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RESEARCH ARTICLE

Pollen-Expressed Plant U-Box Protein, OsPUB14 Involves in Rice Fertility and Degrades OsMTD2

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Abstract

Understanding the intricacies of pollen tube growth in cereal crops, such as rice, is crucial for understanding crossbreeding, seed formation, and crop productivity. In this study, we investigated the molecular mechanisms underlying pollen tube germination and elongation in rice, focusing on the interaction between OsPUB14 and OsMTD2 and its impact on reactive oxygen species (ROS) regulation. Expression studies revealed that *OsPUB14* was highly expressed in pollen and anther tissues, indicating its involvement in pollen function. We demonstrated that OsPUB14 belonging to group II U-box domain proteins, interacts with the kinase domain of OsMTD2 (a pollen-specific CrRLK1L member) and degrades it. This interaction subsequently reduces OsMTD2-mediated ROS generation. Moreover, the overexpression of *OsPUB14* resulted in decreased ROS levels and reduced fertility in rice plants, emphasizing its role in reproductive processes. Yeast two-hybrid screening identified OsCRK10P and OsNET2D as potential interactors of OsPUB14, further expanding our understanding of the regulatory networks associated with pollen development. This study provides insight into the intricate interplay between pollen-specific plant U-box domain proteins (PUBs), demonstrating their roles in regulating ROS levels and ultimately influencing plant fertility.

Keywords Rice · Plant U-box domain protein (PUB) · Reactive oxygen species (ROS) · Protein degradation · Fertility

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Introduction

Understanding the molecular mechanisms underlying pollen tube growth in cereal crops is essential for studying crossbreeding, seed setting, and crop yield. Rice (*Oryza sativa* L.) is one of the world's most important crops and requires several elements for successful pollen tube germination and growth. These elements include proper water content within the stigma (Moon and Jung 2020), calcium levels (Zhao et al. 2002), and reactive oxygen species (ROS) (Kim et al. 2021b).

A previous study reported that *male gene transfer defect 2* (*OsMTD2*) encodes a member of the CrRLK1L family of proteins that primarily function to mediate ROS in rice male gamete transfer. The *osmtd2* mutant exhibited a specific phenotype of premature rupture of pollen tubes before normal elongation (Kim et al. 2021b). In addition, a previous study suggested that the rapid alkalinization factor (OsRALF) members, OsRALF17 and OsRALF19, activate ROS signaling and interact with the extracellular domain of OsMTD2. Exogenous treatment of the mature forms of OsRALF17 and OsRALF19 promotes the internalization of OsMTD2 (Kim et al. 2023a). However, how OsMTD2 is internalized and whether it is degraded has not yet been reported.

Interestingly, the kinase domain of OsMTD2 interacts with an SPL11-like protein, a gene encoding protein homologous to SPL11 (Spotted leaf 11) (Kim et al. 2021b). *SPL11* has been reported to possess E3 ubiquitin ligase activity in vitro and is involved in various cellular processes in rice, including the regulation of cell death and flowering time (Zeng et al. 2004). Since the discovery of plant U-box domain proteins (PUBs) in the Arabidopsis genome (Azevedo et al. 2001), 77 rice PUBs have been identified and classified; as a result, SPL11 was named OsPUB11, and the SPL11-like protein interacting with OsMTD2 was named OsPUB14 (Zeng et al. 2008). Most rice PUBs contain a domain or motif implicated in protein–protein interactions. Furthermore, rice PUBs are grouped into eight classes based on the presence of common motifs or domains other than the U-box [ubiquitin fusion degradation protein-2 (UFD2) specific motif, armadillo (ARM)/HEAT repeats, Glycine (G), Lysine (K)/Arginine (R) (GKL)-box, Kinase, U-box only, tryptophan-aspartic acid (WD40), tetratrico peptide repeat (TPR), both TPR and Kinase, and the middle domain of eukaryotic initiation factor 4G (MIF4G)]. *SPL11* and *OsPUB14* belong to the largest class of rice U-box domain proteins, whereas group II proteins contain ARM repeats (Zeng et al. 2008). It has been reported that the U-box domain interacts with ubiquitin-conjugating enzymes (E2) and is involved in polyubiquitin chain assembly. In addition, it has ubiquitin ligase activity and is related to the E3 protein (Trujillo 2018; Ohi et al. 2003). ARM repeats are involved in protein–protein interactions and cellular functions, including ubiquitination, and were first identified in the segment polarity gene *armadillo* in *Drosophila melanogaster* (Samuel et al. 2006). PUBs interact with other protein targets, are involved in ubiquitination, and are subsequently phosphorylated. However, there have been no reports on the other targets or roles of OsPUB14.

The ARM domain of AtPUB13 is phosphorylated by the barssinosteroid (BR) receptor BRI1-associated receptor kinase (BAK1), which mediates the ubiquitination of flagellin-sensing 2 (FLS2) and targets FLS2 for degradation during flagellin-induced formation of the FLS2-BAK1 complex, resulting in the attenuation of immune signaling (Zhou et al. 2015). The ARM repeat domain of OsPUB15, belonging to the same group as *OsPUB14*, interacts with the PID kinase domain (PID2K) (Wang et al. 2015). The growth of seedlings in *ospub15* knockout plants is impaired, whereas those overexpressing *OsPUB15* exhibit tolerance to elevated salt levels, suggesting that *OsPUB15* might play a role in regulating ROS levels (Park et al. 2011). It has been reported that SPL11-interacting protein1 (SPIN1) is negatively regulated by SPL11 (or OsPUB11) via ubiquitination and might be important for fine-tuning and the timing of flowering in rice (Vega-SáNchez et al. 2008). PUBs act as mediators of ubiquitination and regulate the number of target proteins, thereby regulating biological processes such as immune system and flowering (Mao et al. 2022).

However, few Group II PUBs have been reported to function in plant reproduction. The *AtPUB4* mutant, belonging to group II PUB, showed hypertrophic growth of the tapetum layer and incomplete degeneration of tapetal cells and sterile at 22℃ but showed partial fertility at 16℃, which is a temperature-dependent male-sterility trait (Wang et al. 2013). *BoPUB7*, which is homologous to *AtPUB7* and contains a U-box N-terminal domain, a U-box, and four ARM domains, is expressed in mature pollen, and *pLat52:BoPUB7* Arabidopsis plants show decreased pollen germination rates (Lian et al. 2019).

The focus of our investigation was on the interaction between *OsPUB14* and *OsMTD2* and its effect on the plant rather than on external factors, such as disease or stress. We studied characteristics of *OsPUB14,* such as homologous genes and expression. Biochemical analysis showed that the U-box domain of OsPUB14 degraded OsMTD2 and that the coexistence of OsPUB14 and OsMTD2 reduced ROS. Furthermore, the enhanced expression of *OsPUB14* in plant pollen compared to that in the wild-type (WT) plants resulted in reduced ROS levels. Yeast two-hybrid (Y2H) analysis revealed that OsPUB14 has an interactor partner candidate, OsCRK10P, which is a receptor-like kinase, and OsNET2D.

Materials and Methods

Bioinformatics Analysis

The protein sequences from PUBs were obtained from the Rice Genome Annotation Project website (http://rice.plantbiology.msu.edu/) and the Araport11 version of the Arabidopsis Information Resource website (TAIR, https://www.arabidopsis.org/) and aligned with MAFFT version 7.520 (Katoh and Standley 2013). We generated a phylogenetic tree using FastTree version 2.1.11 (Price et al. 2010) with 1000 bootstrap replicates. The expression data for various tissues used in heatmap analysis was acquired from CAFRI-Rice (Hong et al. 2020) and CAFRI-Arabidopsis (Hong et al. 2024). The tree was visualized using Interactive Tree of Life (iTOL) (Letunic and Bork 2021).

Plant Materials and Growth Conditions

The rice (*Oryza sativa* L. ssp. japonica cultivar Nipponbare) and the generated transgenic plants were grown in under the following settings: 30 °C (14 h light, 12 h light at flowering stage) and 22 °C (10 h dark, 12 h at flowering stage), or in a paddy field. *Nicotiana benthamiana* plants were grown in at 25 °C.

Construction of Vectors and Generation for Transgenic Lines

To construct pOsPUB14-1st exon- *β-glucuronidase* (GUS) vector, the promoter region 2509 bp upstream from the start codon of *OsPUB14* and 1st exon region were amplified by PCR with primers tagged with *Asc*I and *Not*I sites respectively and subcloned into a modified pBlueScript based vector containing *Asc*I, *Not*I, and *Spe*I. *OsPUB14* promoter, 1st exon, and GUS-coding region (between *Not*I and *Spe*I) were cloned into the binary vector pER8, digested with *Asc*I *Spe*I restriction enzymes.

CRISPR/Cas9 mutant plants for *OsPUB14* and *OsPUB13* were generated as previously described (Kim et al. 2024). The pER8-pUBI-OsPUB14-dHA vector was constructed using the method described above for *OsPUB14* overexpression plants. Transgenic rice plants were generated via Agrobacterium-mediated transformation of the LBA4404 strain. The co-cultivated calli were rinsed with water and cultured on 2N6 medium containing 250 mg/L cefotaxime and 30 mg/L hygromycin B. Regenerated shoots were transferred to MSR medium containing 250 mg/L cefotaxime and 30 mg/L hygromycin B.

Gene Expression Analysis

Rice tissues were collected using liquid nitrogen. We collected at least 50 anthers in the early (meiosis-early vacuolated) and late stages (late vacuolated-mature pollen) (Huang et al. 2009). Extracting total RNA and qRT-PCR were performed as previously described (Kim et al. 2024). The supplementary table contains the gene-specific primers used for qRT-PCR.

For histochemical GUS assay, tissue samples were immersed in GUS staining solution (100 mM Sodium phosphate (pH7.0), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.5% Triton X-100, 10 mM EDTA (pH8.0), 0.1% X-Gluc, 2% DMF, and 5% Methanol) and kept in a vacuum condition for 15 min. Subsequently, tissues were incubated at 37 °C for 1 d and decolored, except blue color, using 70% ethanol at 37 °C for 2 to 3 days. The samples were observed under a stereomicroscope and Nikon ECLIPSE 80i microscope.

Biochemistry Assay

To investigate the degradation of OsMTD2-ΔTM, cultured Agrobacteria harboring p35s-OsPUB14-3xHA, p35s-OsPUB14-ARM-3xHA, p35s-OsPUB14-U-Box-3xHA, pUBQ14-H2B-3xHA, and pUBQ14-OsMTD2-ΔTM-3xMYC infiltrated *N. benthamiana* with P19 as a suppressor of gene silencing in infiltration buffer as previously described (Kim et al. 2024). After 1 day of infiltration, 26S proteasome inhibitor MG132 [50 μM MG132, 10 mM $MgCl_2$] was infiltrated in *N. benthamiana* leaf, and 50 μM DMSO was used for control infiltration. After 1 day of MG132 and DMSO infiltration, respectively, the leaves were collected, and total proteins were extracted using immunoprecipitation buffer (IP buffer). Immunoblot analysis was performed using anti-c-Myc horseradish peroxidase and anti-HA-peroxidase, as described previously (Kim et al. 2023a).

Examination of Pollen and Pollen Tube

To observe pollen viability, mature pollen was stained with 1% I2-KI solution. The late-stage anthers from more than 10 flowers were collected and incubated in the solution at room temperature (25 °C) for 1 to 2 h. The pollen samples were collected using a pipette and observed under a Nikon ECLIPSE 80i microscope. Viable pollen was identified as dark red and unviable pollen as light-colored and unstained.

In vitro pollen germination analysis was conducted as previously described (Kim et al. 2021b). Mature pollen grains were collected onto fresh solid pollen germination media (PGM) [20% (w/v) sucrose, 10% polyethylene glycol (PEG) 4000, 3 mM calcium nitrate, 40 mg/L boric acid, 10 mg/L vitamin B1, and 1% agarose]. The samples were

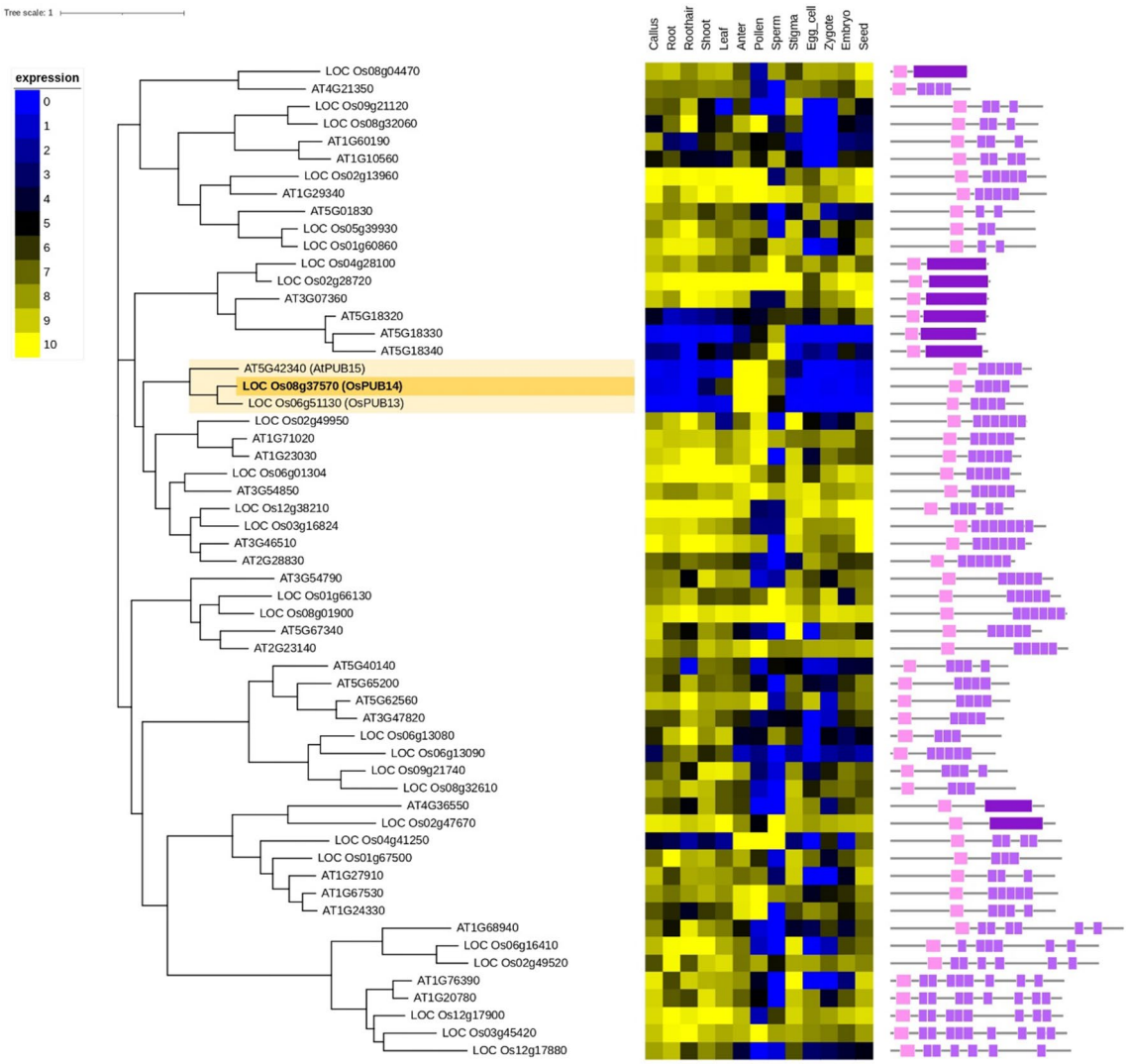

Fig. 1 Integrated bioinformatics analysis of rice and Arabidopsis group II PUBs. Phylogenetic tree, heatmap analysis and predicted protein structure of PUB genes involved group II in rice and Arabidopsis. The phylogenetic tree was displayed using The Interactive Tree of Life (iTOL). Pink box indicates U-box domain from SMART, light purple indicates ARM domain from SMART, and dark purple indicates ARM repeat from SUPERFAMILY

then incubated in a moist chamber at 28 °C in the dark for 20 min. Nitroblue tetrazolium (NBT) staining was performed to visualize hydrogen peroxide. Pollens were germinated in PGM for 5 min and then stained with 0.1 mg ml^{-1} NBT (in liquid PGM solution) for 5 min.

To detect callose in pollen tubes within the pistil using aniline blue, rice spikelets after 5 h of pollination were incubated overnight in Carnoy's solution (30% chloroform, 10% acetic acid, and 57% ethanol) as previously described (Kim et al. 2024). the pistils were mounted on a slide covered with a slip and observed under a Nikon ECLIPSE Ti2 inverted microscope.

Y2H Analysis and Co-IP Analysis

For the Y2H screening, OsPUB14 was cloned into the *Eco*RI/*Bam*HI sites of the pGBKT7 vector. Screening of binding partners using the rice anther c-DNA library for OsPUB14 was conducted by Panbionet Corp. (Pohang, Korea) (http://www.panbionet.com/).

Co-immunoprecipitation (Co-IP) analysis was conducted as described previously (Kim et al. 2023a). Cultured Agrobacteria were infiltrated into *N. benthamiana*, as described above, for the biochemistry assay. Leaves were collected after 2 d of infiltration, and total protein was extracted using IP buffer. Total proteins were incubated with anti-c-MYC magnetic beads for 2 h at 4 °C. The beads were washed

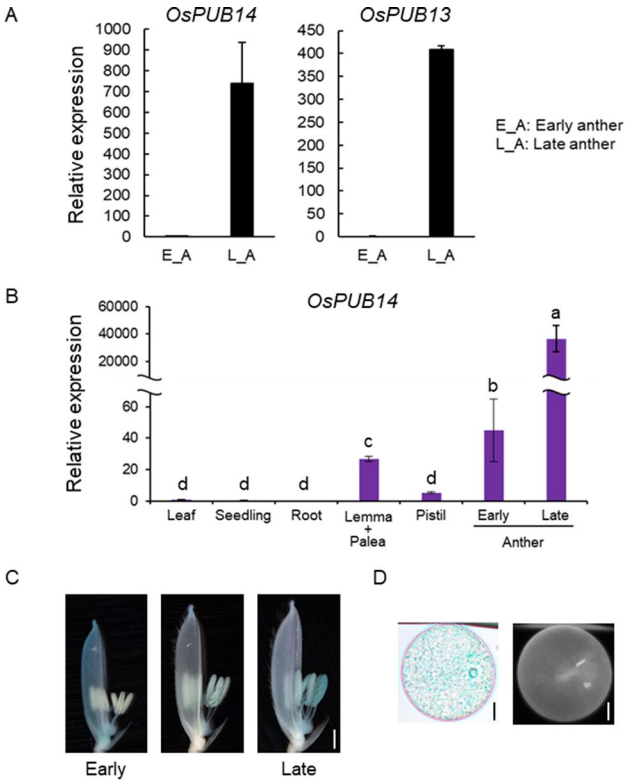

Fig. 2 Expression of *OsPUB14* **A** Quantitative RT-PCR analysis of *OsPUB14* and *OsPUB13* in early anther and late anther. **B** Quantitative RT-PCR analysis of *OsPUB14* using various rice tissues. Duncan's Multiple Range test (DMRT) for comparison of expression of tissues ($\alpha = 0.05$). **C**–**D** GUS staining of pOsPUB14-1st exon-GUS plants. The spatiotemporal expression patterns at various developmental spikelets in rice (**C**) and mature pollen (**D**). Scale bars = 1 mm (C) and 10 μm (**D**). Error bars represent the standard deviation of at least three replicates

thrice with washing buffer (the same as the IP buffer) after incubation. Immunoblot analysis was performed as described above for biochemical assays.

For the bimolecular fluorescence complementation (BiFC) assay, the CDS of *OsPUB14* was fused to the C-terminal fragment of Venus (CV). The fused material was amplified via PCR using gene-specific primers. The amplified CV-fused OsPUB14 was ligated into the *Hin*dIII/*Eco*RI-treated pGREEN vector to generate the OsPUB14-CV vector using an In-fusion HD Cloning Kit (Takara Bio Inc.). The OsRALF17-CV, OsMTD2-NV, and TF-NV vectors have been previously reported (Kim et al. 2023a). Cultured Agrobacteria were infiltrated into *N. benthamiana*, as described above, for the biochemistry assay. One day after infiltration, the leaves were collected and stained with DAB solution (10 mM pH4.35 Tris–HCl, 1 mg ml^{-1} DAB) to visualize the generation of hydrogen peroxide.

Results

Pollen-Specific PUBs in Group II PUB Family

In our previous study, the SPL11-like protein, OsPUB14 (LOC_Os08g37570), was identified as an OsMTD2 interactor using Y2H and Co-IP analyses (Kim et al. 2021b). It was expected that OsPUB14 would have a close phylogenetic relationship with SPL11 (OsPUB11, LOC_Os12g38210), therefore OsPUB14 was considered an SPL11-like protein. According to a classification based on motifs/domains (Zeng et al. 2008), OsPUB14 belongs to group II PUB, which has a domain organization containing a U-box and ARM/HEAT. We performed a phylogenetic analysis of the genes in group II PUB in 28 OsPUBs from rice and 29 AtPUBs from Arabidopsis, compared their expression patterns, and analyzed their protein structures (Fig. 1). In the phylogenetic analysis, OsPUB13 showed the closest phylogenetic distance to OsPUB14 and exhibited high expression in the anthers and pollen, similar to OsPUB14 (Fig. 1). In Arabidopsis, *AtPUB15* exhibits an expression pattern similar to that of *OsPUB14*. In summary, only three genes have been observed to be specifically expressed in pollen or anthers, and even fewer have known functions. In other group II PUBs, no genes were expressed at particularly high levels in specific tissues. *AtPUB16* (*AT5G18330*) displayed particularly high expression in the sperm, but the expression level was slightly low. Some group II PUBs that are ubiquitously expressed exhibit functions and respond to stress. For example, *AtPUB46* (*AT5G18320*) is involved in tolerance to drought and oxidative stress (Adler et al. 2018). Similarly, in rice, *OsPUB8* (*LOC_Os02g28720*), *OsPUB4* (*LOC_Os02g13960*), *OsPUB2* (*LOC_Os05g39930*), and *OsPUB5* (*LOC_Os08g32060*) are upregulated under drought stress (Yoo et al. 2020).

Expression Analysis of *OsPUB14*

The localization of OsPUB14 was found to be predominantly in the plasma membrane (PM) through its expression in tobacco epidermal cells, similar to the localization pattern of OsMTD2 (Kim et al. 2021b). In the heatmap analysis, *OsPUB14* and *OsPUB13* showed specifically high expression in pollen and anthers (Fig. 1). We initially confirmed the expression of *OsPUB14* and *OsPUB13* in early and late-stage of anther by qRT-PCR (Fig. 2A). The results displayed both genes expressed high in late-stage anther and *OsPUB14* showed higher expression than *OsPUB13*.

We performed further analysis of the expression of *OsPUB14* in various rice tissues by qRT-PCR (Fig. 2B). The results revealed that *OsPUB14* was expressed at the highest level in the late-stage anthers, approximately 30,000 times in

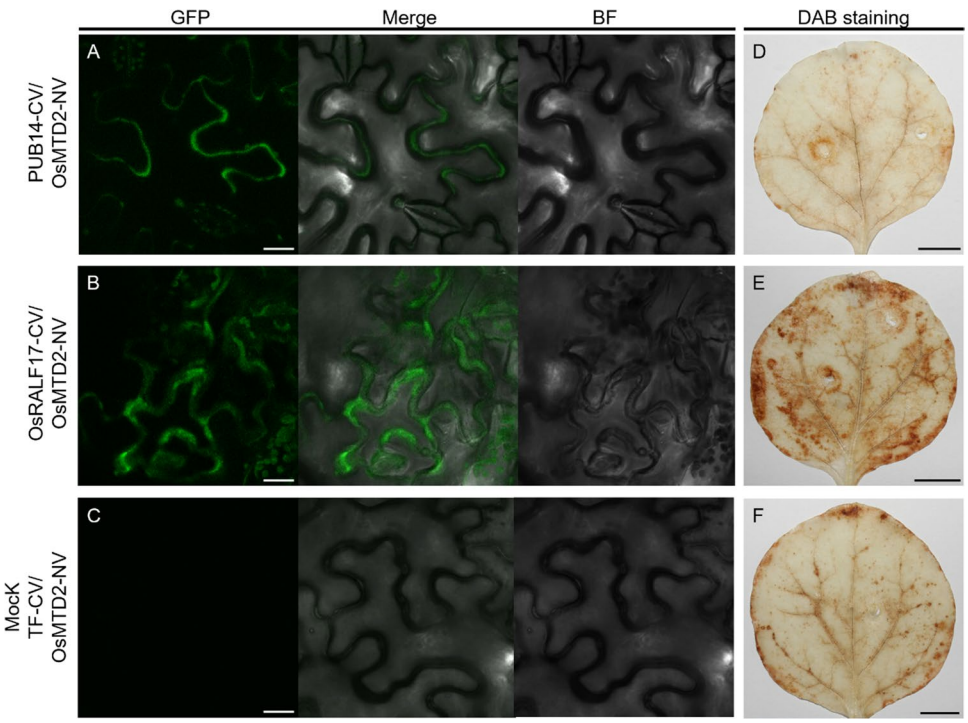

Fig. 3 Coexistence of OsPUB14 and OsMTD2 reduces ROS. **A–C** Bimolecular fluorescence complementation (BiFC) represents the interaction between OsPUB14 and OsMTD2, OsRALF17 and OsMTD2. (D-F) DAB staining indicates OsPUB14 with OsMTD2 reduces ROS compared to Mock. Scale bars = 30 µm (**A–C**) and 1 cm (**D–F**)

the leaves. The Duncan Multiple Range Test (DMRT) confirmed that the expression in the late stage of anther is the highest in *OsPUB14* (Fig. 2B). We generated a transgenic line that expressed the β-glucuronidase (GUS) gene under the *OsPUB14* promoter with 1st exon of *OsPUB14* allowing for a more detailed study of spatial expression patterns. GUS expression was detected in lemmas and palea of young spikelets, late-stages of the anther, and pollen (Fig. 2B-C). Expression analysis of *OsPUB14* suggests that *OsPUB14* may influence pollen function.

Coexistence of OsPUB14 and OsMTD2 Reduces ROS

Our previous study showed that OsPUB14 interacts with OsMTD2 (Kim et al. 2021b). The bimolecular fluorescence complementation (BiFC) assay data also revealed the interaction between OsPUB14 and OsMTD2 (Fig. 3). In a previous study, the coexistence of OsRALF17 and OsMTD2 significantly increased ROS production (Kim et al. 2023a). We confirmed that the co-existence of OsRALF17 and OsMTD2 increased ROS and compared the co-existence of OsMTD2 and OsRALF17 with mock OsMTD2 and OsPUB14 by DAB staining. Interestingly, the coexistence of OsMTD2 and OsPUB14 reduced the ROS levels in *N. benthamiana* leaves (Fig. 3).

Based on the ability of U-box domain known to have E3 ligase activity (Mao et al. 2022), we hypothesized that the coexistence of OsMTD2 and OsPUB14 would lead to a decrease in ROS levels, because OsPUB14 degrades OsMTD2. To confirm this, we first checked whether the protein kinase domain region of OsMTD2 (OsMTD2-ΔTM) interacted with the OsPUB14, OsPUB14-ARM, and OsPUB14-U-Box domains using Co-IP analysis (Supplementary Fig. S1 and Fig. 4A). Consequently, OsMTD2-ΔTM and OsPUB14, OsPUB14-ARM and U-box interacts (Supplementary Fig. S1). Performing western blot analysis of input, we observed that the intensity of bands of OsMTD2-ΔTM-3xMYC was different when it was with OsPUB14, OsPUB14-ARM, and OsPUB14-U-Box (Supplementary Fig. S1). We treated each combination of OsMTD2-ΔTM and domains of OsPUB14 with the 26S proteasome inhibitor MG132 using *N. benthamiana* leaf to check in vivo ubiquitination of OsPUB14. The sample with OsMTD2-ΔTM and OsPUB14-U-Box with MG132 was recovered, the band intensity was approximately twice as high as that observed with DMSO (Fig. 4B–C). OsMTD2 degradation by OsPUB14 and the reduced ROS production by OsMTD2 by OsPUB14 appear to be related.

Overexpression of *OsPUB14* Reduces Fertility in Rice

The coexistence of OsPUB14 and OsMTD2, which reduces ROS, led us to the hypothesis that plants overexpressing *OsPUB14* would exhibit a reduction in ROS or a phenotype similar to that of *osmtd2*. To investigate the biological functions of *OsPUB14*, we generated *OsPUB14* over-expression (OX) and CRISPR/Cas9 mutant lines. Two OX lines, the single mutant line of *OsPUB14*, the double gene *OsPUB14*, and its homologous gene *OsPUB13* edited mutant were used. We were concerned about the potential functional redundancy of

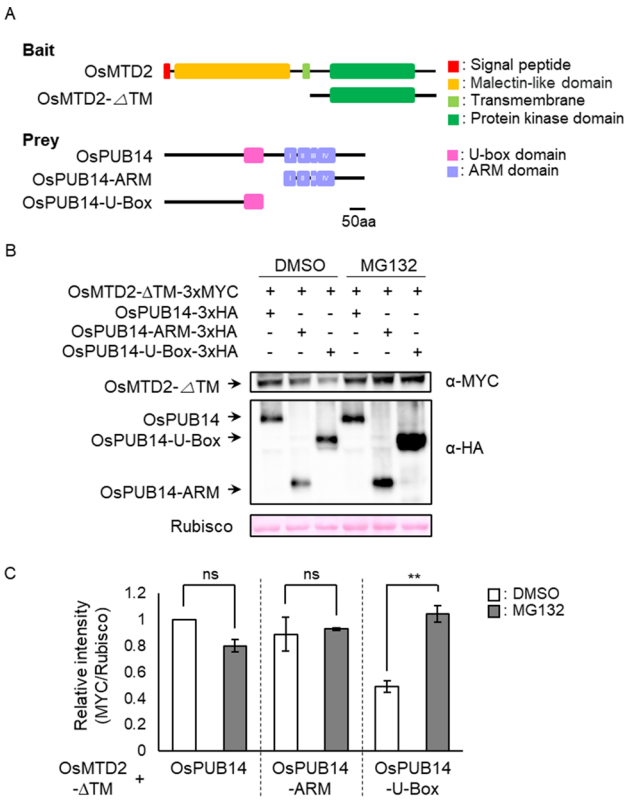

Fig. 4 The proteasome inhibitor MG132 prevents OsMTD2 degradation by OsPUB14. **A** The primary structure of OsMTD2, OsMTD2-ΔTM, OsPUB14, OsPUB14-ARM, and OsPUB14-U-Box proteins. **B** Effect of treatment with the proteasome inhibitor MG132 on the degradation of OsMTD2. **C** Relative intensity of OsMTD2-ΔTM and OsPUB14 proteins with DMSO and MG132 treatment. The DMSO treatments were used as a control. OsMTD2-ΔTM and OsPUB14 with DMSO is used as criteria. ns > 0.05 and $^{**}P < 0.01$. Error bars represent the standard deviation of at least three replicates

OsPUB13 with *OsPUB14*, so we generated double knockout mutants for *OsPUB14* and *OsPUB13*.

Despite the enhanced expression of *OsPUB14* in the OX lines, flower morphology and pollen viability were normal compared to the WT (Fig. 5A–B). Interestingly, ROS production decreased upon NBT staining in the pollen tubes of OX-1 plants (Fig. 5C). Furthermore, the seed-setting rate of the OX lines showed a decreasing tendency, but pollen tube elongation in vivo was normal compared to that of WT (Fig. 5D–E). Between CRISPR/Cas9 mutant lines and WT plants, there were no phenotypic differences in flower morphology, pollen viability, pollen germination, or seed-setting rate (Supplementary Fig. S2). *OsPUB14* OX lines exhibited low seed-setting rates and decreased ROS levels in pollen tubes, whereas the knockout of *OsPUB14* resulted in a normal plant phenotype.

Identification of OsPUB14 Interacting Protein

We performed Y2H screening using OsPUB14 as bait and a cDNA library from rice anther tissues as prey to gain insights into the functions of OsPUB14. Cysteine-rich receptor-like protein kinase 10 precursor (OsCRK10P, LOC_Os06g30130) from 113th aa, 174th aa each and NETWORKED 2D (OsNET2D, LOC_Os01g74510) from 565th aa were identified as OsPUB14 interactors by Y2H screening (Fig. 6A and B). When we confirmed the interaction between OsPUB14 and the interactor candidates by Co-IP analysis, an interaction between OsCRK10P from 174th aa and OsPUB14 was also confirmed (Fig. 6C). Heatmap expression data based on the Rice Male Gamete Expression Database (Chandran et al. 2020) indicated that OsCRK10P is expressed more strongly in spikelets and OsNET2D is expressed preferentially in pollen (Fig. 6D). The interaction between OsPUB14 and OsCRK10P has a protein kinase domain similar to that of OsMTD2.

Discussion

OsPUB14 has a U-box role in E3 ligase activity and ARM domains in protein–protein interactions and belongs to the largest class of rice U-box domain proteins, group II (Zeng et al. 2008). Several PUBs contain specific protein targets that can be ubiquitinated. For example, OsPUB15 is phosphorylated by PID2K and the phosphorylated form of OsPUB15 possesses E3 ligase activity (Wang et al. 2015). PUBs of the same group also varied in their expression in tissues, and a few PUBs of Group II were specific to pollen and anthers (Fig. 1). In rice, *OsPUB14* and *OsPUB13* are specifically expressed in pollen and anthers. qRT-PCR and GUS analyses demonstrated that *OsPUB14* expression was high in pollen and anthers (Fig. 2). These results indicate that *OsPUB14* may function in rice pollen and anthers, along with *OsMTD2*.

In a previous study, *OsMTD2* was specifically expressed in mature pollen. It has been suggested that *OsMTD2* plays a role in maintaining ROS balance and regulating the distribution of highly methylesterified pectin at the apical tube tip and is essential for polar pollen tube growth. This ROS signaling process may be facilitated by OsPUB14, which interacts with OsMTD2 (Kim et al. 2021b). The coexistence of OsPUB14 and OsMTD2 reduced ROS analyzed by DAB staining (Fig. 3). The U-box domain and ARM repeat of OsPUB14 and the protein kinase domain of OsMTD2 interacted, and the amount of OsMTD2 was reduced when the U-box domain region of OsPUB14 and the kinase domain of OsMTD2 were present together (Supplementary Fig. S1 and Fig. 4). Given that treatment with MG132, a 26 s proteasome, prevents OsMTD2 degradation, and that the U-box

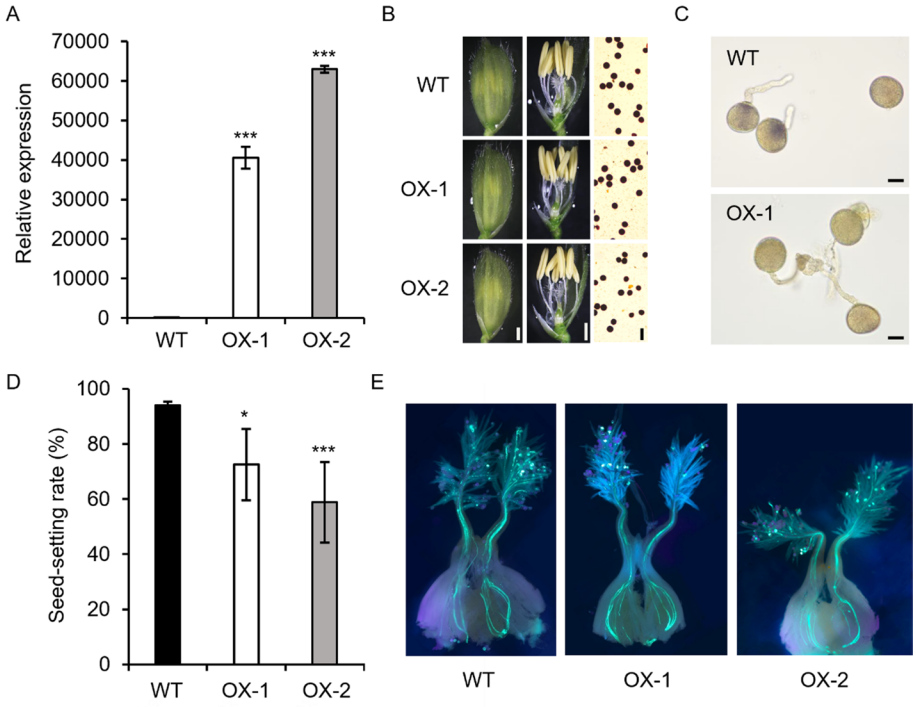

Fig. 5 Phenotypes of *OsPUB14* overexpressing plants. **A** Relative expression of *OsPUB14* in wild type (WT) and overexpressing (OX) lines. **B** The phenotypes of close and open flower structure and pollen viability stained by I2-KI in the WT and OX lines. **C** NBT staining in pollen and pollen tube in vitro. **D** The seed-setting rates of the WT and OX lines. **E** In vivo pollen germination of the WT and OX lines. Scale bars = 1 mm (B, White line), 50 μm (B, Black line), and 10 μm (**D**). $*P < 0.05$ and $***P < 0.001$. Error bars represent the standard deviation of at least three replicates

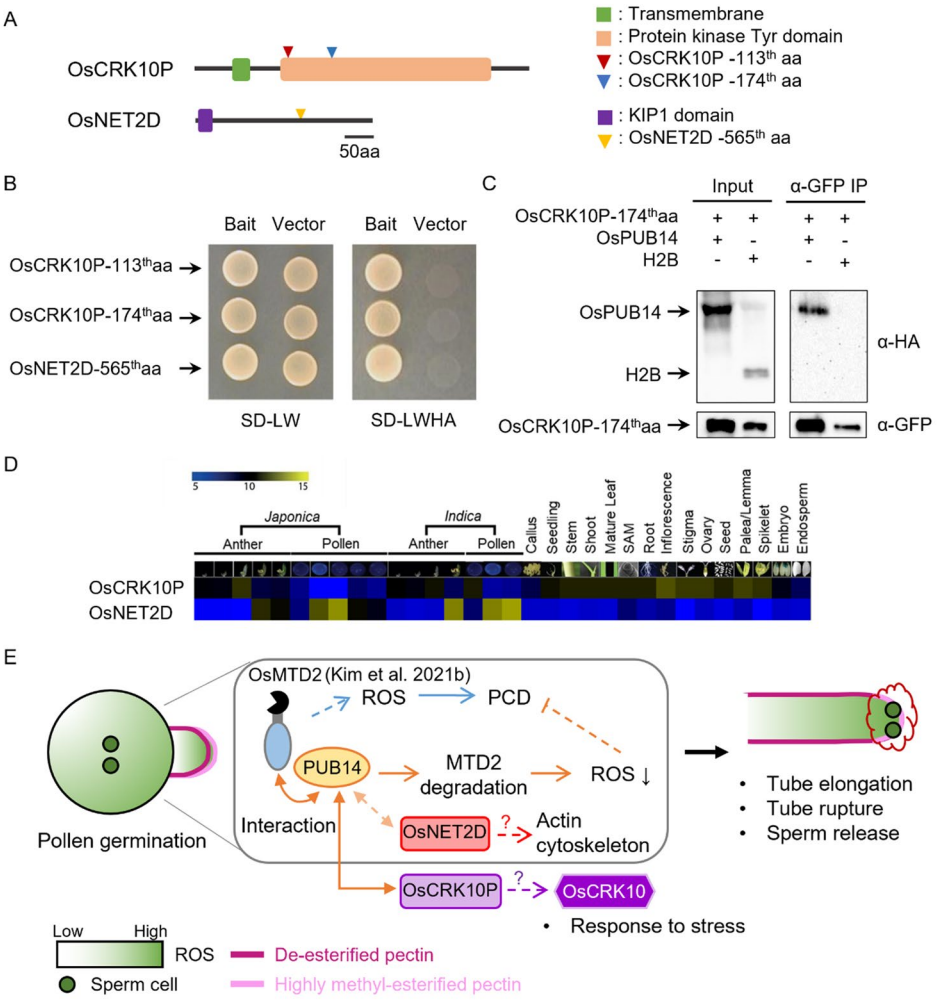

Fig. 6 Identification of proteins interacting with OsPUB14. **A** The primary structure of OsCRK10P and OsNET2D. The triangles indicate where sites Y2H bind from that location. **B** Y2H cDNA library screening of OsPUB14. '-NNthaa' indicates the protein from NNthaa was used. **C** Co-IP analysis of OsPUB14 with OsCRK10P-174th aa. **D** Heat map expression analysis of OsCRK10P and OsNET2D which are interactor candidates. **E** Functional model of OsPUB14 and related genes in rice

domain has E3 ligase activity, it is possible that OsPUB14 acts as an E3 ligase and ubiquitinates OsMTD2, leading to its degradation (Fig. 4). The fact that OsMTD2-ΔTM with OsPUB14 showed lower intensity than with H2B, a negative control, indicates OsPUB14 degrades OsMTD2-ΔTM (Supplementary Fig. S1). However, OsMTD2-ΔTM and the full-length protein of OsPUB14 with MG132 displayed a not significant changes between MG132 treatment and DMSO. Although this experiment suggests that OsMTD2-ΔTM degradation occurs with U-box domain of OsPUB14 and less degradation by full length OsPUB14, further study is required to elucidate the role of OsPUB14 with other interactions for the OsMTD2 degradation in the pollen tube in vivo.

Plants overexpressing *OsPUB14* showed lower fertility than the WT plants, although in vivo pollen germination appeared normal (Fig. 5D and E). In Arabidopsis, ROS play a role in sperm release by facilitating pollen tube rupture during fertilization. Furthermore, the inhibition of ROS production results in the overgrowth of pollen tubes (Duan et al. 2014). This evidence suggests that the observed reduction in ROS levels within the pollen of the OX line was a consequence of a decline in sperm cell delivery, which in turn resulted in a reduction in fertility (Fig. 5). The observation that the *osmtd2* mutant exhibited premature rupture of the pollen tube before elongation, whereas the *OsPUB14* OX lines did not, indicates that other regulatory factors phosphorylate OsPUB14 (Kim et al. 2021b).

In contrast to the OX lines, the CRISPR-Cas9 edited mutant of *OsPUB14* and its homologous gene, *OsPUB13,* showed a non-significant phenotype compared to the WT (Supplementary Fig. S2). Many studies have been conducted on PUBs related to biotic and abiotic stresses. The generated mutants of *OsPUB7*, which belong to the same group as *OsPUB14*, showed improved resistance to drought and salinity stress, suggesting that *OsPUB7* acts as a negative regulator of drought and salinity stress (Kim et al. 2023b). Arabidopsis *PUB48* (*AT5G18340*), which belongs to the same PUB group II and appears to be expressed specifically in sperm, has been demonstrated to be involved in providing resistance to heat stress (Adler et al. 2017). *AtPUB16* (*AT5G18330*) is also shown to be involved in plant defense (Acosta et al. 2012). CRISPR-Cas-edited mutant of *OsPUB14* may exhibit other phenotypes under stress conditions such as heat stress.

The precursor of OsCRK10 was an unveiled interactor of OsPUB14 (Fig. 6A–C). Although OsPUB14 was expressed in the pollen and anthers, OsCRK10P exhibited a generally high level of expression (Fig. 6D). OsCRK10 contributes to OsNPR1 (non-expressor of pathogenesis-related genes 1)-mediated resistance to *Xoo* (Chern et al. 2016). This suggests that OsPUB14 interacts with OsCRK10P and ubiquitinates it, thereby establishing a relationship with OsCRK10 under stress conditions. The expression of *OsPUB8*, which is homologous to *OsPUB7*, was upregulated under drought stress and infection of *Magnaporthe oryzae* treatment showed upregulated levels (Kim et al. 2021a). This suggests that *OsPUB14* may be involved with abiotic and biotic stresses that do not occur under normal conditions. The interactor candidate identified by Y2H screening, OsNET2D, was highly expressed in the anthers and pollen (Fig. 6D). In Arabidopsis, a loss-of-function mutant of the NETWORKED2 (NET2) subfamily of proteins (*net2a/net2b/net2c/net2d*) displayed a disorganized actin cytoskeleton in growing pollen tubes (Duckney et al. 2021). This indicated the possibility that OsPUB14 interacts with OsNET2D and regulates actin cytoskeleton organization in the pollen tube (Fig. 6E).

In conclusion, specifically expressed in pollen and anther tissues, OsPUB14 interacts with OsMTD2, potentially regulating ROS levels that are crucial for polar pollen tube growth. This interaction may involve OsPUB14's E3 ligase activity, leading to OsMTD2 degradation (Fig. 6E). Overexpression of *OsPUB14* resulted in reduced fertility, due to decreased ROS levels**.**

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Data Availability The data presented in this study are available on request.

Declarations

Conflict of Interests All authors declare that they have no conflicts of interest.

Ethics Approval Not applicable.

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