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The N-terminal tetra-peptide (IPDE) short extension of the U-box motif in rice SPL11 E3 is essential for the interaction with E2 and ubiquitin-ligase activity

Hansol Bae, Woo Taek Kim *

Department of Systems Biology, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea

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ABSTRACT

Rice, a monocot model plant, contains at least 77 U-box E3 ubiquitin (Ub)-ligases and 48 E2 Ub-conjugating enzymes. Here, we investigated the minimal binding domain of rice SPL11 U-box E3 to its E2 partners. Serial deletions and site-directed mutagenesis analyses indicated that, in addition to an intact U-box motif, the N-terminal tetra-peptide (IPDE) short extension of the U-box was essential for the interaction of SPL11 with E2s and Ub-ligase activity. The Ile and Pro residues at the –4 and –3 positions of the U-box, respectively, were crucial for this interaction. These results suggest that the N-terminal tetra-peptide extension of the U-box participates in the specific interaction of SPL11 E3 with E2s in a sequence-specific manner in rice.

1. Introduction

The post-translational modification of proteins with ubiquitin (Ub) or poly-Ub chains regulates many cellular processes, including protein degradation, DNA repair, and cellular trafficking [1–4]. Ubiquitination involves a cascade of enzymatic reactions in which Ub is transferred from an E1 Ub-activating enzyme to an E2 Ub-conjugating enzyme, forming a thioester bond with a cysteine residue of E2 [5]. E2 subsequently associates with an E3 Ub-ligase, which recognizes a target protein and facilitates the transfer of Ub from E2 to a lysine residue of the substrate [5].

There are three classes of E3 Ub-ligases, defined by the presence of a HECT, RING, or U-box domain. HECT E3s form a catalytic Ub thioester intermediate before transferring Ub to the protein substrates, whereas RING and U-box E3s catalyze the direct transfer of Ub from E2 to target proteins [6]. Despite the very similar structures of the RING and U-box domains, the U-box does not coordinate Zn^{2+} ion, whereas the RING domain does [7,8]. Higher plants contain a larger number of U-box proteins compared to that of yeasts and mammals. For example, *Arabidopsis* and rice, dicot and monocot model plants, respectively, have at least 64 and 77 U-box motif-containing E3 proteins, with the majority containing the protein–protein interacting ARM repeat motif [9–12]. By contrast, yeasts and humans have 3 and 7 U-box E3s, respectively [13,14]. Plant U-box E3s participate in many diverse plant-specific events, such as responses to phytohormones [15,16], biotic stress [17,18], and abiotic stress [19,20], self-incompatibility [21], and control of flowering time [22]. These results are consistent with the proliferation of U-box proteins in plants relative to yeasts and mammals.

There are at least 37 and 48 E2 Ub-conjugating enzymes in *Arabidopsis* and rice, respectively [23]. Although the U-box proteins have to interact with E2s for their E3 ligase activities and there are large numbers of U-box E3s in plant genomes, studies of the U-box structure or E2-binding requirements are still limited in higher plants [24,25]. We want to understand how the specific interactions between E3s and E2s are controlled in plants. The specific aim of this study was to explore the minimal E2-binding site of the U-box domain in E3. For this purpose, we used SPL11, an ARM repeat-containing U-box E3 Ub-ligase, which plays a role as a negative regulator of programmed cell death in rice (*Oryza* sativa L.) [17]. The yeast two-hybrid assay and studies of *in vitro* self-ubiquitination in conjunction with site-directed mutagenesis indicate that, in addition to an intact U-box motif, an N-terminal tetra-peptide (IPDE) short extension of the U-box is required for the interaction of rice SPL11 E3 with E2s. These results suggest that the short extension of the U-box motif critically affects the interacting interface between the U-box and E2 for the E3 Ub-ligase activity in rice.

2. Materials and methods

2.1. Yeast two-hybrid assay

The cDNAs for full-length rice SPL11 and seven deletion mutants of SPL11were inserted into the pGAD T7 vector (Clontech,

Abbreviations: ARM repeat, armadillo repeat; HECT, homology to E6-AP carboxyl-terminus; MBP, maltose binding protein; PCR, polymerase chain reaction; RING, really interesting new gene; UBC, ubiquitin conjugating; Ub, ubiquitin.

* Corresponding author. Fax: +82 2 312 5657.
E-mail address: wtkim@yonsei.ac.kr (W.T. Kim).

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Mountain View, CA, USA). The gene constructs were then transformed into yeast strain Y187. Full-length rice E2 and human UBCH5b E2 cDNAs were inserted into the pGBK T7 vector, and subsequently transformed into yeast strain AH109. For mating experiments, each pair of individual Y187 and AH109 transformants were inoculated and grown for 4 days at 30 °C in –Leu/–Trp media for diploid selection. The selected diploid cells were inoculated on –Leu/–Trp/–His/–Ade solid medium to analyze the interaction between SPL11 E3 and E2 proteins.

2.2. Construction and expression of MBP-SPL11 E3 Ub-ligases and 6× His-E2 fusion proteins in Escherichia coli

The coding sequences for full-length rice SPL11 and rice E2 proteins (Os09g12230, Os02g16040, and Os06g45000) were cloned from rice leaf cDNA and inserted into pMAL-c2x (New England BioLabs, Ipswich, MA, USA), or pProEX-HTa (Invitrogen, Carlsbad, CA, USA) vectors. These recombinant constructs were then transformed into the *E. coli* strain BL21, expressed, and purified using an amylose affinity resin (New England BioLabs) and IDA Excellose (Takara, Kyoto, Japan) for the *in vitro* ubiquitination assay.

2.3. In vitro self-ubiquitination assay

The purified MBP-SPL11 fusion protein (300 ng) was subjected to *in vitro* self-ubiquitination assays as described previously [16,19]. Protein gel-blot analyses were conducted using anti-MBP antibody (New England BioLabs) or anti-Ub antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described by Kim et al. [26].

2.4. Mutagenesis

Polymerase chain reaction (PCR) was performed using pGAD T7 SPL11$^{U\text{-}box}$N+20/C+10 as a template, various gene-specific primer sets (Supplementary Table S1), and PrimeStar polymerase (Takara). Each PCR mixture was treated with the restriction enzyme *Dpn*I to

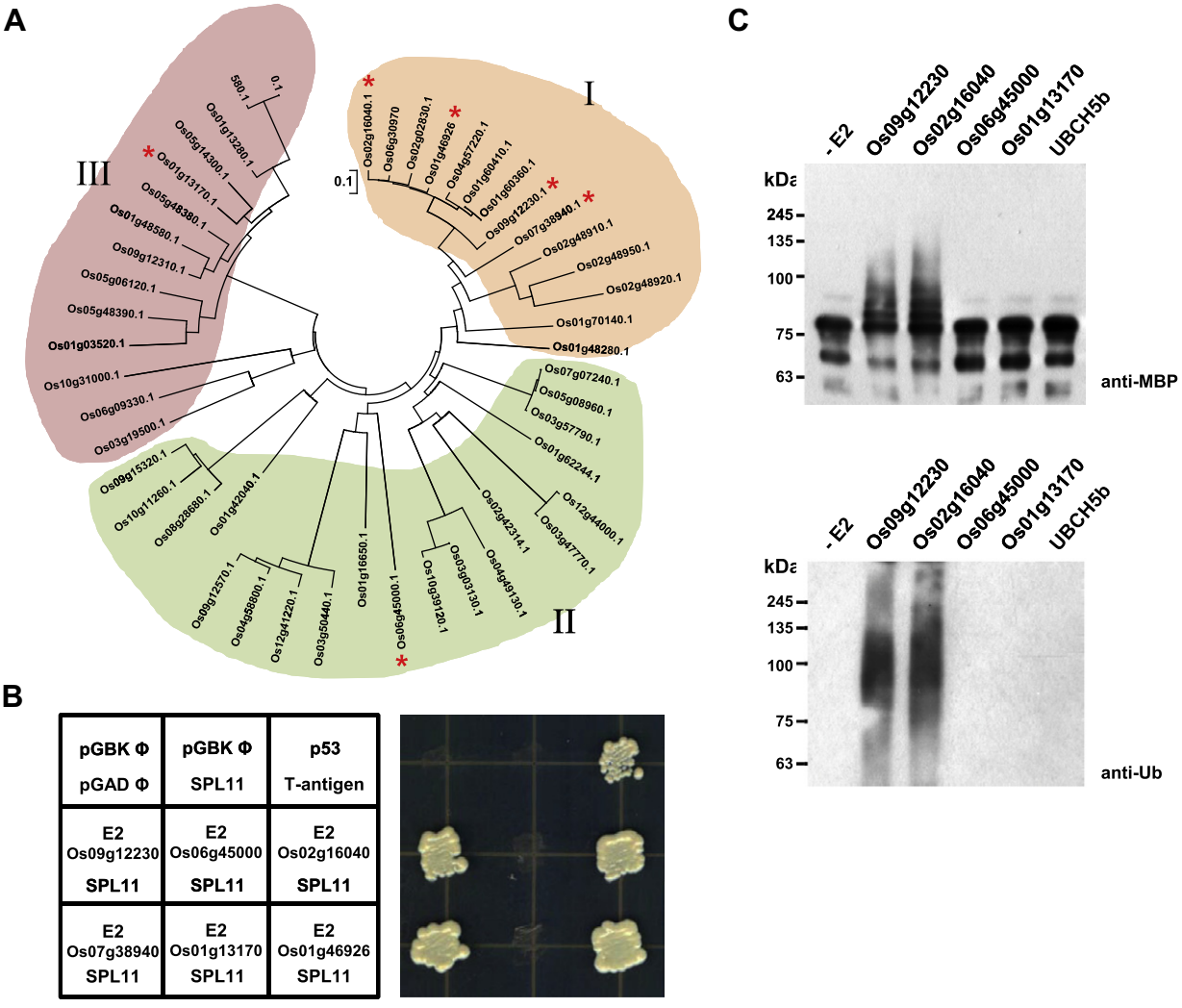

Fig. 1. Interaction of rice SPL11 E3 Ub-ligase and E2s. (A) Phylogenetic tree of rice E2s. A total of 48 rice E2s are aligned by UBC-fold homology using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and MEGA4 software. Asterisks indicate E2s that were randomly selected from sub-groups I, II, and III for subsequent experiments. (B) Interactions of SPL11 E3 with E2s determined by yeast two-hybrid assay. The E2-AH109 and SPL11-Y187 yeast cells were mated under the four-minus (–Leu/–Trp/–His/–Ade) growth conditions at 30 °C for 3 days. Sub-group I E2s: Os09g12230, Os02g16040, Os01g46926, and Os07g38940; sub-group II E2: Os06g45000; and sub-group III E2: Os01g13170. p53 + *T*-antigen was used as a positive control; pGBK + pGAD and pGBK + SPL11 were used as negative controls. (C) *In vitro* SPL11 self-ubiquitination assay. Bacterially-expressed MBP-SPL11 fusion protein was incubated at 30 °C for 1 h with ATP, Ub, and E1 (*Arabidopsis* His-UBA1) in the presence or absence of one of the four different rice E2s. UBCH5b is a human E2 for a negative control. Reaction mixtures were subjected to immuno-blot analysis with anti-MBP (upper panel) or anti-Ub (lower panel) antibody. High-molecular-mass ladders indicate self-ubiquitinated forms of SPL11. (For interpretation of color in this figure, the reader is referred to the web version of this article.)

remove a template plasmid, and then transformed into *E. coli* strain DH5α. The recombinant constructs were subjected to DNA sequencing to verify the DNA sequence.

3. Results

3.1. Identification of the minimal E2-binding site of the U-box domain in rice SPL11 E3 Ub-ligase

An interacting network analysis of human E2-RING E3 Ub-ligase revealed that only 10 of 35 active E2s were able to account for more than 80% of the total E2–E3 interactions. This indicates that a relatively small number of E2s are closely connected with a larger number of E3s [27,28]. These studies suggest that E2–E3 interactions are not uniformly distributed, but there are E2 hubs for the interaction with their E3 partners in humans. To identify which E2 proteins interact with SPL11 U-box E3 in rice, we first generated phylogenetic trees of E2 proteins based on the UBC-fold domain. Fig. 1A shows that the 48 known rice E2s could be classified into three sub-groups. E2s belonging to sub-group I show significant sequence identities with those of human that interact with RING E3 Ub-ligases, whereas sub-groups II and III E2s are more distantly related to the human E2 hub [27] (Supplementary Fig. S1). Therefore, we randomly selected four E2s (Os09g12230, Os02g16040, Os01g46926, and Os07g38940) from sub-group I, one E2 (Os06g45000) from sub-group II, and one E2 (Os01g13170) from sub-group III for subsequent experiments.

Full-length cDNAs for each of the six selected rice E2s were transformed into the AH109 yeast strain. The E2-AH109 cells were then mated with Y187 cells that were transformed with a full-length SPL11 E3 cDNA under the four-minus (–Leu/–Trp/–His/–Ade) growth conditions. The results show that all of the yeast cells containing SPL11 E3 + sub-group I E2s grew efficiently in the four-minus medium (Fig. 1B). By contrast, cells that harbor SPL11 + sub-group II E2 and SPL11 + sub-group III E2 failed to grow in the four-minus medium. These results indicate that SPL11 interacts with E2s that belong to sub-group I, which are homologous to the human E2 hub.

We next carried out *in vitro* self-ubiquitination assays. MBP-SPL11 fusion protein was expressed in *E. coli*, purified, and incubated in the presence of ATP, Ub, E1 (*Arabidopsis* His-UBA1), and one of the four different bacterially-expressed rice E2s. The reaction mixtures were then analyzed by immuno-blotting using anti-MBP and anti-Ub antibodies. As shown in Fig. 1C, MBP-SPL11 yields high-molecular-mass ubiquitinated bands with Os09g12230 and Os02g16040 E2s, both of which belong to sub-group I (Fig. 1C). However, MBP-SPL11 failed to produce ubiquitinated ladders in the presence of sub-group II Os06g45000 and sub-group III Os01g13170 rice E2s and human UBCH5b E2 (Fig. 1C). These results suggest that rice SPL11 E3 Ub-ligase interacts with E2s that are homologous to the human E2 hub.

We asked whether the U-box motif of SPL11 E3 is sufficient for the interaction with E2 and the Ub-ligase activity. To investigate the minimal requirement of SPL11 for E2 binding, seven deletion mutants of SPL11 were generated and tested for E2 binding using the yeast two-hybrid assay (Fig. 2A). Fig. 2B shows that serial deletions of the C-terminal region of SPL11 did not affect the binding activity between SPL11 and E2, as evidenced by the equal growth of yeast cells harboring full-length SPL11, SPL11$^{U\text{-}box}$C+10, and SPL11$^{U\text{-}box}$C0 in the presence of the sub-class I E2 Os09g12230.

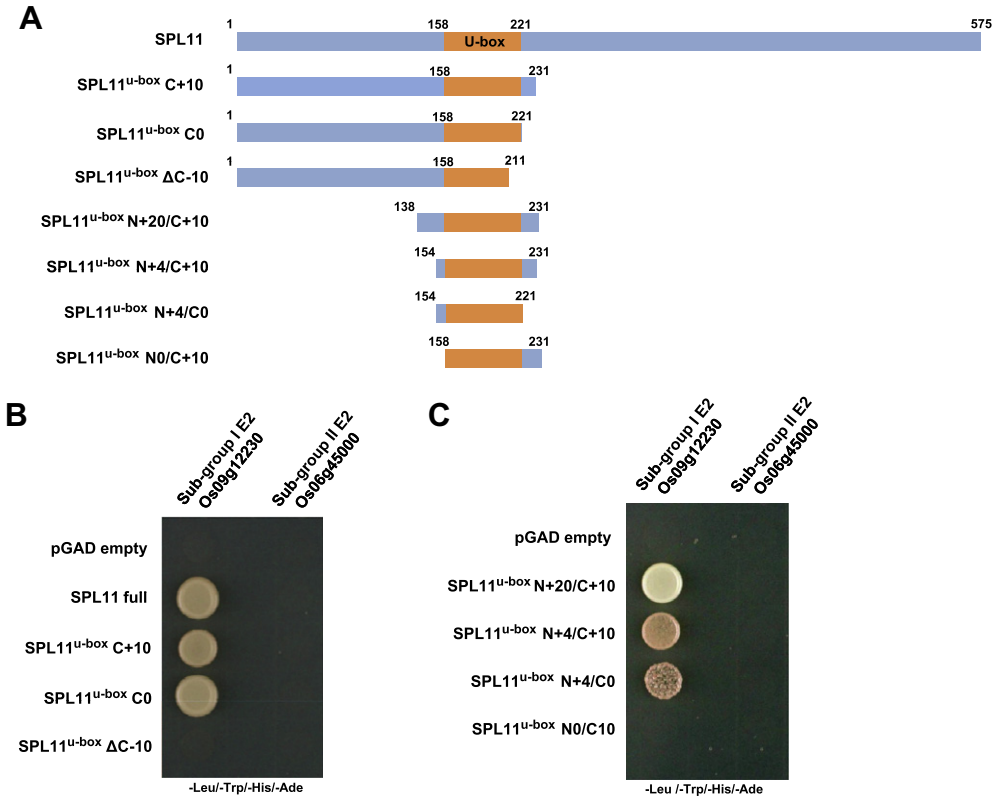

Fig. 2. Identification of minimal E2-binding domain of rice SPL11 E3. (A) Schematic representation of full-length and seven deletion mutants of SPL11. Three C-terminal deletion mutants (SPL11$^{U\text{-}box}$C+10, SPL11$^{U\text{-}box}$C0, and SPL11$^{U\text{-}box}$ΔC-10) and four N-terminal deletion mutants (SPL11$^{U\text{-}box}$N+20/C+10, SPL11$^{U\text{-}box}$N+4/C+10, SPL11$^{U\text{-}box}$N+4/C0, and SPL11$^{U\text{-}box}$N0/C+10) were constructed. Location of the U-box motif and amino-acid residue numbers in SPL11 are indicated. (B) and (C) Yeast two-hybrid assay. Sub-group I E2 Os09g12230 and sub-group II E2 Os06g45000 were used as bait. Full-length and three C-terminal deletion mutants (B), and four N-terminal deletion mutants (C) of SPL11 E3 were used as prey. Yeast cells were plated onto –Leu/–Trp/–His/–Ade medium and allowed to grow for 3 days at 30 °C. (For interpretation of color in this figure, the reader is referred to the web version of this article.)

By contrast, the growth of yeast cells that contain SPL11$^{U\text{-}box}$ΔC-10, in which ten amino-acid residues were removed at the C-terminal end of the U-box, was completely diminished in the presence of E2, confirming that an intact U-box motif is required for the interaction of SPL11 E3 and E2 (Fig. 2B). On the other hand, serial deletions of the N-terminal region of the SPL11 E3 resulted in a gradual decrease in the binding activity between E3 and E2 (Fig. 2C). Further, deletion of the four amino-acid residues at the positions from −4 and −1 of the U-box effectively abolished the interaction of SPL11 with E2 (Fig. 2C). These results indicate that the N-terminal tetra-peptide short extension of the U-box motif in rice SPL11 E3 is essential for its interaction with sub-class I E2 Os09g12230. Thus, an intact U-box motif is necessary but not sufficient for the binding of SPL11 E3–E2. As expected, all of the deletion constructs of SPL11 were unable to interact with sub-class II E2 Os06g45000 (Fig. 2B and C), which is consistent with the results of Fig. 1B and C.

3.2. The N-terminal four amino-acid (IPDE) short extension of the U-box domain in SPL11 is required for the interaction between SPL11 E3 and E2

Aforementioned results indicate that the N-terminal tetra-peptide (IPDE) short extension of the U-box motif in rice SPL11 E3 is critical for the interaction with its E2 partner (Fig. 2). SPL11 is one of the 28 class II rice U-box E3 Ub-ligases [10]. Amino-acid sequence alignment of rice class II U-box E3s reveals that, among four amino-acid residues (IPDE), the Pro residue at the −3 position is highly conserved (Fig. 3A). Therefore, the N-terminal IPDE four amino-acid residues may be important for the interaction of SPL11 E3 with E2 in a sequence-specific manner. Alternatively, it is possible that this short extension simply participates in the binding to E2 in a length-dependent manner. To investigate these possibilities, amino-acid substitution mutants were generated using SPL11$^{U\text{-}box}$N+20/C+10 as template and analyzed for their binding activities to the isolated sub-group I E2 Os09g12230 by yeast two-hybrid assays. The results show that single substitutions of the Ile and Pro residues to Thr and Ala at the −4 and −3 positions of the U-box, respectively, markedly reduced the binding activities of SPL11 E3–E2 (Fig. 3C). Consistently, interaction of SPL11 with E2 was almost completely abolished when both Ile and Pro residues were double-mutated (Fig. 3D). In contrast, AspAla and GluAla substitutions at the −2 and −1 positions, respectively, did not significantly affect the binding activity of SPL11 E3–E2. To confirm these results, we carried out *in vitro* self-ubiquitination assays using the amino-acid substitution derivatives of SPL11. As expected, the E3 Ub-ligase activity of SPL11 was completely lost

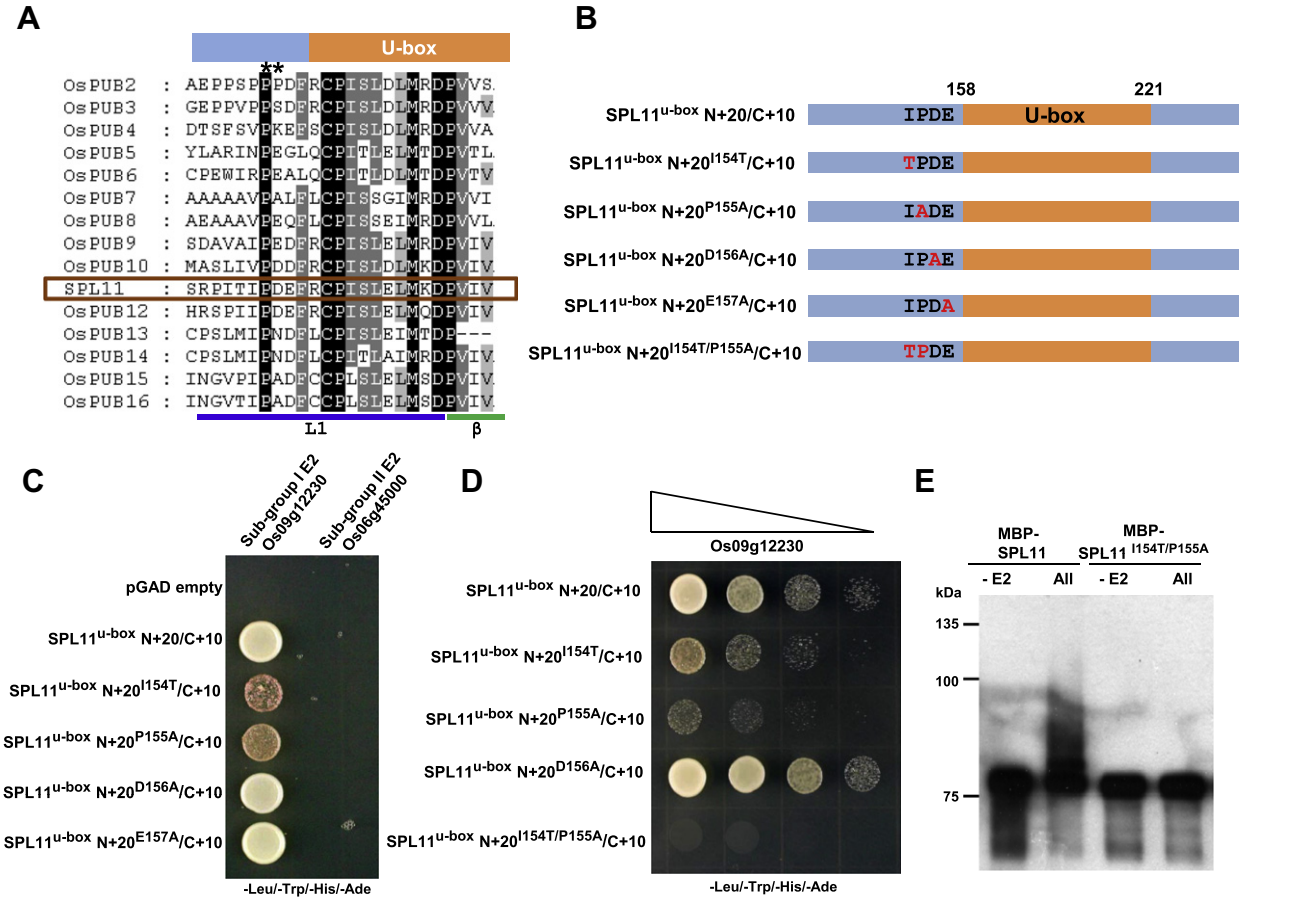

Fig. 3. The N-terminal tetra-peptide (IPDE) short extension of the U-box domain is required for the interaction between SPL11 E3 and E2. (A) Amino-acid sequence alignment of the N-terminal region of the U-box from 15 class II U-box E3s in rice. The predicted secondary structures, such as loop (L1) and β-sheet (β), and the U-box motif are indicated. Asterisks indicate amino-acid residues at the −4 and −3 positions of the U-box. Amino-acid sequences conserved in all 15 proteins are shown in black; amino-acid residues identical in at least 9 of the 15 sequences are shaded. (B) Schematic representation of wild-type (SPL11$^{U\text{-}box}$N+20/C+10) and five amino-acid substitution mutants of the N-terminal extension region of the U-box. Substituted amino-acid residues are indicated by red letters. (C) and (D) Interactions between N-terminal extension mutants of SPL11 and E2s. Sub-group I E2 Os09g12230 and sub-group II E2 Os06g45000 were used as bait. Wild-type and four single-amino-acid (C) and one double-amino-acid (D) substitution mutants of SPL11$^{U\text{-}box}$N+20/C+10 were used as prey. The pGAD empty vector was a negative control. (E) *In vitro* self-ubiquitination assay. Purified MBP-SPL11 and MBP-SPL11$^{I154T/P155A}$ mutant proteins were subjected to *in vitro* self-ubiquitination assays in the presence or absence of sub-group I E2 Os09g12230 with anti-MBP antibody. (For interpretation of color in this figure, the reader is referred to the web version of this article.)

when the Ile and Pro residues at the –4 and –3 positions of the U-box were double-mutated (Fig. 3E). These results suggest that both Ile and Pro residues at the –4 and –3 positions of the U-box are essential for the binding of SPL11 E3–E2 and for its Ub-ligase activity.

3.3. The C-terminal ten amino-acid residues of the U-box domain in SPL11 E3 are critical for its binding to E2 and Ub-ligase activity

The results in Fig. 2A show that the SPL11$^{U\text{-}box}$ΔC-10 construct, in which ten amino-acid residues at the C-terminal end of the U-box are deleted, lacks both binding activity to E2 and Ub-ligase activity. These ^{212}VLRSLISQWC^{221} sequences form the helix-2 structure at the C-terminal end of the U-box (Fig. 4A) [24,29]. This led us to repeat the yeast two-hybrid and self-ubiquitination assays using a series of single- and double-amino-acid substitution derivatives of SPL11 E3 to examine their binding activities to E2 (Fig. 4B). As demonstrated in Fig. 4C, all of the substitution mutants, including ^{212}ValLeu^{213} → ^{212}ThrThr^{213}, ^{214}Arg → ^{214}Ala, ^{216}Leulle^{217} → ^{216}ArgThr^{217}, and ^{220}TrpCys^{221} → ^{220}LeuGly^{221}, failed to interact with sub-group I E2 Os09g12230. Further, the mutant proteins were unable to display *in vitro* Ub-ligase activity in the presence of E2 (Fig. 4D). These results suggest that the helix-2 structure in the C-terminal end of the U-box motif is crucially involved in the binding of SPL11 E3–E2 and its Ub-ligase activity.

4. Discussion

Plant U-box E3 Ub ligases regulate diverse plant-specific cellular processes [1–4]. However, structural and functional relationships between U-box E3s and their E2 partners are largely unknown in higher plants [24,25]. In this study, we investigated the minimal binding domain of rice SPL11 U-box E3–E2 Ub-conjugating enzyme by yeast two-hybrid assay and analysis of *in vitro* self-ubiquitination in combination with site-directed mutagenesis. Our results revealed that SPL11 U-box E3 interacted with sub-group I E2s, which were homologous with a hub-group of human E2s [27] (Fig. 1 and Supplementary Fig. S1). SPL11 failed to bind to E2s belonging to the sub-groups II and III E2s, both of which were more distantly related to the human hub. Consistent with these results, SPL11 exhibited Ub-ligase activity with sub-group I E2s, but not with sub-groups II and III E2s (Fig. 1). Recently published results showed that large numbers of *Arabidopsis* RING and U-box E3s displayed *in vitro* Ub-ligase activities with AtUBC8 and AtUBC10 E2s, which were similar to the human E3 hub group [19,26,30,31]. Thus, it could be possible to speculate that, similar to human systems, E2–E3 interactions in rice do not depend on a uniform distribution, but E3 ligases are active within specific E2 hubs. However, more detailed investigations must be performed to examine this possibility.

The U-box motif was necessary but not sufficient for the interaction of SPL11 E3 with its E2 partners (Fig. 2). Deletion of the en-

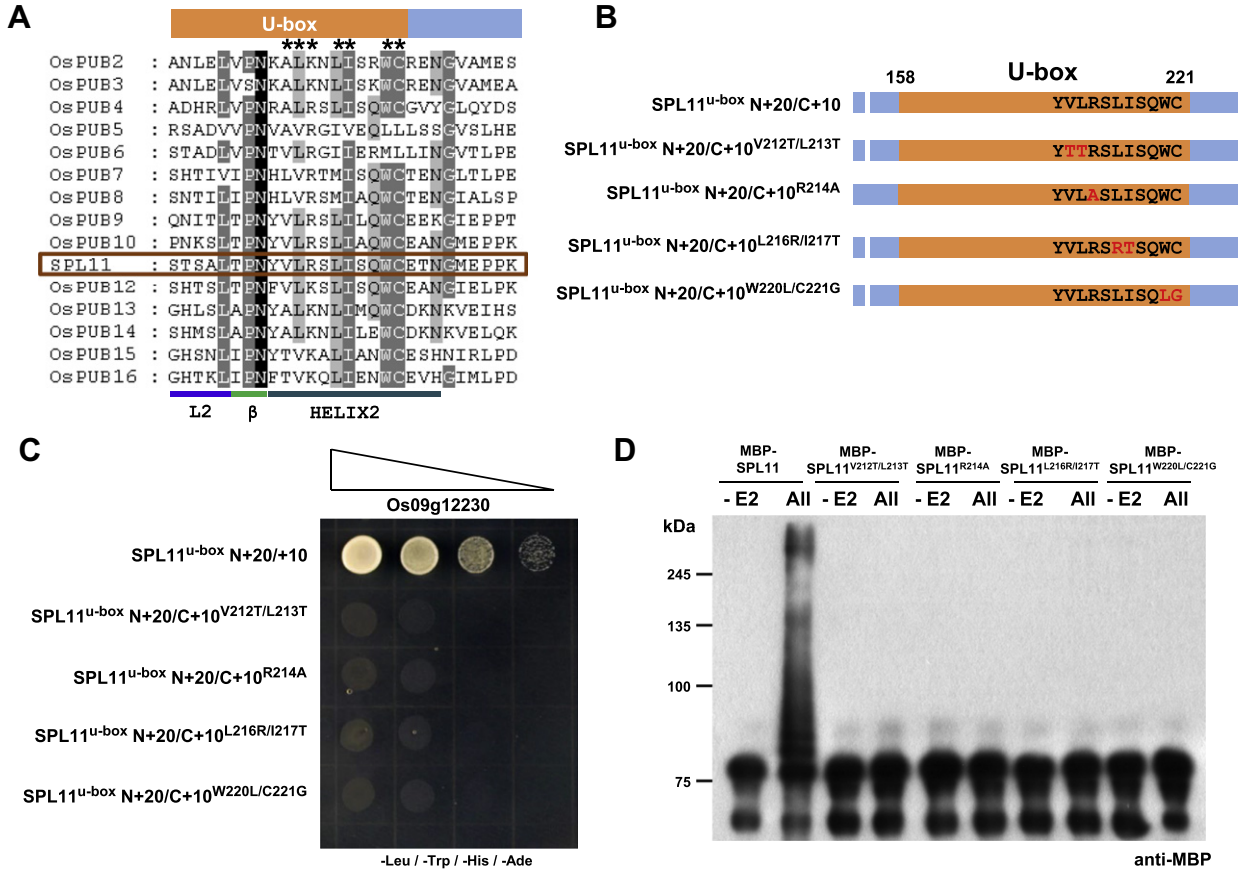

Fig. 4. The C-terminal 10 amino-acid residues of the U-box domain in SPL11 E3 are critical for its binding to E2 and Ub-ligase activity. (A) Amino-acid sequence alignment of the C-terminal region of the U-box from 15 class II U-box E3s in rice. The predicted secondary structures, such as loop (L2), β-sheet (β), and α-helix (HELIX2), and the U-box motif are indicated. Asterisks indicate amino-acid residues used for site-directed mutagenesis. (B) Schematic representation of wild-type (SPL11$^{U\text{-}box}$N+20/C+10) and four C-terminal helix mutants of the U-box. Substituted amino-acid residues are indicated by red letters. (C) Interactions between C-terminal helix mutants of SPL11 and E2. Sub-group I E2 Os09g12230 was used as a bait. Wild-type and four amino-acid substitution mutants of SPL11$^{U\text{-}box}$N+20/C+10 were used as prey. (E) *In vitro* self-ubiquitination assay. Purified MBP-SPL11, MBP-SPL11$^{V212T/L213T}$, MBP-SPL11^{R214A}, MBP-SPL11$^{L216R/I217T}$, and MBP-SPL11$^{W220L/C221G}$ fusion proteins were subjected to *in vitro* self-ubiquitination assay in the presence or absence of sub-group I E2 Os09g12230 with anti-MBP antibody. (For interpretation of color in this figure, the reader is referred to the web version of this article.)

tire C-terminal region (amino-acid residues 222–575) of SPL11 did not significantly affect its binding activity with E2 (Fig. 2A and B). In contrast, serial deletions of the N-terminal region resulted in the gradual decrease in binding activity between SPL11 and its E2 partner (Fig. 2C). This suggests that the N-terminal extension of the U-box is involved, at least in part, in the binding of SPL11 E3 and E2s. When the short N-terminal extension that consists of the IPDE tetra-sequence from –4 to –1 position of the U-box was removed, the binding capacity of SPL11 to E2 completely disappeared (Fig. 2). Of these four amino-acid residues, Ile and Pro residues at the –4 and –3 positions were essential to SPL11 for both its interaction with E2 and its Ub-ligase activity (Fig. 3). The Pro residue is conserved in approximately 90% of total rice U-box proteins. The N-terminal extension, along with eleven amino-acid residues in the U-box motif, was predicted to form the loop-1 structure (Fig. 3A) [29]. This raised the possibility that, although the N-terminal short extension of the U-box motif may not directly participate in the binding to the E2 partner, it critically affected the interacting interface between the U-box and E2 and the Ub-ligase activity of rice SPL11 E3.

In contrast to the N-terminal extension, no C-terminal extension is necessary for the function of the U-box in SPL11 (Fig. 2). However, deletion of the C-terminal ten amino-acid residues of the U-box resulted in complete loss of binding activity to E2 (Fig. 2). This region is involved in the formation of the helix-2 structure (Fig. 4A). This helix-2 structure forms a three-dimensional hydrophobic core with the loop-1 in the N-terminal region of the U-box [24,29]. Thus, deletion of the C-terminal ten amino-acid residues of the U-box may cause disruption of the hydrophobic core surrounding the U-box. This result indicates its critical role in the interaction between the U-box and E2.

Although the U-box E3 Ub-ligases from rice and *Arabidopsis* have different structures (e.g., locations of the U-box motif and presence of additional functional domains), all these plant U-box E3s contain well-conserved tetra-peptide N-terminal extension. On the other hand, the amino-acid sequences of the N-terminal extension of the human and yeast U-box E3s are relatively divergent. Of seven U-box E3s in human, three do not have a Pro residue at the –3 position of the U-box (Supplementary Fig. S2). Furthermore, the human and yeast Prp19 U-box protein homologs contain neither the N-terminal extension nor the C-terminal helix of the U-box domain with functional E3 Ub-ligase activity [24]. Thus, it may be proposed that the N-terminal tetra-peptide extension, along with the greater number of U-box E3s, may be correlated not only with their association with E2 but also with the diverse cellular functions of U-box E3s in higher plants. Additional functional and structural studies will be necessary to further investigate the plant-specific roles of U-box E3 Ub-ligases in higher plants.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.005.

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