mutant lines in *Arabidopsis*, researchers have begun to explore the possible functions of PUM proteins in plants. *Arabidopsis* pumilio 1, 2, and 3 (APUM1, 2, 3) have been reported to play roles in maintaining plant stem cell identity and differentiation[6]. Specific expression of APUM10 in the apical cell layers of embryo occur at the heart-shape stage[7]. Using a similar approach, APUM16 and APUM17 showed specific expression in the sperm cells, suggesting their role in cell division[8]. Furthermore, APUM23 is critical for normal growth patterning and development in *Arabidopsis*[9]. However, in monocot plants such as rice, the functions of PUM proteins are not well understood.

The yeast two-hybrid system (Y2H) is one of the most powerful and versatile methods to study protein-protein interaction as well as characterizing protein function. This system identifies interacting proteins through the functional restoration of the yeast GAL4 transcriptional activator in vivo[10]. In the study of PUM proteins, the Y2H system has been utilized for screening the interacting partners of PUM2 in human[5].

In this study, the Y2H system was employed to identify proteins that interact specifically with rice Pumilio 1 (OsPUM1). The N-terminal region was used as a bait for screening, and a rice cDNA library was utilized as the prey. The primary information obtained from this study will provide new insights for further studies.

MATERIALS AND METHODS

Sequence and phylogenetic tree analysis

The nucleotide and amino acid sequences of OsPUM1 were retrieved from the Knowledge-based Oryza Molecular Biology Encyclopedia[11] (cdna01.dna.affrc.go.jp/cDNA/). The accession number of *OsPUM1* cDNA was AK070870. Alignment between cDNA and genomic DNA was observed using SPIDEY[12]

(www.ncbi.nlm.nih.gov/spidey/). Molecular weight and isoelectric point (pI) was calculated using the prediction site[13]: web.expasy.org/compute_pi/. In addition, protein domain features were determined using Prosite[14] (www.expasy.org/prosite/). The phylogenetic tree was constructed using algorithms from CLUSTALW in EMBL-EBI website (www.ebi.ac.uk).

Yeast two-hybrid assay

For bait vector construction, the 5′ terminal region of *OsPUM1* was PCR-amplified using *Pfu* DNA polymerase with the following primers: 5′-AATGGC TAC AGA GAG TGC TCG GC-3′ and 5′-TGT CGA CGG TTG GAA TCC CTT GAC AT-3′ (the start codon and *Sal*I site is underlined). The pGBT9 vector was sequentially digested with *Sma*I and *Sal*I. Subsequently, the PCR-amplified product of *OsPUM1* was digested with *Sal*I and ligated into pGBT9 vector to create pGBT9-*OsPUM1*. The cDNA library (prey) was made from rice panicle according to the HybriZAP two-hybrid cDNA Gigapack cloning kit manual (Stratagene, La Jolla, CA, USA). Total cDNA of the phagemid form was obtained by the mass in vivo excision method. The yeast (*Saccharomyces cerevisiae*) strain YRG-2 (genotype: *MATa*, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3*, *112*, *gal4-542*, *gal80-538*, LYS::UAS_{GAL1} - $TATA_{GAL1}$ - HIS3, URA3::$UAS_{GAL417mers(x3)}$-$TATA_{CYC1}$-*lacZ*) was transformed with the binding domain pGBT9-*OsPUM1*. The yeast cells containing the binding domain were co-transformed with 100 µg of the HybriZAP cDNA library plasmid DNA and salmon sperm carrier DNA by the lithium acetate method[15]. Transformants were selected on the synthetic dropout medium lacking tryptophan, leucine, and histidine (SD-TLH). The transformants, which appeared after 3–5 days incubation at 30 °C, were then grown on an SD-TLH plate containing 5 mM and then 25 mM 3-aminotriazole (3-AT). Yeast colonies which still survived on media containing 25 mM 3-AT were tested for the beta-galactosidase activity by the filter assay[16]. The colonies that turned blue in less than 6 h were collected and used as the template in colony PCR reaction as described previously[17]. The primers for amplification were GAL4 AD-forward and -reverse with following PCR procedure: the denaturation condition was 7 min at 95 °C, followed by the amplification reaction for 1 min at 95 °C, 1 min at 55 °C, and 3 min at 72 °C for 35 cycles, prior to a final extension for 7 min at 72 °C and storage at 4 °C. The PCR product was then purified with a QIAEX II kit (Qiagen, Valencia, CA, USA) and confirmed by DNA sequencing. Nucleotide sequences obtained from DNA sequencing were used for gene annotation by BLAST against NCBI (www.ncbi.nlm.nih.gov) and rice genome database (rice.plantbiology.msu.edu).

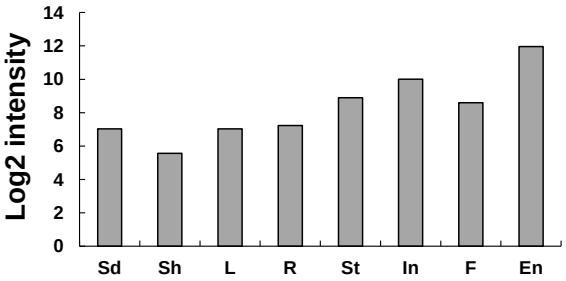

Fig. 1 Expression profile of the *OsPUM1* collected from public microarray database (www.ricearray.org). The expression was obtained from Affymatrix meta-analysis using Os.13 605.1.S1_at as a main probe set. Sd, seedling; Sh, shoot; L, leaf; R, root; St, stem; In, internode; F, flower; En, endosperm.

RESULTS

Expression profile, molecular structure and phylogenetic tree of *OsPUM1*

OsPUM1 expression was observed from the rice oligonucleotide array database (www.ricearray.org). Through Affymatrix microarray meta-analysis using a main probe specific to *OsPUM1* (Os.13 605.1.S1_at), the expression was determined in several organs that represents a whole life cycle of rice. *OsPUM1* was expressed in all organs throughout the life cycle of rice (Fig. 1). The highest transcript level was found in the endosperm, while moderate levels were observed in the internode, stem, and flower. Low expression was detected in the root, seedling, leaf, and shoot (Fig. 1).

The structure of the *OsPUM1* gene was determined by the sequence information from KOME full-length cDNA clone (Accession AK070870) and rice genome database (rice.plantbiology.msu.edu). *OsPUM1* was located at chromosome number 9 within the rice genome. This gene comprises nine exons and eight introns. The open reading frame of the *OsPUM1* cDNA consists of 2967 nucleotides and encodes a protein consisting of 988 amino acids with a predicted molecular mass (Mw) of 107.77 kDa and isoelectric point (pI) of 6.1 (Fig. 2a, b). Eight repeats of the Pumilio domain were located between amino acids 651 and 945 at the C-terminal region of the OsPUM1 protein (Fig. 2b). In contrast, no conserved motif was observed in the N-terminal region. When the OsPUM1 amino acid sequence was compared with known sequences from other plants, it had ap-

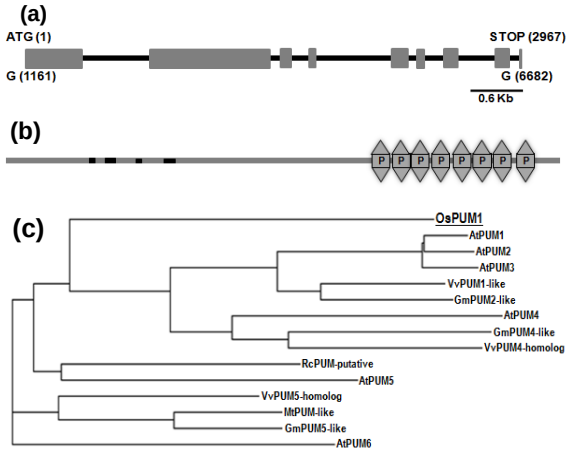

Fig. 2 Structure of the rice OsPUM1 gene and protein and relationship of OsPUM1 to other proteins; (a) molecular structure of the *OsPUM1* gene with nine exons (grey-filled boxes) and eight introns (inter-lines); (b) map of the OsPUM1 protein showing pumilio motif (hexagons with P letters) in C-terminal region and the segments of low-complexity regions (black colours) in N-terminal region; (c) phylogenetic relationships between OsPUM1 (underlined) and other PUM proteins. Phylogenetic tree shows a graphical representation of evolutionary relationships and was constructed with the EBI-CLUSTALW algorithm. Species names, proteins and accession numbers are *Arabidopsis thaliana* pumilio 1, AtPUM1 (NP_180483); *A. thaliana* pumilio 2, AtPUM2 (NP_180482); *A. thaliana* pumilio 3, AtPUM3 (NP_180478); *A. thaliana* pumilio 4, AtPUM4 (NP_187647); *A. thaliana* pumilio 5, AtPUM5 (NP_188660); *A. thaliana* pumilio 6, AtPUM6 (NP_567733); *Glycine max* pumilio 2, GmPUM2 (XP_003537980); *G. max* pumilio 4, GmPUM4 (XP_003535521); *G. max* pumilio 5, GmPUM5 (XP_003547443); *Medicago truncatula* pumilio, MtPUM (XP_003594978); *Ricinus communis* pumilio, RcPUM (XP_002513314); *Vitis vinifera* pumilio 1, VvPUM1 (XP_002283191); *V. vinifera* pumilio 4, VvPUM4 (XP_002273503); *V. vinifera* pumilio 5, VvPUM5 (XP_002268751).

proximately 28–39% identity. OsPUM1 shared 39 and 35% amino acid sequence identity with RcPUM and AtPUM5, respectively, and shared lower identity (28%) with AtPUM4 and GmPUM4. The evolutionary relationship between these proteins is illustrated in the neighbour joining phylogenetic tree in Fig. 2c.

Screening proteins that could interact with OsPUM1

The yeast two-hybrid screening was conducted to identify proteins that interact with rice pumilio 1 (OsPUM1). The fusion between the GAL4 binding domain and the N-terminal region of OsPUM1 was prepared as a bait construct. Subsequently, the rice cDNA library was introduced into the YRG-2 yeast strain harbouring the bait protein. Selection was performed in SD-Trp-Leu-His (SD-TLH) agar media containing 25 mM 3-aminotriazole (3-AT). The transformants were also tested for *LacZ* activation. Out of the 14 colonies grown in the high stringency media, only 11 colonies appeared in blue colour (*LacZ* activation). The colonies putatively harbouring the sequences of interest were then isolated and annotated according to the BLASTn results against NCBI nucleotide database or the rice genome database (rice.plantbiology.msu.edu/) and summarized in Table 1. These proteins are sigma factor F inhibitor (LOC_Os01g55770), 60S ribosomal protein L18 (RPL18C, LOC_Os03g22180), 60S ribosomal protein L24 (RPL24A, LOC_Os07g12250), polyubiquitin (RUBQ2, LOC_Os02g06640), ania-6a type cyclin (RCY1, LOC_Os01g27940), small nuclear ribonucleoprotein G (LOC_Os07g41790), dormancy-associated protein (LOC_Os11g44810), transferase hexapeptide repeat-containing protein (LOC_Os01g18070), and two expressed proteins (LOC_Os12g37650 and LOC_Os01g70670). Among these, the dormancy-associated protein appeared twice during the screening process.

DISCUSSION

The expression pattern of OsPUM1 was observed from the rice microarray database. From several organs or tissues examined, the expression was relatively high in the endosperm and internodes. This result indicates that OsPUM1 might play a potential role not only during the vegetative stage but also during the period of reproductive development. In addition, the expression of OsPUM1 was detected in all organs or tissues examined, suggesting a broad extent of its function.

Typically, proteins interact with other molecules to regulate their functions. These molecules can be proteins, nucleic acids, lipids, or any other biomolecules that may serve as ligand, substrate, inhibitor, cofactor, co-enzyme or activator. Since the C-terminal region of OsPUM1 is highly conserved among plant species, the N-terminal part was utilized as bait construct in yeast two-hybrid (Y2H) analysis. By using this strategy, several proteins that interact with OsPUM1 were isolated. One of the proteins that interact with OsPUM1 in rice is a homologue of sigma factor F (SigF) inhibitor. In bacteria, this protein had been reported to be involved in the regulation of tolerance and susceptibility to rifampin, a drug

Table 1 List of proteins interact with OsPUM1.

Colony number	Gene annotation/putative role	Locus number	Accession number (protein)
1	Sigma factor F inhibitor	Os01g55770	NP_001044334
2	60S ribosomal protein L18 (RPL18C)	Os03g22180	NP_001050069
3	Expressed protein	Os12g37650	NP_001067038
5	Polyubiquitin (RUBQ2)	Os02g06640	NP_001045980
7	60S ribosomal protein L24 (RPL24A)	Os07g12250	NP_001059209
8	Ania-6a type cyclin (RCY1)	Os01g27940	NP_001043083
10	Small nuclear ribonucleoprotein G	Os07g41790	AAK55776
11	Dormancy-associated protein	Os11g44810[a]	NP_001068430
12	Transferase hexapeptide repeat-containing protein	Os01g18070	NP_001042763
13	Expressed protein	Os01g70670	EEE55952
14	Dormancy associated protein	Os11g44810[a]	NP_001068430

[a] Identical protein.

Table 2 Prediction of molecular characters of OsPUM1-interacting proteins.

Interacting-protein	Mw (kDa)	pI	Localization[†]
Sigma factor F inhibitor	9.37	15.44	Chloroplast
60S ribosomal protein L18 (RPL18C)	11.43	21.07	Nucleus/Cytoplasm
Expressed protein	5.71	26.81	Chloroplast
Polyubiquitin (RUBQ2)	7.05	51.19	Cytoplasm
60S ribosomal protein L24 (RPL24A)	10.72	18.23	Cytoplasm
Ania-6a type cyclin (RCY1)	8.93	49.13	Nucleus
Small nuclear ribonucleoprotein G	5.10	8.09	Chloroplast
Dormancy-associated protein	9.45	13.57	Nucleus/Chloroplast
Transferase hexapeptide repeat-containing protein	6.36	28.47	Cytoplasm
Expressed protein	5.56	89.31	Nucleus

[†] pSORT prediction.

molecule[18]. However, no characterization of the rice SigF orthologue has been reported. Two ribosomal proteins, RPL18C and RPL24A, were also detected as OsPUM1 partners. In *Arabidopsis*, the RPL18C interacts with open reading frame VI product (P6) of cauliflower mosaic virus (CaMV)[19]. Interestingly, OsPUM1 also interacts with RUBQ2, a polyubiquitin. Ubiquitin has been reported to be involved in cellular processes such as ribosome biosynthesis, chromatin structure and cell cycle regulation[20,21]. Ubiquitin is also involved in the degradation of phytochrome, plant senescence and stress response[22–25]. RCY1, a cyclin, was also a candidate partner for OsPUM1. In almost all organisms, cyclins play a role in cell cycle regulation, especially during mitotic division. In yeast, overexpression of RCY1 could induce tolerance to LiCl and NaCl[26].

The pSORT software predicted that OsPUM1 is localized in the cytoplasm. In most cases, two interacting proteins are co-localized to perform their action. To confirm the localization of these interacting proteins, the amino acid sequence of each protein was predicted using the identical strategy. As shown in Table 2, the interacting proteins are found in different locations in the plant cell. Three proteins were predicted to be localized in the cytoplasm, RUBQ2, RPL24A, and transferase hexapeptide repeat-containing protein, while RPL18C is a ribosomal protein, so it will also function in the cytoplasm, once the mature ribosomal subunits are translocated there from the nucleus. This in silico study indicates that these four proteins are strong candidates to interact with OsPUM1. However, further analysis such as co-immunoprecipitation (co-IP) or pull down assay is required to confirm this possibility.

In summary, this study underlines the critical function of *OsPUM1* that might be involved in a broad extent of biological processes, including not only those related with morphology determination, but is also associated with protein folding and plant immunity. Further study is required to determine the functional role of *OsPUM1* and/or interacting genes by using knockout mutants as well as over-expression lines. In addition, subsequent studies for in vitro

and in planta interaction between OsPUM1 and its partners are justified.

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