Rice OsPUB16 modulates the ‘SAPK9-OsMADS23-OsAOC’ pathway to reduce plant water-deficit tolerance by repressing ABA and JA biosynthesis

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Abstract

Ubiquitin-mediated proteolysis plays crucial roles in plant responses to environmental stress. However, the mechanism by which E3 ubiquitin ligases modulate plant stress response still needs to be elucidated. In this study, we found that rice PLANT U-BOX PROTEIN 16 (OsPUB16), a U-box E3 ubiquitin ligase, negatively regulates rice drought response. Loss-of-function mutants of OsPUB16 generated through CRISPR/Cas9 system exhibited the markedly enhanced water-deficit tolerance, while OsPUB16 overexpression lines were hypersensitive to water deficit stress. Moreover, OsPUB16 negatively regulated ABA and JA response, and ospub16 mutants produced more endogenous ABA and JA than wild type when exposed to water deficit. Mechanistic investigations revealed that OsPUB16 mediated the ubiquitination and degradation of OsMADS23, which is the substrate of OSMOTIC STRESS/ABA-ACTIVATED PROTEIN KINASE 9 (SAPK9) and increases rice drought tolerance by promoting ABA biosynthesis. Further, the ChIP-qPCR analysis and transient transactivation activity assays demonstrated that OsMADS23 activated the expression of JA-biosynthetic gene OsAOC by binding to its promoter. Interestingly, SAPK9-mediated phosphorylation on OsMADS23 reduced its ubiquitination level by interfering with the OsPUB16-OsMADS23 interaction, which thus enhanced OsMADS23 stability and promoted OsAOC expression. Collectively, our findings establish that OsPUB16 reduces plant water-deficit tolerance by modulating the ‘SAPK9-OsMADS23-OsAOC’ pathway to repress ABA and JA biosynthesis.

Author summary

Plants face various adverse environmental stimuli during their growth, such as drought, salinity and extreme temperatures. Different from animals, plants cannot move, so they must endure abiotic stresses, which greatly limit the distribution of plants, alter their
growth and development, and reduce crop productivity. To adapt to the major adverse environmental conditions, plants have developed multifaceted strategies at different levels. Among these, protein post-translational modifications play crucial roles. However, how protein post-translational modifications in response to environmental stress hormones modulate plant stress response still needs to be elucidated. Here, our results showed that the two types of post-translational modifications, phosphorylation and ubiquitination, regulate the balance between plant growth and stress response. Furthermore, there is the close link between the two types of post-translational modifications, which was affected by environmental stress hormones. The research will help us understand the mechanisms underlying the “trade-off” between plant growth and stress response, and engineer stress-resistant and high-yielding crops.

Introduction

Drought stress inhibits plant growth and severely limits crop production [1]. Phytohormones abscisic acid (ABA) and jasmonic acid (JA) play important roles in promoting plant drought tolerance [2,3]. ABA is considered to be the main hormone that enhances plant drought tolerance through various morpho-physiological and molecular processes including stomata regulation, root development, and initiation of ABA-dependent pathway [4]. There is empirical evidence that the core ABA signaling components including PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE/REGULATORY COMPONENTS OF ABA RECEPTORS (PYR/PYL/RCARs), type 2C PROTEIN PHOSPHATASES (PP2Cs) and SNF1-RELATED KINASES 2 (SnRK2s) play pivotal roles in response to drought stress [5–7]. In the presence of ABA, ABA binding to PYR/PYL/RCAR receptors initiates deactivation of the co-receptor PP2Cs, thus resulting in the release of positive effector SnRK2s and subsequently activating downstream AREB/ABF transcription factors by mediating their phosphorylation [5,6,8]. During the process, the degradation of PP2Cs and PYR/PYL/RCARs mediated by E3 ubiquitin ligases finely modulates ABA signaling [9]. JA signaling regulates a broad range of JA-dependent responses including plant growth and stress response, and JASMONATE ZIM-DOMAIN (JAZ) proteins are primary transcriptional repressors in JA signaling pathway [10–12]. Like ABA and other phytohormone signaling pathways, JA signaling also follows a ‘Relief of Repression’ module that degrades the negative regulators via receptor-SCF-26S proteasome-mediated proteolysis. Briefly, JA is perceived by F-box protein CORONATINE INSENSITIVE1 (COI1), which recruits the E3 ubiquitin ligase to form complex SCF<sup>COI1</sup> to target JAZ proteins, resulting in SCF<sup>COI1</sup>-dependent ubiquitination and degradation of JAZ proteins through the 26S proteasome [13,14]. The degradation of JAZ repressors alleviates the inhibition of downstream transcription factors, thus activating JA signaling [15,16].

A series of studies have shown that JA signaling can interact with ABA signaling to synergistically modulate multiple physiological processes including seed germination and biotic/abiotic stress response in plants [17–20]. In rice, OSMOTIC STRESS/ABA-ACTIVATED PROTEIN KINASEs (SAPKs), the SnRK2 homologues, are activated by ABA and hypertonic stress, and play essential roles in the transduction of ABA signaling [21,22]. Recent research has shown that ABA promotes JA biosynthesis through the ‘SAPK10-bZIP72-OsAOC’ pathway to synergistically inhibit rice seed germination [22]. In Arabidopsis, JAZ proteins modulate seed germination through the interaction with ABA-signaling transcription factors ABOCISIC ACID-INSENSITIVE 3 (ABI3) or ABI5 and thus repress their transactivation activity [20,23]. As a negative regulator of ABA signaling, GbWRKY1 reduces cotton salt and drought
tolerance through an interaction network involving JAZ1 and ABI1 [24]. Although previous efforts have provided clues that ABA signaling coordination with JA signaling modulates plant seed germination and abiotic stress response, the molecular mechanism of the synergetic effect of ABA on JA signaling during plant drought response is still largely elusive.

The ubiquitin-proteasome system plays important roles in plant growth and adaptation to environmental stress [25]. Protein ubiquitination is a multistep reaction, requiring ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3 [26]. Among these, the ubiquitin ligase E3 is critical in determining the specificity for degradation of target proteins [27]. The E3 ubiquitin ligases can be classified into HECT, RING, and U-box types based on the mechanisms for target recognition, ubiquitin tagging and specific domains [28–30]. Plant U-box (PUB) E3 ubiquitin ligases modulate various physiological processes and stress responses by regulating phytohormone signaling pathways [31,32]. In Arabidopsis, PUB12/13 modulate stomatal movement and confer drought response by mediating the degradation of ABI1, a key co-receptor of ABA [33], while they regulate cell differentiation and growth through a mechanism of membrane receptor kinase BRASSINOSTEROID-INSENSITIVE 1 (BRI1) internalization mediated by ubiquitination [34]. PUB18/PUB19 are negative regulators in ABA-mediated stomatal closure and drought stress responses [35]. PUB11 negatively modulates ABA-mediated drought response by the degradation of receptor-like protein kinases LEUCINE RICH REPEAT PROTEIN 1 (LRR1) and KINASE 7 (KIN7) [36]. In rice, OsPUB67 enhances drought tolerance by mediating a multilayered complex mechanism in an ABA-dependent manner [37]. Recently, PalPUB79 was indicated to enhance ABA-dependent drought tolerance via ubiquitination of PalWRKY77 in *Populus* [38]. Despite the recent advances, the knowledge about E3 ubiquitin ligases in monocot crops modulating abiotic stress response via phytohormone signalings is still limited.

Our previous study demonstrated that SAPK9-mediated phosphorylation on OsMADS23 conferred rice water-deficit tolerance in an ABA-dependent manner [39]. In this current study, we discovered that rice U-box E3 ubiquitin ligase OsPUB16 negatively regulates plant water-deficit tolerance via the 'SAPK9-OsMADS23-OsAOC' module by mediating ubiquitination and degradation of OsMADS23. OsMADS23 binds to the promoter region of JA-biosynthetic gene OsAOC to regulate JA biosynthesis, and SAPK9 enhances OsMADS23 protein stability as well as the transactivation activity by interfering with OsPUB16-OsMADS23 interaction via phosphorylation in an ABA-dependent manner. Thus, our findings provide a mechanistic understanding of how OsPUB16 modulates both ABA and JA signaling pathways that are integrated by the 'SAPK9-OsMADS23-OsAOC' module during the water-deficit response in rice.

**Results**

**OsPUB16 is a U-box/ARM repeat protein localized in the cytoplasm and nucleus**

To gain insight into the roles of rice U-box E3 ubiquitin ligases in plant growth and adaption to environmental stress, the phylogenetic analysis of a subset of U-box E3 proteins from different plants was performed. The result showed that the class-II U-box protein OsPUB16 shares high identity with OsPUB15 (S1 Fig), which regulates plant oxidative stress response and immunity in rice [40,41]. We further examined OsPUB16 expression in response to MV (methyl viologen), an oxidative stress inducer in plants, as well as ABA and MeJA, which are two well-recognized environmental stress hormones. We found that the mRNA levels of *OsPUB16* were rapidly, strongly induced by MV and the two hormones (Fig 1A–1C). These results suggest that OsPUB16 is likely to play essential roles in plant response to oxidative...
stress caused by adverse environmental stimuli. In addition, analysis of the spatio-temporal expression profile using the PlaNet Browser [42] showed that OsPUB16 was expressed in various tissues, but preferentially in the shoot apical meristem and young leaf (S2 Fig), implying that OsPUB16 may play important roles in plant growth. We investigated the subcellular localization of OsPUB16 by transiently expressing OsPUB16-GFP fusion protein in the epidermal cells of *Nicotiana benthamiana* leaves, and the result showed that OsPUB16 was mainly localized in the nucleus and cytoplasm (Fig 1D).

**OsPUB16 is required for plant growth in rice**

The CRISPR/Cas9-mediated knockout mutants (*ospub16-1* and *ospub16-2*) were generated for investigating the biological functions of OsPUB16. The mutant *ospub16-1* harbored a one-nucleotide (T) insertion and *ospub16-2* harbored a 25-nucleotide deletion in the first exon of OsPUB16, respectively, which might result in frameshift mutations and thus early termination of protein translation (S3A and S3B Fig). Knockout of OsPUB16 led to plant growth inhibition...
Notably, the homozygous ospub16-2 mutant was lethal, probably due to earlier termination of protein translation caused by the large DNA fragment deletion in ospub16-2 genome than that in ospub16-1, while the heterozygous ospub16-2 mutant could survive with the reduced growth (S3C–S3F Fig). We then investigated OsPUB16 expression in ospub16 mutants, and the result showed that low levels of OsPUB16 mRNA exist in ospub16-2 (+/-) (S3G and S3H Fig), which is consistent with its reduced growth. Little mRNA of OsPUB16 was detected in ospub16-1 (S3G and S3H Fig). Together, these findings strongly demonstrated that OsPUB16 is indispensable for plant growth and development in rice. We generated rice transgenic plants overexpressing OsPUB16. Contrary to ospub16 mutants, the OsPUB16-OX plants (OX-4 and OX-10) exhibited the enhanced growth (S3I–S3K Fig).

OsPUB16 acts as a negative regulator of plant water-deficit tolerance

Next, the water-deficit stress response in wild type (WT), ospub16 mutants and OsPUB16-OX plants was examined. Here, given that ospub16-2 (-/-) mutants were lethal, the ospub16-2 (+/-) mutants were used for the evaluation of water-deficit tolerance. After water withholding and rewatering, the survival rates of ospub16-1 and ospub16-2 (+/-) were 51.3% and 19.2%, respectively, which were significantly higher than that of WT (5.4%), whereas few OsPUB16-OX plants survived (Fig 2A and 2B). To further confirm the water-deficit tolerance in ospub16 mutants was caused by OsPUB16 knockout, we performed a functional complementation test by introducing the coding sequence of OsPUB16 driven by its own promoter into the ospub16-1 mutant, and the positive transgenic lines harboring the OsPUB16pro:OsPUB16 (C-1, C-2) showed WT phenotypes, indicated by the reduced water deficit tolerance (Figs 2C, 2D and S4). Further, the water loss rates of detached leaves showed that ospub16 mutants lost water more slowly but overexpression lines lost water faster than WT (Fig 2E), which supported the result that ospub16 mutants were more tolerant but overexpression lines were more sensitive to water deficit stress. In agreement, the complementary lines of ospub16-1 restored the rapid water loss (Fig 2F). The findings suggest that OsPUB16 negatively regulates the water-deficit tolerance in rice.

Stomatal density, size and morphology in leaf are closely associated with the leaf water loss rate under water deficit conditions [43]. To explore the possible cellular processes regulated by OsPUB16 during plant water-deficit response, we then examined the influence of OsPUB16 knockout or overexpression on the stomatal development in rice leaves. Interestingly, we found that the stomatal density was increased in ospub16-1, but reduced in OX-4 leaves, in comparison with that in WT (Fig 3A and 3B). However, a significant decrease in stomatal length and width was observed in ospub16-1 mutant (Fig 3C and 3D), which supports that ospub16-1 lost water more slowly when water was withheld (Fig 2E). By contrast, overexpression of OsPUB16 led to the increased stomatal size (Fig 3C and 3D), suggesting a rapid water loss in OsPUB16-OX plants under water deficit conditions. ABA-induced stomatal closure is a well-recognized model system for the study of plant response to drought stress [44]. We then compared the ABA-induced stomatal movement in WT, ospub16 mutant and OsPUB16-OX leaves. Under daylight conditions, stomata in rice leaf can be classified into three typical status categories: completely open, partially open, and completely closed (Fig 3E). Without ABA, there was little difference in the proportions of these three categories of stomata between WT and ospub16-1 plants (Fig 3F). However, in the presence of ABA, more stomata were completely closed and fewer stomata were completely open in ospub16-1 leaves than that in WT. Expectedly, stomatal movement exhibited the opposite trend in OsPUB16-OX leaves (Fig 3F). Overall, these results demonstrate that OsPUB16 negatively modulates plant water-deficit tolerance, possibly through regulating stomatal development and ABA-induced stomatal movement.
OsPUB16 attenuates the ABA and JA response by repressing their biosynthesis. ABA and JA are considered as the main hormones that promote drought tolerance in plants through regulating various physiological responses and molecular processes [4,44,45]. Given the high expression of OsPUB16 induced by ABA and MeJA (Fig 1B and 1C), we asked whether OsPUB16 regulates ABA and JA responses in plants. We first investigated the

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ABA and JA are considered as the main hormones that promote drought tolerance in plants through regulating various physiological responses and molecular processes [4,44,45]. Given the high expression of OsPUB16 induced by ABA and MeJA (Fig 1B and 1C), we asked whether OsPUB16 regulates ABA and JA responses in plants. We first investigated the
response of different genotypes to exogenous ABA and MeJA, respectively, through the primary root growth inhibition assay. As shown in Fig 4A–4D, the primary root elongation in ospub16-1 mutant was inhibited more severely by ABA treatment, indicated by the higher decrease rate in root length than that in WT; however, the inhibitory effects of ABA on the root growth of OsPUB16-OX plants were poorer than that on WT. Similar response was observed when plants were exposed to exogenous MeJA (Fig 4E–4H). The ABA and JA sensitivity of ospub16 mutants was further confirmed by the seed germination inhibition assay (S5 Fig). Thus, these results demonstrated that ospub16 mutants were hypersensitive, while OsPUB16-OX plants were hypo-sensitive to ABA and JA, suggesting that OsPUB16 inhibits the ABA and JA response. Further, the endogenous ABA and JA levels in WT and ospub16 plants exposed to water deficit stress were evaluated. As shown in Fig 4I and 4J, after water withholding for 5 days, both ABA and JA-Ile levels were much higher in ospub16 plants than that in WT. Expectedly, the expression of ABA and JA biosynthetic genes in ospub16 mutants was significantly increased when exposed to drought stress (Fig 4K and 4L). These results imply that OsPUB16 represses plant ABA and JA response possibly through inhibiting the biosynthesis of two phytohormones.

OsPUB16 physically interacts with a subset of JAZ proteins, and mediates the OsJAZ9 protein ubiquitination and degradation

JA signaling is associated with plant drought stress response, and the JAZ proteins act as key repressors of JA signaling to block the downstream responses through the interaction with the
Fig 4. OsPUB16 reduced the ABA and JA response in rice. (A, C) Images showing the responses of ospub16-1 mutant, wild type (WT, Nipponbare) and OsPUB16 overexpression lines (OX-4 and OX-10) to exogenous ABA. Scale bars, 2 cm. Uniformly germinated seeds were grown on 1/2MS medium supplemented with or without 3 μM ABA for 3 days. (B, D) Measurement of the primary root length of plants as described in (A) and (C), respectively. Data are means ± SD (n = 20). (E, G) Images showing the responses of different genotypes to exogenous MeJA. Scale bars, 2 cm. Uniformly germinated seeds were grown on 1/2MS medium with or without 2 μM MeJA for 3 days. (F, H) Measurement of the primary root length of plants as described in (E) and (G), respectively. Data are means ± SD (n = 15). (I, J) ABA and JA-ile content in WT and ospub16 mutants after exposed to water deficit stress. Two-week-old plants were subjected to water deficit stress for 5 days, and leaves were collected for measurement of bioactive ABA and JA-ile content. Data are means ± SD (n = 3). (K, L) Expression of key genes involved in ABA (K) and JA (L) biosynthesis in ospub16 mutants and WT under normal conditions and drought.
transcription factors [46]. To further explore the roles of OsPUB16 in JA signaling, we checked whether OsPUB16 interacts with JAZ proteins through the yeast two-hybrid (Y2H) assay. Interestingly, we found that OsPUB16 interacted with multiple members of the JAZ family proteins (Fig 5A), suggesting that OsPUB16 is involved in JA signaling. Here we selected OsJAZ9 as the representative of JAZ family members for further validation for the interaction of OsPUB16 with JAZ proteins. In the bimolecular fluorescence complementation (BiFC) assay, strong YFP fluorescence signal was observed in the nucleus of epidermal cells of N. benthamiana leaves co-expressing OsPUB16-cYFP and OsJAZ9-nYFP, but no signal was detected when each construct was co-expressed with an empty vector (Fig 5B). Next, we used the firefly luciferase complementation imaging (LCI) assay to confirm the interaction. Consistently, no luciferase signal was detected in N. benthamiana leaves harboring each construct and an empty vector. However, we detected a robust luciferase signal in the presence of OsPUB16-nLUC and cLUC-OsJAZ9 (Fig 5C). The GST pull-down assay also demonstrated that GST-OsPUB16, but not GST alone, pulled down a significant amount of His-OsJAZ9 (Fig 5D). Based on these observations, we conclude that OsPUB16 interacts with OsJAZ9 in vitro and in vivo.

It is recognized that E3 ubiquitin ligases mediate the ubiquitination and subsequent degradation of substrate proteins by 26S proteasome system. The OsPUB16-OsJAZ9 interaction suggests that OsJAZ9 might be a substrate of OsPUB16. Next, we investigated whether OsPUB16 mediates the ubiquitination on OsJAZ9, and how JA affects the ubiquitination process. Recombinant His-OsJAZ9 was incubated with protein extracts from WT or OsPUB16-OX plants, with or without 100 μM MeJA, and then immunoprecipitation was performed with anti-His beads. Detection of the immunoprecipitated His-OsJAZ9 by anti-Ubi antibody showed that, compared to protein extracts from WT plants, the extracts from OsPUB16-OX plants increased the ubiquitination on His-OsJAZ9, and the ubiquitination levels were further enhanced in the presence of MeJA (Fig 5E). These findings demonstrated that OsPUB16 mediated the ubiquitination on OsJAZ9 in a JA-dependent manner. We then performed the cell-free degradation assay by using His-OsJAZ9 protein. His-OsJAZ9 protein was incubated with equal amount of total protein extracts from WT or OsPUB16-OX plants, in the presence of ATP. Immunoblot analysis showed that His-OsJAZ9 was degraded much faster in OsPUB16-OX extracts than that in WT extracts (Fig 5F), indicating that OsPUB16 mediates His-OsJAZ9 degradation. However, there was no significant difference in the degradation rate for His-OsJAZ9 in ospub16-1 extracts, compared to that in WT (S6 Fig), probably due to the compensational effects by other U-box E3 ubiquitin ligases in ospub16-1 mutant. His-OsJAZ9 degradation in WT extracts was largely blocked by an addition of MG132, a 26S proteasome inhibitor (Fig 5G), indicating that OsPUB16 mediates His-OsJAZ9 degradation mainly through the 26S proteasome system. These results indicate that OsPUB16 can regulate JA signaling by mediating JAZ protein degradation.

OsPUB16 physically interacts with OsMADS23, leading to its ubiquitination and degradation

OsPUB16 functions as a negative regulator in the water-deficit tolerance (Fig 2), and our recent study revealed that SAPK9-mediated phosphorylation on OsMADS23 confers rice drought tolerance [39], which urges us to examine whether OsMADS23 or SAPK9 acts a
OsPUB16 modulates the ‘SAPK9-OsMADS23-OsAOC’ pathway

Fig 5. OsPUB16 interacts with rice JAZ proteins, and mediates ubiquitination and degradation of OsJAZ9. (A) Yeast two-hybrid assays to test the interaction of OsPUB16 with rice JAZ proteins. Yeast cells were grown on synthetic defined (SD)/-Ade-/His-/Leu-/Trp (-LWHA) medium for 2 days. (B) Bimolecular fluorescence complementation (BiFC) assay for the interaction of OsPUB16 with OsJAZ9 in

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**A**

| Yeast Cells | BD-OsPUB16 | BD |
|-------------|------------|----|
| AD-OsJAZ5   | +          | -  |
| AD-OsJAZ6   | +          | -  |
| AD-OsJAZ7   | +          | -  |
| AD-OsJAZ8   | +          | -  |
| AD-OsJAZ9   | +          | -  |
| AD-OsJAZ10  | +          | -  |
| AD-OsJAZ11  | +          | -  |
| AD-OsJAZ12  | +          | -  |
| AD-OsJAZ13  | +          | -  |
| AD           | +          | -  |

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**B**

- **Input**
  - His-OsJAZ9
  - GST-OsPUB16

- **Pull down**
  - +
  - -

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**C**

- nLUC + cLUC
- nLUC + cLUC-OsJAZ9
- OsPUB16-nLUC + cLUC
- OsPUB16-nLUC + cLUC-OsJAZ9

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**D**

- Time/ min: 0, 30, 60, 120
- His-OsJAZ9
  - 1.00, 0.45, 0.32, 0.14

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**E**

- MG132: +, Me-JA: +, His-OsJAZ9: +
- α-UBi: 50 kD, 35 kD, 35 kD
- α-GFP: 119 kD

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**F**

- Time/ h: 0, 1, 2, 4
- His-OsJAZ9
  - 1.00, 0.88, 0.45, 0.28

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**G**

- CBB
  - MG132: -, Me-JA: -, His-OsJAZ9: -
Nicotiana benthamiana epidermal cells. YFP, yellow fluorescent protein. RFP, red fluorescent protein. HYS-RFP, the nuclear-localized marker. Scale bars, 20 μm. (C) OsPUB16 interacts with OsJAZ9 assessed by firefly luciferase complementation imaging (LCI) assays in N. benthamiana leaves. (D) OsPUB16 interacts with OsJAZ9 indicated by the GST Pull-down assay. GST-OsPUB16 was used as bait, and pull-down of His-OsJAZ9 was detected by anti-His antibody. GST-OsPUB16 and GST were detected by anti-GST antibody. (E) Semi-endogenous ubiquitination analysis showing the ubiquitination of OsJAZ9 mediated by OsPUB16, with or without MeJA. Briefly, the total protein extracts from wild type (WT) or OsPUB16-overexpressing plants (OX-4) were incubated with His-OsJAZ9 with or without 100 μM MeJA, and then the immunoprecipitation was performed with anti-His antibodies. The immunoprecipitated proteins were detected with different antibodies. Three independent experiments were performed, and representative pictures were shown. (F) Cell-free degradation assays for recombinant His-OsJAZ9 protein in the protein extracts from WT or OsPUB16-overexpressing plants (OX-4), in the presence of ATP. The numbers at indicated times show the relative levels of the recombinant protein. Three independent replicate experiments were performed, and representative pictures were given. The Coomassie blue-stained ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (RbcL) was used as a loading control. (G) Cell-free degradation assays for recombinant His-OsJAZ9 in the protein extracts from WT with or without protease inhibitor MG132, in the presence of ATP. The numbers at indicated times indicate the relative levels of the recombinant protein from three independent replicate experiments. In cell-free degradation assays, equal amount of His-OsJAZ9 protein was incubated with equal amount of plant protein extracts, in the presence of 10 mM ATP. His-OsJAZ9 was detected by immunoblot analysis with anti-His antibody. Three independent replicate experiments were performed, and representative pictures were given.

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substrate of OsPUB16. We first tested whether OsPUB16 interacts with OsMADS23 in the Y2H assay, with a subset of other rice U-box E3 proteins as control. The result showed that OsPUB16, but not other U-box proteins, specifically interacts with OsMADS23 (Figs 6A and S7A). Notably, OsPUB16 does not interact with SAPK9 in the Y2H assay (S7B Fig). Then, the GST pull-down assay showed that GST-OsPUB16, but not GST alone, pulled down His-OsMADS23 (Fig 6B), indicating that OsPUB16 physically interacts with OsMADS23 in vitro. The interaction of OsPUB16-OsMADS23 was further verified by BiFC, illustrated by the strong YFP fluorescence signal in the epidermal cells of N. benthamiana leaves harboring both OsPUB16-cYFP and OsMADS23-nYFP (Fig 6C). Consistently, the LCI assay also confirmed the interaction of OsPUB16 with OsMADS23 (Fig 6D). These results demonstrate that OsPUB16 interacts with OsMADS23 in vivo and in vitro, suggesting OsMADS23 might act as one of the substrates of OsPUB16.

We then tested whether OsPUB16 could ubiquitinate OsMADS23 by using a semi-endogenous ubiquitination assay. Total protein extracts from WT or OsPUB16-OX plants were incubated with GST-OsMADS23, in the presence of MG132, and then the immunoprecipitation was performed with anti-GST beads. We used anti-Ubi antibody to detect the immunoprecipitated GST-OsMADS23 and found that the abundance of poly-ubiquitinated GST-OsMADS23 was increased in OsPUB16-OX plant extracts (Fig 6E), indicating that OsPUB16 could mediate OsMADS23 ubiquitination. A close link between phosphorylation and ubiquitination has been implicated, and the E3 ligase-substrate association is regulated by phosphorylation [34]. Thr-20 and Ser-36 in OsMADS23 are main phosphorylation sites recognized by SAPK9 [39]. We next checked whether phosphorylation status of OsMADS23 affects its ubiquitination level. Interestingly, we found that the mimicked phosphorylation markedly blocked ubiquitination of the protein when GST-OsMADS23T20D,S36D was incubated with protein extracts from OsPUB16-OX plants (Fig 6E). Then, we investigated whether the phosphorylation status of OsMADS23 influences its interaction with OsPUB16, and thus affects its ubiquitination level. Expectedly, our GST pull-down assay showed that, compared to native GST-OsMADS23, the mimicked dephosphorylation (GST-OsMADS23T20A,S36A) strengthened its interaction with His-OsPUB16, while the mimicked phosphorylation (GST-OsMADS23T20D,S36D) reduced their interaction (Fig 6F). These results demonstrate that OsPUB16 targets OsMADS23 for ubiquitination, which is regulated by the phosphorylation status of OsMADS23.

Next, we investigated the influence of OsPUB16 on the stability of OsMADS23 by the cell-free degradation assay. GST-OsMADS23 protein was incubated with equal amount of protein
Fig 6. OsPUB16 mediates the ubiquitination and degradation of OsMADS23 via the physical interaction. (A) Yeast two-hybrid assays showing the interaction between OsPUB16 and OsMADS23. Yeast cells were grown on synthetic defined (SD)/-Leu-Trp (-LW) medium and
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SD/-Ade-His-Leu-Trp (-LWHA) medium. The AD-T + BD-P33 was used as a positive control, and AD-T + BD-lam as a negative control. (B) GST Pull-down assay showing the interaction of OsPUB16 with OsMADS23. GST-OsPUB16 was used as bait, and pulled-down His-OsMADS23 was detected by anti-His antibody. GST-OsPUB16 and GST were detected by anti-GST antibody. (C) Bimolecular fluorescence complementation (BiFC) assay for the interaction of OsPUB16 with OsMADS23 in Nicotiana benthamiana epidermal cells. YFP, yellow fluorescent protein. RFP, red fluorescent protein. HY5-RFP, the nuclear-localized marker. Scale bars, 20 μm. (D) Firefly luciferase complementation imaging (LCI) assay showing the interaction of OsPUB16 with OsMADS23 in N. benthamiana leaves. (E) Semi–endogenous ubiquitination analysis showing that phosphorylation of OsMADS23 blocks its ubiquitination mediated by OsPUB16. Briefly, the total protein extracts from wild type (WT) or OsPUB16-overexpressing plants (OX-4) plants were incubated with GST-OsMADS23 or GST-OsMADS23T20D S36D for 5 hours, in the presence of 50 μM MG132, and then the immunoprecipitation was performed with anti-GST beads. The immunoprecipitated proteins were detected with anti-Ubi and anti-GFP antibodies, respectively. The immunoblot analysis of Histone H3.1 (H3.1) was used as a loading control of protein extracts. Three independent experiments were performed, and representative pictures were shown. (F) GST Pull-down assays showing the interaction of GST-OsMADS23 or its different mutated versions (GST-OsMADS23T20A S36A and GST-OsMADS23T20D S36D) with His-OsPUB16. (G-I) OsPUB16 promotes the degradation of OsMADS23 in the cell-free degradation assay. Recombinant GST-OsMADS23 or its different mutated versions was incubated in equal amount of total protein extracts from two-week-old wild type (WT) and ospub16-1 mutant in the presence of 10 mM ATP. GST-OsMADS23 and its mutated versions were detected by immunoblot analysis using anti-GST antibody. The Coomassie blue-stained ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (Rbc L) was used as a loading control. Representative pictures were shown in (G) and relative protein levels were analyzed in (H, I). In (H) and (I), data are means ± SD (n = 3, three independent replicate experiments). The significant difference between WT and ospub16-1 mutant, or native GST-OsMADS23 and its different mutated versions was determined by Student’s t test. *p < 0.05, **p < 0.01 or ***p < 0.001.

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extracts from WT or ospub16-1 mutant. Immunoblot analysis showed that GST-OsMADS23 degradation was much slower in the extracts from ospub16-1 mutant than that in WT (Fig 6G and 6H), manifesting that OsPUB16 mediates OsMADS23 degradation. ABA-induced phosphorylation of OsMADS23 mediated by SAPK9 is critical for OsMADS23 stability [39]. We found that the degradation of GST-OsMADS23T20A S36A mediated by OsPUB16 was much faster, while degradation of GST-OsMADS23T20D S36D was slower than its native form (Fig 6G and 6I), suggesting that phosphorylation increased OsMADS23 stability. These data demonstrate that OsPUB16 mediates the ubiquitination and degradation of OsMADS23 in plants, which is controlled by its phosphorylation status. These observations also confirm the close link between the two types of post-translational modifications, phosphorylation and ubiquitination.

OsMADS23 promotes the JA response

The interaction of OsPUB16 with OsMADS23 as well as JAZ proteins urges us to examine whether OsMADS23 interacts with these JAZ proteins. Unexpectedly, the result of Y2H assay showed that OsMADS23 does not interact with this subset of JAZ proteins (S8 Fig). Given that OsPUB16 modulates JA and ABA response (Fig 4), and OsMADS23 regulates ABA response [39], we queried whether OsMADS23 also regulates JA response. Next, we evaluated the JA sensitivity of the loss-of-function mutant osmads23-1, OsMADS23-overexpressing lines (OE13, OE14) and their corresponding wild type through the primary root elongation inhibition assay. We found that, similar to their response to exogenous ABA [39], OsMADS23-overexpressing lines were more sensitive but osmads23-1 mutant was more tolerant to MeJA than their corresponding wild type (Fig 7), indicating that overexpression of OsMADS23 increases plant JA response. These data suggest a positive regulatory role of OsMADS23 in the interplay between ABA and JA signaling pathways.

OsMADS23 directly activates OsAOC transcription by binding to its promoter

Considering the fact that OsMADS23 positively regulates JA response, we then examined the transcription levels of a couple of JA-biosynthetic genes in osmads23-1 mutant, OsMADS23-overexpressing lines and wild type. Intriguingly, the expression of JA biosynthesis step-limiting genes OsAOC and OsLOX1 [47,48] was significantly downregulated in osmads23-1, but...
upregulated in OsMADS23-overexpressing plants (Fig 8A and 8B), suggesting that OsAOC and OsLOX1 are likely to be the downstream targets of OsMADS23. MADS-box transcription factors regulate gene transcription through binding to the elements containing CArG-box in the promoter region of target genes [49]. One CArG-box cis-element was found in 2 kb-promoter region of OsAOC (Fig 8C). Thus, we performed ChIP-qPCR assays in OsMADS23-GFP plants to test whether OsMADS23 binds to the promoter region of OsAOC in vivo. Substantial DNA enrichment was observed in the OsAOC promoter region containing CArG-box, while little enrichment was found in other regions (Fig 8D), hinting that OsMADS23 directly regulates the transcription of OsAOC, the master regulator of JA biosynthesis in the production of JA precursor 12-oxophytodienoic acid (12-OPDA). To further verify the regulation of OsAOC by OsMADS23, we performed a transient transactivation assay in N. benthamiana leaves through the luciferase reporter assay. The promoter region of OsAOC (pOsAOC) was fused to the LUC gene to generate the reporter, while the effector construct contained OsMADS23 driven by the CaMV 35S promoter (Fig 8E). In support of the ChIP-qPCR result, the LUC/REN ratio as well as the luciferase fluorescence signal intensity was remarkably increased in N. benthamiana leaves co-expressing the reporter carrying the OsAOC promoter driving LUC with the effector containing OsMADS23, in comparison with the negative control (Fig 8F and 8G), demonstrating that OsMADS23 significantly induces the expression of LUC driven by OsAOC promoter. Further, to examine the impact of ABA signaling on OsAOC transcription regulated by OsMADS23, OsMADS23 was co-expressed with SAPK9 in N. benthamiana leaves as effectors. Notably, the expression of LUC was significantly upregulated in leaves co-expressing OsMADS23 with SAPK9 as effectors, compared to expressing OsMADS23 alone (Fig 8F and 8G). Thus, we conclude that OsMADS23 is an upstream transcriptional activator of OsAOC.
and regulates its expression \textit{in vivo}, and SAPK9 enhances the transactivation activity of OsMADS23, and thus elevates OsAOC expression. Here, we identified a ‘SAPK9-OsMADS23-OsAOC’ pathway, through which ABA promotes JA biosynthesis.

**SAPK9 interferes with OsPUB16-OsMADS23 interaction through the phosphorylation on OsMADS23**

Our previous findings showed that SAPK9 interacts with OsMADS23, resulting in the increased stability of OsMADS23 via phosphorylation \cite{39}. Our current observation showed...
OsPUB16 interacts with OsMADS23 to promote its ubiquitination and degradation (Fig 6), but OsPUB16 does not physically interact with SAPK9 (S7B Fig). These observations suggest that SAPK9 might interfere with the OsPUB16-OsMADS23 interaction via the phosphorylation on OsMADS23. To verify our hypothesis, we co-transformed OsPUB16-nYFP and OsMADS23-cYFP with or without SAPK9 into N. benthamiana leaves and analyzed the OsPUB16-OsMADS23 interaction by confocal microscopy. The result showed that, compared to co-expressing OsPUB16-nYFP and OsMADS23-cYFP alone, the fluorescent signal drastically decreased when co-expressing OsPUB16-nYFP and OsMADS23-cYFP with SAPK9--FLAG (S9 Fig), suggesting that SAPK9 interferes with the OsPUB16-OsMADS23 interaction. Further, ABA treatment markedly enhanced the interference effect of SAPK9 on the OsPUB16-OsMADS23 interaction (S9 Fig). We then examined the effect of SAPK9 on OsPUB16-OsMADS23 interaction by investigating OsMADS23 transcription regulation of the downstream target gene through transient transactivation assays. Compared to OsMADS23 alone, co-expression of OsPUB16 with OsMADS23 repressed LUC expression driven by the OsAOC promoter, while the LUC/REN ratio was enhanced when SAPK9 was added (Fig 9A), confirming the interference of SAPK9 on OsPUB16-OsMADS23 interaction. Notably, after ABA treatment, SAPK9 drastically increased LUC expression (Fig 9A), implying that SAPK9-mediated phosphorylation on OsMADS23 interferes OsPUB16-OsMADS23 interaction, and thus enhances OsMADS23 stability. This observation was further verified by using the mutated forms of OsMADS23 protein. Different from co-expression of OsPUB16 with native OsMADS23 repressing LUC expression, the transactivation activity of OsMADS23 T20D S36D was not greatly reduced by OsPUB16, while the LUC/REN ratio from OsMADS23 T20A S36A significantly decreased when OsPUB16 was added (Fig 9B). These results demonstrate that OsMADS23 phosphorylation reduces its ubiquitination level, and thus promotes its stability, indicated by its transactivation activity.

**Discussion**

Plants tightly control intracellular protein levels via various strategies to adapt to various adverse conditions. Emerging evidence indicates that the E3 ubiquitin ligase-mediated degradation pathway is one of the most important mechanisms maintaining the homeostasis of intracellular proteins [50]. The U-box E3 ubiquitin ligases are widely present in eukaryotic organisms. Arabidopsis, a dicot model plant, contains 64 U-box E3 ubiquitin ligases, whereas the monocot model crop rice harbors 77 U-box proteins [51,52]. To date, a few U-box E3 ubiquitin ligases have been identified to be involved in plant environmental stress responses by modulating ABA signaling [33,35,38]. Whether E3 ubiquitin ligases modulate plant stress response via the interplay between ABA and other phytohormone signaling pathways still needs to be elucidated. In our study, we investigated how OsPUB16 integrates ABA and JA signaling pathways to regulate plant water-deficit response in rice by using CRISPR/Cas9-mediated knockout mutant ospub16-1 and ospub16-2 (+/−). ospub16-1 could grow, but ospub16-2 (−/−) was lethal. As to this, there might be two reasons. The large DNA fragment deletion in ospub16-2 genome caused earlier termination of protein translation than that in ospub16-1, which might be responsible for the lethality of the ospub16-2 mutant. It has been reported that, in addition to causing frameshift mutations, CRISPR/Cas9-mediated sequence alterations in the coding region of the target gene may also result in altered splicing of the respective pre-mRNA, and such altered splicing products also give rise to aberrant protein [53–55]. In the ospub16-1 mutant, some kind of functional mRNA of OsPUB16 might be expressed, perhaps by the altered splicing, which ensures its survival.
OsPUB16 negatively regulates plant water-deficit tolerance by modulating the ‘SAPK9-OsMADS23-OsAOC’ pathway

Crosstalk among phytohormones is crucial for plant growth as well as adjustment to various environments, and previous studies have provided the evidence that ABA interacts with JA signaling to synergistically regulate plant multiple physiological processes [17,20,22]. In this current study, our findings demonstrate that OsPUB16 negatively modulates rice water-deficit tolerance by repressing ABA and JA biosynthesis (Figs 2–4). The increased ABA/JA sensitivity and ABA/JA levels in ospub16 mutants (Fig 4) urge us to ask a critical question whether OsPUB16 modulates the interplay between ABA and JA signaling pathways. If it is, how does it achieve? Here, our findings provide the convincing evidence that OsPUB16 regulates the
link between JA and ABA signaling pathways during plant water-deficit response. First, biochemical evidence demonstrated OsPUB16 interacts with a subset of JA signaling repressors (Fig 5A–5D); meanwhile, OsPUB16 interacts with OsMADS23 (Fig 6A–6D), a transcription factor that promotes ABA signaling by activating ABA-biosynthetic genes OsNCEDs [39]. These physical interactions reveal a direct link between JA and ABA signaling at the molecular level. Second, OsMADS23 acts as an upstream activator to regulate the transcription of JA-biosynthetic gene OsAOC, and SAPK9-mediated phosphorylation on OsMADS23 increases its transcription activity (Fig 8E–8G). Finally, ABA-induced SAPK9-mediated phosphorylation on OsMADS23 interferes with the OsPUB16-OsMADS23 interaction (Fig 9), which reduces OsMADS23 ubiquitination and degradation and therefore enhances its regulatory effect on OsAOC (Fig 9). Therefore, our results strongly demonstrate that OsPUB16 represses ABA and JA biosynthesis by regulating the module ‘SAPK9-OsMADS23-OsAOC’.

Previous efforts have provided clues that MYC2 as well as JAM1 might serve as the linkers of ABA and JA signaling pathways [18,56], and recent studies have shown that the ABA promotes JA biosynthesis through a novel pathway ‘SAPK10-bZIP72-OsAOC’ [22]. Nevertheless, the exact mechanisms underlying the link between ABA and JA signaling still remain to be elucidated, particularly during plant water-deficit response. Here, our study provides the new evidence of the link between JA and ABA signaling. In the ‘SAPK9-OsMADS23-OsAOC’ pathway, ABA promotes the JA biosynthesis to synergistically promote plant water-deficit tolerance.

As key suppressors in JA signaling, JAZ proteins modulate JA signaling through a negative regulatory feedback loop involving MYC2, suggesting a mechanism for the rapid switching on and off of JA pathway in response to a JA pulse [57,58]. In our study, characterization of the physical interactions between OsPUB16 and a subset of JAZ proteins (Fig 5A) may shed light on the molecular basis of the regulation by U-box E3 ubiquitin ligases.

Interestingly, OsPUB16 mediates the ubiquitination and subsequent degradation of JAZ proteins in a JA-dependent manner (Fig 5E–5G), suggesting that OsPUB16 is likely to promote JA response; on the other hand, OsPUB16 represses JA response by inhibiting JA biosynthesis (Fig 4E–4H and 4J). These observations seem to be contradictory, but actually reflect the negative regulatory feedback on the increased JA response. How OsPUB16 finely tunes the JA response switching on and off in the changing environment is a challenging but interesting research, which needs to be explored in the future work.

The phosphorylation status of OsMADS23 modulates its ubiquitination level and stability

The current study elaborates a key module ‘SAPK9-OsMADS23-OsAOC’ regulated by OsPUB16 during plant water-deficit response. In the module, there is a connection between the phosphorylation and ubiquitination of OsMADS23, which acts a central regulator to determine plant water-deficit response. Our findings showed that, compared to the native form, the phosphorylated OsMADS23 (OsMADS23\T20D\S36D) reduced its interaction with OsPUB16, and thus was less ubiquitinated by OsPUB16 (Fig 6E and 6F), demonstrating that phosphorylation of OsMADS23 regulates its ubiquitination. Consistently, phosphorylated OsMADS23 (OsMADS23\T20D\S36D) was more stable, while the dephosphorylated OsMADS23 (OsMADS23\T20A\S36A) was labile than the native form (Fig 6G–6I). This observation was further confirmed by the fact that OsMADS23\T20D\S36D had increased transactivation activity, compared to its native form (Fig 9B). OsPUB16 reduces the ABA and JA biosynthesis via mediating OsMADS23 ubiquitination and degradation, whereas SAPK9 enhances the ABA and JA signaling via mediating OsMADS23 phosphorylation and stabilization. Although
‘SAPK9-OsMADS23-OsAOC’ is identified as a pathway that links ABA and JA signaling pathways, how OsPUB16 precisely regulates the balance between plant growth and environmental adaption through the module still needs to be revealed.

Based on our results, we propose a model to elucidate how OsPUB16 interacts with its partners to modulate the water-deficit response in rice (Fig 10). Under normal growth conditions, SAPK9 is deactivated, and therefore OsPUB16 interacts with OsMADS23, resulting in the OsMADS23 ubiquitination and degradation through the 26S proteasome system, and thus repressing of JA- and ABA-biosynthetic gene expression (Fig 10, left). Under water deficit conditions, rapid ABA accumulation activates SAPK9, which then interferes with OsPUB16-OsMADS23 interaction via the phosphorylation on OsMADS23, leading to enhanced OsMADS23 stability and subsequent ABA and JA biosynthesis, thus promoting ABA- and JA-mediated water-deficit tolerance (Fig 10, right).

Materials and methods

Plant materials and growth conditions

Rice (Oryza sativa ssp. japonica) cv Nipponbare was used in this study. The knockout mutants of OsPUB16 were generated by CRISPR-Cas9 system from Nipponbare. For generating OsPUB16-overexpressing lines, the open reading frame (ORF) of OsPUB16 fusion with GFP (green fluorescence protein) driven under the 35S promoter was cloned into pCAMBIA1301, and then transformed into rice calli by Agrobacterium tumefaciens-mediated transformation [59]. For the complementation test, the OsPUB16 coding sequence driven by its promoter region (2,509 bp) was cloned into the binary vector pCAMBIA1301, and the resulting construct was introduced into ospub16-1 mutant. The seeds of homozygous transgenic plants were used for the research. T-DNA insertion mutants of OsMADS23 and its overexpression lines were used [39]. Plants were grown in the growth chamber or greenhouse with a 14-hour
light (30˚C) /10-h dark (25˚C) cycle (300 μmol photons m⁻²s⁻¹) with 60% humidity. Primers for vector construction and transformant identification are listed in S1 Table.

**Phytohormone sensitivity assessment**

For seed germination inhibition assay, sterilized seeds were sown on 1/2MS medium supplemented with different concentration of ABA or MeJA at 28˚C with a 12-hour photoperiod for germination. Germinated seeds were counted every day. Complete germination was defined as the growth of the coleoptile to a length of 5 mm. For the primary root elongation inhibition assay, uniformly germinated seeds were grown on 1/2MS medium supplemented with 3 μM ABA or 2 μM MeJA for 3 days at 28˚C, and the primary root length of these seeding were measured.

**Water-deficit tolerance and water loss assays**

To evaluate the water-deficit tolerance of different genotypes, plants were grown in pots filled with equal amount of soil and cultivated under normal growth conditions for 3 weeks, with equal irrigation. Then, these plants were exposed to the same severity of soil drying by withholding water for 12 days under the same growth environment. Survival rates were calculated after 5 days of rewatering. For the water loss assay of leaf, the detached leaves from 2-month-old plants were placed at room temperature under dim light, and the fresh weight of leaves was monitored at the indicated time points. Water loss rate (WLR) was calculated from the decrease in weight at indicated time points compared with time zero, based on the following formula: WLR (‰) = (initial weight–final weight) / initial weight × 100%. The average survival rate and water loss rate were calculated from three independent experiments.

**Stomatal observation**

The stomatal movement observation was performed as described previously [39]. Two-week-old seedlings were subjected to light for at least 2 hours to ensure stomata open. Then the fully expended young leaves were floated in the buffer (1 mM MES-NaOH, 20 mM KCl, pH 6.0) with 0 or 30 μM ABA for 3 hours. After treatment, the samples were frozen in liquid nitrogen immediately. Then the stomata were detected with a Hitachi TM3000 scanning electron microscope with a -35˚C cold stage. At least 200 stomata from 5 independent plants were observed in each treatment of each line. Three independent experiments were repeated.

**RNA extraction and quantitative real-time PCR (qRT-PCR) analysis**

Total RNA was extracted from 2-week-old seedlings using TRIzol reagent (Takara). The qRT-PCR analysis was performed with OsActin1 as the internal control. Relative changes in gene expression level were quantified based on three biological replicates via the 2⁻ΔΔCT method [60]. Primers used for expression analysis are given in S1 Table. Three independent experiments were repeated.

**Subcellular localization assay**

The ORF of OsPUB16 fused with GFP was cloned into pCAMBIA1301 (for primers, in S1 Table), and the construct was transformed into 4-week-old Nicotiana benthamiana leaves with an efficient agroinfiltration expression system [61]. After infiltration, N. benthamiana grew at 25˚C for 48–72 hours, and the leaf epidermis was used for detecting GFP fluorescence signals under confocal laser scanning microscope (Leica SP8).
Yeast two-hybrid assay
We used the Matchmaker Gold Yeast Two-Hybrid System (Clontech) to perform the yeast two-hybrid assay, according to the manufacturer’s instructions. The ORF of OsPUB16 was cloned into pGBK7T vector, and JAZs or OsMADS23 was cloned into pGADT7 vector, respectively. Then the corresponding plasmids were co-transformed into the yeast strain Y2HGold for two-hybrid assay. The transfected yeast cells were plated on synthetic defined (SD)/-Leu-Trp (-LW) or SD/-Ade-His-Leu-Trp (-LWHA) medium, and incubated at 28˚C for 2 days. Primers for these constructs are listed in S1 Table.

Bimolecular fluorescence complementation (BiFC) assay
BiFC vectors pFGC-nYFP and pFGC-cYFP [62] were used. The ORF of OsPUB16 was cloned into pFGC-cYFP and OsJAZ9 or OsMADS23 was cloned into pFGC-nYFP. The generated cYFP and nYFP constructs were transformed into Agrobacterium GV3101, respectively, and then co-infiltrated into 4-week-old N. benthamiana leaves with an efficient agroinfiltration expression system [61], with HY5-RFP as a nuclear-localized marker. After infiltration, N. benthamiana grew at 25˚C for 48–72 hours, and YFP and RFP fluorescence signals were visualized using the confocal microscope (Leica SP8). The primers used for these constructs are listed in S1 Table.

Firefly luciferase complementation imaging (LCI) assay
JW771 and JW772 vectors were used, and the LCI assay was performed in N. benthamiana leaves as described previously [63]. Briefly, the ORF of OsPUB16 or OsMADS23 was fused with the N- and C-terminal parts of the luciferase reporter gene LUC (nLUC and cLUC), respectively, to generate the constructs OsPUB16-nLUC and cLUC-OsMADS23. The resulting constructs were transformed into N. benthamiana leaves with the agroinfiltration expression system. After 36 hours, the leaves were sprayed with 0.32-mg/ml D-Luciferin potassium salt in 0.1% (v/v) Triton X-100, and the LUC signal was observed under low-light cooled CCD imaging apparatus (Alliance, UK). The primers used for BiFC assay are listed in S1 Table.

GST pull-down assay
The ORF of OsPUB16 was cloned into pGEX-4T-1 and transformed into DE3 to produce GST-OsPUB16, and OsMADS23 or OsJAZ9 into pET-28a to produce His-OsMADS23 or His-OsJAZ9 (for primers, in S1 Table). For the pull-down assay, GST-OsPUB16 or GST was incubated with GST Bind Resin overnight at 4˚C for 2 hours, and then purified His-OsMADS23 or His-OsJAZ9 was added for incubation overnight. The beads were washed with pull-down buffer for 3 times, and then the bounded proteins were finally eluted. The pulled-down proteins were analyzed by immunoblot analysis with the anti-His antibody (ProteinTech, 66005-1-Ig) and anti-GST antibody (ProteinTech, 66001-2-Ig), respectively.

Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) analysis
The EpiQuik Plant ChIP Kit (Epigentek) was used for ChIP assays. Briefly, 10-day-old OsMADS23-GFP seedlings were harvested and fixed in 1% formaldehyde, and chromatin was isolated from 2 g crosslinked leaves. Isolated chromatin was sonicated for DNA fragmentation ranging from 200 to 500 bp. Subsequently, the DNA/protein complex was immunoprecipitated with anti-GFP antibody (Abm, M20004) or IgG. Then the immunoprecipitated DNA was purified with phenol/chloroform after reverse crosslinking and proteinase K treatment.
The immunoprecipitated DNA was used for qPCR analysis. The primers for ChIP-qPCR used are listed in S1 Table.

**Semi-endogenous ubiquitination assay**

Briefly, the total proteins were extracted from 10-day-old wild type or OsPUB16-OX plants by using the NP40 lysis buffer (Beyotime Biotechnology, P0013F). Then the protein extracts were incubated with GST-OsMADS23, GST-OsMADS23$_{T20D \ S36D}$ or His-OsJAZ9 for 5 hours, in the presence of 50 μM MG132, and then the immunoprecipitation was performed with anti-GST or anti-His beads. The immunoprecipitated proteins were detected with anti-Ubi antibody (Santa Cruz Biotechnology, sc-8017) and anti-GFP antibody, respectively. The immunoblot analysis of Histone H3.1 (H3.1) with anti-H3.1 antibody (Solarbio, K800007P) was used as a loading control of protein lysates. Three independent experiments were repeated, and representative pictures were shown.

**Cell-free degradation assay**

Cell-free degradation assays were performed as previously described [64]. Two-week-old seedlings were used to extract protein in the buffer (25 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl$_2$, 5 mM DTT). Purified recombinant GST-OsMADS23, OsMADS23$_{T20A \ S36A}$, or His-OsJAZ9 was incubated with equal amount of protein extracts for different time, in the presence of 10 mM ATP. Anti-His antibody (Proteintech, 66005-1-Ig) or anti-GST antibody (Proteintech, 66001-2-Ig) were used to detect recombinant protein level by immunoblot analysis.

**Accession numbers**

*OsPUB16* (LOC_Os01g66130), *OsMADS23* (LOC_Os08g33488), *SAPK9* (LOC_Os12g39630), *OsAOC* (LOC_Os03g32314), *OsLOX3* (LOC_Os03g49260), *OsJAZ5* (LOC_Os04g32480), *OsJAZ6* (LOC_Os03g28940), *OsJAZ7* (LOC_Os07g42370), *OsJAZ8* (LOC_Os09g26780), *OsJAZ9* (LOC_Os03g08310), *OsJAZ10* (LOC_Os03g08330), *OsJAZ11* (LOC_Os03g08320), *OsJAZ12* (LOC_Os10g25290), *OsJAZ13* (LOC_Os10g25230).

**Supporting information**

**S1 Fig. Alignment of amino acid sequence of OsPUB16 with other U-box E3 ubiquitin ligases.** (A) The phylogenetic analysis of a subset of PUB proteins from different plants. (B) Alignment of OsPUB16 with other plant U-box proteins. The solid red line indicates the conserved U-box domain, and solid block line indicates the ARM repeats domain. The accession numbers are as follows: OsPUB16 (LOC_Os01g66130.1), OsPUB15 (LOC_Os08g01900.1), SB03g37980 (Sobic.003G379800.1), SB07g011400 (Sobic.007G011400.2), ATPUB4 (AT2G23140.1), SIU-box9 (Solyic01g014230.2.1), SIU-box62 (Solyic12g100000.1.1), MDP000221363, MDP0000411998, StPUB40 (PGSC0003DMT400046296), GrPUB55 (Gotai.008G079700.1), GrPUB53 (Gorai.007G219900.1). (C) Schematic representation of the U-box (amino acids 269 to 332) domain and the ARM repeats near the C-terminus in OsPUB16 protein.

**S2 Fig. The spatio-temporal expression profile of OsPUB16 analyzed using the PlaNet Browser.**

(TIF)
S3 Fig. Morphological phenotypes of the ospub16 mutants and OsPUB16 overexpression lines. (A) Schematic presentation of the gene structure of OsPUB16 and CRISPR-cas9 editing site. White boxes: untranslated regions; Black boxes: exons; black line: intron. TGG: PAM (protospacer adjacent motif). The CRISPR-cas9 target site and mutation were shown in homozygous mutants of ospub16. The letter in red represents the nucleotide insertion, and the red dots represent nucleotide deletion. (B) Frameshift mutations in ospub16 mutants in (A) resulting in early termination of protein translation. (C) Two-week-old wild type (WT, Nip) and ospub16 mutants. Scale bars, 2 cm. (D) Shoot length measurement for 2-week-old seedlings. Data are means ± SD (n = 10). (E) Three-month-old WT and ospub16 mutants. Scale bars, 10 cm. (F) Plant height measurement for 3-month-old plants. Data are means ± SD (n = 10). (G) Analysis of OsPUB16 by quantitative real-time PCR analysis. The transcript levels in wild type were defined as “1”. Error bars indicate SD (n = 3 biological replicates). OsActin1 was used as the internal control. (H) Analysis of OsPUB16 by semi-quantitative PCR analysis. The red arrows are the positions of primers for quantitative real-time PCR and semi-quantitative PCR analysis. (I) Four-month-old WT and OsPUB16 overexpression (OX-4, OX-10) plants. Scale bar, 10 cm. (J) Expression analysis of OsPUB16 independent OsPUB16 overexpression transgenic lines (OX-4, OX-10). The transcript level in the wild type (Nip) was defined as “1”. Data are means ± SD (n = 3). (K) Plant height measurement for 4-month-old plants. Data are means ± SD (n = 10). In (D), (F), (G), (I) and (K), the significant difference between transgenic plants and wild type was determined by Student’s t test. *p < 0.05, **p < 0.01 or ***p < 0.001.

(TIF)

S4 Fig. OsPUB16 expression levels in WT, ospub16-1 mutant and complementary lines (C-1, C-2). (A) Construction of the complementary vector. The coding sequence of OsPUB16 driven by its own promoter was cloned into pCAMBIA1301. (B) Analysis of OsPUB16 by quantitative real-time PCR analysis in WT, ospub16-1 mutant and complementary lines. The transcript levels in WT were defined as “1”. Error bars indicate SD (n = 3 biological replicates). OsActin1 was used as the internal control.

(TIF)

S5 Fig. OsPUB16 mediates ABA and JA sensitivity indicted by seed germination inhibition. (A, C) Seed germination of WT and ospub16-1 mutant on 1/2MS medium without or with different concentrations of ABA (A) or MeJA (B) at 4 DAG (day after germination). Bars = 1 cm. (B, D) Seed germination rate on 1/2MS medium without or with 5 μM ABA (J) or 3 μM MeJA (L) at 4 DAG. Data are means ± SD with biological triplicates (n = 3, each replicate containing 50 seeds). In (B) and (D), the significant difference between the treated and untreated plants was determined by Student’s t test. *p < 0.05.

(TIF)

S6 Fig. The cell-free degradation assay for recombinant His-OsJAZ9 in the protein extracts from wild type (WT) and ospub16-1 mutant. (A) Representative pictures showing the cell-free degradation assay for recombinant His-OsJAZ9, in the presence of 10 mM ATP. Recombinant His-OsJAZ9 was detected by anti-His antibody. The Coomassie blue–stained ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (Rbc L) was used as a loading control. (B) Relative recombinant protein levels of His-OsJAZ9 at indicated time points in the cell-free degradation. Data are means ± SD (n = 3, three independent replicate experiments). The significant difference between the treated and untreated was determined by Student’s t test. ns, not significant.

(TIF)
S7 Fig. Yeast two-hybrid assays to test the interaction of OsPUB16 with OsMADS23 (A) or SAPK9 (B). Yeast cells were grown on synthetic defined (SD)/-Leu-Trp (-LW) medium and SD/-Adet-His-Leu-Trp (-LWHA) medium. The AD-T + BD-P53 was used as a positive control, and AD-T + BD-lam as a negative control.

(TIF)

S8 Fig. Yeast two-hybrid assays to test the interaction of OsMADS23 with rice JAZ proteins. Yeast cells were grown on synthetic defined (SD)/-Leu-Trp (-LW) medium and SD/-Ade-His-Leu-Trp (-LWHA) medium.

(TIF)

S9 Fig. SAPK9 interferes with the OsPUB16-OsMADS23 interaction. Confocal images showing the interference SAPK9 with the OsPUB16-OsMADS23 interaction, particularly in the presence of ABA. OsPUB16-cYFP and OsMADS23-nYFP were co-expressed in the leaves of Nicotiana benthamiana with or without the presence of SAPK9. Strong YFP fluorescent was detected when OsPUB16-cYFP and OsMADS23-nYFP were co-expressed, but the fluorescent signals were reduced in the presence of SAPK9-FLAG, not FLAG alone. ABA treatment further reduced the YFP fluorescent signals in the leaves co-expressing OsPUB16-cYFP and OsMADS23-nYFP with SAPK9-FLAG, but not in the leaves containing OsPUB16-cYFP and OsMADS23-nYFP. HY5-RFP was used as a nuclear-localized marker. YFP and RFP fluorescent signals were visualized using the confocal microscope (Leica SP8). YFP, fluorescent channel in yellow and RFP fluorescent channel in red.

(TIF)

S1 Table. List of primers.

(XLSX)

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