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A transposon insertion in the OsUBC12 promoter enhances cold tolerance during germination in japonica rice (Oryza sativa)

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Abstract

Low-temperature germination (LTG) is an important agronomic trait for rice (*Oryza sativa*). *Japonica* rice generally has greater capacity for germination at low temperatures than the *indica* subpopulation. However, the genetic basis and molecular mechanisms underlying this complex trait are poorly understood. Here, we report that *OsUBC12*, encoding an E2 ubiquitin-conjugating enzyme, increases low-temperature germinability in *japonica*, owing to a transposon insertion in its promoter enhances its expression. Natural variation analysis revealed transposon insertion in the *OsUBC12* promoter mainly in the *japonica* lineage. Notably, the genomic region carrying the *japonica OsUBC12* locus (with the transposon insertion) has been introgressed into the modern elite *indica* two-line male sterile lines Y58S and J4155S. Further molecular analysis showed that OsUBC12 may negatively regulate ABA signaling. OsUBC12-regulated seed germination and ABA signaling mainly depend on a conserved active site required for ubiquitin-conjugating enzyme activity. Furthermore, OsUBC12 directly associates with rice SUCROSE NON-FERMENTING 1-RELATED PROTEIN KINASE 1.1 (OsSnRK1.1), promoting its degradation. In contrast to OsUBC12, OsSnRK1.1 inhibits LTG by enhancing ABA signaling. These findings shed light on the possible genomic contributions of introgressions of the *japonica OsUBC12* locus to trait improvements of *indica* rice cultivars and provide genetic reference points for improving LTG in *indica* rice.

Introduction

Seed germination is a complex trait influenced by many genes and environmental conditions [1,2]. Optimal rice (*Oryza sativa*) germination temperature ranges from 25°C to 35°C; temperatures below 17°C cause cold stress, with low germination rates, germination delay, retarded growth and seedling mortality [3,4]. Moreover, low-temperature germinability is a prerequisite for modern direct-seeding cultivation, an alternative to conventional transplanting that effectively reduces rice production costs [5,6]. Although rice is generally sensitive to low temperatures, artificial human selection and cultivation in different geographic regions have given rise to two distinct rice varietal groups with significantly different chilling tolerances [7,8]. *Indica* subspecies are more sensitive to cold stress than *japonica* subspecies [9–11], and most cold-tolerant alleles identified in previous studies belong to *japonica* [12–14].

Ubiquitin (Ub) is a highly conserved 76-amino-acid polypeptide that can be conjugated to target proteins by the sequential action of three classes of enzymes: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin-ligase enzymes [15]. Ubiquitin harbors seven lysine (K) residues: K6, K11, K27, K29, K33, K48 and K63. Polyubiquitin (polyUb) chains are formed when multiple Ub moieties are linked by one of the seven lysine residues in a ubiquitin molecule, or by the N-terminal methionine residue in the form of head-tail linear repeats [16–19]. PolyUb chains exhibit different topologies and are associated with diverse biological functions [20]. K48-linked polyubiquitination, the most common type, triggers the degradation of target proteins by the 26S proteasome [21]. Atypical K63-linked polyubiquitination alters the activity of target proteins, primarily in a proteolysis-independent manner [22].

Much less is known about the other polyUb chain linkages. Ubiquitin-conjugating (UBC) E2 enzymes play important roles in protein ubiquitination by mediating the formation of the polyubiquitin chain and transferring it to the target protein [23]. In general, E2s have a conserved UBC domain that harbors the active-site cysteine residue required for enzyme-ubiquitin thioester bond formation [24]. UBC enzymes are widespread in eukaryotes. Rice has 48 UBC members, although only 39 are predicted to contain cysteine active sites [25].

Studies of plant E2 function are lacking and mainly relate to Arabidopsis (*Arabidopsis thaliana*). Among the 37 UBCs in Arabidopsis, ubiquitin-conjugating enzyme activity has been detected for 17, including AtUBC1, AtUBC2 and AtUBC32 [26,27]. Further functional analyses reveal that AtUBCs play diverse roles. For instance, the double loss-of-function Arabidopsis mutant *atubc1-1 atubc2-1* shows a dramatically reduced number of rosette leaves and an early-flowering phenotype [28]. AtUBC32 regulates brassinosteroid (BR)-mediated salt tolerance [29], while AtUBC27 modulates ABA signaling and drought tolerance by promoting the degradation of the ABA co-receptor ABA INSENSITIVE 1 (ABI1) [30]. UBC13 is the only known E2 that can catalyze K63-linked polyubiquitination, which is closely related to iron metabolism [31], auxin signal transduction [32], and low-temperature and pathogen stress responses [33] in Arabidopsis. Overall, however, knowledge regarding E2s in plants is limited, with little in-depth analysis of the biological functions and regulatory mechanisms of E2s having been performed in rice and other crops.

The phytohormone abscisic acid (ABA) plays pivotal roles in seed germination. Accordingly, mutation or overexpression of genes involved in ABA biosynthesis or signaling often results in abnormal germination phenotypes [34–36]. For example, a loss-of-function mutation in the 14-3-3 family gene *GF14h* enhances ABA signaling and reduces seed germination rate in rice [37]. *Seed Dormancy 6* (*SD6*), encoding a basic helix-loop-helix (bHLH) transcription factor in rice, influences seed dormancy and germination by directly regulating the ABA catabolism gene *ABA8OX3* [38]. Moreover, we identified a series of regulatory factors controlling rice seed germination and dormancy by large-scale screening of mutant rice populations. Notably, the majority are related to ABA accumulation or signaling [39–45].

The core components of the ABA signaling network have been identified in recent decades. ABA is perceived by the ABA receptors PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCARs). The binding of ABA to these receptors promotes their interaction with the subclass A type 2C protein phosphatases (PP2Cs), which releases the SNF1-related protein kinase 2s (SnRK2s), members of the plant serine/threonine protein kinase family [46]. SnRK2s activate basic leucine zipper (bZIP) transcription factors, including ABSCISIC ACID INSENSITIVE 5 (ABI5), to regulate the expression of ABA-responsive genes such as *RAB21* [47–49]. It's also reported that the related SnRK1.1 positively regulates ABA signaling in Arabidopsis; accordingly, its overexpression causes ABA hypersensitivity [50].

In this work, we examined *indica-japonica* rice chromosome segment substitution lines (CSSLs) and identified a transposon insertion in the *japonica OsUBC12* promoter that activates *OsUBC12* expression and increases cold tolerance during germination. Natural variation analysis revealed that the transposon in the *OsUBC12* promoter is found mainly in the *japonica* lineage and is absent in wild and *indica* accessions. Notably, the *japonica OsUBC12* locus harboring the inserted transposon has been introgressed into the modern elite *indica* two-line male sterile lines Y58S and J4155S. Molecular and biochemical analyses showed that OsUBC12 may negatively regulate ABA signaling by promoting the proteasomal degradation of OsSnRK1.1, thus accelerating LTG. These results broaden our understanding of the regulatory functions of rice E2 UBC enzymes involved in ABA signaling and LTG, and provide avenues to improve the LTG of *indica* rice via molecular breeding.

Results

A transposon insertion in the *OsUBC12* promoter promotes seed germination at low temperature

To identify genes regulating low-temperature germination, an advanced mapping population of CSSLs [51] derived from a cross between IR64 (*Oryza sativa* ssp. *indica*, cold-susceptible) [52] and Koshihikari (*Oryza sativa* ssp. *japonica*, cold-tolerant) [53] was generated and subjected to germination tests at two different immersion temperatures: 15 °C for 60 h and 30 °C for 24 h. Among the 36 CSSLs with Koshihikari introgression in an IR64 background, three (SL2116, SL2117 and SL2130) attracted our attention, as they consistently exhibited the highest increases in germination rates at both 15 °C and 30 °C when compared with IR64 (Fig. S1a, b).

The introgression segments on chromosome 9 of Koshihikari in SL2130 contained a previously identified bZIP transcription factor-coding gene, *bZIP73* (*LOC_Os09g29820*). It has been reported that the *japonica* allele of bZIP73 ($bZIP73^{Jap}$) could improve rice cold stress tolerance [8]. Therefore, the more vigorous LTG of SL2130 may be partially attributable to the introgression of $bZIP73^{Jap}$. Interestingly, the Koshihikari introgression segments of SL2116 and SL2117 include overlapping regions of Chr. 5 (Fig. S2a, b), implying that these introgression segments, and especially the overlapping section, might control low-temperature germination.

To determine which genes contribute to the LTG trait, we carried out fine-mapping for a population obtained by crossing SL2117 and IR64 (Fig. S2c). Genetic analysis showed that the LTG phenotype of SL2117 segregated as a semi-dominant trait (Fig. S2d). We generated a segregating F_2 population to screen for fixed homozygous recombinant individuals using twelve molecular markers covering the target region (specific primers are listed in Supplemental Table S1). Finally, progeny phenotyping of fixed homozygous recombinant individuals narrowed the candidate gene to a ~40-kb region between markers M5-22.59 and M5-22.63, containing five predicted open reading frames (ORFs) (Fig. 1a, upper panel). Notably, the locus *LOC_Os05g38550*, encoding an E2 ubiquitin-conjugating enzyme OsUBC12, is localized to this region, and the -542 site of its promoter was inserted a transposon (LOC_Os05g38540; about 6 kb) in *japonica* Koshihikari background compared to that in *indica* IR64 (Fig. 1a, bottom panel). In the ORF,

only a T/C synonymous mutation without changing the amino acid residue was detected between Koshihikari and IR64 (Fig. S3). We therefore hypothesized that the transposon insertion in *pOsUBC12*^{Koshihikari} may affect the expression level of *OsUBC12*, and thus cause the change of germinability.

To test our hypothesis, we examined the transcription level of *OsUBC12* in the SL2116 and SL2117 lines. Compared to IR64, the transcription of *OsUBC12* was significantly higher in SL2116 and SL2117 (Fig. 1b). We then performed a dual-luciferase reporter assay to elucidate whether the transposon insertion could change the transcriptional activity of the *OsUBC12* promoter. We cloned *pOsUBC12*^{IR64} (2 kb) or *pOsUBC12*^{Koshihikari} (7.9 kb, containing the inserted transposon) to drive firefly luciferase, and used Renilla luciferase as an internal reference for transfection efficiency (Fig. S4). Compared with the rice protoplasts transfected with *p35S:REN–pOsUBC12*^{IR64}*:LUC*, those transfected with *p35S:REN–pOsUBC12*^{Koshihikari}*:LUC* displayed significantly increased promoter activity (greater LUC/REN ratios) (Fig. 1c). These results confirm that the insertion of the transposon in *pOsUBC12*^{Koshihikari} could up-regulate the expression of *OsUBC12.* The Germination tests of SL2116 and SL2117 lines also showed that they had higher germination rates than the IR64 control both at low temperature (15 °C) and at 30 °C (Fig. 1d, S5a), with higher relative germination fold changes at 15 ° C (Fig. 1e).

To further determine whether OsUBC12 influences low-temperature germination, we generated *OsUBC12* homozygous knockout mutants (*osubc12-2*, *osubc12-3*, and *osubc12-10*) using a clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) system in the Kongyu 131 (KY131) background (a *japonica* cultivar from a cold region). The *osubc12-2* mutant contained a 3-bp deletion in the *OsUBC12* coding region, leading to a premature stop codon (early termination, 38/170 amino acids), whereas the *osubc12-3* and *osubc12-10* mutants contained a 1-bp insertion and a 7-bp deletion in the coding region, resulting in a frameshift in the ORF and an early termination codon, respectively (Fig. S6). When we tested the germination of these knockout mutants at 15 °C and 30 °C, the *osubc12* mutant lines showed clearly decreased germination rates at low temperature (15°C) (Fig. 1f), but only a slight reduction in germination at 30°C (Fig. S5b), confirming that OsUBC12 preferentially accelerates seed germination at low temperature, but fine-tunes seed germination at 30 °C. These results suggest that the transposon insertion in the *OsUBC12* promoter might promote low-temperature germination.

Natural variations and potential application of *OsUBC12* in rice

To study the natural variation in *pOsUBC12* among rice populations, we investigated a rice diversity panel consisting of 69 cultivated accessions selected to represent nine rice subpopulations from the 3,000 rice genomes and six wild accessions [54]. We assessed the presence/absence of the transposon in 62 accessions and found that it was absent in all wild and *indica* accessions, but show high frequency in *japonica* accessions (14/16), suggesting that the transposon insertion occurred in the *japonica* lineage (Fig. 2a). The fixation index (F_{st}) among these subpopulations was 0.7798, suggestive of significant genetic divergence among them (Fig. 2a).

Hybridization between different rice groups has been a common practice in rice breeding, and genomic loci conferring favorable agronomic traits are often introgressed across different rice subgroups [55-57]. Several recent studies have identified extensive introgressions between modern GJ (Geng/*japonica*) and XI (Xian/*indica*) rice varieties during modern rice breeding [58,59]. Since two-line hybrid rice has been used for large-scale grain production in China, benefiting from the continuous improvement of two-line male sterile lines, we checked for the transposon insertion in eight representative two-line male sterile lines, along with IR64 and Koshihikari as negative and positive controls. Nongken 58S is the first two-line male sterile line developed from *japonica* rice; Peiai 64S is the first commercialized *indica* two-line male sterile line, which was transferred from Nongken 58S and then transferred to the second generation of the main *indica* two-line male sterile line Guangzhan 63S; Zhu 1S is a two-line male sterile line from *indica* rice, which was transformed into Xiangling 628S and Longke 638S and finally developed into the modern elite *indica* two-line male sterile lines Y58S and Jing 4155S [60,61]. The results showed that the transposon insertion normally exits in two *japonica* lines, Koshihikari and Nongken 58S (Fig. 2b). Interestingly, the *japonica OsUBC12* locus (transposon insertion) has been introgressed into Y58S and Jing 4155S, from Nongken 58S, Zhu 1S, Peiai 64S, Guangzhan 63S, Xiangling 628S, Longke 638S (Fig. 2b), indicating that this *japonica*-derived locus (harboring the transposon insertion) has been introgressed into modern cultivars of an *indica* genomic background, possibly through artificial selection to improve the LTG of these *indica* cultivar. Furthermore, a simulated rice direct seeding experiment at low temperature (18 °C) at soil depths of 1, 2 and 3 cm showed that the seedlings of introgression lines SL2116 and SL2117 emerged faster than those of IR64, while *osubc12* mutants emerged more slowly than WT (KY131) seedlings (Fig. 2c). These results suggest that *japonica OsUBC12* locus introgressions into *indica* might have been used to improve the low-temperature germinability of *indica* rice.

OsUBC12 accelerates low-temperature germination by negatively regulating ABA signaling

To elucidate the molecular mechanism by which OsUBC12 regulates seed germination, we examined differences in gene expression in *osubc12* mutant plants compared to WT using transcriptome deep sequencing (RNA-seq). We identified 671 differentially expressed genes (DEGs) (403 up-regulated and 268 down-regulated) in *osubc12* mutants compared to the WT using a 1.5-fold change in expression as the threshold (Figs. 3a, S7). Among the DEGs, *OsUBC12* was significantly down-regulated in *osubc12* mutants compared with WT (Fig. 3b), supporting the reliability of the transcriptome data. Moreover, many ABA-related genes showed differential expression between WT and *osubc12* mutants, such as *OsHVA22* (LOC_Os09g27730), *OsABCG25* (LOC_Os05g48180), *OsMLP423* (LOC_Os04g39150), *OsAO2* (LOC_Os03g58372), *OsRab17* (LOC_Os11g26570), *OsGL1-3* (LOC_Os06g44300), *OsABI5* (LOC_Os01g64000) and *OsRAB21* (LOC_Os11g26790) (Fig. 3b). Of the ABA-related genes, *OsABI5* functions as a negative regulator of seed germination [62], and *RAB21* is a commonly used marker gene for ABA response [63]. We therefore examined the expression of *OsABI5* and *OsRAB21* in WT and *osubc12* mutants by qRT-PCR analysis. Both *OsABI5* and *OsRAB21* expression was significantly higher in *osubc12* mutants compared to WT, consistent with the RNA-seq data (Fig. 3c).

We also studied the effect of ABA on the germination of WT and *osubc12* mutant seeds by quantifying germination rates in response to 0, 1 or 2 μM ABA (Fig. 3d-f). In the absence of ABA, the *osubc12* mutant lines exhibited delayed germination (Fig. 3d). Treatment with 1 or 2 μM exogenous ABA produced a more pronounced inhibitory effect on *osubc12* mutants than on WT (Fig. 3e, f). To better quantify the ABA sensitivity of WT and *osubc12* mutants, we analyzed their ABA-mediated germination inhibition rates. After 1 or 2 μM ABA treatment, the germination inhibition rate was significantly higher in *osubc12* mutants in WT, indicating that *OsUBC12* knockdown increases ABA sensitivity (Fig. 3g, h). Taken together, these findings indicate that knockdown of *OsUBC12* enhances ABA signaling, and thus that knockdown of *OsUBC12* enhances ABA signaling.

OsUBC12's role in regulating LTG mainly depends on its conservatived ubiquitination function

Sequence analysis showed that OsUBC12 contains a highly conserved UBC domain harboring an active-site cysteine residue (Fig. S8). In general, the active site cysteine residue is required for enzyme-ubiquitin thioester bond formation [24]. To determine whether OsUBC12 has ubiquitin-conjugating enzyme activity and its active site, we mutated its active-site cysteine residue to alanine (OsUBC12^{C92A}) and performed an *in vitro* ubiquitin thioester formation assay. In the presence of the E1 and Ub, OsUBC12-His generated abundant poly-Ub conjugates (Fig. 4a, lane 4), while the mutated OsUBC12^{C92A}-His variant did so very slightly (Fig. 4a, lane 5). These results indicate that OsUBC12 has ubiquitin-conjugating enzyme activity, which requires the conserved cysteine residue in its UBC domain.

To analyze the relationship between OsUBC12-mediated ubiquitination and its biological function, we produced transgenic rice expressing *p35S:OsUBC12* (*OsUBC12-OE*) or *p35S:OsUBC12^{C92A}* (*OsUBC12^{C92A}-OE*), using enhanced green fluorescent protein (EGFP) as a selectable marker for germination assays at 15 °C and 30 °C. Fluorescence screening (Figs. S9a, b) and RT-qPCR (Fig. 4c) confirmed the overexpression of *OsUBC12* and *OsUBC12^{C92A}* in three independent T_3 *OsUBC12-OE* lines (*OsUBC12-OE1–3*) and T_3 *OsUBC12^{C92A}-OE* lines (*OsUBC12^{C92A}-OE1–3*), respectively. Compared with WT, *OsUBC12-OE* lines showed early germination at 15 °C. In contrast, the germination of *OsUBC12^{C92A}-OE* lines at 15 °C was more similar to that of WT (Fig. 4b), whereas only a slight effect was observed at 30 °C (Fig. S5c). Moreover, *OsABI5* and *OsRAB21* expression was significantly lower in *OsUBC12-OE* lines compared to WT. However, the reduction was less pronounced in the *OsUBC12^{C92A}-OE* lines (Fig. 4d, e). Additionally, *OsUBC12-OE* lines exhibited early germination in the absence of ABA (Fig. 4f, g) and were less sensitive to ABA compared to WT. In contrast, *OsUBC12^{C92A}-OE* lines showed decreased insensitivity combining ABA sensitivity phenotypes (Fig. 4h, i) and germination inhibition rate analysis (Fig. 4j, k). These results suggest that the conserved ubiquitination function of OsUBC12 is required to regulate LTG and ABA responses in rice.

OsUBC12 interacts with OsSnRK1.1

To further understand the molecular regulatory mechanism of OsUBC12 protein, we attempted to identify its ubiquitination targets, using OsUBC12 as bait to screen a yeast two-hybrid (Y2H) library generated

from rice seed cDNA, and isolated 67 candidate clones. Expressed sequence tags (ESTs) from partial candidate genes encoding proteins are listed in Supplemental Table S2. Among them, RING-BOX1 (OsRBX1), OsSnRK1.1, OsUBC12 and OsWRKY42 were verified in a point-to-point yeast two-hybrid system. Among the different combinations tested, only yeast cells co-expressing *pGBD-OsUBC12* and *pGAD-OsSnRK1.1* grew well on screening medium (QDO) and showed α-galactosidase activity (Fig. 5a), indicating that OsUBC12 interacts with OsSnRK1.1 in yeast cells.

We performed an *in vitro* pull-down assay to validate the interaction between OsUBC12 and OsSnRK1.1. OsSnRK1.1-His, OsUBC12-GST and GST alone were all detected in whole-cell lysates (Input). OsSnRK1.1-His was not detected in the control sample (GST protein alone) but was pulled down by OsUBC12-GST, suggesting that OsUBC12 directly interacts with OsSnRK1.1 (Fig. 5b). We further confirmed the OsUBC12–OsSnRK1.1 interaction *in planta* using firefly luciferase complementation imaging (LCI) (Fig. 5c). Collectively, the three independent assays demonstrate that OsUBC12 directly interacts with OsSnRK1.1 both *in vitro* and *in vivo*.

OsUBC12 mainly catalyzes K48-linked polyubiquitination and promotes OsSnRK1.1 degradation

SnRK1s are serine/threonine protein kinases that influence ABA signaling [64]. The OsUBC12–OsSnRK1.1 interaction prompted us to explore the potential protein modifications occurring between them. We asked whether OsSnRK1.1 phosphorylates OsUBC12, or OsSnRK1.1 is a target of OsUBC12-mediated ubiquitination. To this end, we first performed an *in vitro* kinase assay using Phos-tag Biotin BTL-104, finding that OsSnRK1.1 had auto-phosphorylation activity, but did not phosphorylate OsUBC12 (Fig. 6a); this suggests that OsSnRK1.1 might be a target of OsUBC12-mediated ubiquitination. However, it should be noted that E2s exert different effects on target proteins by mediating different polyubiquitination modifications [23].

We then explored the type of polyubiquitination mediated by OsUBC12. We detected polyUb conjugates when using a typical Ub or a Ub-K63R variant that lacks K63 (Fig. 6b, lanes 2 and 3), but not a Ub-K48R variant lacking K48 (Fig. 6b, lane 4), indicating that OsUBC12 mainly catalyzes K48-linked polyubiquitination. Therefore, we examined whether OsUBC12 mediates the proteasomal degradation of OsSnRK1.1. To this end, we performed a cell-free degradation assay with immunoblot analysis to measure OsSnRK1.1-His abundance. Compared to WT extract, the degradation rate of OsSnRK1.1 was significantly decreased in *osubc12* extract (Fig. 6c, upper panel). Notably, OsSnRK1.1 degradation was significantly inhibited the proteasome inhibitor MG132, irrespective of whether protein extracts from WT or *osubc12* mutants were assayed (Fig. 6c, bottom panel). These data indicated that OsUBC12 promotes the degradation of OsSnRK1.1, possibly via the 26S proteasome pathway.

Furthermore, overexpression of *OsUBC12*, but not of a *OsUBC12*^{C92A} mutant, enhanced the degradation of OsSnRK1.1 (Fig. 6d). This provided additional evidence that OsUBC12 promotes OsSnRK1.1 degradation and requires its active-site cysteine residue to do so. We also analyzed *OsSnRK1.1* expression in *osubc12* knockout mutants and transgenic OE lines, and showed that the presence of the

functional *OsUBC12* allele does not significant affect *OsSnRK1.1* transcript levels (Fig. S10a, b), indicating that the regulatory effects of OsUBC12 on OsSnRK1.1 likely occur mainly at the post-translational rather than the transcriptional level.

OsSnRK1.1 functions oppositely to OsUBC12 in controlling LTG and ABA signaling

We then explored the possible role of OsSnRK1.1 in controlling LTG by analyzing the germination rates of *OsSnRK1.1-OE* lines at 15 °C and 30 °C. The *OsSnRK1.1-OE* transgenic lines were examined by fluorescence screening (Fig. S9c) and qRT-PCR (Fig. S10c). Compared with WT, the germination of *OsSnRK1.1-OE* lines was strongly delayed at 15 °C (Fig. 7a) but only slightly delayed at 30 °C (Fig. S5d). Accordingly, *OsABI5* and *OsRAB21* expression levels were higher in *OsSnRK1.1-OE* lines than in WT (Fig. 7b, c). Moreover, ABA sensitivity assays showed that *OsSnRK1.1-OE* lines exhibited delayed germination in the absence of ABA (Fig. 7d) and were more sensitive than WT to ABA (Fig. 7e, f). The germination inhibition rates of *OsSnRK1.1-OE* lines were also significantly higher than in WT after 1 or 2 μM ABA treatment (Fig. 7g, h), indicating that *OsSnRK1.1* overexpression increased seed sensitivity to ABA. These results indicate that OsSnRK1.1 may inhibit LTG by enhancing ABA signaling, a functionality opposite to that of OsUBC12. Taken together, our results suggest that OsUBC12 may negatively regulate ABA signaling by promoting the degradation of OsSnRK1.1, thus accelerating LTG.

Discussion

Although the control of seed germination is important for plant adaptability, knowledge of the genetic basis and molecular mechanisms regulating this trait remains limited. In the present study, OsUBC12 is genetically and molecularly identified as a previously unrecognized master regulator of seed germination, preferentially functioning at 15°C, but performing a fine-tuning at 30°C. We also revealed that OsUBC12-mediated ubiquitination controls LTG by affecting ABA signaling, and provided a genetic reference for improving the low-temperature germinability of *indica* rice.

Arabidopsis encodes 37 E2 ubiquitin-conjugating enzymes (UBCs) [26], many of whose functions have been elucidated. However, rice encodes 48 UBCs [25], and in-depth studies of their functions are lacking. Here, we found that OsUBC12 accelerated seed germination at low temperature. *osubc12* mutants generated via CRISPR/Cas9-mediated genome editing showed considerably decreased germinability under low temperature (15°C) (Fig. 1f), but only slightly slower germination at 30°C (Fig. S5b), implying that OsUBC12 preferentially accelerates seed germination at low temperature, but fine-tunes it at 30°C. Moreover, the expression levels of *OsABI5* and *OsRAB21* were higher in *osubc12* mutants than in WT (Fig. 3c). Knockdown of *OsUBC12* also increased ABA sensitivity (Fig. 3d-h). These findings indicate that OsUBC12-regulated LTG may be associated with enhanced ABA signaling. Furthermore, the results obtained by overexpression analysis of transgenic rice lines showed an agreement with that of *osubc12* mutants, and blocking the 92th cysteine site of OsUBC12 could significantly suppress its regulatory effect on germination (Fig. 4b-k). Our results thus provide evidence that OsUBC12 accelerates LTG by repressing ABA signaling via its conserved ubiquitination function.

Several E2s play specific roles in ABA signaling in Arabidopsis. For example, AtUBC26 forms complexes with the ABA receptors PYR1 and PYL4 to negatively regulate ABA signaling [65], while AtUBC32, AtUBC33 and AtUBC34 negatively regulate ABA-mediated stomatal closure and drought tolerance [66]. Our findings broaden the knowledge of E2-regulated ABA signaling. More importantly, we revealed that OsUBC12 regulates LTG, a key trait in rice, by negatively regulating ABA signaling.

Moreover, E2s directly bind target proteins to regulate specific functions. In Arabidopsis, the E2 conjugase PHOSPHATE 2 (PHO2) directly binds and modulates the stability of the phosphate transporter PHO1 in phosphate homeostasis [67]. In addition, UBC27 promotes the degradation of the ABA co-receptor ABI1 via the 26S proteasome, likely through K48-linked polyubiquitination, to regulate ABA signaling and drought tolerance [30]. Similarly, we demonstrated that OsUBC12 interacts with OsSnRK1.1 *in vitro* and *in vivo* (Fig. 5). OsUBC12 mainly catalyzes K48-linked polyubiquitination and promotes the degradation of OsSnRK1.1, with the latter process requiring the OsUBC12 active-site cysteine residue (Fig. 6). SnRK1s and SnRK2s, core components of ABA signaling, both belong to the plant serine/threonine protein kinase family [46]. In Arabidopsis, SnRK1.1 positively regulates ABA signaling, and its overexpression delays germination and growth [68] and causes ABA hypersensitivity [50]. Consistent with these results, germination of *OsSnRK1.1-OE* seeds was delayed substantially at 15°C (Fig. 7a) and slightly at 30°C (Fig. S5d). *OsSnRK1.1* overexpression enhanced ABA signaling, as evidenced by the increased *OsABI5* and *OsRAB21* expression (Fig. 7b, c) and greater ABA sensitivity of *OsSnRK1.1-OE* lines (Fig. 7d-h). These results indicate that OsSnRK1.1 inhibits LTG by enhancing ABA signaling. Thus, SnRK1.1 plays an opposite role to OsUBC12, suggesting that OsUBC12 may negatively regulate ABA signaling by promoting OsSnRK1 degradation, thereby accelerating LTG.

Notably, we found that a transposon insertion in the *OsUBC12* promoter promotes seed germination at low temperatures (Fig. 1). Natural variation analysis revealed that transposon insertion in the *OsUBC12* promoter mainly occurred in the *japonica* lineage, and that the transposon is absent in wild and *indica* accessions (Fig. 2a). Indeed, the *japonica* subpopulation in general has a greater low-temperature germinability than *indica*, and most alleles with increased low-temperature germinability belong to *japonica* [4,13,14,69,70]. This finding is consistent with the notion that *japonica* is adapted to the low temperatures occurring at high latitudes and higher elevations, while *indica* is adapted to low-latitude regions [11], suggesting that *japonica* varieties contain alleles that would be useful to enhance the low-temperature germinability of *indica*.

Furthermore, extensive introgressions have occurred between modern GJ (Geng/*japonica*) and XI (Xian/*indica*) rice varieties during modern rice breeding, and introgression from GJ into XI was much greater than introgression from XI into GJ [58,59,71]. Interestingly, we demonstrated that the *japonica* *OsUBC12* locus (transposon insertion) has been introgressed into the modern elite *indica* two-line male sterile lines Y58S and Jing 4155S (Fig. 2b). Under direct-seeding conditions at low temperature (18°C), seedlings of the introgression lines SL2116 and SL2117 emerged faster than those of IR64, while *osubc12* mutants emerged slower than WT (Fig. 2c). These results underline the potential applicability of

japonica OsUBC12 in improving the low-temperature germinability of *indica* rice. Our study not only sheds light on the possible genomic contributions of *japonica OsUBC12* locus introgressions to trait improvements of *indica* rice cultivars, but also provides a genetic reference for improving the LTG of *indica* rice.

Taking these results together, we propose a model to explain how OsUBC12 regulates LTG (Fig. S11). According to our model, OsSnRK1.1 functions as a downstream key regulator to enhance ABA responses by upregulating the expression of ABA-signaling-related genes such as *OsABI5* and *OsRAB21*, thus inhibiting LTG. OsUBC12, an E2 enzyme for K48-linked polyubiquitination, recruits and degrades OsSnRK1.1. Compared with *indica* rice, a transposon insertion in the *japonica OsUBC12* promoter activates its expression. Increased OsUBC12 levels further promote the degradation of OsSnRK1.1, thereby weakening OsSnRK1.1-regulated ABA signaling and enhancing the low-temperature germinability of *japonica* rice. Although we revealed natural variations and potential applications of *OsUBC12* in rice, more details regarding its possible molecular mechanism need to be uncovered in the future study. For example, in addition to directly recruiting targets, E2s interact with E3 ubiquitin ligases, which specifically bind substrates [23]. It remains unclear whether specific E3 ligases participate in the OsUBC12-mediated degradation of OsSnRK1.1. Further studies are needed to identify other OsUBC12 ubiquitination targets and interacting partners, which would reveal its substrate diversity and functional specificity. Moreover, given the complex nature of the LTG trait in rice, determining how to use the *japonica OsUBC12* locus to efficiently improve the LTG of *indica* rice remains a major goal.

Methods

Plant materials and growth conditions

The chromosome single-segment substitution line (CSSL) with Koshihikari introgression in IR64 was grown in a growth chamber under a 10 h light/14 h dark cycle. The F_2 population was grown in Hainan province, China (winter), under natural conditions. Transgenic *OsUBC12* and *OsSnRK1.1* plants in the KY131 background were grown in the field at the experimental stations of the Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, in Harbin, Heilongjiang province, China.

Evaluation of germination rate

Germination rate was evaluated as described by Yoshida *et al.* [37] and Fujino *et al.* [3] with minor modifications. Seeds of each rice line were collected at 45 days after flowering, air-dried, and stored at 45 °C for three days to break dormancy. To evaluate the seed germination of the CSSLs, 30 seeds per line were placed on filter paper in a 9-cm Petri dish, 10 mL of distilled water was added, and plates were incubated at 30 °C for 24 h or 15 °C for 60 h under dark conditions to induce germination. To evaluate *OsUBC12* and *OsSnRK1.1* transgenic lines, 30 seeds per line were incubated for seven days at 30 °C or 15 °C under dark conditions. Germination was considered to have occurred when the epiblast was broken and the white embryo had emerged to a certain length [37]. Germinated seeds were counted

and the germination rates (%) were calculated by dividing them with those germinated at 30 °C for 48 h, under which all viable seeds were thought to have germinated.

Cloning of *OsUBC12*

To clone *OsUBC12*, SL2117 was backcrossed with IR64 to construct an F_2 segregating population. Fixed homozygous recombinant plants were screened by molecular markers covering the target genomic region. The target gene was then identified by repeated phenotypic characterization of these fixed recombinants. The primer sequences are listed in Supplemental Table S1.

RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

Total RNA was isolated from seeds using TRIzol reagent and reverse transcription was performed using the ReverTra Ace Kit (Toyobo) according to the manufacturer's instructions. RT-qPCR was employed to measure gene expression levels using the SYBR qPCR Mix kit and a LightCycler® 96 System (Roche). The gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method with *OsGAPDH* (LOC_Os02g38920) or *OsUBQ5* (LOC_Os01g22490) as the internal control. The primers used for expression analysis are shown in Supplemental Table S1.

Transient dual-luciferase (dual-LUC) assays

The $OsUBC12^{IR64}$ (2 kb) or $OsUBC12^{Koshihikari}$ (7.9 kb, containing the inserted transposon) promoter sequence was cloned into pGreenII-0800-LUC, and subsequently transformed into rice protoplasts. The Renilla luciferase (REN) gene directed by the *35S* promoter in the pGreenII 0800-LUC vector was used as an internal control. Firefly LUC and REN activities were measured using the Dual-Luciferase reporter assay kit (Beyotime) and a GloMax 20/20 luminometer (Promega). LUC activity was normalized to REN activity and LUC/REN ratios were calculated. The data presented are the averages of at least three independent replicates.

Plasmid construction and genetic transformation of rice

Knockout *osubc12* mutants were generated by CRISPR/Cas9-mediated genome editing [72]. The *OsUBC12* guide RNA sequence (Supplemental Table S1) was introduced into the CRISPR/Cas9 binary vector pYLCRISPR/Cas9P_{ubi}-H, and the recombinant vector was transformed into *Agrobacterium tumefaciens* strain EHA105-pSOUP for rice genetic transformation. To generate overexpression lines, the coding sequences (CDS) of *OsUBC12* and *OsSnRK1.1* were amplified from *japonica* cv. Nipponbare cDNA. The active-site cysteine residue of *OsUBC12* was mutated by site-directed mutagenesis to generate $OsUBC12^{C92A}$, which was subsequently cloned into modified pCAMBIA2300 with the GFP gene (as the selectable marker) under the control of the *Actin1* promoter. KY131 was used for *Agrobacterium*-mediated transformation. Primers used for gene editing and plasmid construction are listed in Supplemental Table S1.

Natural variations of *OsUBC12*

To determine whether the transposon was present in the promoter, the 1-kb promoter and gene sequence from the Nipponbare genome (containing the transposon insertion) were used as a probe to query against genome assemblies. If the full probe sequence mapped to the genome assembly, the transposon was deemed present in that assembly. In contrast, if the first ~400 bp of the probe sequence were missing from the alignment, the transposon was deemed absent from that assembly. Assemblies with ambiguous alignments were called "NA" (not determined).

RNA sequencing (RNA-seq) analysis

Three independent *osubc12* mutant and wild-type (WT, KY131) seeds were collected 12 h after imbibition for RNA-seq analysis. The extraction and examination of total RNA, library preparation and Illumina sequencing were performed by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using the Illumina HiSeq 2500 platform. The reference genome of the rice cultivar Nipponbare and gene information were downloaded from IRGSP-1.0 (http://rapdb.dna.affrc.go.jp/download/irgsp.html). Differentially expressed genes (DEGs) between samples were defined by DESeq, based on fold change $|\log_2_\text{ratio}| > 1.5$ and false discovery rate-adjusted P value < 0.05. Gene Ontology (GO) enrichment analysis of the DEGs was performed using the GOseq R package based on the Wallenius noncentral hypergeometric distribution, which adjusts for gene length bias in DEGs.

ABA sensitivity assays

Rice seeds were placed on half-strength Murashige and Skoog (½-MS) agar medium containing 0, 1, and 2 μM ABA and incubated at 30 °C. Germination rates were assessed every 12 h for 120 h. The formula for calculating the germination inhibition rate by ABA at designated timepoints is as follows: Inhibition rate (%) = [(Number of germinated seeds at 0 μM ABA – Number of seeds germinated at 1 or 2 μM ABA)/Number of germinated seeds at 0 μM ABA] * 100%.

Ubiquitin-conjugating enzyme activity assays

The E2 ubiquitin-conjugating activity assay was conducted as described by Zhao *et al.* [73] with minor modifications. *OsUBC12* and *OsUBC12*^{C92A} were individually cloned into the pET29b(+) expression vector. The fusion proteins OsUBC12-His and OsUBC12^{C92A}-His were purified at 4 °C and quantified according to the pET System Manual. Buffer, ubiquitin, E1 enzyme, OsUBC12-His or OsUBC12^{C92A}-His and other components were combined in the reaction tube to prepare the reaction system. The reaction was performed at 37 °C for 3 h. Subsequently, *in vitro* ubiquitin thioester bond formation was detected using anti-His and anti-Ub antibodies.

Yeast two-hybrid assays

The *OsUBC12* CDS was cloned into the pGBKT7 (bait) vector, and the recombinant plasmid and pGADT7 (prey) empty vector were co-transformed into the Y2HGold yeast strain to test for self-activation. Subsequently, OsUBC12 was used as the bait protein to screen against a yeast two-hybrid cDNA library, and candidate clones were obtained through sequencing and alignments. The CDS of candidate clones was independently cloned into the pGADT7 vector. These prey plasmids and the *OsUBC12* bait vector were co-transformed, and interactions were verified by a point-to-point yeast two-hybrid system. The yeast cells were selected on SD/-Leu/-Trp (DDO), and interaction was assessed based on their ability to grow on SD/-Leu/-Trp/-His/-Ade (QDO) or SD/-Leu/-Trp/-His/-Ade/+X-α-Gal for three days at 30 °C.

In vitro pull-down assays

The *OsSnRK1.1* CDS was cloned into the pET29b(+) expression vector. The recombinant protein was purified at 4 °C and quantified according to the pET System Manual. The *OsUBC12* CDS was inserted into the pGEX-4T-1 expression vector and expressed in Rosetta (DE3) *Escherichia coli* cells. The target protein OsUBC12-GST was purified with GST resin (GE Healthcare). The pulled-down proteins were eluted and detected by immunoblotting using anti-GST and anti-His antibodies, respectively.

Firefly luciferase complementation imaging (LCI) assays

OsUBC12 and *OsSnRK1.1* were cloned in-frame with the N-terminal and C-terminal fragments of the luciferase reporter gene to generate pCAMBIA1300-*OsUBC12nLUC* and pCAMBIA1300-*OsSnRK1.1*cLUC, respectively. *Agrobacteria* harboring the these constructs were co-infiltrated into *Nicotiana benthamiana* leaves, which were subsequently sprayed with luciferin (1 mM luciferin and 0.01% Triton X-100) and photographed using Chemiluminescence imaging (Tanon 5200) at 72 h after infiltration.

In vitro kinase assays

The purified proteins (OsUBC12-GST + SnRK1.1-His) or (GST + SnRK1.1-His, negative control) were incubated in phosphorylation buffer (25 mM Tris-HCl at pH 7.4, 12 mM $MgCl_2$, 1 mM DTT and 1 mM ATP). The reactions were incubated at 30 °C for 45 min, and then boiled in 1× SDS loading buffer. The phosphorylation signal was detected by Phos-tag Biotin BTL-104 (Wako).

Analysis of polyubiquitination

Ubiquitin and the Ub-K63R and Ub-K48R variants were used as basic components to prepare reaction systems with E1, OsUBC12-His, etc. The polyubiquitination type of OsUBC12 was assessed by immunoblotting using anti-His and anti-Ub antibodies.

Cell-free protein degradation assays

Cell-free protein degradation assays were performed as described by Kong *et al*. [74] with some modifications. Total proteins were extracted from transgenic lines and WT with degradation buffer. Each cell-free protein degradation reaction contained 500 μg total protein and 100 ng of OsSnRK1.1-His

purified from *E. coli* Rosetta (DE3) cells. For the proteasome inhibitor experiments, 100 μM MG132 was added to the total proteins 60 min prior to the cell-free degradation experiment. The reactions were incubated at 22 °C. The mixed solutions were collected at designated time points (0, 0.5, 1, and 3 h) and examined by immunoblotting using an anti-His antibody (Abmart). Results were quantified using ImageJ software (https://imagej.nih.gov/ij/index.html).

Statistics and reproducibility

All experiments were performed at least three times. Numbers (n) of samples or replicates are indicated in figure legends. The data are presented as mean values ± standard error (SE). Data were statistically analyzed using Student's t-test. A difference was considered statistically significant when $*P < 0.05$ or $**P < 0.01$.

Reporting summary

Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

Declarations

Data availability

All data supporting the findings of this study are available in the main text and its supplementary information. Sequence data from this study can be accessed from the Rice Genome Annotation Project website (http://rice.plantbiology.msu.edu/) under the following accession numbers: *OsUBC12* (*LOC_Os05g38550*), *OsSnRK1.1* (*LOC_Os03g17980*), *OsHVA22* (*LOC_Os09g27730*); *OsABCG25* (*LOC_Os05g48180*), *OsMLP423* (*LOC_Os04g39150*), *OsAO2* (*LOC_Os03g58372*), *OsRab17* (*LOC_Os11g26570*), *OsGL1-3* (*LOC_Os06g44300*), *OsABI5* (*LOC_Os01g64000*), *OsRAB21* (*LOC_Os11g26790*), *OsGAPDH* (*LOC_Os02g38920*) and *OsUBQ5* (*LOC_Os01g22490*).

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Author contributions

JF and CZ designed the experiments. CZ, HW, XT and XL performed the experiments. CZ, YH, ZH, HS and JL analyzed the data. CZ and JF wrote the manuscript. JL, JZ and QB revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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Figures

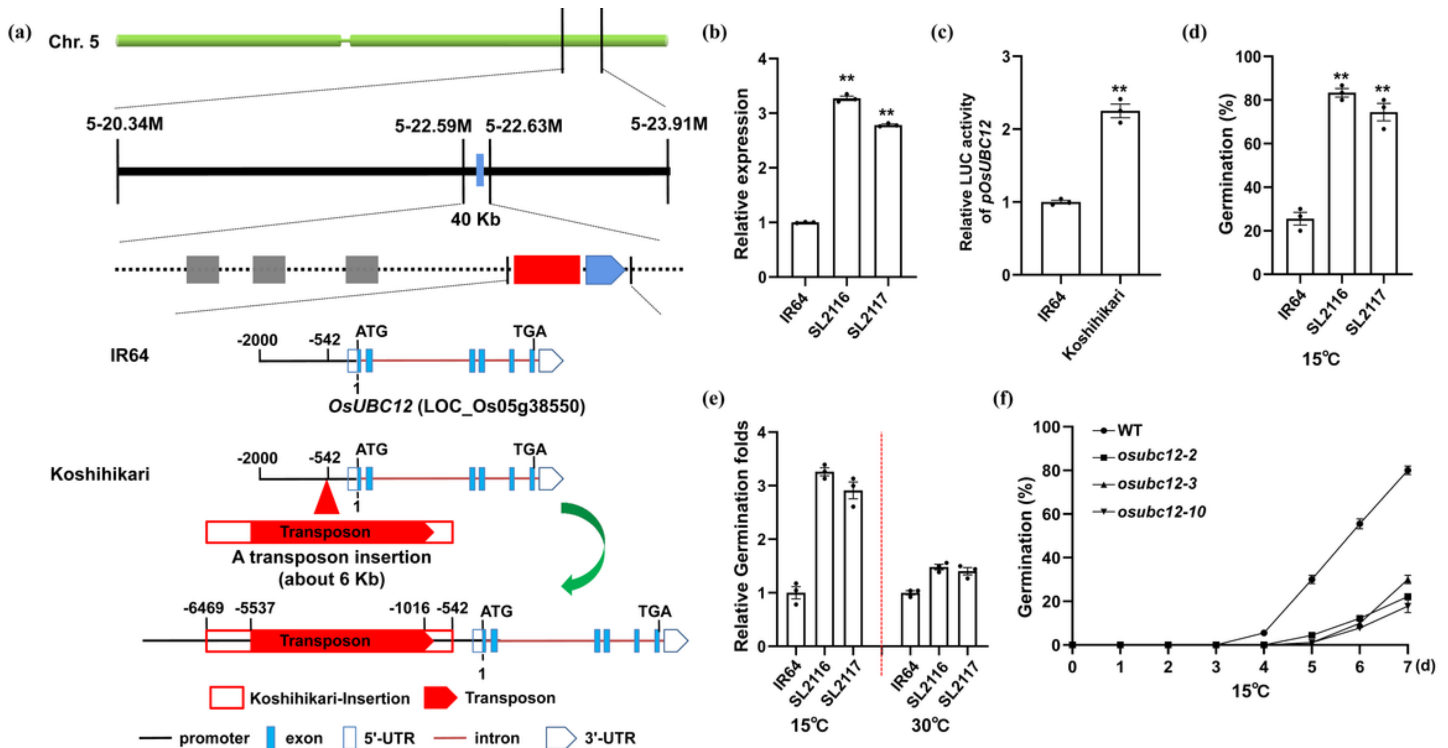

Figure 1

A transposon insertion in the *OsUBC12* promoter promotes seed germination at low temperatures. (a) Map-based cloning of *OsUBC12* and the transposon insertion in the *OsUBC12*^{Koshihikari} promoter as compared to IR64. Specific primers used for mapping are listed in Supplemental Table S1. **(b)** Relative expression of *OsUBC12* in SL2116 and SL2117 seeds. The expression level of the control sample (WT) was set to 1. Values are means ± SE from three individual replicates (n = 3). The housekeeping gene *OsGAPDH* was used as an internal control to normalize the data. **(c)** Relative LUC activity in rice protoplasts transformed with the recombinant *pOsUBC12*^{IR64} or *pOsUBC12*^{Koshihikari} vector. The relative LUC activity of *pOsUBC12*^{IR64} was set to 1. Values are means ± SE from three individual replicates (n = 3). The data were statistically analyzed by Student's t-test ($*P < 0.05$, $**P < 0.01$). **(d)** Germination rates of SL2116 and SL2117 after 60 h at 15 °C. **(e)** The relative germination fold changes of SL2116 and SL2117 after 60 h at 15 °C or 24 h at 30 °C. The germination of IR64 after 60 h at 15 °C or 24 h at 30 °C was set to 1, respectively. **(f)** Time-course germination analysis of *osubc12* mutants at 15 °C. Values are means ± SE from three individual biological replicates (30 seeds per biological replicate).

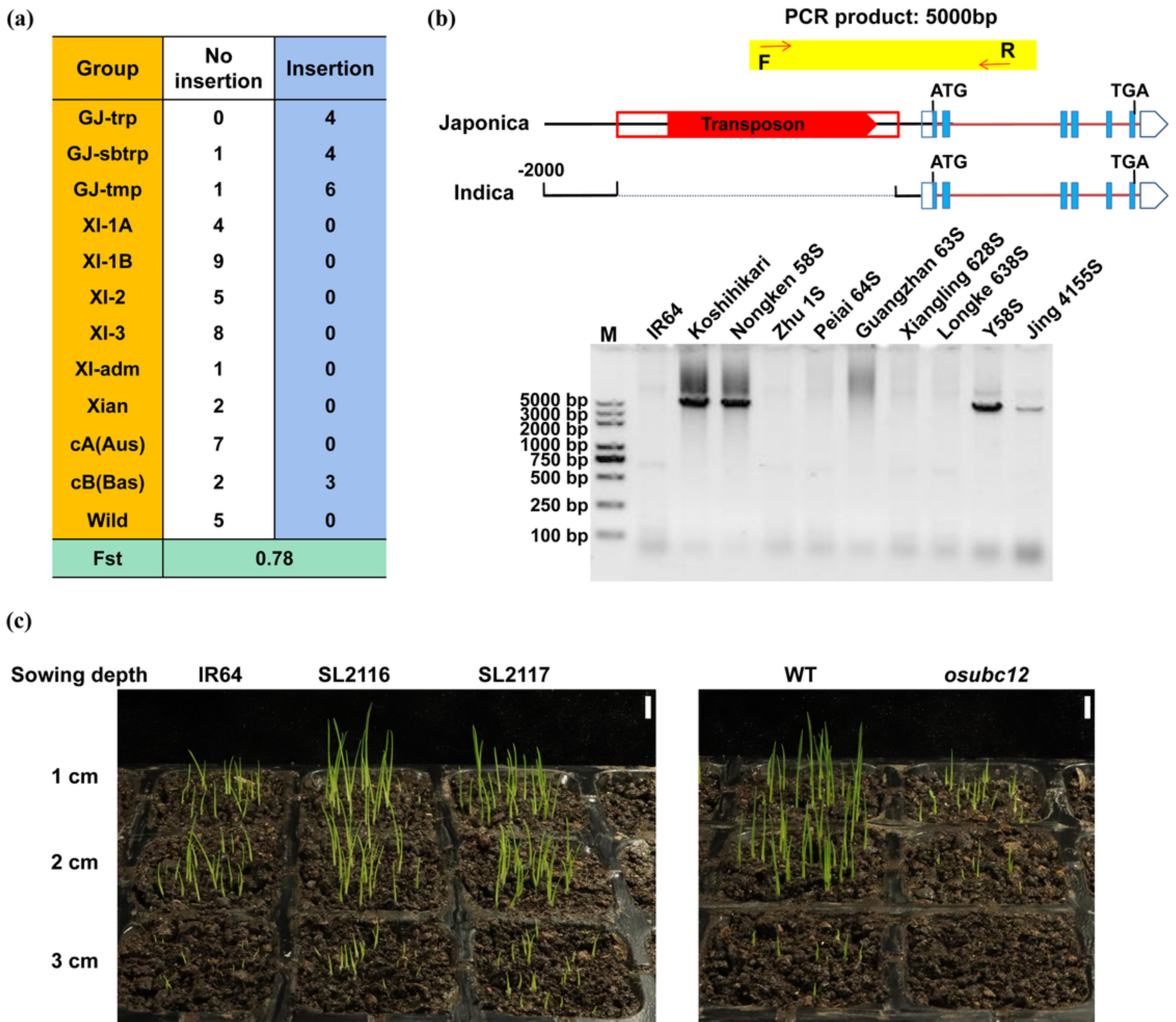

Figure 2

Natural variation and potential application of *OsUBC12* in rice. (a) The presence or absence of the transposon in the *OsUBC12* promoter is shown in a rice diversity panel. **(b)** The presence of the transposon insertion in eight representative two-line male sterile lines. M, DL2000 Plus DNA Marker. To detect the transposon insertion, we used forward (F) and reverse (R) primers located downstream of the transposon gene and upstream of *UBC12*, respectively; the PCR products are sequenced correctly. Specific primers for transposon insertion detection are listed in Supplemental Table S1. **(c)** Seedlings of the introgression lines SL2116 or SL2117, or *osubc12* mutants, after 8 d at 1, 2 or 3 cm sowing depth under direct-seeding conditions at low temperature (18 °C). Scale bars, 1 cm.

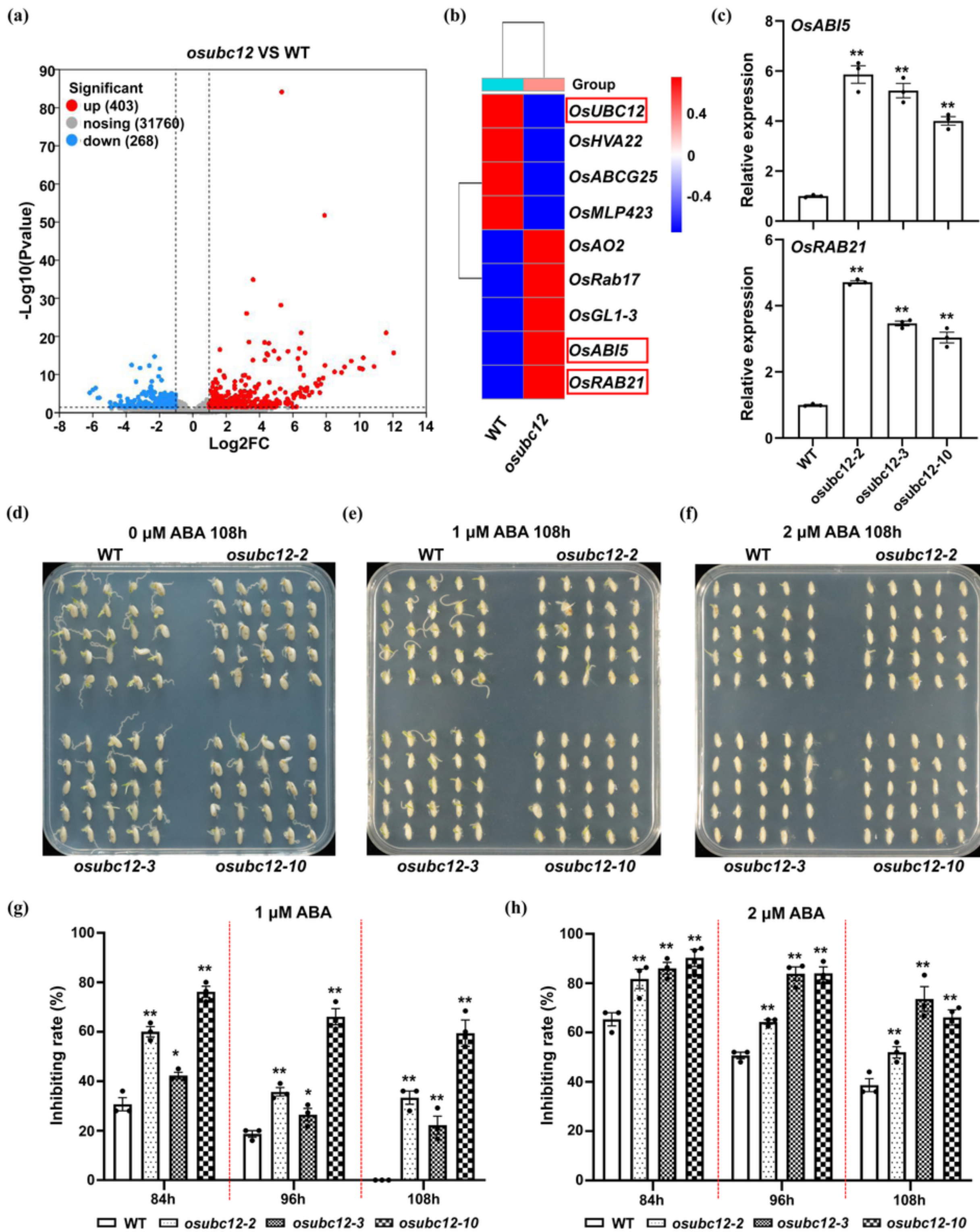

Figure 3

OsUBC12 accelerates low-temperature germination by negatively regulating ABA signaling. (a) Volcano plots of the significantly differentially expressed genes in *osubc12* mutants vs. WT seeds detected by RNA-seq analysis. **(b)** Heat map of microarray expression profiles for ABA-signaling-related genes in WT and *osubc12* mutants. **(c)** Relative expression of *OsABI5* and *OsRAB21* in seeds of *osubc12* mutants. The expression level of the control sample (WT) was set to 1. Values are means ± SE from three

individual replicates (n = 3). The housekeeping gene *OsUBQ5* was used as an internal control to normalize the data. **(d), (e), (f)** Germination performance of WT and *osubc12* mutant seeds after 108 h on ½-MS agar medium containing 0 μM (d), 1 μM (e) or 2 μM (f) ABA. **(g), (h)** The germination inhibition rate of WT and *osubc12* mutant seeds under 1 μM **(g)**or 2 μM **(h)** ABA at three timepoints. Values are means ± SE from three individual biological replicates (25 seeds per biological replicate). The data were statistically analyzed using Student's t-test ($*P < 0.05$, $**P < 0.01$).

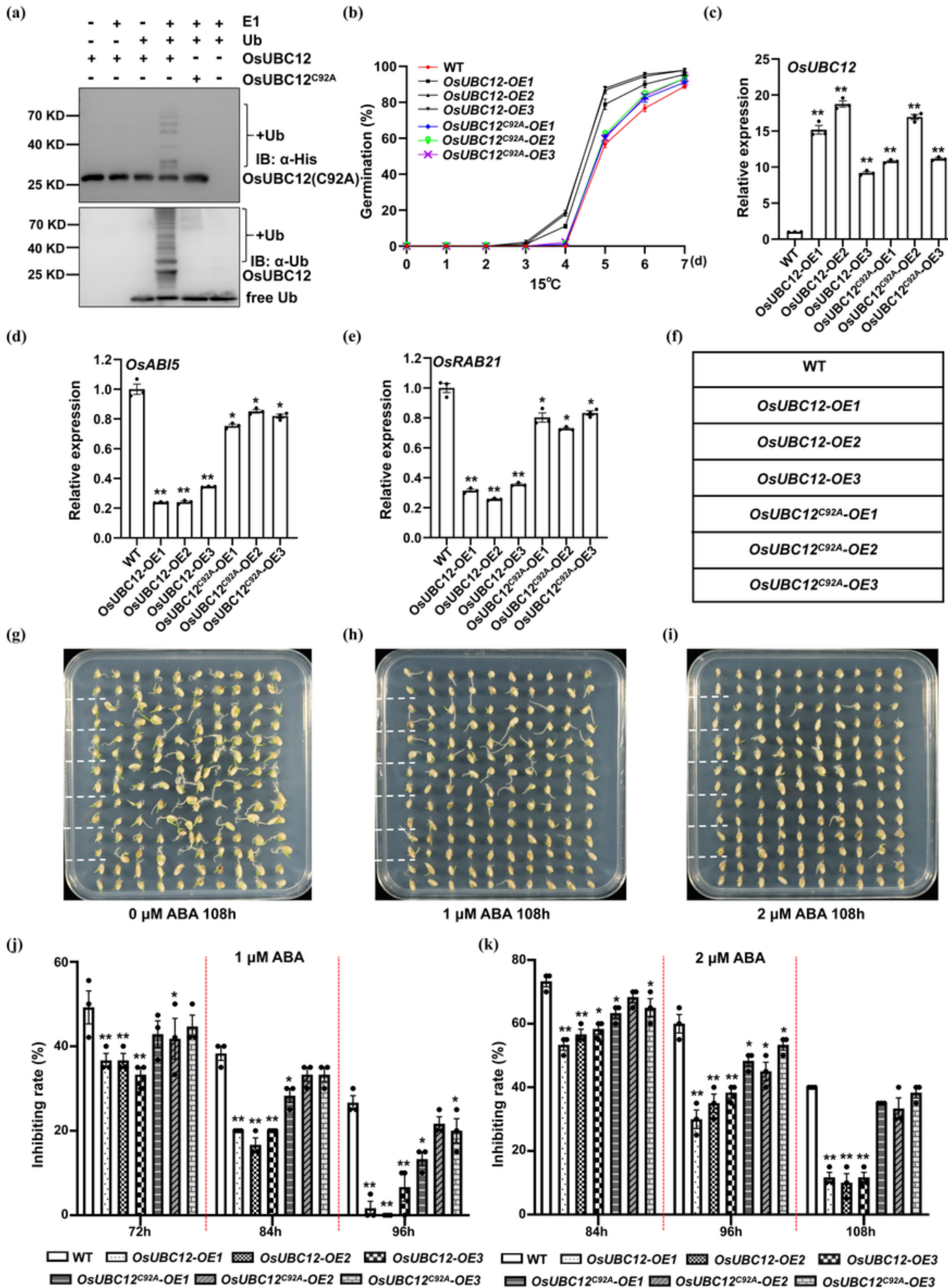

Figure 4

OsUBC12's role in regulating LTG mainly depends on its conservatived ubiquitination function. (a) Formation of the ubiquitin thoester bond was analyzed for OsUBC12 and OsUBC12^{C92A} using anti-His and anti-Ub antibodies. **(b)** Time-course germination analysis of *OsUBC12-OE* and *OsUBC12^{C92A}-OE* lines over seven days at 15 °C. Values are means ± SE from three individual biological replicates (30 seeds per biological replicate). **(c), (d), (e)** Relative expression of *OsUBC12* **(c)**, *OsABI5* **(d)** and *OsRAB21* **(e)** in seeds of *OsUBC12-OE* and *OsUBC12^{C92A}-OE* lines. The expression levels of the control samples (WT) was set to 1. Values are means ± SE from three individua replicates (*n* = 3). The housekeeping gene *OsUBQ5* was used as an internal control to normalize the data. **(f), (g), (h), (i)** Germination performance of *OsUBC12-OE* and *OsUBC12^{C92A}-OE* seeds after 108 h on ½-MS agar medium containing 0 μM **(g)**, 1 μM **(h)** or 2 μM **(i)** ABA. **(j), (k)** The germination inhibition rates of *OsUBC12-OE* and *OsUBC12^{C92A}-OE* seeds under 1 μM **(k)** or 2 μM **(l)** ABA at three timepoints. Values are means ± SE from three individual biological replicates (20 seeds per biological replicate). The data were statistically analyzed using Student's *t*-test (*$*P < 0.05$, **$*P < 0.01$).

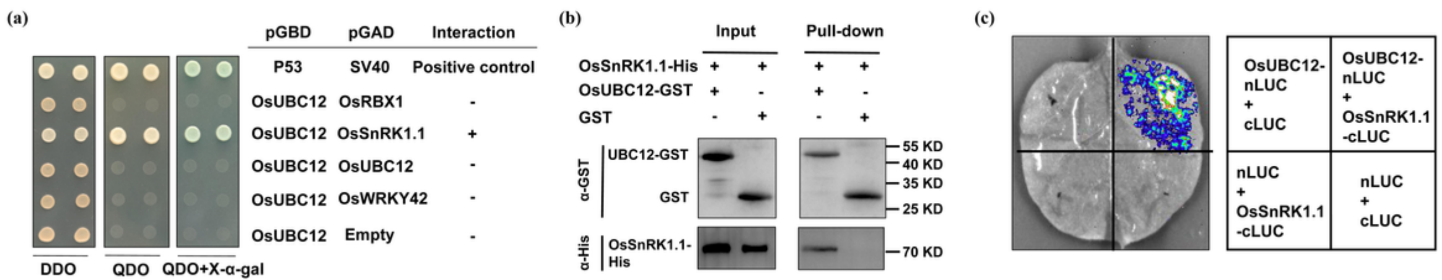

Figure 5

OsUBC12 interacts with OsSnRK1.1. (a) OsUBC12 interacts with OsSnRK1.1 in a yeast two-hybrid system. The yeast cells harboring the bait and prey vectors were selected on SD medium lacking Leu and Trp (DDO). Interaction was assessed based on the growth of yeast cells on selective SD medium lacking Leu, Trp, His and Ade (QDO) or on SD medium lacking Leu, Trp, His and Ade (QDO) but containing X-α-Gal for three days at 30 °C. The pGBD-P53 + pGAD-SV40 combination was used as a positive control. X-α-Gal represents 5-bromo-4-chloro-3-indolyl-α-D-galactoside. **(b)** *In vitro* pull-down assays showing the interaction of OsUBC12 with OsSnRK1.1. His-tagged SnRK1.1 was incubated with immobilized GST or GST-tagged UBC12, and immunoprecipitated fractions were detected using an anti-His antibody. **(c)** Interaction between OsUBC12 and OsSnRK1.1 in luciferase complementation imaging (LCI) assays. Co-transformation of OsUBC12-nLUC and OsSnRK1.1-cLUC led to reconstitution of the LUC signal, whereas no signal was detected upon co-expression of OsUBC12-nLUC and cLUC, nLUC and OsSnRK1.1-cLUC, or cLUC and nLUC.

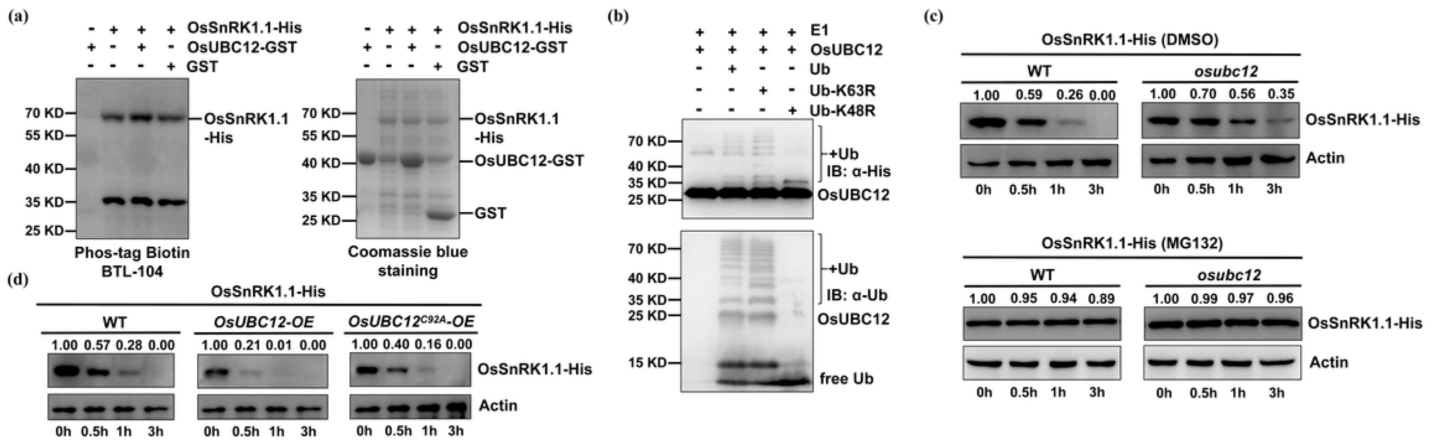

Figure 6

OsUBC12 mainly catalyzes K48-linked polyubiquitination and promotes OsSnRK1.1 degradation. (a) *In vitro* phosphorylation assays using OsUBC12 and OsSnRK1.1. Phosphorylation reactions were performed using the different combinations of purified proteins indicated within the figure. Left, phosphorylation detected by Phos-tag Biotin BTL-104; right, Coomassie-blue-stained SDS-PAGE gel used to monitor equal protein loading. **(b)** The polyubiquitination type of OsUBC12. Ubiquitin and two mutated Ub variants (Ub-K63R and Ub-K48R) were used in the reactions. Poly-Ub conjugates were detected using anti-His and anti-Ub antibodies. **(c)** Cell-free degradation assays of OsSnRK1.1-His incubated with protein extracts from WT or *osubc12* mutants following a 1-h treatment with DMSO (top) or 100 μM MG132 (bottom). DMSO is the solvent used for MG132 preparation. Protein extracts from WT and *osubc12* mutants were incubated with OsSnRK1.1-His for the indicated durations. OsSnRK1.1-His levels were visualized by immunoblotting using an anti-His antibody. OsActin was used as the loading control. To quantify relative protein band intensity, the protein band at 0 h was set to 1.00. **(d)** Cell-free degradation assays of OsSnRK1.1-His incubated with protein extracts from WT and *OsUBC12-OE* or *OsUBC12^{C92A}-OE* transgenic rice for the indicated durations. OsSnRK1.1-His levels were visualized by immunoblotting using an anti-His antibody. OsActin was used as the loading control. To quantify relative protein band intensity, the protein band at 0 h was set to 1.00.

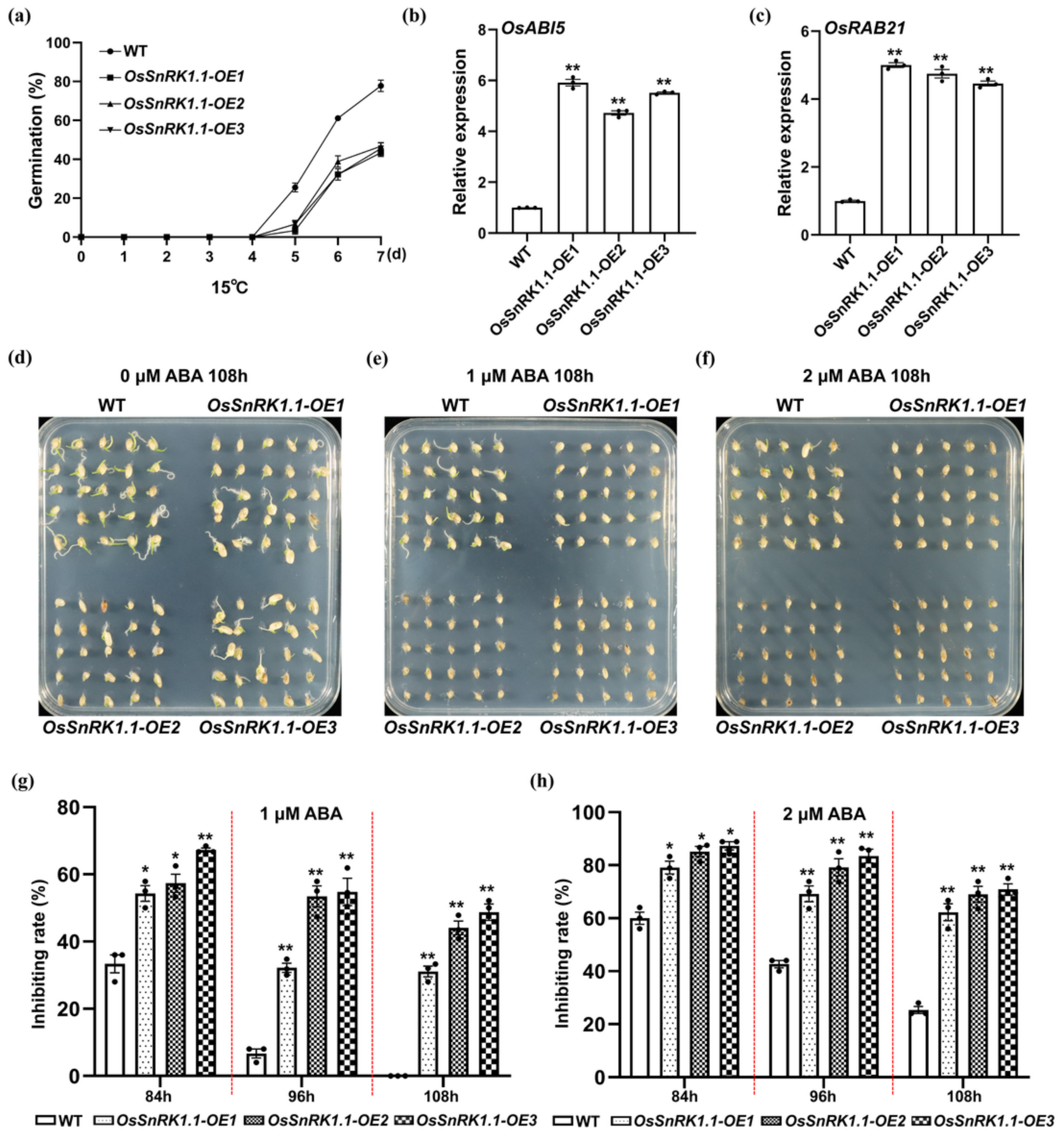

Figure 7

OsSnRK1.1 functions opposite to OsUBC12 in controlling LTG and ABA signaling. (a) Time-course germination analysis of *OsSnRK1.1-OE* lines at 15 °C. Values are means ± SE from three individual biological replicates (30 seeds per biological replicate). **(b), (c)** Relative expression of *OsABI5* **(b)** and *OsRAB21* **(c)** in seeds of *OsSnRK1.1-OE* lines. The expression level of the control samples (WT) were set to 1. Values are means ± SE from three individual replicates (n = 3). The housekeeping gene *OsUBQ5* was

used as an internal control to normalize the data. **(d), (e), (f)**Germination performance of *OsSnRK1.1-OE*seeds after 108 h on ½-MS agar medium containing 0 μM **(d)**, 1 μM **(e)**or 2 μM **(f)** ABA. **(g), (h)** The germination inhibition rates of WT and *OsSnRK1.1-OE*seeds under 1 μM **(j)** or 2 μM **(k)** ABA at three timepoints. Values are means ± SE from three individual biological replicates (25 seeds per biological replicate). The data were statistically analyzed using Student's *t*-test (*P < 0.05, **P < 0.01).

Supplementary Files

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