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Classification and interaction modes of 40 rice E2 ubiquitin-conjugating enzymes with 17 rice ARM-U-box E3 ubiquitin ligases

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 crop, contains at least 48 putative E2 ubiquitin (Ub)-conjugating enzymes. Based on homology comparisons with 40 *Arabidopsis* E2 proteins and 35 human E2s, 48 rice E2s were classified into 15 different groups. Yeast two-hybrid analyses using the U-box-domain regions of armadillo (ARM)-U-box E3 Ub-ligases and the Ub-conjugating (UBC) domains of E2s showed that, among 40 rice E2s, 11 E2s accounted for 70% of the interactions with 17 ARM-U-box E3s. Thus, a single E2 could interact with multiple ARM-U-box E3s, suggesting the presence of E2 hubs for E2–E3 interactions in rice. Rice SPL11 ARM-U-box E3 displayed distinct self-ubiquitination patterns, including poly-ubiquitination, mono-ubiquitination, or no ubiquitination, depending on different E2 partners. This suggests that the mode of ubiquitination of SPL11 E3 is critically influenced by individual E2s.

1. Introduction

Ubiquitination is a well-characterized post-translational modification that is critical for regulating a wide range of cellular processes in eukaryotic organisms, including higher plants [1–3]. The conjugation of ubiquitin (Ub) to target proteins is sequentially carried out by E1 Ub-activating enzymes, E2 Ub-conjugating enzymes, and E3 Ub-ligases [4–6].

In plant genomes, there are few E1s, approximately 40 E2s, and more than 1000 E3s [7,8]. With their abundance and target-binding activities, E3s generally determine the specificity of the ubiquitination pathway. In humans, however, E2s not only interact with E1s and E3s to receive and transfer Ub, respectively, but also regulate, at least in part, the length and topology of the poly-Ub chain and the efficiency of poly-ubiquitination conducted by E3s [5,9]. Thus, E2s are not simply involved in ubiquitination, but are one of the regulators in the ubiquitination pathway.

E3s contain one of three distinct functional domains: HECT, RING, or U-box [10–12]. The U-box motif shares a similar structure with the RING domain, but does not bind zinc ions to act as an E3 Ub-ligase [11,12]. Compared to humans and yeast, higher plants have a large number of U-box motif-containing E3 Ub-ligases. *Arabidopsis*, a dicot model plant, contains 64 U-box genes [13] and the monocot model crop rice possesses at least 77 U-box genes [14]. Based on their primary sequences and presence of specific domains, the U-box E3s are divided into nine different classes [14]. Class II and III U-box E3s are the most abundant E3s and are typified by the presence of a protein–protein interacting armadillo (ARM) repeat domain [13–15]. Plant U-box proteins have a role in diverse plant-specific phenomena, including hormone signaling [16,17], responses to abiotic/biotic stress [18–20], self-incompatibility [21], and flowering time control [22]. For instance, rice ARM-U-box E3 SPL11 negatively regulates programmed cell death [20]. SPL11 plays an additional role in regulating flowering time by mono-ubiquitinating its target protein SPIN1 that represses flowering by down-regulating the flowering promoter gene *HD3A* [22]. *OsPUB15*, which encodes a class II ARM-U-box E3, regulates oxidative stress and cell death responses by reducing reactive oxygen species in rice [19].

To function as Ub-ligases, E3 proteins must interact with E2s. As compared to the extensive studies on E3s, functional studies on E2s are relatively rudimentary in higher plants. We previously reported that there are 48 genes encoding Ub-conjugating (UBC) fold-containing putative E2 proteins in the rice genome that are divided into three classes [23]. In another study, Kraft et al. [24] sorted 37 *Arabidopsis* E2s into 14 groups (groups III–XVI) based on detailed sequence homology analyses. In addition, *Arabidopsis* has a single SUMO-conjugating enzyme (AtSCE1a) and two Related to Ub-conjugating (RUB) enzymes (RCE1 and RCE2) that were classified into groups I and II, respectively [24].

Abbreviations: HECT, homology to E6-AP carboxyl-terminus; MBP, maltose binding protein; PCR, polymerase chain reaction; RING, really interesting new gene.

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

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In this report, we further classified the 48 rice E2s into 15 different groups. Yeast two-hybrid (Y2H) assays were performed to examine the interaction profiles of 40 E2s with 17 ARM-U-box E3s in rice. Of the 40 E2s, 11 E2s belonging to groups VI, VII, and VIII accounted for 70% of the E2–E3 interactions. These E2–E3 interactions were further validated by *in vitro* self-ubiquitination assays with rice SPL11 ARM-U-box E3 and various E2 partners. SPL11 E3 displayed distinct self-ubiquitination patterns, including poly-ubiquitination, mono-ubiquitination, and no ubiquitination, depending on the various E2s. Overall, these results suggest that the mode of ubiquitination of SPL11 E3 is critically influenced by individual E2s.

2. Materials and methods

2.1. Phylogenetic analysis and classification of rice UBC E2 proteins

Forty-eight rice UBC domain-containing proteins and 40 *Arabidopsis* E2s, including SUMO and RUB E2s, were aligned using MEGA 5 software with the ClustalW algorithm with gap-open and gap-extension penalties of 10 and 0.1, respectively. Aligned sequences were used to construct an unrooted phylogenetic tree based on the neighbor-joining method [25] or maximum likelihood method [26] after bootstrap analysis for 10,000 replicates. Rice UBC E2s were classified into 15 classes based on their homology with 16 previously-classified groups of *Arabidopsis* E2s [24].

2.2. Y2H assays

cDNAs encoding the UBC-fold region of 40 rice E2 proteins were inserted into the pGBK vector and transformed into the yeast strain AH109 as recently described [27]. The U-box motif + N-terminal short extension [23] of SPL11 and class III ARM-U-box E3s were cloned into pGAD T7 vector and transformed into the yeast strain Y187. Mating of E2-AH109 and E3-Y187 yeast cells was performed by pinning each transformant at the same position on solid -Trp/-Leu medium. After 3 days at 30 °C, the acquired diploid cells were transferred to -Trp/-Leu or -Trp/-Leu/-His/-Ade plates to visualize E2-ARM-U-box-E3 interactions.

2.3. In vitro self-ubiquitination assays

The expression and purification of MBP–SPL11 fusion protein and *in vitro* self-ubiquitination assays were performed as described previously [23]. Immuno-blot analysis was conducted using anti-MBP antibody (New England BioLabs, Ipswich, MA, USA) or anti-Ub antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described by Kim et al. [28].

2.4. In vitro E2 activity assays

In vitro E2 activity assays of rice UBC fold-containing proteins were performed in total volumes of 40 μl, including 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM ATP, 20 μg Ub, 200 ng $(His)_6$-tagged E1 (*Arabidopsis* UBA1), and 200 ng E2. After incubation for 15 min at 30 °C, reactions were stopped by addition of SDS sample buffer without reducing agents. Ub-conjugated E2s were separated by SDS–PAGE and detected with anti-Ub antibody.

3. Results

3.1. Classification of 48 rice UBC fold-containing proteins

Since the UBC domain of E2s is highly conserved, we previously used the amino-acid sequence of the UBC domain in OsUBC8 to search for E2 proteins in the rice genome and identified 48 UBC domain-containing putative E2 Ub-conjugating enzymes [23]. For more detailed classification and characterization, the 48 rice E2s were subjected to homology comparison with 40 *Arabidopsis* E2s that were already classified into 16 groups [24] and with 35 functional human E2s [29] using the neighbor-joining [25] and maximum-likelihood methods [26]. These two different methods resulted in phylogenetic trees with very similar topologies. Homology analyses showed that the 48 putative rice E2s had significant sequence identities to corresponding groups of *Arabidopsis* E2s with one exception (Fig. 1 and Supplementary Table S1). There was no rice homolog of the group XIII *Arabidopsis* UBC31 E2. Thus, the 48 putative rice E2s were classified into 15 groups (groups I–XII and groups XIV–XVI).

Among the 15 E2 groups, group VI with 12 E2s is the largest in rice, as is the case in *Arabidopsis* (Fig. 1 and Supplementary Table S1 and Fig. S1). There are 10 rice E2s in group XI. On the other hand, groups IV, IX, X, XII, XV, and XVI contain only a single E2 protein. Group VI E2s contain the catalytic UBC domain without other functional domains and are closely related to the human hub E2 proteins UBE2D1/2/3/4, UBE2E1, and UBE2E3 [30].

Among the five E2s in group VIII, four proteins (OsUBC28, OsUBC29, OsUBC30, and OsUBC31) have a UBC domain without an active site Cys residue. Thus, these E2s could be considered ubiquitin E2 variants (UEVs) [31,32]. In humans and *Arabidopsis*, there are seven and eight UEVs, respectively. The cellular functions of rice UEVs are currently unknown.

Unlike other groups, the 10 rice E2s in group XI share very low sequence identities (less than 30%) with human E2s (Supplementary Fig. S2). This raises the possibility that group XI E2s may have plant-specific roles. For example, group XI rice OsPHO2 (OsUBC35) and *Arabidopsis* PHO2 (UBC24) E2s are crucial components of phosphate starvation signaling [33,34]. However, the cellular functions of the remaining rice group XI E2s are unknown.

3.2. The modes of interaction of five rice ARM-U-box E3s with 40 E2 Ub-conjugating enzymes as determined by Y2H assays

We previously demonstrated that the N-terminal short extension in addition to the U-box motif is essential for the interaction between rice SPL11 E3 and its E2 partners [23]. To further uncover the modes of interaction between E3s and E2s, class II (SPL11) and four class III (OsPUB33, OsPUB36, OsPUB38, and OsPUB41) rice ARM-U-box E3s were selected and their cDNAs encoding the U-box-motif region were individually transformed into the yeast Y187 strain (Fig. 2A). E3-Y187 cells were then mated with yeast AH109 cells that contained the UBC domain of 40 different E2s under four-minus (-Leu/-Trp/-His/-Ade) growth conditions. The Y2H analysis showed that OsPUB33, OsPUB36, and OsPUB38 ARM-U-box E3s interacted strongly with group VI E2s (OsUBC14, OsUBC15, OsUBC16, OsUBC17, OsUBC18, OsUBC19, and OsUBC24), group VII E2s (OsUBC25 and OsUBC26), group VIII E2s (OsUBC29 and OsUBC30), and, to a lesser extent, the group XII E2 OsUBC44 (Fig. 2B). However, these three rice ARM-U-box E3s did not bind or only very weakly bound to other E2 groups including groups I, II, III, IV, V, IX, X, XI, XIV, XV, and XVI. SPL11 and OsPUB41 ARM-U-box E3s also exhibited similar binding modes to E2s (Fig. 2B). These results indicate that five rice ARM-U-box E3s did not randomly interact with E2s, but exhibited selective binding activities toward specific E2s. Thus, among 40 rice E2s, class VI, VII, and VIII E2s were selectively involved in the interactions with five ARM-U-box E3s. It is worth noting that rice group VI and VII E2s are significantly homologous to human hub E2s.

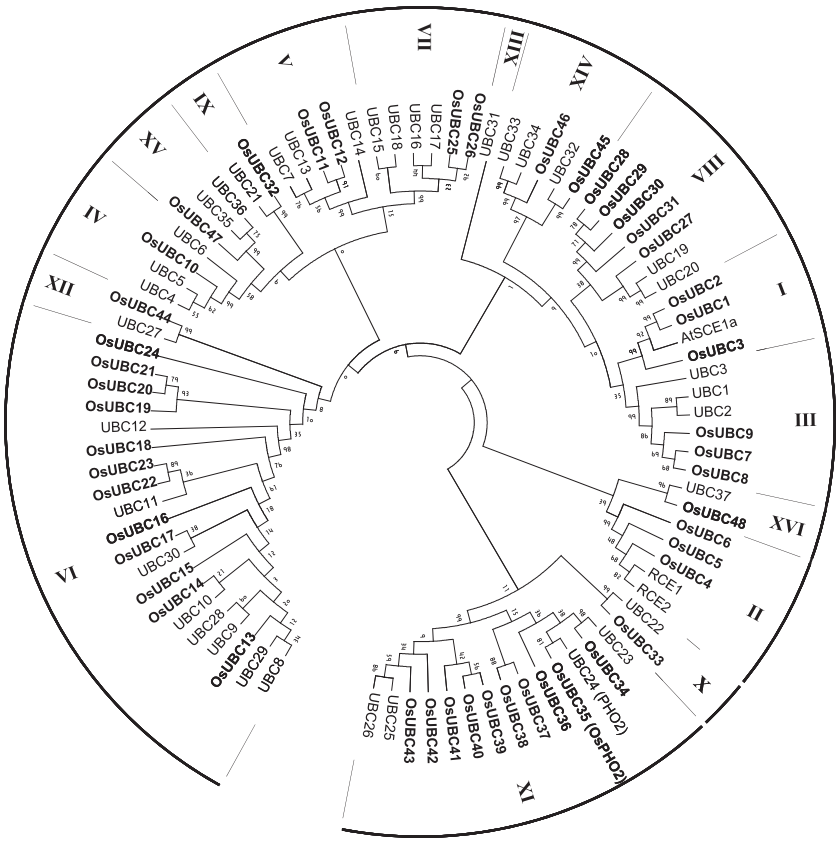

Fig. 1. Phylogenetic tree of rice and *Arabidopsis* UBC domain-containing E2 proteins. Full-length amino-acid sequences of 48 rice UBC proteins and 40 *Arabidopsis* UBC proteins, including SUMO and RUB E2s, were aligned using the ClustalW algorithm (MEGA 5 software). The tree was constructed by neighbor-joining method after bootstrap analysis for 10,000 replicates. Group numbers are indicated by Roman numerals and follow the previously described *Arabidopsis* E2 group designations [24]. Rice UBCs are indicated in bold letters.

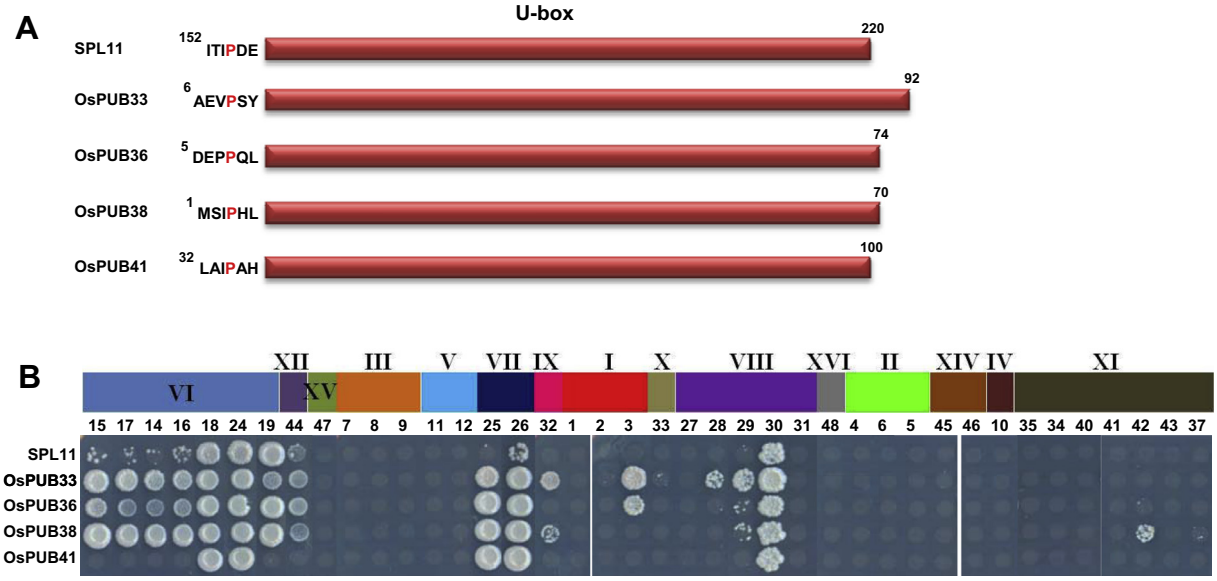

Fig. 2. Interactions between five rice ARM-U-box E3s and 40 rice E2s. (A) Schematic representation of the N-terminal six-amino-acid short extension + U-box motif of five rice ARM-U-box E3s (SPL11, OsPUB33, OsPUB36, OsPUB38, and OsPUB41) used in Y2H assays. Conserved Pro residues essential for E2 interactions [23] are shown in red. (B) Y2H assays. Interactions between the U-box-motif region of five rice ARM-U-box E3s and the UBC domain of 40 rice E2s were analyzed by Y2H. Rice E2s are sorted by their classified groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. The modes of interaction of 40 rice E2s with 17 ARM-U-box E3s

To further examine the interaction profiles between E3s and E2s in rice, Y2H assays were repeated with the U-box-motif region of class II SPL11 and 16 class III ARM-U-box E3s with the UBC domain of 40 E2s. As shown in Fig. 3, the interaction profiles of 17 ARM-U-box E3s with 40 E2s were highly reminiscent of those of five E3s and E2s. Most interactions of the 17 E3s with the E2s occurred with

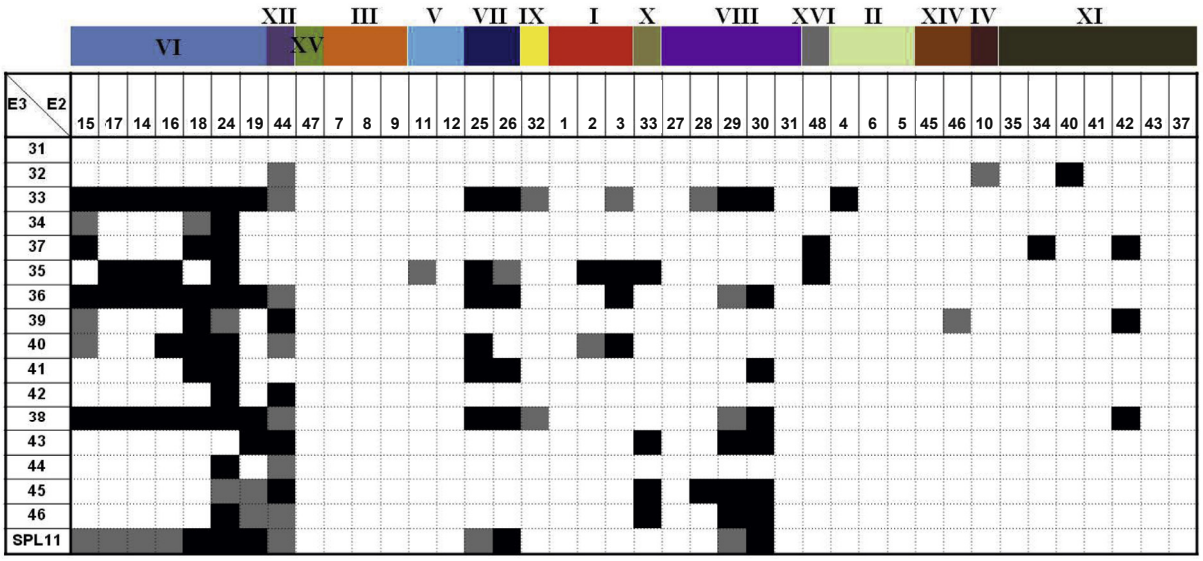

Fig. 3. Interaction modes of 17 rice ARM-U-box E3s and 40 rice E2s. Interactions between the U-box-motif region of 17 rice ARM-U-box E3s and the UBC domain of 40 rice E2s were analyzed by Y2H. Rice E2s are sorted by their classified groups. Black boxes and gray boxes indicate strong interacting and weak interacting E2–E3 pairs, respectively.

class VI, VII, and VIII E2s (Fig. 3). Among the 40 E2s, 12 E2s that belong to class VI, VII, and VIII accounted for 70.6% of the E2–E3 interactions. As mentioned above, rice group VI and VII E2s are highly homologous to the human E2 hub. These results raise the possibility that group VI and VII E2s may also function as hub E2s for interactions with E3s in rice. Group VIII E2s (OsUBC29 and OsUBC30), which displayed significant interactions with E3s (Fig. 3), lack an active site Cys in the UBC domain. These UEVs are considered to be inactive E2s and, therefore, their strong interactions with E3s were somewhat unexpected. In humans, UEVs participate in hetero-dimer formation with other functional E2s [31,32].

3.4. Rice SPL11 E3 displayed distinct self-ubiquitination patterns depending on its interactions with different E2s

To test whether interactions between E2 and E3 proteins in yeast cells affects E3 Ub-ligase activity, *in vitro* self-ubiquitination assays were performed using full-length SPL11 E3. As a control experiment, bacterially-expressed $(His)_6$-tagged full-length OsUBC1, OsUBC8, OsUBC9, and OsUBC11 E2 proteins, all of which failed to interact with SPL11 (Fig. 3), were incubated individually with MBP–SPL11 fusion protein in the presence of ATP, Ub, and E1 (*Arabidopsis* UBA1). The reaction products were assayed by immuno-blot analysis with anti-MBP antibody. As expected, MBP–SPL11 was unable to display *in vitro* self-ubiquitination activity with OsUBC1, OsUBC8, OsUBC9, and OsUBC11 E2s (left panel in Fig. 4A).

MBP–SPL11 was then incubated with OsUBC14, OsUBC15, OsUBC16, OsUBC18, OsUBC19 (group VI E2s), and OsUBC25 (group VII E2) individually and subjected to *in vitro* self-ubiquitination assays. All of these rice E2s interacted with SPL11 in yeast cells (Fig. 3). SPL11 E3 had different self-ubiquitination patterns when incubated with different E2s. SPL11 had poly-ubiquitination activity when incubated with OsUBC14, OsUBC15, OsUBC16, and OsUBC18 (left panel in Fig. 4A), whereas it had mono-ubiquitination activity when incubated with OsUBC25 (right panel in Fig. 4A). In contrast, SPL11 failed to display detectable self-ubiquitination activity when incubated with OsUBC19. Thus, the mode of *in vitro* self-ubiquitination of SPL11 E3 is influenced by individual E2s.

We next performed *in vitro* E2 activity assays. Bacterially-expressed $(His)_6$-tagged OsUBC14, OsUBC15, OsUBC16, OsUBC18, OsUBC19, or OsUBC25 E2s were incubated with ATP, Ub, and E1 without an E3. The E2 Ub-conjugating activity was then examined by immuno-blot analysis with anti-Ub antibody. OsUBC14, OsUBC15, OsUBC16, OsUBC18, and OsUBC25 E2s showed *in vitro* E2 Ub-conjugating activities (Fig. 4B). In contrast, OsUBC19 did not exhibit *in vitro* E2 activity. Thus, although OsUBC19 E2 interacted with numerous ARM-U-box E3 Ub-ligases (Figs. 2 and 3), it may be an inactive E2 (Fig. 4B). This supports the finding that SPL11 E3 failed to display Ub-ligase activity with OsUBC19 E2 (Fig. 4A).

Amino-acid sequence alignment of the group VI and VII E2s indicated that the Cys residue in the UBC motif was well-conserved in OsUBC14, OsUBC15, OsUBC16, OsUBC18, and OsUBC25 (Fig. 4C). In contrast, the active site Cys residue was changed to Ala at the 107 position of OsUBC19 E2. Thus, a single amino-acid substitution of the Cys residue to Ala in the UBC motif resulted in the loss of E2 activity of OsUBC19.

4. Discussion

Our results based on the Y2H analysis between the U-box-motif region of E3s and the UBC domain of E2s showed that, among 40 rice E2s, 11 E2s accounted for 70% of the interactions with 17 ARM-U-box E3 Ub-ligases (Figs. 2 and 3). These 11 E2s belong to groups VI, VII, and VIII (Fig. 1 and Supplementary Table S1 and Fig. S2). Thus, a single E2 could interact with multiple ARM-U-box E3s and a relatively small number of E2s interact with a large number of functional ARM-U-box E3 Ub-ligases. This supports the hypothesis that the rice system contains E2 hubs for E2–E3 interactions. There are significant sequence identities between rice group VI and VII E2s and human E2s that function as hubs for interactions with E3s [30]. These results suggest that the human E2 hub system may be conserved in rice. However, Y2H results obtained in our current study were based on the interactions between the U-box-motif region of E3s and the UBC domain of E2s. Therefore, we could not exclude the possibility that interactions between full-length E2s and E3s would give rise to different interaction patterns.

Unexpectedly, two group VIII E2s (OsUBC29 and OsUBC30), which lack the catalytic Cys residues in their UBC domain and have no Ub-conjugating activity, interacted with numerous ARM-U-box E3s (Fig. 2 and 3). The human UEV protein MMS2 (UBE2V2) forms a functional E2 complex through hetero-dimerization with Ubc13 E2 [31,32]. Thus, future studies will examine if rice UEVs are involved

in the regulation of E3 Ub-ligase activity by dimerization with other E2s.

The rice SPL11 ARM-U-box E3 displayed distinct self-ubiquitination patterns based on its different E2 partners (Fig. 4). SPL11 exhibited poly-ubiquitination activity with group VI E2s (OsUBC14, OsUBC15, OsUBC16, and OsUBC18), mono-ubiquitination activity with group VII OsUBC25, and no activity with group VI OsUBC19. The interaction between SPL11 and OsUBC25 was relatively weak compared to other SPL11–E2 interactions (Figs. 2 and 3). Thus, the mono-ubiquitination pattern of SPL11 with OsUBC25 may be due to the weak interaction. An alternative possibility is that SPL11 E3 and OsUBC25 E2 specifically mono-ubiquitinates substrate proteins. This possibility is supported by UBE2W, a human homolog of OsUBC25, that mono-ubiquitinates substrate proteins with its E3 partner [35,36]. A more detailed functional relationship between SPL11 and OsUBC25 remains to be elucidated.

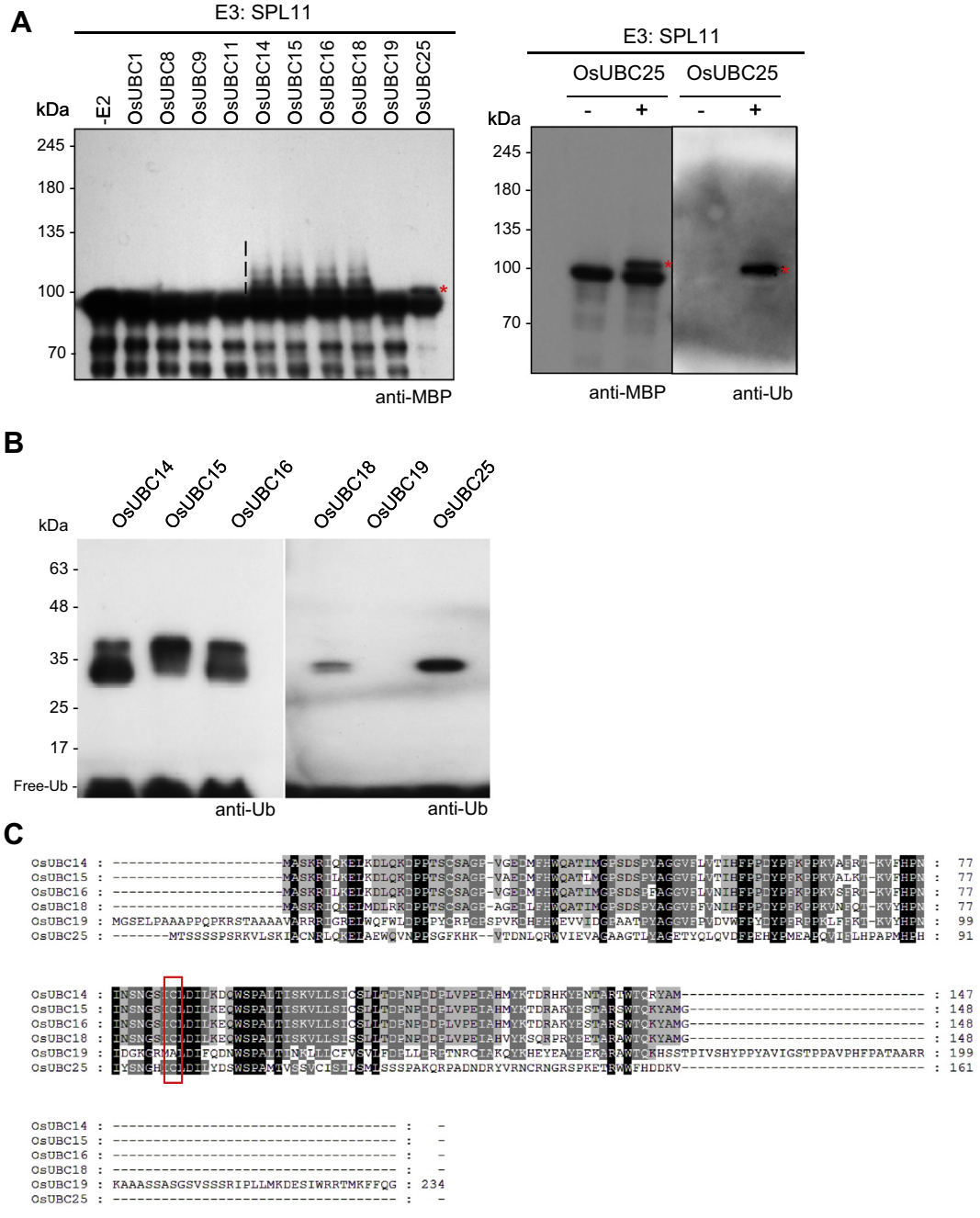

Fig. 4. The self-ubiquitination pattern of SPL11 E3 is critically influenced by different E2s. (A) *In vitro* self-ubiquitination assay of rice SPL11 ARM-U-box E3 Ub-ligase. Bacterially-expressed MBP–SPL11 recombinant protein was incubated with one of ten $(His)_6$-tagged full-length E2 proteins as indicated in the presence of Ub, ATP, and E1 (*Arabidopsis* UBA1). Reaction products were assayed by immuno-blot analysis with anti-MBP antibody or anti-Ub antibody. MBP–SPL11 E3 was mono-ubiquitinated with OsUBC25 E2. Vertical dashed line indicates poly-ubiquitinated MBP–SPL11. Red asterisk indicates mono-ubiquitinated MBP–SPL11. (B) *In vitro* E2 activity assay. $(His)_6$-tagged full-length OsPUB14, OsPUB15, OsPUB16, OsPUB18, OsPUB19, and OsPUB25 E2s were incubated at 30 °C for 15 min in the presence of E1, Ub, and ATP. Reactions were stopped by non-reducing SDS sample buffer. Reaction products were analyzed by immuno-blotting with anti-Ub antibody. OsPUB19 E2 failed to display the Ub-conjugated form. (C) Amino-acid sequence alignment of rice OsPUB14, OsPUB15, OsPUB16, OsPUB18, OsPUB19, and OsPUB25 E2s. Amino-acids conserved in all six E2 proteins are highlighted in black. Amino-acid residues identical in at least four of the six E2s are shaded. The conserved active site Cys residue is indicated by a red box. OsPUB19 E2 lacks the catalytic Cys residue at the active site. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Arabidopsis COP10, which is homologous to rice VI E2s, lacks the active site Cys residue and is an inactive E2 [37]. Instead of functioning as an active E2, COP10 interacts with other active E2s and enhances their Ub-conjugating activities. This suggests that seemingly inactive E2s may still participate in ubiquitination regulation. Similarly, rice group VI UBC19 E2 that also lacks the catalytic Cys residue interacts with several ARM-U-box E3s (Figs. 2 and 3) without detectable E2 activity (Fig. 4). Thus, a possible function of OsUBC19 is interacting with other E2s during ubiquitination activity in rice.

In conclusion, our results suggest the possible presence of E2 hubs in rice, a monocot model crop. Furthermore, ARM-U-box E3 Ub-ligase activities are significantly influenced by different E2 Ub-conjugating enzymes.

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.2014.01.098.

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