Structural analyses of the plant PRT6-UBR box in the Cys-Arg/N-degron pathway and insights into the plant submergence response
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Abstract
The submergence response in higher plants is highly dependent on the protein stability of group VII ethylene response factors, which are primarily degraded through the oxygen-dependent Cys-Arg branch of the N-degron pathway of targeted proteolysis. Knockout of PRT6, an E3 ligase and a vital component of the N-degron pathway, improves submergence tolerance in Arabidopsis and barley but is associated with side effects such as germination deficiency. In this study, we determined structures of rice and Arabidopsis PRT6-UBR box in complex with various Arg/N-degron related peptides. We identified two highly conserved motifs in the plant PRT6-UBR box, which is responsible for Cys-Arg/N-degron recognition. Structural and mutagenesis studies revealed the importance of two conserved motifs for Cys-Arg/N-degron recognition. The phenotype of Arabidopsis seedlings with PRT6-UBR mutants in these newly identified conserved motifs showed superior submergence survival suggesting that rational manipulation of the PRT6-UBR box can improve flood tolerance. Our results provide an engineering platform for generating crops with improved submergence tolerance.

Introduction
Although oxygen produced by plants is the major source released into the atmosphere, oxygen is also essential for plant for respiration to generate energy and to conduct some cellular functions. Plant submergence limits oxygen exchange, resulting in cellular oxygen reduction, in turn threatening survival. In many places around the
world, climate change has transformed once-in-a-lifetime heavy rainfall into frequent occurrences thus increasing the likelihood of submergence and, along with it, devastating agricultural economic loss and global food security risk\textsuperscript{1}. Accordingly, it is critical to develop strategies to improve plant submergence tolerance.

Submergence is a complex stress that affects hormone signalling, carbohydrate metabolism, redox balance, cytosolic pH, reactive oxygen species (ROS) level, and other plant physiology\textsuperscript{2}. Recent studies have shown a direct association between submergence and the group VII ethylene response factors (ERF-VIIIs). The expression and degradation of ERF-VII proteins is highly correlated with plant submergence survival\textsuperscript{3-10}. One of the characteristic features of ERF-VIIIs is their conserved N-terminal domain, which possesses Met-Cys-Gly-Gly as the first four amino acid residues. This conserved N-terminal MCGG motif is the N-degron for the oxygen-dependent Cys-Arg/N-degron pathway of targeted proteolysis, which leads to destabilization of ERF-VIIIs the presence of oxygen. As the initial step in this pathway, the first methionine is removed by methionine aminopeptidase, exposing the cysteine residue at the N-terminus\textsuperscript{11}. This N-terminal cysteine is then oxidized in the presence of oxygen by plant cysteine oxidases (PCOs) converting it into a negatively charged cysteine sulfonate (CysO\textsubscript{2})\textsuperscript{-}; subsequent arginylation by the arginyl-tRNA protein transferase 1 (ATE1) creates the unique Cys-Arg/N-degron\textsuperscript{12,13}. This N-degron possesses a positively charged arginine and a negatively charged cysteine sulfinate at the first and second positions, respectively. The N-terminal Arg residue is the primary destabilizing residue in the Arg/N-degron pathway\textsuperscript{12} and, in this report, we use the term ‘secondary destabilizing residue’ to refer to the second residue of the Arg/N-degron (cysteine sulfinate). Once formed, the Cys-Arg/N-degron is recognized by the UBR box of PRT6, an E3 ubiquitin ligase for polyubiquitylation, resulting in targeted proteolysis by the ubiquitin–proteasome system (UPS)\textsuperscript{12,14,15}.

Overexpression of Arabidopsis ERF-VIIIs often upregulates downstream genes for hypoxia responses and improves tolerance to submergence, suggesting that higher levels of ERF-VIIIs enhance plant submergence survival\textsuperscript{4}. Indeed, endogenous ERF-VIIIs accumulation in barley, achieved by lowering PRT6 levels to impede the Arg/N-degron pathway, improves tolerance to waterlogging\textsuperscript{4,11}. Similarly, prt6 knock-out in Arabidopsis enhances tolerance to submergence\textsuperscript{16}. The prt6 mutant allele, ged1, which possesses a non-functional PRT6 protein, shows high tolerance to starvation during submergence or prolonged darkness\textsuperscript{17}. Knockout of prt6 in barley and Arabidopsis also enhance tolerance to other abiotic stresses, such as salinity and drought\textsuperscript{11,15}. All these findings suggest the possibility of introducing non-functional prt6 mutants into crops for enhanced tolerance to several abiotic stresses. However, prt6 knockout lines are also associated with disadvantages\textsuperscript{15}. For example, prt6 mutants show defects in the
development of shoots and leaves, and are hypersensitive to abscisic acid (ABA), resulting in slower germination and lipid breakdown\textsuperscript{18,19}. A recent study suggested that PRT6 activities are required to optimize seed storage reserves to coordinate germination and seedling establishment\textsuperscript{20}. In short, while developing flood-resistant crops based on PRT6 modulation may be a promising strategy to improve submergence tolerance, careful manipulation of PRT6 activities is required to avoid unwanted effects.

Herein, we report the crystal structures of the UBR boxes of \textit{Oryza sativa} (rice) PRT6 and \textit{Arabidopsis thaliana} PRT6 (\textit{OsPRT6-UBR} and \textit{AtPRT6-UBR}, respectively) in complex with different Arg/N-degron related peptides at 1.5–2.4 Å resolution. Our sequence and structural studies showed that two highly conserved motifs near the Arg/N-degron binding pocket are critical for recognition of the oxidized cysteine (Cys\textsubscript{O2}). Mutagenesis of these conserved motifs of \textit{OsPRT6-UBR} led to a dramatic reduction of binding affinity to the Cys-Arg/N-degron mimic, an Arg–Asp-containing peptide. Furthermore, overexpression of these PRT6-UBR mutants in \textit{prt6}-knockout \textit{Arabidopsis} seedlings showed much better submergence tolerance compared to those overexpressing wild-type PRT6-UBR. Our study thus demonstrates that structure-guided modification of PRT6-UBR provides a possibility to improve submergence tolerance in crops.

Results

Motifs that are highly conserved in plant PRT6-UBR box

Phylogenetic analyses of UBR box motifs from plant PRT6s, \textit{S. cerevisiae} UBR1, and animal UBRs showed that they all share a common ancestor, but evolved into three distinct clades (Supplementary Fig. 1), a finding that is consistent with a previous report\textsuperscript{21}. Amino acid sequence alignment showed that the residues to form a three-zinc-stabilized heart-shaped fold\textsuperscript{22} and the Nt-Arginine of Arg/N-degron binding are highly conserved, except for one C-terminal histidine in plants and animals that holds structural zinc; in yeast an N-terminal histidine fulfills the same purpose. Based on the reported structures of yeast and human UBR boxes\textsuperscript{23,24}, residues for Arg/N-degron second destabilizing residue recognition can be predicted. Of note, surprisingly, our phylogenetic analyses showed that two motifs near the Arg/N-degron second destabilizing residue binding site are only conserved within plant PRT6, suggesting its role in Cys\textsubscript{O2} recognition (Fig. 1 and Supplementary Fig. 1).

Unique structural features of \textit{OsPRT6-UBR}

To further investigate the structural uniqueness near the secondary destabilizing (Cys\textsubscript{O2}) binding site of the UBR box in plants, we determined the structure of the \textit{OsPRT6-UBR} at 1.6 Å resolution. \textit{OsPRT6-UBR} shares 38 and 36% amino acid
sequence identity, and 54 and 46% sequence similarity with \( Hs\)UBR1-UBR and \( Sc\)UBR1-UBR, respectively. \( Os\)PRT6-UBR structurally resembles \( Hs\)UBR1-UBR and \( Sc\)UBR1-UBR with \( Ca\) root-mean-square deviations (RMSD) of 1.5 and 1.8 Å, respectively\(^{23,24}\). Therefore, the folding pattern, secondary structural elements, and zinc-coordinating residues of \( Os\)PRT6-UBR are virtually the same as the human and yeast UBR box; however, there is significant structural deviation in the loop region (Tyr165–Gly169), which is only conserved among plant PRT6-UBRs (Fig. 1a). This loop is right after the \( \beta3\) strand (Tyr160–Met164) and adopts a 90 degree turn to connect to the highly conserved Asp172, which interacts with the positively charged \( \alpha\)-amino group of the N-degron. The Tyr165–Gly169 loop, which is one residue shorter than that of \( Hs\)UBR1-UBR and three residues shorter than that of \( Sc\)UBR1-UBR, forms part of the binding pocket for the Arg/N-degron secondary destabilizing residue (Fig. 1a and Supplementary Fig. 1). Because the His156 and the Cys171 are coordinating a zinc atom, the position of the \( \beta3\) strand and key recognizing Asp172 residue are fixed. Therefore, the shorter Try165–Gly169 loop forms a sharp turn (Fig. 1b) resulting in a narrower Arg/N-degron secondary destabilizing residue binding pocket (7.8 Å in length and 6.4 Å in width) as compared to the \( Sc\)UBR1-UBR (PDB ID: 3NIL), which has modest-sized pocket (12.2 Å in length and 6.8 Å in width) and \( Hs\)UBR1-UBR (PDB ID: 3NY1), which has the largest pocket (14.4 Å in length and 10.2 Å in width) (Fig. 1c, d).

The other difference is the conserved Arg134–Glu139 sequence (Arg-Cys-Arg-Thr-Cys-Glu). Cys135 and Cys138 are part of the C2H2 zinc finger and thus are conserved in all UBR boxes but the unique salt bridge between Arg136 and Glu139 is only found in plant PRT6-UBR. The Cys135 carboxyl oxygen forms a 4-residue \( \beta\)-hairpin with the Asp139 amide, forcing both positively-charged side chains of Arg134 and Arg136 to face the same side. Therefore, the Arg134 side chain points toward the Arg/N-degron secondary destabilizing residue (CysO\(_2\)) binding pocket (Fig. 1b).

**Structure of \( Os\)PRT6-UBR in complex with N-degron peptides**

The crystal structure of \( Os\)PRT6-UBR in complex with a tripeptide, Arg-Asp-Gly, a mimic of the Cys-Arg/N-degron physiological substrate\(^{25,26}\), was determined at 1.6 Å resolution (Fig. 2a and Supplementary Table 1). The arginine, the Arg/N-degron primary destabilizing residue, is surrounded by a negatively charged environment that includes Asp141, Asp172 and Asp175, which are highly conserved in all UBR boxes (Fig. 1a and Supplementary Fig. 1). This arginine also interacts with Asp141 and Thr177 via water molecules. The backbone of the N-degron packs tightly into the groove by forming three coordinating hydrogen bonds with the backbone carbonyl group of Cys170 and the backbone amide nitrogen of Cys170 and Thr143 (Fig. 2b and...
3a). The aspartate residue at the second position residue of Arg/N-degron forms a salt bridge with Arg134 at the distance of 2.8 Å. When the aspartate, the Arg/N-degron secondary destabilizing residue, is replaced with serine, which possesses a shorter and polar side chain, the Arg134 and serine can also form a hydrogen bond with a distance of 3.1 Å (Figs. 2c and 3d).

During hypoxia, the cytosolic environment changes from neutral to acidic27-29 and the recombinant UBR box binds to the Arg/N-degron better at acidic pH23,30. Therefore, we determined OsPRT6-UBR structures in complex with the RDG peptide at pH 5.5 and pH 8.5. Compared to pH 8.5, at pH 5.5, we observed that the arginine side chain of the N-degron moves closer to Asp141, by 1.2 Å, to form an additional hydrogen bond network via water molecules (Figs. 2b, 2d and 3a).

We also noticed two alternative conformations of the Arg136 side chain (Fig. 3b-d). In one conformation, the Arg136 side chain, tilting away from Arg134, forms a salt bridge with the Asp160 side chain and a hydrogen bond with the Ser162 hydroxyl group (Fig. 3b-d). In the other conformation, the Arg136 side chain tends to form a salt bridge with Glu141 and hydrogen bonds with the carbonyl backbone of Pro142, moving closer to Arg134 from a distance of 7.8 to 5.9 Å. Based on the electron density and side-chain occupancy, we observed that the Arg136 side chain prefers to move toward Arg134 at pH 5.5. This long range charge–charge repulsion can be strong even at distances of 5–10 Å31. This Arg136 side chain movement towards to Arg134 results in the Arg134 side chain entering the binding pocket of the Arg/N-degron secondary destabilizing residue, which may enhance the binding of OsPRT6-UBR to the negatively-charged Arg-CysO₂ residue.

**Recognition of the second position of the Cys-Arg/N-degron by OsPRT6-UBR**

To examine the preference for the secondary destabilizing residue – the residue right after the N-terminal Arg of OsPRT6-UBR – we measured the binding affinities of RXG where X can be Arg, Asp, Ser and Leu as representatives of residues with positively charged, negatively charged, polar, or hydrophobic side-chains, respectively. As described above, Asp can mimic CysO₂25,26. Geometries of both the sulfinate (–SO₂) and carboxylate (–CO₂) groups are approximately trigonal planar, and the distance of the S–O bond is 0.1 Å longer than a C–O bond. The atomic radius of sulfur is 0.2 Å larger than that of carbon. The pKa values of cysteine sulfinic acid (CysO₂H) and carboxylic acid of Asp (CO₂H) are ~2.0 and 3.9, respectively32. Based on geometry and pKa, the negatively charged Cys-sulfinate could have a stronger interaction with positively charged residue(s) of OsPRT6-UBR compared to the carboxylate side chain of Asp as a mimic of Cys-Arg/N-degron in this study.
To our surprise, rice PRT6-UBR prefers Leu as secondary destabilizing residue and shows relatively weak preference for Asp at this position (Fig. 4a). The weak binding must be still strong enough to be recognized and to allow the protein to undergo subsequent ubiquitination and degradation. To further dissect the specific determinants for the recognition of CysO2 by plant PRT6-UBR, we determined the crystal structures of AtPRT6-UBR in complex with RR- and RL-peptides, two representative Arg/N-degrons (Supplementary Table 2 and Supplementary Fig. 2). Our structural analyses showed that the movement of key active site residues, in particular AtPRT6-UBR Arg133 (corresponding to OsPRT6-UBR Arg134), creates space to accommodate different secondary destabilizing residues, which is similar to yeast UBR1.

**Two plant-conserved motifs for Arg-CysO2 recognition**

To reveal the role of the conserved motifs we had identified, residues 136–139 (R<sup>136</sup>-T-C-E<sup>139</sup>) and 161–168 (Y<sup>161</sup>SIMYT---GG<sup>168</sup>) were replaced by the corresponding residues from ScUBR1-UBR: HECG (named 136H) and VCTIDCTEFTS (named 161V), respectively. In addition, a double mutant, containing both 136H and 161V, was generated (136H+161V). The binding affinity between wild-type OsPRT6-UBR and RD-peptides, a Arg-CysO2 mimic was approximately 137.2 μM (Fig. 4a). However, all three mutants showed a drastic decrease in binding affinity. There was essentially no detection of binding affinity using fluorescence polarization methods. In contrast, the RL-peptide bound to the wild type protein with 25.8 μM affinity (Fig. 4b). Although the 136H+161V double mutant shows reduced binding with Kd of 709 μM, the 136H and 161V mutants showed Kds of 58.9 and 41.4 μM, respectively, which are slightly lower than the wild type protein (Fig. 4b). This clearly shows that the conserved mutated region surrounding the negatively charged residue at the second position in plants is particularly sensitive to any variation.

It is worth mentioning that despite there being weak detection of binding of OsPRT6-UBR to RD peptide in vitro and no detect of binding of OsPRT6-UBR mutants to RD peptide in vitro, we did see ex vivo activity (see next section), suggesting that even extremely weak binding can generate a physiological consequence. Alternatively, the real substrate, Arg-CysO2-protein, may have higher binding affinity to the wild-type and mutant PRT6-UBR proteins.

**Two plant-specific motifs for submergence survival in Arabidopsis**

Knockout of prt6 can significantly enhance submergence survival by inhibiting the Arg/N-degron pathway<sup>17</sup>. To determine whether the highly conserved motifs in plant PRT6-UBR boxes are important for submergence survival (Fig. 1a and Supplementary Fig. 1), different 14-day seedlings, including Columbia-0 (Col-0), prt6-5 (prt6
knockout)\textsuperscript{33}, and \textit{prt6-5} knockout with overexpressed \textit{OsPRT6} variants, were subjected to 32–33 hours of dark submergence followed by four days of recovery. We determined the damage index based on the ratio of chlorotic leaves (over 1/3 of leaf area) to overall leaves for each seedling\textsuperscript{34}. For Col-0, only 10\% of seedlings had minor damage (less than 25\% damage), but 40\% of seedlings had severe damage (more than 50\% damage). On the other hand, whereas the \textit{prt6-5} line (\textit{prt6} knock-out) showed better submergence tolerance with 45\% of seedlings showing minor damage and less than 20\% of seedlings showing severe damage, which is consistent with previous reports\textsuperscript{16}. Previously, we identified \textit{OsERF66} and \textit{OsERF67}, the rice ERF-VIIs involved in the SUB1A-1 regulatory cascade\textsuperscript{35}. When \textit{OsERF66} is overexpressed in the \textit{prt6-5} line, we saw no significant improvement in the damage index compared with \textit{prt6-5} line. Thus, overexpression of \textit{OsERF66} in the \textit{prt6-5} line (\textit{prt6-5}/\textit{ERF66} line) had a negligible effect in our rescue experiment. Overexpressing wild-type \textit{OsPRT6} in the \textit{prt6-5}/\textit{ERF66} lines (three different lines) showed a similar damage index to Col-0 (Fig. 5). In short, \textit{OsPRT6} can rescue \textit{AtPRT6} function in \textit{prt6} line as a positive control in the rescue experiment. The overexpression of \textit{OsPRT6-136H}, \textit{OsPRT6-161V} or double 136H+161V mutants defective in the binding of Arg-CysO\textsubscript{2} peptide mimic show a significant submergence-resistant phenotype similar to the \textit{prt6-5} lines, suggesting loss of PRT6 function in those mutants.

To further examine whether the degradation of the Cys-Arg/N-degron substrate is reduced in the mutant lines, an \textit{ex vivo} protein stability assay using protoplasts was established (Fig. 5a). RT-qPCR analyses confirmed that HA-tagged \textit{prt6} was transcribed in our overexpressed lines (Supplementary Fig. 3). Using protoplast transient assays, we overexpressed luciferase with the Cys-Arg/N-degron in \textit{PRT6-WT} and \textit{PRT6-double mutant overexpression lines with similar overexpression levels of PRT6}. Cycloheximide chase experiments showed that luciferase with the Cys-Arg/N-degron was degraded faster in the \textit{PRT6-WT} overexpression line compared to the \textit{PRT6-double mutant overexpression line (Fig. 6 and Supplementary Fig. 4)}. More quantitatively, approximately 30\% of the model Cys-Arg/N-degron substrate was degraded in the wild-type protoplast line within 10 min, whereas less than 10\% of the substrate was degraded in the mutant line. Taken together, our phenotype and \textit{ex vivo} data suggest that the highly conserved motifs in \textit{PRT6-UBR} have critical roles in the Cys-Arg/N-degron pathway and the corresponding mutants can manipulate Cys-Arg/N-degron activities, resulting in enhanced plant submergence resistance.

\textbf{Concluding Remarks}

This work reveals that our two newly-identified motifs in \textit{PRT6-UBR} that are conserved only in plants are responsible for the Cys-Arg/N-degron. The oxygen-
dependent Cys-Arg/N-degron pathway specifically generates a negatively charged cysteine sulfinic acid at the second position after the primary Arg, and is functionally critical for oxygen level sensing and responses in flowering plants\textsuperscript{11,36}. In animals and yeast, oxygen levels are sensed by the proline hydroxylation and heme generation, respectively\textsuperscript{37,38}. These functional differences show the uniqueness of the Cys-Arg/N-degron secondary destabilizing residue in the plant Arg/N-degron pathway. These conserved motifs can be rationalized by the requirement of the plant PRT6 to recognize the oxidized N-degron, N-terminal Arg-Cys\textsubscript{O2}, and be evolution of the plant PRT6-UBR to enable submergence sensing. Our work thus provides important new insights into key residues with critical roles in N-terminal Arg-Cys\textsubscript{O2} peptide binding in plant PRT6, and reveals further structural and mechanistic insight into how PRT6 interacts with the oxidized N-degron substrate.

Given that plant PRT6 regulates ERF-VIIs levels, and the importance of ERF-VIIs in adapting to submergence, manipulation of PRT6 activity may be an effective strategy to enhance flood tolerance in plants as shown in plant model systems\textsuperscript{11,15,17,39}. The ability to manipulate PRT6 is important because the loss of PRT6 activity alters ABA sensitivity and causes germination defects in flowering plants\textsuperscript{15,18-20}, as shown in similar concept on PCO manipulation\textsuperscript{40,41}. However, PRT6 is essential in the plant Arg/N-degron pathway whereas PCO contains five isoforms, which makes PRT6 a more feasible target. In this study, we demonstrate that two highly conserved regions of the plant PRT6-UBR box evolved for Cys\textsubscript{O2} recognition. Mutations in these regions reduce PRT6 function \textit{in vitro} and plants with mutated PRT6 show a better submergence resistance phenotype. This structural work lays the foundations for structure-guided manipulation of PRT6 to balance benefit and harm. As climate change is increasing, the likelihood of submergence that threatens food security is increases. This work underscores the feasibility of altering PRT6 can to improve plant submergence resistance, with applications in agriculture.
**Materials and Methods**

**Phylogenetic analysis**

Phylogenetic analysis was conducted using MEGA 11 software. The phylogenetic tree was built using the neighbor-joining method, inferred from 1000 bootstrap replicates\(^4^2\). The following PRT6/UBR1 were compared: *Gm* PRT6 (GM07G15840, *Glycine max*), *Eg* PRT6 (EG0001G10280, *Eucalyptus grandis*), *Cs* PRT6 (CS00454G00020, *Citrus sinensis*), *St* PRT6 (ST10G024170, *Solanum tuberosum*), *Pr* PRT6 (PT06G08570, *Populus trichocarpa*), *Md* PRT6 (MD04G009580, *Malus domestica*), *Egu* PRT6 (EGU2028G0858, *Elaeis guineensis*), *Pequ* PRT6 (PEQU_18062, *Phalaenopsis equestris*), *Os* PRT6 (LOC_Os01G05500, *Oryza sativa*), *Ph* PRT6 (PH1004749G0010, *Phyllostachys edulis*), *Bradi* PRT6 (Bradi2G03180, *Brachypodium distachyon*), *Tae* PRT6 (TAE37857G001, *Triticum aestivum*), *At* PRT6 (AT5G02310, *Arabidopsis thaliana*), *Dr* UBR1 (XP_021323192.1, *Danio rerio*), *Bp* UBR1 (XP_020775514.1, *Boleophthalmus pectinurostris*), *Cc* UBR1 (XP_023783982.1, *Cyanistes caeruleus*), *Pt* UBR1 (XP_026565212.1, *Pseusonaja textillis*), *Hs* UBR1 (NP_777576.1, *Homo sapiens*), *Mm* UBR1 (NP_033487.2, *Mus musculus*), *Rn* UBR1 (NP_001171543.1, *Rattus norvegicus*), *Sc* UBR1 (NP_011700.1, *Saccharomyces cerevisiae*). Structure alignment was calculated by PROMALS3D using *Hs* UBR1-UBR (PDB ID: 3NY1), *Hs* UBR2-UBR, (PDB ID: 3NY2), *Sc* UBR1-UBR (PDB ID: 3NIL), *Os* PRT6-UBR and *At* PRT6-UBR as inputs\(^4^3\).

**Cloning and sample production**

The UBR domain of PRT6 from *Oryza sativa indica* (residues Ala120–Gly190) was cloned into a pET 15b vector by Ligation Independent Cloning (LIC) to construct a N-terminal fusion tag containing a Nt-hexahistidine, glutathione S-transferase and tobacco etch virus (TEV) protease cutting site, which leaves an additional serine residue after TEV protease treatment. The plasmid containing the gene *Os* PRT6-UBR was transformed into *E. coli* BL21 (DE3) for recombinant protein production. The transformed cells were cultured in a rich LB medium at 37°C in the presence of 100 μg/ml ampicillin and were induced at the optical density (O.D.) of 1.0 by 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) at 16°C for 16 h. The harvested cells were collected by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl at pH 8.0, 250 mM NaCl, 25 mM imidazole, 10% (w/v) glycerol and 10 mM β-mercaptoethanol) supplemented 1 mM PMSF and DNase followed by sonication. After centrifugation at 20,000g for 30 min, the supernatant was loaded onto a column containing NiNTA resin pre-equilibrated with lysis buffer. The column was washed with lysis buffer followed
by 5 column volumes (CVs) of wash buffer supplemented with an additional 25 mM imidazole. Proteins were eluted with elution buffer (50 mM Tris-HCl at pH 8.0, 250 mM NaCl and 500 mM imidazole). After removing the N-terminal fusion tag by treating with TEV protease during the dialysis against 50 mM Tris buffer at pH 5.0 and 250 mM NaCl overnight at 4°C, OsPRT6-UBR was loaded onto a Superdex 75 column pre-equilibrated with 20 mM Tris at pH 7.5, 10 mM TCEP, 10 mM NaCl and 10 μM ZnCl₂ for final purification.

The UBR box domain of PRT6 from Arabidopsis thaliana (residues Gly119-Gly189) was cloned into KpnI and XhoI restriction sites of a modified pET-His-LC3B vector. For crystallization, we inserted N-degron sequences (RRGSGG and RLGSGG) between the LC3B sequence and the ApRT6 UBR box sequence. The plasmid containing the ApRT6-UBR box gene was transformed into E. coli BL21(DE3) cells for recombinant protein production. The plasmid-transformed E. coli BL21(DE3) cells were incubated in LB medium at 37°C in the presence of 100 μg/ml kanamycin until O.D. of 2.0 and then induced with a final concentration of 0.5 mM IPTG at 18°C with 500 μM ZnSO₄ for 20 h. The harvested cells were collected by centrifugation and resuspended in a lysis buffer containing 50 mM Tris pH 8.0, 200 mM NaCl, and 1 mM Tris(2-carboxyethyl)phosphine (TCEP). Cell lysis was performed using ultrasonication supplemented with 1 mM PMSF and a tablet of protease inhibitor cocktail. The insoluble fraction was removed by centrifugation at 17,000 rpm for 2 h, and the supernatant was loaded onto a pre-equilibrated HisTrap™ column (GE Healthcare, 17-5255-01) and eluted by gradually increasing the concentration of imidazole to 500 mM. Eluted samples were further purified using the HiTrap™ Q HP column (GE Healthcare, 17-1154-01). The His-LC3B-tag was cleaved using human ATG4B protease at 25°C overnight. Samples with 30 mM imidazole were loaded onto the HisTrap™ column to remove the His-LC3B-tag and human ATG4B protease. Loading through samples were concentrated by ultrafiltration (Amicon Ultra 3K NMWL, Millipore) and loaded onto a HiLoad™ 16/600 Superdex™ 75 pg (GE Healthcare, 28-9893-33) column equilibrated with 20 mM Tris-HCl pH 8.0, 100 mM NaCl, and 1 mM TCEP.

For fluorescence polarization assays (FP-assays), the same construct of the UBR box domain of OsPRT6 was cloned into His-MBP-vectors. The HisMBP-OsPRT6 UBR box plasmids were transformed into E. coli BL21(DE3) cells. Cell lysates were applied to a column containing amylose resin (New England Biolabs). The beads were washed with at least ten column volumes of buffer containing 50 mM Tris-HCl pH 8.0, 100 mM NaCl, and 1 mM TCEP, and the MBP-OsPRT6 UBR box was eluted with the same buffer supplemented with 10 mM maltose. Eluted samples were further purified using the HiTrap™ Q HP column, concentrated by ultrafiltration (Amicon Ultra 10K NMWL, Millipore), and then loaded onto a HiLoad™ 16/600 Superdex™ 200 pg (GE


Healthcare, 28-9893-35) gel filtration column pre-equilibrated with 50 mM HEPES pH 7.0, 10 mM NaCl, and 1 mM TCEP.

Protein crystallization and structure determination

OsPRT6-UBR was concentrated to 8–9 mg/ml prior to crystallization. Crystals were grown at 18°C by the sitting drop vapor diffusion method. Apo-form protein was crystallized in 8% (v/v) Tacsimate pH 6.0 and 20% PEG3350. An additional 20% (w/v) glycerol was added to crystallization drops and crystals were flash-cooled in liquid nitrogen at 100 K prior to data collection. For RDG/RSG peptide soaking, apo OsPRT6-UBR was crystallized in 1.0 M sodium citrate, 200 mM NaCl and 0.1 M Tris-HCl at pH 7.0. Crystals were transferred to 300 mM citrate/glycine/HEPES buffer at the desired pH, 1.0 M sodium citrate and 200 mM NaCl with saturated peptide for 1 h followed by flash-freezing in liquid nitrogen with an additional 25%(w/v) glycerol as a cryoprotectant. X-ray diffraction data were collected at beamline BL13B1 and TPS05A at the National Synchrotron Radiation Research Center (NSRRC, Hsinchu, Taiwan). Data were processed using the HKL2000 program suite. Data processing and refinement statistics are summarized in Supplementary Table 1. Apo-form structures were determined by zinc phasing using Phenix and the peptide-bound structure was determined by molecular replacement using the apo structure as the search model. Models with peptides were iteratively rebuilt using Coot and refined with using REFMAC5. The bound-peptides were built only after the R-free value decreased below 30% and were guided by Fo–Fc electron density maps contoured at 3σ.

AtPRT6-UBR box was concentrated to 7–15 mg/ml prior to crystallization. Crystals were grown at 22°C by the sitting drop vapor diffusion method. RRGSGG-AtPRT6 UBR box (P 2 2 1 2, PDB ID: 7XWE) was crystallized in 0.2 M MgCl2 hexahydrate, 0.1 M sodium HEPES pH 7.5, and 30% (v/v) PEG 400; RRGSGG-AtPRT6 UBR box (I 2 2 2, PDB ID: 7Y6W) in 0.2 M calcium acetate hydrate and 20% (w/v) PEG 3,350, and RRGSGG-AtPRT6 UBR box (P 3 2, PDB ID: 7Y6X) in 1.2 M DL-malic acid pH 7.0 and 0.1 M BIS-Tris pH 7.0. RLGSGG-AtPRT6 UBR box (I 2 2 2, PDB ID: 7XWF) was crystallized in 0.2 M calcium acetate hydrate and 17% (w/v) PEG 3,350; RLGSGG-AtPRT6 UBR box (I 2 2 2, PDB ID: and 7Y6Z) was crystallized in 0.2 M calcium acetate hydrate and 19% (w/v) PEG 3,350; RLGSGG-AtPRT6 UBR box (C 1 2 1, PDB ID: 7Y6Y) in 0.2 M calcium acetate hydrate and 20% (w/v) PEG 3,350, and RLGSGG-AtPRT6 UBR box (P 4 3 2, PDB ID: 7Y70) in 1.3 M ammonium sulfate, 0.1 M Tris pH 8.5, and 12% (w/v) glycerol. Apo-AtPRT6 (PDB ID: 7XWD) was crystallized in 1.6 M ammonium sulfate, 0.1 M sodium HEPES pH 7.5, and 0.1 M NaCl. Crystals were usually cryoprotected by adding 10–25% (w/v) glycerol and then frozen in liquid nitrogen. X-ray diffraction data were collected at beamline 5C and 11C.
at the Pohang Accelerator Laboratory, South Korea, and BL44XU at the Spring-8, Japan. Data sets were processed using the HKL2000 program suite\textsuperscript{46}. Statistics for the collected data are summarized in Supplementary Table 2. All structure figures were generated by PyMOL and LigPlot\textsuperscript{+51,52}.

**Fluorescence polarization assay**

Solutions of FITC-labeled RDAAK, RRAAK, RLAAK, and RSAAK peptides were prepared at 500 nM concentration in 50 mM MES pH 6.0, 10 mM NaCl, and 1 mM TCEP. Purified MBP-\textit{Os}PRT6 UBR box WT and the respective mutants were serially diluted in the same buffer. Fluorescence measurements to detect the change in fluorescence polarization of the FITC-labeled peptide were performed in a 384-well format on a Corning black plate reader with excitation and emission wavelengths of 485 and 525 nm, respectively. GraphPad Prism 7 software was used for calculations and generation of graphs.

**Plant materials**

The T-DNA insertion lines of \textit{prt6-5} (SALK\_051088) were obtained from the Arabidopsis Bioglobal Resoruce Center, Ohio State University. The \textit{Os}ERF66 overexpression lines (\textit{prt6-5}/\textit{ERF66} lines) were generated by transforming the 35S::\textit{Os}ERF66-GFP/pCAMBIA1302 vector into \textit{prt6-5}. The HA-PRT6 WT, 136H, 161V, and 136H+161V overexpression lines were generated by transforming the 35S::HA-PRT6 WT, 136H, 161V, 136H+161V cloned pEarleyGate 201 vector into \textit{prt6-5}/\textit{ERF66} transgenic plants.

**Damage index after submergence**

\textit{Arabidopsis} seeds were sterilized with 1.2\% (v/v) sodium hypochlorite for 15 min and washed with sterilized water. Seeds were sown on plates with 0.57\% Phytagel (Sigma-Aldrich) in half-strength Murashige and Skoog (MS) medium (Duchefa Biochemie) containing 0.5\% sucrose at pH 5.7 and kept at 4°C in the dark for three days to achieve uniform germination, and then the plates were transferred to a growth chamber and grown at 22°C with a 16-h-light (81 \textmu mol m\textsuperscript{-2} S\textsuperscript{-1})/8-h-dark cycle for four days. To obtain 14-d-old plants for submergence treatment, 7-d-old seedlings were transplanted onto fresh plates, and then the plates were placed vertically to prevent roots from growing into the medium. The transplanted seedlings were grown in the growth chamber until they were 14-d-old. For submergence treatment of 14-d-old \textit{Arabidopsis} seedlings, plates with plants on the surface of the medium were placed into sterilized water for 32–33 h. After that, the plates were taken out from the water and transferred back into the growth chamber for 4 days. The damage index\textsuperscript{34} was quantified based on
the percentage of chlorotic leaves (>1/3 leaf area) out of total number of leaves for each seedling.

**Ex vivo protein stability assay**

The cycloheximide (CHX) chase assay for monitoring *ex vivo* protein stability was conducted by using an *Arabidopsis* protoplast system. The RAP2.12(1-28)-luciferase chimeric protein was used as the substrate to indicate the activity of PRT6 variants. The *Arabidopsis* UBQ10 promoter, RAP2.12(1-28), and luciferase DNA fragments were cloned into pUC18 at the same time by using NEBuilder HiFi DNA Assembly Cloning Kit (M5520, NEB) to form the substrate plasmid, pUC18_AtUP::RAP2.12(1-28)-Luc. The *Arabidopsis* protoplast preparation and transformation were conducted according to a published protocol with minor modifications. The substrate plasmids were transformed into the protoplasts prepared from PRT6-WT and PRT6-136H+161V overexpression line. After incubation at 22°C for 4 h, the transformed protoplasts were aliquoted followed by buffer exchange containing 100 μM CHX to stop the protein translation process. The 90% buffer was removed and protoplasts were flash-frozen in liquid nitrogen to quench the protein degradation process followed by luciferase activity assay (E1500, Promega) to determine the relative amount of RAP2.12(1-28)-luciferase chimera protein remaining at various time points. The protein levels of overexpressed PRT6-WT and PRT6-136H+161V were determined by Western blot using anti-HA antibody (16B12, BioLegend).

**Data availability**

Atomic coordinates have been deposited in the PDB under the accession codes: 7WUK, 7WUL, 7WUM, and 7WUN for *Os*PRT6-UBR; 7XWD, 7XWE, and 7XWF for *At*PRT6-UBR

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Author Contributions

Competing interests
The authors declare no competing interests.

Figure Legends
Figure 1. Structural features of plant PRT6-UBR box. (a) Structure-based sequence alignment with secondary structure was calculated by PROMALS3D using OsPRT6-UBR (this study. PDB ID: 7KUW), AtPRT6-UBR (this study, PDB ID: 7XWD), HsUBR1-UBR (PDB ID: 3NY1), HsUBR2-UBR (PDB ID: 5TDA) and ScUBR1-UBR (PDB ID: 3NIH). The yellow highlight indicates the structurally most divergent region among the various UBR boxes. (b) Overlay of the Tyr165–Gly169 region and nearby key Arg residues (Arg134/Arg136 in OsPRT6-UBR; Arg136/His138 of ScUBR1-UBR). The OsPRT6-UBR and ScUBR1-UBR are shown as a cartoon diagram in gray and yellow, respectively. The bound N-degron peptide (Arg-Asp-Ala-Ala) in ScUBR1-UBR is shown as a green stick to indicate the binding location of the Arg/N-degron secondary destabilizing residue. The arrows show the structural deviations.
between OsPRT6-UBR and ScUBR1-UBR. (c, d) Surface map of OsPRT6-UBR and ScUBR1-UBR boxes are shown. Color scheme: red, negatively charged residues; blue, positively charged residues; gray (OsPRT6-UBR)/yellow (ScUBR1-UBR), neutral. The binding pocket for Arg/N-degron secondary destabilizing residues are shown by the black boxes.

**Figure 2. The interaction between OsPRT6-UBR and Arg/N-degron peptides.** (a) The OsPRT6-UBR is drawn as a surface presentation. The key interacting residues (Arg134 and Tyr165-Gly169) and RDG peptide are shown as a yellow stick model. (b-d) The RDG-OsPRT6 at pH 5.5 (b), RSG-OsPRT6 at pH 5.5 (c), and RDG-OsPRT6 at pH 8.5 (d) interaction diagrams are displayed by LIGPLOT+. The N-degron peptides and OsPRT6 residues are shown as purple and orange lines. Bound N-degron peptides and OsPRT6-UBR protein residues are labeled in blue and green colors, respectively. The water molecules are shown as cyan balls. The hydrogen bonds are shown as green dashed lines and the spoked arcs represent van der Waals interactions.

**Figure 3. The dual conformations of two Arg residues of OsPRT6-UBR with various Arg/N-degron peptides.** (a) The Arg of Arg/N-degron conformation changes upon pH change. The OsPRT6-UBR and bound RDG peptide at pH 5.5 are shown as cyan and green sticks, respectively, and at pH 8.5, they are shown as yellow sticks and orange sticks, respectively. The water molecules are shown as red balls. (b) The conformations of Arg136 and Arg134 in OsPRT6-UBR/RDG complex at pH 5.5. (c) The conformations of Arg136 and Arg134 in OsPRT6-UBR/RDG complex at pH 8.5. (d) The conformations of Arg136 and Arg134 in OsPRT6-UBR/RSG complex at pH 5.5. (b-d) OsPRT6-UBR is shown as gray sticks. The bound RDG peptide at pH 5.5 and pH 8.5, and the bound RSG peptide at pH 5.5 are shown as green, orange, and purple sticks respectively. Ser162 side chain in OsPRT6-UBR also has dual conformations. The hydrogen bond and charge-charge interactions are indicated as black and green dashed lines. The orange dashed line indicates key distance. Key residues are labeled in black.

**Figure 4. The binding affinity of measurement between OsPRT6-UBR proteins and Cys-Arg/N-degron peptides.** (a) Measurements of fluorescence polarization of FITC-labeled RX-peptide with increasing concentrations of MBP-OsPRT6-UBR proteins. Measurements of fluorescence polarization of FITC-labeled RDAAK-peptide (b) and RLAAK-peptide (c) with increasing amount of wild-type, 136H, 161V, or 136H+161V mutants. For a clear signal, His-MBP-OsPRT6-UBR protein was used. In contrast to the wild-type protein, mutants at the putative determinant regions possess no binding affinity (N.B.) when RDAAK, a mimic of the RCO₂-peptide, was used. The
RL-peptide did not show significant discrepancy among the proteins. The error bars represent standard error of the mean of more than three independent experiments.

**Figure 5. The damage index of Col-0 and\(^{prt6-5}\) lines with overexpressed \(OsPRT6\) variants after submergence and recovery.** (a) Bar graph summarizing the damage index score of over 100 seedlings for three repeats. (b) Representatives of each line after recovery placed in Petri dishes for index score examination. After submergence and recovery, the number of chlorotic leaves over total leaves were counted for each plant as the damage index. The data represent means ± SD from three independent replicates. Statistical differences between Col-0 and\(^{prt6-5}\) with overexpressed \(OsPRT6\) variants were determined by Student’s \(t\) test. *, \(P < 0.01\).

**Figure 6. Ex vivo protein stability CHX chase assay.** (a) Schematic procedure for ex vivo assay. The plasmid contained the model protein substrate, luciferase bearing Cys-Arg/N-degron sequence derived from the first 28 amino acids of \(AtRAP2.12\) which is a well-known ERF-VII in Arabidopsis. (b) The assay shows luciferase with Cys-Arg/N-degron degrades faster in the \(PRT6\)-WT overexpression line (blue) than the \(PRT6\)-double mutant (136H+161V) overexpression line (red). The data represent means ± SD from four independent replicates. Statistical differences between \(PRT6\)-WT and \(PRT6\)-double mutnat were determined by Student’s \(t\) test. *, \(P < 0.01\).
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