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Molecules and Cells

Structure and Expression of OsUBP6, an Ubiquitin-Specific Protease 6 Homolog in Rice (*Oryza sativa* L.)

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Although the possible cellular roles of several ubiquitin-specific proteases (UBPs) were identified in *Arabidopsis*, almost nothing is known about UBP homologs in rice, a monocot model plant. In this report, we searched the rice genome database (http://signal.salk.edu/cgi-bin/RiceGE) and identified 21 putative *UBP* family members (*OsUBPs*) in the rice genome. These *OsUBP* genes each contain a ubiquitin carboxyl-terminal hydrolase (UCH) domain with highly conserved Cys and His boxes and were subdivided into 9 groups based on their sequence identities and domain structures. RT-PCR analysis indicated that rice *OsUBP* genes are expressed at varying degrees in different rice tissues. We isolated a full-length cDNA clone for *OsUBP6*, which possesses not only a UCH domain, but also an N-terminal ubiquitin motif. Bacterially expressed OsUBP6 was capable of dismantling K48-linked tetra-ubiquitin chains *in vitro*. Quantitative real-time RT-PCR indicated that *OsUBP6* is constitutively expressed in different tissues of rice plants. An *in vivo* targeting experiment showed that OsUBP6 is predominantly localized to the nucleus in onion epidermal cells. We also examined how knock-out of *OsUBP6* affects developmental growth of rice plants. Although homozygous T3 *osubp6* T-DNA insertion mutant seedlings displayed slower growth relative to wild type seedlings, mature mutant plants appeared to be normal. These results raise the possibility that loss of *OsUBP6* is functionally compensated for by an as-yet unknown *OsUBP* homolog during later stages of development in rice plants.

INTRODUCTION

The ubiquitination pathway plays a critical role in cellular processes as diverse as cell division, differentiation, hormonal responses, transcriptional regulation, and stress responses (Dreher and Callis, 2007; Glickman and Ciechanover, 2002; Moon et al., 2004; Smalle and Vierstra, 2004; Vierstra, 2009). Ubiquitin is a highly conserved 76-amino acid polypeptide that is conjugated to substrate proteins by the serial actions of three enzymes, E1 (a ubiquitin-activating enzyme), E2 (a ubiquitin-conjugating enzyme), and E3 (a ubiquitin ligase) (Kraft et al., 2005; Stone et al., 2005; Vierstra, 2003). Poly-ubiquitinated proteins are often degraded by the 26S proteasome complex, while multi- and mono-ubiquitination of target proteins results in changes in their activity, lipidation, cellular localization, and interactions with other proteins (Mukhopadhyay and Riezman, 2007; Pickart and Eddins, 2004).

Removal of ubiquitin from proteins has also been implicated in many aspects of cellular functions (Bonnet et al., 2008; Love et al., 2007). Ubiquitin-specific proteases (UBPs), also known as USPs in mammalian systems, are critically involved in de-ubiquitination processes (Komander et al., 2009; Nijman et al., 2005; Vierstra, 2009). In the *Arabidopsis* genome, there are at least 27 putative *UBP* genes (Yan et al., 2000). Among these UBPs, UBP1 and UBP2 may eliminate damaged nonfunctional proteins. *In vitro* and *in vivo E. coli* assays showed that *Arabidopsis* AtUBP3, AtUBP4, and AtUBP5 possess de-ubiquitination activity (Rao-Naik et al., 2000). Subsequently, it was shown that *atubp14* mutants are embryonic lethal, indicating that AtUBP14 is essential for early embryo development (Doelling et al., 2001). Although *atubp3* and *atubp4* single knock-out mutant plants were phenotypically normal, *atubp3atubp4* double knock-out mutants displayed severe phenotypes, including anomalous mitosis, reduced efficiency of pollen germination, unusual vacuole and endomembrane structures, and defects in pollen development (Doelling et al., 2007). These results suggest that AtUBP3 and AtUBP4 participate in pollen development in *Arabidopsis*. Ubiquitin C-terminal hydrolases 1 and 2, which belong to a subset of *Arabidopsis* de-ubiquitinating proteases, are shown to participate in shoot architecture modification and auxin signaling (Yang et al., 2007). AtUBP26 that de-ubiquitinates histone H2B is involved in seed development and proper regulation of flowering (Luo et al., 2008; Schmitz et al., 2009). Mutations in *AtUBP15*

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resulted in pleiotropic phenotypes, including narrow and flat rosette leaves, defective cell proliferation, early flowering, reduced apical dominance, and decreased fertility (Liu et al., 2008). Collectively, these results indicate that UBPs are critical for a variety of cellular processes in *Arabidopsis*.

In this report, we searched the rice genome database (http://signal.salk.edu/cgi-bin/RiceGE) for homologs of *Arabidopsis UBP* genes and identified 21 putative *UBP* family members (*OsUBPs*) in the rice genome. The predicted 21 OsUBP proteins were grouped into 9 sub-families (G1-G9) on the basis of their primary structures. RT-PCR analysis indicated that rice *OsUBP* genes are expressed at varying degrees in different rice tissues. We then cloned the *OsUBP6* gene and showed that OsUBP6 was capable of dismantling K48-linked tetra-ubiquitin chains *in vitro*. We next examined the effect of *OsUBP6* knock-out on the development of rice plants. Although homozygous T3 *osubp6* T-DNA insertion mutant seedlings displayed defective phenotypes, their mature forms appeared to be normal. Based on these results, we discuss possible functional redundancies among *OsUBP* family members in the development of rice plants.

MATERIALS AND METHODS

Plant materials and experimental treatments

Dry wild-type and *osubp6* knock-out mutant rice (*Oryza sativa* var. japonica cv. Dongjin) seeds were germinated on Murashige and Skoog medium containing MS basal salt (Wako Pure Chemical, Japan), 3% sucrose, 0.2% phytogel, and 0.55 mM *myo*-inositol (Hong et al., 2007). The seedlings were grown for 2 d, 5 d, 10 d, and 2 weeks at 28°C under continuous light in a growth chamber, transplanted to soil, and raised to maturity in a greenhouse, as described by Byun et al. (2008).

Rice seedlings were subjected to various abiotic stresses and hormones as described previously (Hong et al., 2005), with slight modifications. For salt stress treatment, 10-day-old wild-type rice seedlings were soaked in solution containing 200 mM NaCl for 2 h. The 10-day-old whole seedlings were treated with 100 μM ABA, 100 μM SA, and 20 μl/L ethylene, wounded with a razor blade, and then harvested at suitable time points. For cold stress treatment, whole seedlings were transferred to an incubator in which the temperature was set at 4°C. Rice seedlings were dehydrated as described previously (Cho et al., 2008; Lee et al., 2009). Briefly, seedlings were dehydrated on Whatman 3MM filter paper at room temperature for 1-6 h. At the end of each treatment, the tissues were immediately frozen in liquid nitrogen and stored at -80°C until use.

Identification of rice *UBP* gene families

Rice homologs of *Arabidopsis UBP* genes, which contain ubiquitin carboxyl-terminal hydrolase (UCH) motif, were found in the rice genome database (http://signal.salk.edu/cgi-bin/RiceGE) using the BLASTN program and an E-value cut-off less than 10^{-14}. Partial cDNAs for all 21 rice *UBP* genes were obtained by RT-PCR using gene specific primers (Table 1) and confirmed by DNA sequencing of PCR products.

Total RNA isolation and RT-PCR

Total RNA was prepared from various organs, including leaves, stems, roots, and flowers, and stress- and hormone-treated seedlings, such as drought, salt, cold, wounding, ABA, SA, and ethylene, by a method described previously (Hong et al., 2007). Isolated total RNA was quantified using a BioPhotometer (Eppendorf, Germany) and confirmed by visualizing ethidium-bromide stained ribosomal RNA content. First-strand cDNA was synthesized from 5 μg of total RNA and amplified by PCR using oligonucleotide primers specific for the *OsUBP* genes (Table 1). PCR was performed in a total volume of 50 μl using Ex-Tag polymerase (Takara, Japan) with 25 cycles, each consisting of 30 s at 94°C, 30 s at 65°C and 90 s at 72°C, as described by Seo et al. (2008). The A-1 (5′-TTGAACGGATCCTTACGGAG-3′), A-2 (5′-AACGGCGTCAAATTAGAACG-3′), B-1 (5′-CTGTTGTCTTTAAGACCCAGC-3′), and B-2 (5′-GAAGCCTTCTCAAGTTCTGAC-3′) primers were used to examine the expression level of *OsUBP6* in wild-type and *osubp6* mutant rice plants. The RT-PCR products were separated on a 1% agarose gel by electrophoresis and visualized by the Molecular Image Gel Doc XR System (Bio-Rad, USA). RT-PCR for rice actin was used as a loading control.

Table 1. Primers for RT-PCR

Gene name	Primers
Os01g08200	5′-ATCCACAAGGACGAGTGCTG-3′
	5′-CTAGCTGCTTCAGATCCATA-3′
Os01g36930	5′-ATAATGTGAGGGGTAATGGTGTT-3′
	5′-AGCCTTCTCAAGTTCTGACTTC-3′
Os01g56490	5′-ATGAAAACGACATACCAGAACA-3′
	5′-TTACACTTGGCTCGAACATTAC-3′
Os02g36400	5′-GTCTCCAAGCTCGAGAAGGC-3′
	5′-TCTTCATTCTCTTGTGTGCTTC-3′
Os02g14730	5′-GAAGATTTACATTGAGAAGGGC-3′
	5′-TATTCCCCTGAAGTTCTAGTGC-3′
Os02g55180	5′-GCACTGGTGTAATATTGGAGAA-3′
	5′-ATTTCTCTTAAACCCCTTGTGA-3′
Os03g09080	5′-GAGCTCTAGGCTGGAGAAGG-3′
	5′-TAAGTTTAAGCTCCAGAGGGAA-3′
Os04g37950	5′-ATAACAAAACTCCTGGAGATGC-3′
	5′-AAAGCTTCTTATACCGACCAAG-3′
Os05g43480	5′-ATCTAATGAGCCCCTGAAGATA-3′
	5′-CAGTGAAAATTTGTCCTGGTAA-3′
Os06g08530	5′-CATTATTTGAAGAAAGATGGCA-3′
	5′-TCACAAACTACAGACCGTCTTC-3
Os06g44380	5′-GTTTTGGTCCCATCTCATTAGT-3′
	5′-GCACATCCTCTTGTATCAAATG-3′
Os07g46660	5′-GACGATCAGATCCTTCTGTTT-3′
	5′-GATGAAACAATCATACCCTCCT-3′
Os08g41540	5′-AGAATCTGGATTAGCAAAGAGG-3′
	5′-TCCCTAAATCATCAGCCTTAGT-3′
Os08g41620	5′-GTTGATCAAAAACAATCCGAG-3′
	5′-CTCCTCACGTAAACTGATGAAA-3′
Os08g41640	5′-GTGTGATTTTGGTGTGATGAAC-3′
	5′-ACTGACTCAGATGCATAACCAC-3′
Os08g37350	5′-GAAAACATAGCTGAAACCAACA-3′
	5′-TGAAAAGCTCATATGGAAAATG-3′
Os08g41530	5′-GAGTAACACCGAAATTGTCAAA-3′
	5′-GGAAGGCAACTAGATACAGCTT-3′
Os08g41630	5′-AGTGTGCTGCTTTGTGTGTAAA-3′
	5′-GACCTTATCCTTCTGAATCTGC-3′
Os09g28940	5′-TGATTCTGATACCTTCAAGTGC-3′
	5′-AAGCCTGTTTGTTAGAATGGTT-3′
Os10g07270	5′-TACAAAAGAATGGAGTTTGTGG-3′
	5′-TGTTCTCTATCAGACCAGTCCA-3′
Os12g30540	5′-TGAAGCTCATCTCTACACCATC-3′
	5′-AAGCCTACCAACAAAACAAAGT-3′

Isolation of a full-length *OsUBP6* cDNA clone

To isolate full-length coding region of *OsUBP6* cDNA, total RNA extracted from wild-type rice leaves was subjected to RT-

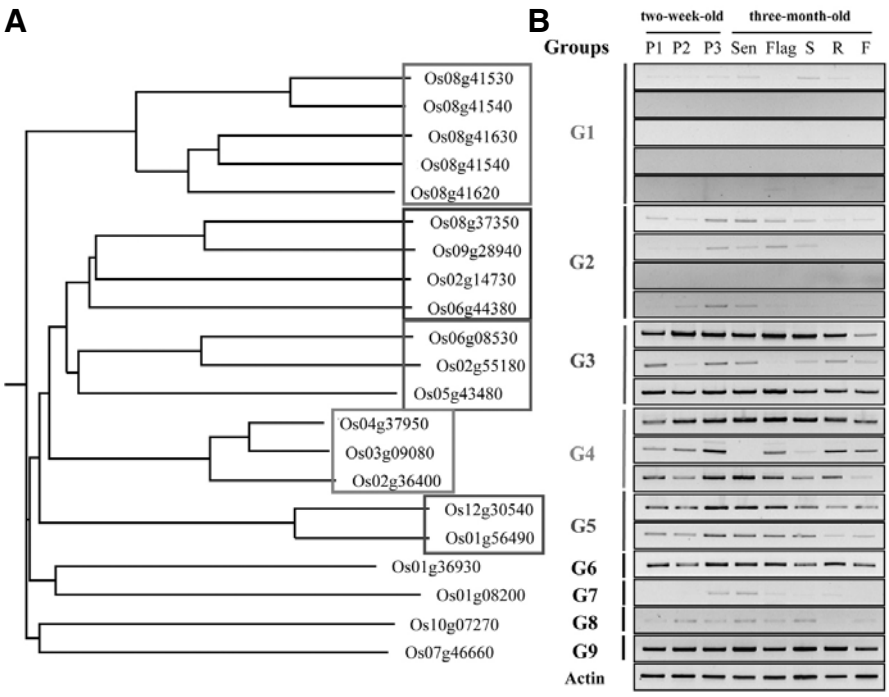

Fig. 1. Identification and expression of 21 putative *OsUBP* family members in rice. (A) Phylogenetic analysis of 21 *OsUBP* genes in rice. The dendrogram was generated by MEGA4 software using the neighbor-joining method. Based on the predicted amino acid sequence identities, the 21 rice *OsUBP* genes can be subdivided into 9 groups (G1-G9). (B) RT-PCR analysis of 21 *OsUBP* genes in different tissues using gene-specific primer sets (Table 1). P1, P2, and P3 indicate the first, second, and third leaf, respectively, in two-week-old seedlings. Sen, Flag, S, R, and F indicate senescence leaves, flag leaves, stems, roots, and flowers in mature, three-month-old rice plants. The actin gene was used as a loading control.

PCR using *OsUBP6* full-length primers (5′-ATGCCGACCGTAAGCGTGAAA-3′ and 5′-TCAGATAACACGAGCTTTGTA-3′) by a method described by Kim et al. (2008). The PCR products were purified by the Nucleotide Removal kit (Qiagen, USA) following the manufacturer's protocol and confirmed by DNA sequencing analysis. Full-length coding region of *OsUBP6* cDNA was used in a de-ubiquitination enzymatic assay and subcellular localization experiments.

Quantitative real-time RT-PCR

Quantitative real-time PCR was carried out with the IQ5 Multicolor Real-time PCR Detection System (Bio-Rad, USA) using the 2X SYBR® *Premix Ex* Taq™ II (Takara, Japan) and *OsUBP6* gene-specific primer set (5′-TTTCTCAAACTCTTACATCAG-3′ and 5′-CCACTTTCTGCACAATGGACC-3′). To determine transcript levels, Ct values for *OsUBP6* in wild-type rice plants were normalized to that of the rice *UBQ10* gene, as described by Seo et al. (2009).

Enzymatic assay of OsUBP6

For the *in vitro* de-ubiquitination enzymatic assay, MBP-OsUBP6 fusion protein was expressed in *E. coli* and purified using Poly-Prep® chromatography columns (Bio-Rad, USA), as described previously (Seo et al., 2008). The purified MBP-OsUBP6 protein was co-incubated with K48-linked tetra-ubiquitin (Boston Biochem, USA) as a substrate for various lengths of time (0-3 h) at 30°C. The reaction products were resolved on an 8% SDS-PAGE gel and transferred to an Immobilon-P Transfer Membrane (Millpore, USA). The membrane was analyzed by Western blot with anti-ubiquitin antibody (Sigma, USA), as described previously (Lee et al., 2006).

***In vivo* localization of OsUBP6**

The full-length *OsUBP6* cDNA was ligated into a soluble-modified green fluorescent protein (smGFP) plasmid (Son et al., 2009). Transient expression of a *35S:OsUBP6-smGFP* fusion construct was examined by introducing the DNA into onion (*Allium cepa*) epidermal cells via particle bombardment, according to the manufacturer's protocol (Bio-Rad, USA) with some modifications (Lee et al., 2004). After 18 h of transformation, the epidermal cells were viewed by a cooled CCD camera and a BX51 fluorescence microscope (Olympus, Japan), as described previously (Hong et al., 2007).

Identification and characterization of *osubp6* T-DNA insertion knock-out rice plants

Total leaf genomic DNA was isolated from wild-type and *osubp6* T-DNA insertion knock-out mutant plants (An et al., 2003; Jeon et al., 2002), as described previously (Byun et al., 2008) with some modifications. Mature leaves were harvested in 2.0 ml microcentrifuge tubes (Eppendorf, Germany) and tungsten beads added. Each tube was frozen in liquid nitrogen and vibrated at 3,000 rpm for 1 min with a Grinding Mixer Mill MM300 (Retsch, Germany). The pulverized tissue was suspended in CTAB buffer (2% [w/v] CTAB, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris-HCl [pH 8.0], 2% [w/v] polyvinylpyrrolidone-40, and 5 mM ascorbic acid) and 10 μl of RNaseA (10 mg/ml). The leaf extract was then incubated at 65°C for 10 min, vigorously mixed, and centrifuged. After centrifugation, the supernatant was harvested and the DNA was precipitated with isopropanol and centrifugation. The DNA pellet was washed with 70% ethanol and resuspended in 50 μl of TE buffer. To isolate heterozygous and homozygous *osubp6* mutants, multiplex PCR was performed in 50 μl total volume using genomic DNA as a template, as described by Lee et al. (2008). The primers used were A-1 (5′-TTGAACGGATCCTTACGGAG-3′), A-2 (5′-AACGGCGTCAAATTAGAACG-3′), and RB-1 (5′-CAAGTTAGTCATGTAATTAGCCAG-3′).

RESULTS

Identification and characterization of *OsUBP* genes encoding ubiquitin-specific protease homologs in rice

The cellular roles of UBPs (also called USPs in mammals)

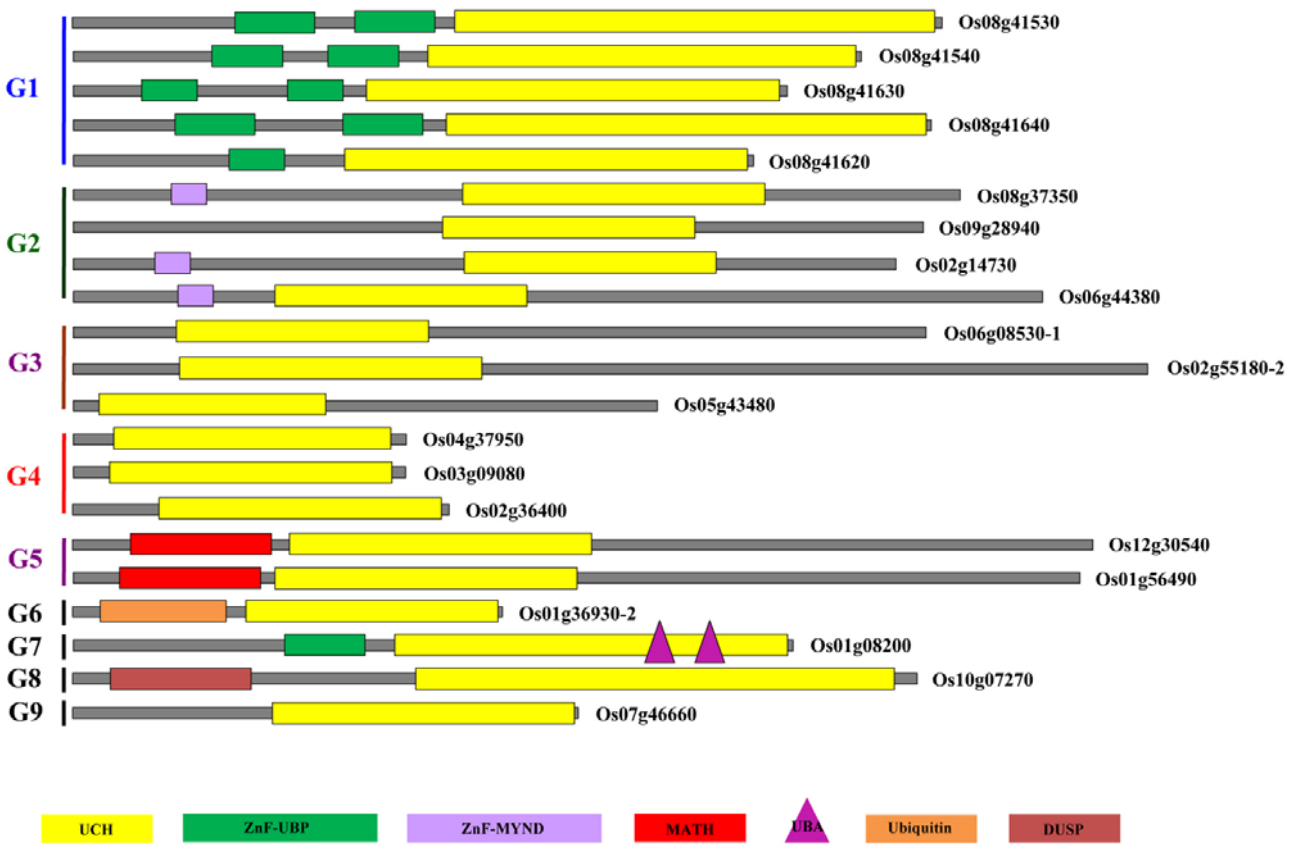

Fig. 2. Domain architectures of putative 21 OsUBPs. Analysis of predicted rice OsUBPs reveals that these OsUBPs possess various domains and subdivided into 9 groups based on their sequence identities and domain structures. The G1 members (Os08g41530, Os08g41540, Os08g41630, Os08g41640, and Os08g41620) contain 1 or 2 zinc-finger ubiquitin-specific protease (ZnF-UBP) domains, three out of four G2 members (Os08g37350, Os02g14730, and Os06g44380) contain myeloid, nervy and DEAF1 (MYND)-type zinc finger (ZnF-MYND) motif, and two G5 OsUBPs (Os12g30540 and Os01g56490) have a single meprin and TRAF homology (MATH) domain in their N-terminal regions. Ubiquitin and DUSP (domain in USPs) motifs are present in the G6 (Os01g36930) and G8 (Os10g07270) OsUBPs, respectively, in their N-terminal regions. The G7 (*Os01g08200*) member contains ZnF-UBP, UCH, and two ubiquitin-associated (UBA) domains. The G3 (Os06g08530, Os02g55180, and Os05g43480), G4 (Os04g37950, Os03g09080, and Os02g36400), and G9 (Os07g46660) OsUBP proteins have a single UCH motif. UCH, ZnF-UBP, ZnF-MYND, MATH, UBA, Ubiquitin, and DUSP domains are indicated in the bottom of the figures.

were recently described in animals (Bonnet et al., 2008; Komander et al., 2009; Nijman et al., 2005; Routenberg et al., 2007). In contrast, although the possible functions of UBPs were studied in *Arabidopsis* (Doelling et al., 2007; Liu et al., 2008; Vierstra, 2009), almost nothing is known about UBPs in rice, a monocot model plant. To identify rice *UBP* genes, we searched the rice genome database (http://signal.salk.edu/cgi-bin/RiceGE) for homologs of *Arabidopsis UBP* genes that contain ubiquitin carboxyl-terminal hydrolase (UCH) motif. Our database search yielded 21 putative *UBP* family members in the rice genome, which we called *OsUBPs* (*Oryza sativa Ubiquitin-Specific Proteases*). We next carried out the phylogenetic analysis. As shown in Fig. 1A, the predicted OsUBPs were grouped into 9 sub-families (G1-G9) on the basis of their sequence identities and domain architectures (see below).

The domain architectures of 21 putative OsUBPs are also analyzed. As shown in Fig. 2, all of the predicted OsUPBs contain a single ubiquitin carboxyl-terminal hydrolase (UCH) motif (Fig. 2). In addition, these OsUBPs possess various domains. For example, there are 1 or 2 zinc-finger ubiquitin-specific protease (ZnF-UBP) domains in five G1 OsUBP members (Os08g41530, Os08g41540, Os08g41630, Os08g41640, and Os08g41620), while myeloid, nervy and DEAF1 (MYND)-type zinc finger (ZnF-MYND) motif is found in three out of four G2 members (Os08g37350, Os02g14730, and Os06g44380) (Fig. 2). In addition, two G5 OsUBPs (Os12g30540 and Os01g56490) have a single meprin and TRAF homology (MATH) domain in their N-terminal regions. The G6 (Os01g36930) and G8 (Os10g07270) OsUBPs possess ubiquitin and DUSP (domain in USPs) motifs, respectively, in their N-terminal regions. Interestingly, the G7 member (Os01g08200) contains three different motifs, including ZnF-UBP, UCH, and a couple of ubiquitin-associated (UBA) domains. Finally, only UCH motif is present in G3 (Os06g08530, Os02g55180, and Os05g43480), G4 (Os04g37950, Os03g09080, and Os02g36400), and G9 (Os07g46660) OsUBP proteins (Fig. 2).

Expression of 21 *OsUBP* genes in different rice tissues

Because almost nothing is known about rice *OsUBPs*, we investigated whether these 21 putative *OsUBP* genes are expressed in rice plants. Total RNA was obtained from young leaves (first, second, and third leaves) of two-week-old rice seedlings and from senescent leaves, flag leaves, shoots, roots, and flowers of three-month-old mature rice plants. Total RNA was

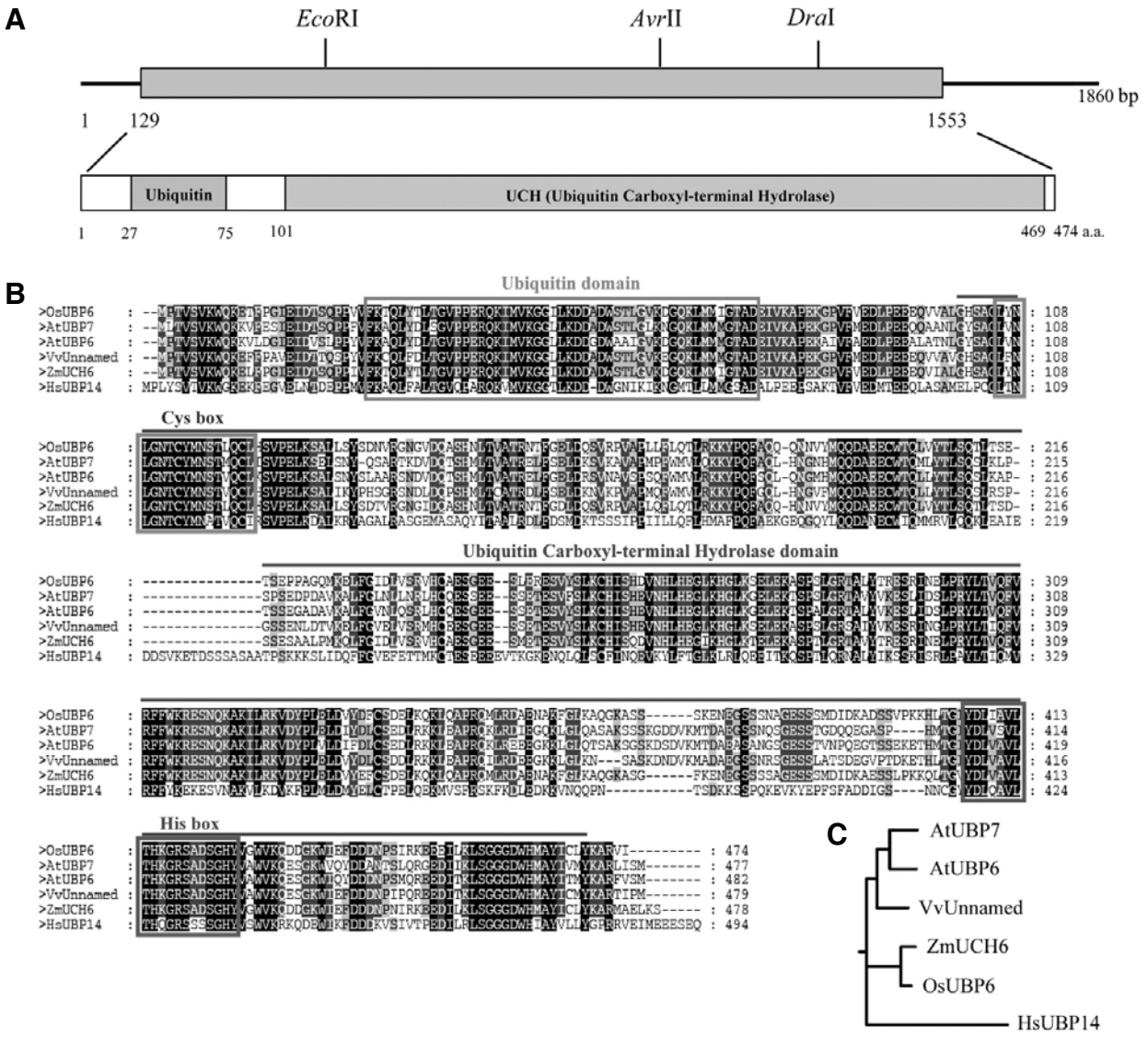

Fig. 3. Sequence analysis of rice *OsUBP6*. (A) Restriction enzyme analysis and the deduced protein structure of a *OsUBP6* cDNA clone (Genbank database under accession number NP_001043299.1). The solid bar depicts the coding region and solid lines represent 5′- and 3′- untranslated regions. The ubiquitin domain and Ubiquitin Carboxyl-terminal Hydrolase (UCH) domains are indicated. (B) Comparison of the derived amino acid sequence of OsUBP6 with *Arabidopsis* AtUBP6 (NP_564596.1) and AtUBP7 (NP_566680.2), maize ZmUCH6 (NP_001148768.1), grape VvUnnamed protein (XP_002281000.1), and human HsUBP14 (NP_001032411.1). Amino acid residues that are conserved in at least four of the six sequences are shaded, while amino acids identical in all six proteins are shown in black. The ubiquitin domain, Cys box, UCH domain, and His box are indicated. (C) Phylogenetic analysis of rice, *Arabidopsis*, maize, grape, and human UBP6 homologs.

then subjected to RT-PCR analysis using gene specific primer sets (Table 1). Figure 1B shows that most of the 21 *OsUBP* genes are expressed at varying degrees in different tissues of rice plants. For example, under our experimental conditions, five G1 genes (*Os08g41530*, *Os08g41540*, *Os08g41630*, *Os08g 41640*, and *Os08g41620*) are expressed at very low levels in every tissue examined, including leaves, stems, roots, and flowers. Their transcripts were barely detectable after prolonged cycles (more than 40 amplification cycles) of RT-PCR. By contrast, the levels of G3 (*Os06g08530*, *Os02g55180*, and *Os05g43480*), G4 (*Os04g37950*, *Os03g09080*, and *Os02g36400*), G5 (*Os12g 30540* and *Os01g56490*), G6 (*Os01g36930*), and G9 (*Os07g 46660*) genes were high in most tissues. On the other hand, expression of G2 (*Os08g37350*, *Os09g28940*, *Os02g14730*, and *Os06g44380*), G7 (*Os01g08200*), and G8 (*Os10g07270*) genes were weak but detectable in rice tissues. These results indicate that *OsUBP* genes are differentially expressed in various tissues of rice plants.

Isolation and characterization of *OsUBP6* full-length cDNA

Among the 21 putative OsUBPs, the Os01g36930 protein (the G6 member) is predicted to contain a unique ubiquitin domain at the N-terminal region (Fig. 2). We proceeded to isolate full-length *Os01g36930* cDNA by RT-PCR using gene-specific primers. Full-length *Os01g36930* cDNA is 1,860 bp long and contains a 128 bp 5′-untranslated region, a 1,425 bp coding region (474 amino acids), and a 306 bp 3′-untranslated region (Fig. 3A). The Os01g36930 protein has a predicted molecular mass of

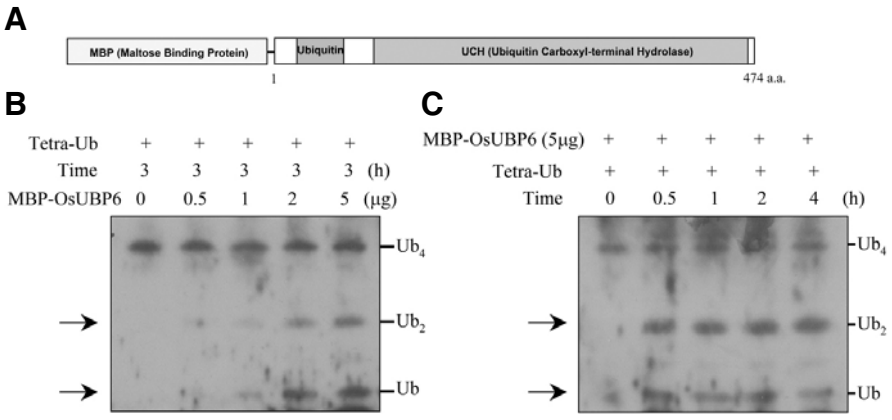

Fig. 4. *In vitro* de-ubiquitination assay of OsUBP6 protein. (A) Schematic representation of MBP-OsUBP6 fusion protein. (B-C) *In vitro* de-ubiquitination assay of MBP-OsUBP6 fusion protein. Bacterially expressed MBP-OsUBP6 protein (0-5 μg) was purified and co-incubated with K48-linked tetra-ubiquitin for 3 h at 30°C (B), and 5 μg MBP-OsUBP6 was co-incubated with tetra-ubiquitin for different lengths of time (0-4 h) at 30°C (C). The samples were resolved on an 8% SDS-PAGE gel and subjected to immunoblotting with anti-ubiquitin antibody. Arrows indicate ubiquitin monomer and dimer.

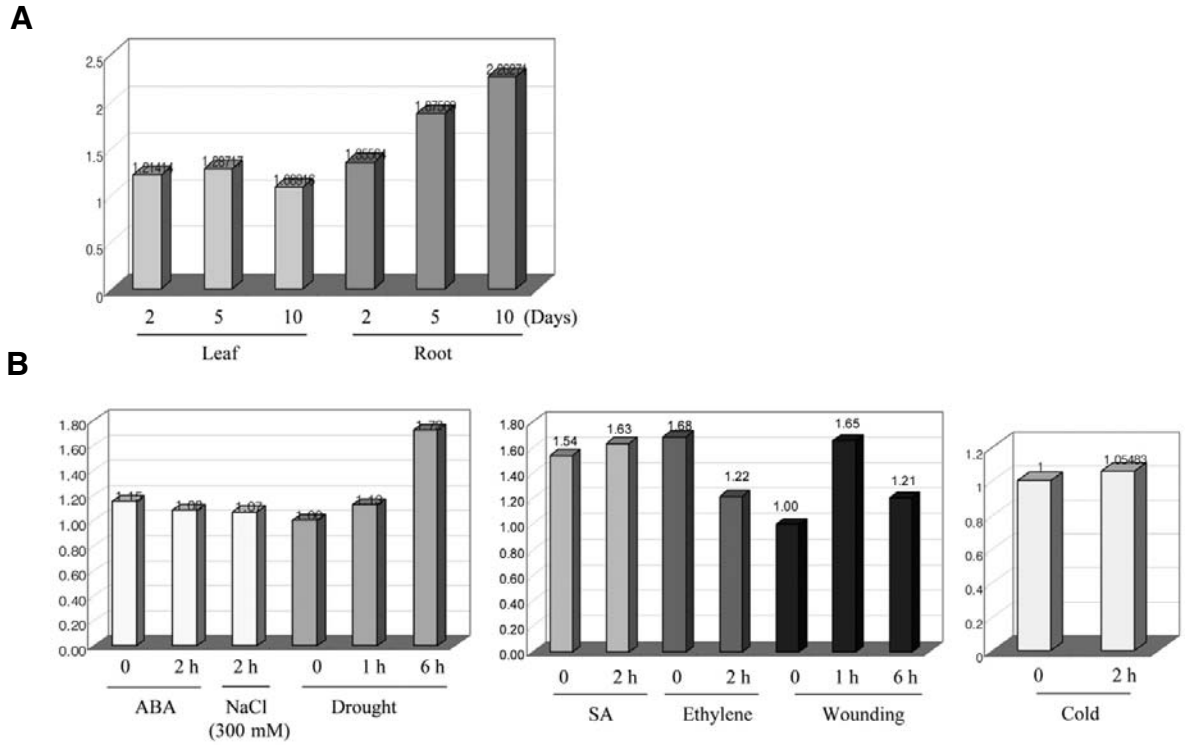

Fig. 5. Quantitative real-time PCR analysis of the *OsUBP6* gene. (A) Expression levels of *OsUBP6* mRNA in the leaves and roots of 2-, 5-, and 10-day-old rice seedlings. (B) Expression of *OsUBP6* in response to various stresses (high salinity, drought, cold, and wounding) and hormone treatments (ABA, SA, and ethylene). Results are average values obtained from two independent experiments.

53.2 kDa and a calculated pI of 5.73. The rice Os01g36930 protein contains a single ubiquitin domain at the N-terminal region and a UCH domain encompassing the middle and C-terminal regions. The UCH domain possesses highly conserved Cys and His boxes, both of which may be essential for its enzymatic activity (Fig. 3B). Os01g36930 protein is 92%, 74%, 70%, and 69% identical at the amino acid level to maize (*Zea mays*) ZmUCH6 (GenBank accession number NP_001148768), grape (*Vitis vinifera*) unnamed protein (GenBank accession number CAO45730), and *Arabidopsis* AtUBP6 (GenBank accession number NP_594596) and AtUBP7 (GenBank accession number NP_566680) proteins, respectively (Figs. 3B and 3C). Therefore, we named rice Os01g36930 protein OsUBP6. OsUBP6 shares relatively lower sequence identity (42%) with human ortholog HsUBP14 (GenBank accession number NP_005142.1) (Figs. 3B and 3C).

OsUBP6 possesses de-ubiquitination enzymatic activity *in vitro*

To investigate whether OsUBP6 possesses de-ubiquitination activity, MBP (maltose binding protein)-fused *OsUBP6* construct was prepared and expressed in *E. coli* cells (Fig. 4A). The MBP-OsUBP6 fusion protein was then purified using the amylose resin-affinity chromatography. The purified protein (0-5 μg) was co-incubated with K48-linked tetra-ubiquitin at 30°C for 3 h, and subjected to immuno-blotting using anti-ubiquitin antibody. As shown in Fig. 4B, MBP-OsUBP6 produced ubiquitin monomer and dimer in a dose-dependent manner. We next incubated 5 μg of MPB-OsUBP6 with K48-linked tetra-ubiquitin for different

lengths of time (0-4 h) and found that 30 min is sufficient for OsUBP6 to produce ubiquitin monomer and dimer from tetra-ubiquitin substrate (Fig. 4C). Collectively, these results indicate that rice OsUBP6 has de-ubiquitination enzyme activity *in vitro*.

Real-time RT-PCR analysis of *OsUBP6* at different developmental stages and in response to various abiotic stresses in rice plants

To examine the spatial and temporal expression pattern of *OsUBP6*, we monitored *OsUBP6* transcript levels in leaf and root tissues at different developmental stages by quantitative RT-PCR. Total RNA was prepared from the leaves and roots of light-grown 2-, 5-, and 10-day-old seedlings and was subjected to real-time RT-PCR analysis. The results of Fig. 5A reveal that, although slightly different levels of mRNA were detected during root development, *OsUBP6* transcript appeared to be constitutively expressed in both leaf and root tissues at all developmental stages examined. We next subjected 2-week-old rice whole seedlings to various environmental stresses, including drought, cold, salt, and wounding, as well as several stress-related plant hormones, such as ethylene, SA, and abscisic acid (ABA) (Joo and Kim, 2007; Kim, 2007). After treatment, total RNA was extracted from tissues and analyzed by quantitative real-time RT-PCR. As shown in Fig. 5B, basal level of *OsUBP6* expression remained unchanged in response to various abiotic stresses and hormones. Thus, *OsUBP6* appears to be constitutively expressed in rice plants.

***In vivo* sub-cellular localization of OsUBP6 in onion cells**

To determine the sub-cellular localization of OsUBP6, we carried out an *in vivo* targeting experiment in which OsUBP6-fused soluble-modified fluorescent protein (smGFP) was transiently expressed in onion (*Allium cepa* L.) epidermal cells. The full-length *OsUBP6* gene was fused to the 5′-end of *smGFP* gene in-frame under the control of the cauliflower mosaic virus (CaMV) 35S promoter, and the resulting construct (*35S:OsUBP6-smFGP*) was transfected into onion epidermal cells by particle bombardment. Localization of the fusion protein was then visualized with a fluorescence microscope. The results show that OsUBP6-smGFP is primarily localized to the nucleus (Fig. 6, lower panels), while control smGFP is found to be present throughout the cell (Fig. 6, upper panels). To confirm the nuclear localization, onion cells were stained with DAPI, a nuclear marker. As shown in Fig. 6, blue DAPI fluorescence overlapped completely with the green fluorescent signal of OsUBP6-smGFP. Based on these *in vivo* targeting results, we conclude that rice OsUBP6 is predominantly localized to the nucleus.

Isolation and analysis of *OsUBP6* T-DNA insertion mutants

To further investigate the physiological roles of OsUBP6 in rice plants, we searched the rice T-DNA insertion sequence database (http://www.postech.ac.kr/life/pfg/risd) and identified one *OsUBP6* T-DNA insertion mutant line (3A-09178). This mutant line was referred to *osubp6*. The T-DNA was inserted into the sixteenth exon of the *OsUBP6* gene, which resides on chromosome 1 (Fig. 7A). To isolate homozygous mutant plants, we performed multiplex PCR using gene-specific primers A-1 and A-2 and the T-DNA-specific primer RB-1 (Fig. 7B). RT-PCR analysis using various primers shows that full-length (with primers B-1 and A-2) and 3′-end (with primers A-3 and A-2) *OsUBP6* transcript was absent in *osubp6* mutant seedlings, but a partial mRNA transcript was still detectable with a primer set (B-1 and B-2) that amplified a region upstream of the T-DNA insertion site in *OsUBP6* (between exon 2 and exon 12) (Fig. 7C). This indicates that although there are some truncated transcripts, *osubp6* does not contain functional, full-length *OsUBP6* mRNA.

To examine the phenotypic differences of *osubp6* compared to wild-type rice, homozygous T2 seeds were germinated and T3 seedlings were obtained. As shown in Fig. 7D, under our growth conditions, 7-day-old, light-grown homozygous T3 *osubp6* seedlings exhibited significantly retarded root and shoot growth compared to wild-type seedlings. The heights of shoots and roots in mutant seedlings were approximately 40-55% those of wild-type organs. In contrast, 3-month-old mature *osubp6* plants were phenotypically indistinguishable from wild-type plants (Fig. 7E), suggesting that loss of *OsUBP6* may be functionally compensated for by other *OsUBP* family members during development.

DISCUSSION

Although the possible cellular roles of several UBPs were identified in *Arabidopsis* (Doelling et al., 2007; Liu et al., 2008; Vierstra, 2009), almost nothing is known about UBP homologs in rice, a monocot model plant. In this report, we identified 21 putative *OsUBP* genes in the rice genome that each contains a UCH domain with highly conserved Cys and His boxes. These predicted 21 UBP proteins contain various domains and further divided into 9 groups based on their sequence identities and domain structures (Figs. 1 and 2). The five G1 family members contain 1 or 2 ZnF-UBPs in their central regions, whereas three out of four G2 members have ZnF-MYND (Fig. 2). Two G5 members possess a single MATH domain. In addition, Ubiquitn, UBA, and DUSP motifs are also present in G6, G7, and G9 OsUBPs, respectively. On the other hand, all of the three G3 and three G4 UBPs as well as G9 member contain only a single UCH motif (Fig. 2). The mRNA expression analysis suggests that genes belonging to the same group show similar expression patterns. For example, transcript levels for the G1 family members, *Os08g41530*, *Os08g41540*, *Os08g41630*, *Os08g41640*, and *Os08g41620*, are extremely low in most rice tissues, while four G2 genes (*Os08g37350*, *Os09g28940*, *Os02g14730*, and *Os06g44380*) show similar low expression levels. In addition, all G3 genes (*Os06g08530* and *Os02g55180*), G4 genes (*Os04g37950*, *Os03g09080*, and *Os02g36400*), and G5 genes (*Os12g30540* and *Os01g56490*) are expressed at high levels in most tissues examined (Fig. 1). Thus, it is likely that there is a relationship between the primary structures and expression patterns of *OsUBP* family members. These results raise the possibility that *OsUBP* genes in different groups may have unique roles in rice plants, while there may be functional redundancies among genes belonging to the same group. This notion is consistent with the fact that mature loss-of-function *osubp6* mutant plants are phenotypically normal (Fig. 7).

We isolated a full-length cDNA clone for *OsUBP6*, which possesses not only a UCH domain but also an N-terminal ubiquitin motif (Fig. 3). As expected, bacterially expressed MBP-OsUBP6 fusion protein has de-ubiquitination enzymatic activity (Fig. 4). Under our *in vitro* experimental conditions, the de-ubiquitination activity of OsUBP6 was clearly detected, as 30 min was sufficient for MBP-OsUBP6 to produce ubiquitin monomer and dimer from K48-linked tetra-ubiquitin substrate (Fig. 4C). Quantitative real time RT-PCR indicates that *OsUBP6* is expressed constitutively in rice plants (Fig. 5).

We next examined the effect of *OsUBP6* knock-out on developmental growth of rice plants. Although homozygous *osubp6* T-DNA knock-out mutant seedlings show some defects, by the time they reach maturity, they appear to be normal (Fig. 7). Recently, Doelling et al. (2007) found that single *atubp3* and *atubp4* knock-out *Arabidopsis* mutants are normal, while *atubp3atubp4* double

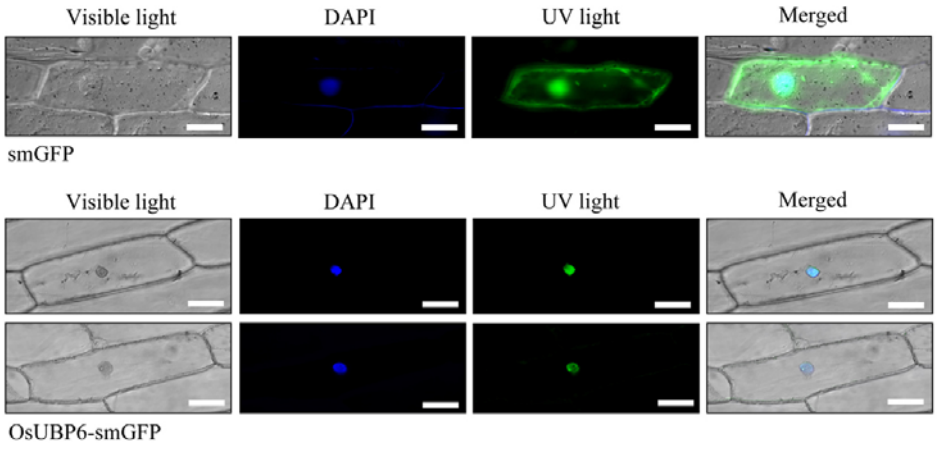

Fig. 6. Subcellular localization of OsUBP6 protein in onion epidermal cells. *35S:smGFP* and *35S:OsUBP-smGFP* plasmids were introduced into onion epidermal cells via particle bombardment. Transient expression of transgenes was viewed after 18 h by fluorescence microscopy under UV or visible light. Blue DAPI staining marks the nuclei. The green fluorescence signal of OsUBP6-GFP overlapped with the blue DAPI signal. Scale bars, 50 μm.

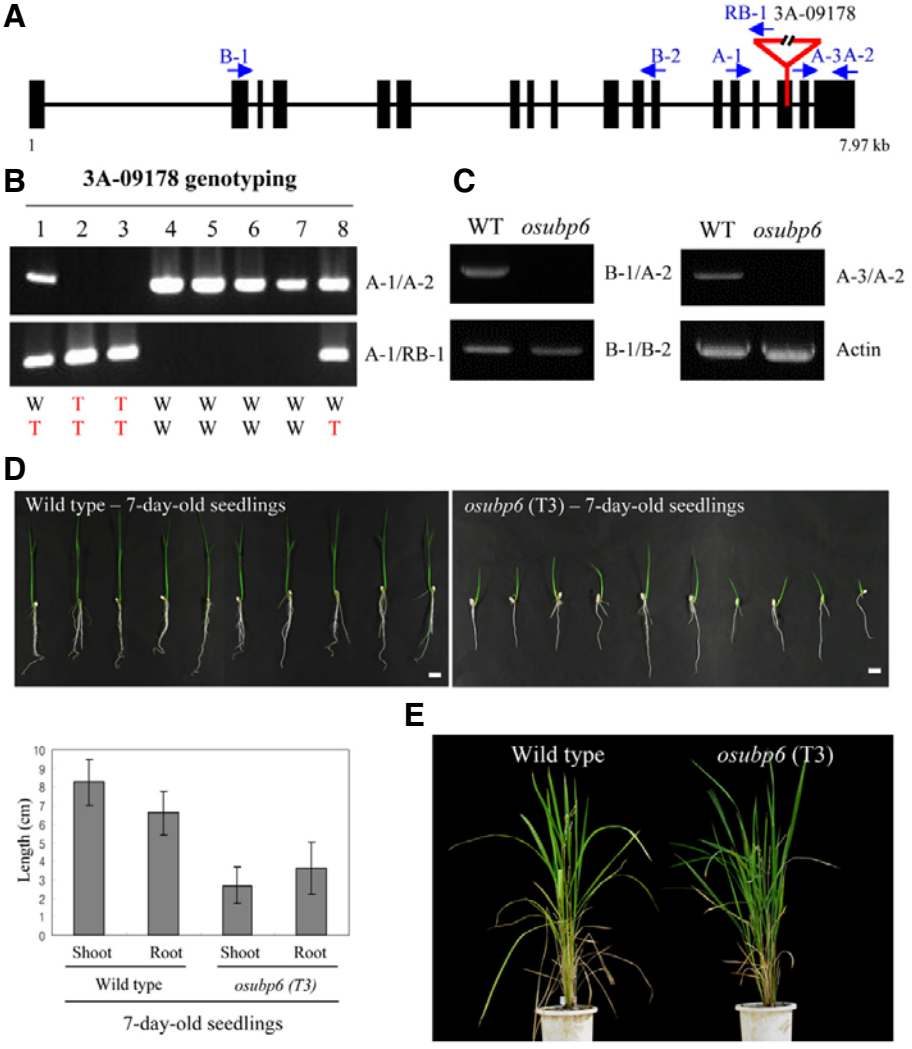

Fig. 7. Identification and characterization of *osubp6* mutant plants. (A) Diagram of *osubp6* mutant with the position of the inserted T-DNA (triangle) in the *OsUBP6* gene. Dark bars show exons, and solid lines indicate introns. Gene-specific (A-1, A-2, A-3, B-1, and B-2) and T-DNA specific (RB-1) primers used for PCR genotyping and RT-PCR analyses are represented by arrows. (B) PCR genotyping of *osubp6* mutant plants. The gene-specific and T-DNA-specific primer sets used for genomic PCR are indicated in the agarose gel image in the right panel. W, wild type; T, T-DNA insertion. (C) RT-PCR analysis of *OsUBP6* in wild-type and *osubp6* mutant rice plants. The gene-specific primer sets used for RT-PCR are indicated in the agarose gel image in the right panel. The actin gene was used as a loading control. (D) Morphology of 7-day-old light-grown wild-type and homozygous T3 *osubp6* mutant rice seedlings. Scale bar, 1 cm. The values are means ± SD (n = 10). (E) Morphology of three-month-old mature wild-type and homozygous T3 *osubp6* mutant rice plants.

mutants exhibit a severely defective phenotype, suggesting that AtUBP3 and AtUBP4 may work together in the process of growth and development. In addition, it was shown that AtUBP15 and AtUBP16 may play redundant roles in *Arabidopsis* (Liu et al., 2008). Thus, the normal phenotype of mature *osubp6* rice mutant plants could be due to functional complementation by an as-yet unknown *OsUBP* homolog(s) during later stages of development.

The *Arabidopsis OsUBP6* homolog, AtUBP6, was shown to interact with calmodulin, which raises the possibility that calcium signaling via calmodulin participates in ubiquitin-mediated protein degradation and/or stability in *Arabidopsis*. We are currently conducting yeast two-hybrid screens to identify proteins

that may interact with OsUBP6. HsUSP14 is a human homolog of OsUBP6 (Fig. 3C). HsUSP14 was previously reported to interact with 26S proteasome complex and this interaction enhanced the catalytic activity of HsUSP14 (Hu et al., 2005). OsUBP6 also possesses a ubiquitin-like motif, and thus it is required to investigate whether OsUBP6 is associated with 26S proteasome complex in rice nucleus. In *Arabidopsis*, histone H2B is mono-ubiquitinated and the levels of ubiquitinated H2B and trimethyl H3 are upregulated in *sup32* mutant plants (Sridhar et al., 2007). In addition, SUP32/UBP26 can de-ubiquitinate H2B (Luo et al., 2008; Schmitz et al., 2009). These results suggest that H2B de-ubiquitination by SUP32/UBP26 is involved in heterochromatic histone H3 methylation. Because rice OsUBP6 is exclusively localized to the nucleus (Fig. 6), it would be intriguing to investigate whether OsUBP6 is involved in the de-ubiquitination of nuclear proteins in rice plants.

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