


ORIGINAL ARTICLE

The *Arabidopsis* U-box E3 ubiquitin ligase PUB30 negatively regulates salt tolerance by facilitating BRI1 kinase inhibitor 1 (BKI1) degradation

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Abstract

The *Arabidopsis* U-box E3 ubiquitin ligases play an important role in the ubiquitin/26S proteasome-mediated protein degradation pathway. Recently, PUB30 has been reported to participate in the salt stress response during seed germination stage in abscisic acid (ABA)-independent manner, but the molecular mechanism remains to be elucidated. Here, we displayed that the *pub30* mutant was more tolerant to salt stress during seed germination, whereas the mutant of its closest homologue PUB31 showed mild sensitivity to salt stress. PUB30 exhibited E3 ubiquitin ligase activity in vitro. PUB30 specifically interacted with BRI1 kinase inhibitor 1 (BKI1), a regulator playing dual roles in brassinosteroids signaling, in vitro and in vivo. We found that BKI1 protein was ubiquitinated and degraded by the 26S proteasome. The degradation of BKI1 was slowed down in the *pub30-1* mutant compared with that in the wild type. The *bki1* mutant was sensitive to salt, whereas the transgenic plants overexpressing *BKI1* showed salt tolerant phenotype. All these results indicate that PUB30 negatively regulates salt tolerance probably through regulating the degradation of BKI1 and brassinosteroids signaling in *Arabidopsis*.

KEYWORDS

BKI1, degradation, PUB30, salt tolerance, U-box E3 ubiquitin ligases

*Ming Zhang and Jinfeng Zhao contributed equally to this work.

1 | INTRODUCTION

Salinity is one of the major threats affecting land use capability and crop yield worldwide. Understanding the molecular mechanism governing the salt stress response will be a great asset to develop salt resistant crop plants (Ji et al., 2016). Salinity causes both ion toxicity and osmotic stress, and plants have developed a very complex salt tolerance mechanism during the course of evolution (Zhu, 2002). The salt overly sensitive (SOS) signaling pathway is crucial for maintaining ion homeostasis under salt stress. SOS1, SOS2, SOS3, and SOS3-like calcium binding protein8 (SCaBP8) are major components in this pathway. SOS1 encodes a plasma membrane-localized Na^+/H^+ antiporter that functions in maintaining the ion homeostasis in the cell (Qiu, Guo, Dietrich, Schumaker, & Zhu, 2002; Shi, Ishitani, Kim, & Zhu, 2000). SOS2 is a serine/threonine protein kinase that phosphorylates and activates SOS1 (Liu, Ishitani, Halfter, Kim, & Zhu, 2000; Qiu et al., 2002). SOS3 encodes a calcium-binding protein that interacts with SOS2, recruiting it to the plasma membrane and activating it in calcium-dependent manner that further activates SOS1 in plasma membrane. (Albrecht, Ritz, Linder, Harter, & Kudla, 2001; Guo, Halfter, Ishitani, & Zhu, 2001; Halfter, Ishitani, & Zhu, 2000; Quintero et al., 2011; Quintero, Ohta, Shi, Zhu, & Pardo, 2002). SCaBP8/Calcineurin B-like 10 is a homologous protein of SOS3 and also crucial for SOS2 activation. SCaBP8 mainly functions in the shoot, whereas SOS3 functions in the root. These two proteins share the same properties in the regulation of SOS2 (B. G. Kim et al., 2007; Quan et al., 2007).

The ubiquitin/26S proteasome-mediated pathway is involved in selective degradation of proteins in cells where E3 ubiquitin ligases are responsible for substrate specificity. According to the mode of action and the presence of specific domains, E3 ligases are categorized into four classes: RING, HECT, F-box, and U-box (Yee & Goring, 2009). In the *Arabidopsis* genome, 64 plant U-box (PUB) genes have been predicted, and they function in diverse biological processes (Yee & Goring, 2009). PUB22 and its homologous protein PUB23 coordinately regulate plant response to drought by ubiquitinating cytosolic RPN12a, a subunit of the 19S regulatory particle in the 26S proteasome (Cho, Ryu, Song, Kwak, & Kim, 2008). Additionally, PUB22 was reported to attenuate the pathogen-associated molecular patterns (PAMPs)-induced signaling by mediating degradation of Exo70B2, a subunit of the exocyst complex that regulates vesicle tethering during exocytosis (Stegmann et al., 2012). PUB12 and PUB13 positively regulate ABA signaling by ubiquitinating ABI1 only when it interacts with ABA receptors (Kong et al., 2015). The *pub18 pub19* double mutant was found less sensitive to ABA and sodium chloride (NaCl) stress compared to the wild type during seed germination stage (Bergler & Hoth, 2011). Recently, PUB30 was reported to be induced specifically by high salinity and participated in the salt stress response as a negative factor in seed germination, but the molecular mechanism is yet to be completely elucidated (Hwang, Seo, Kang, Kwak, & Kim, 2015).

Brassinosteroids (BRs) are a group of important plant hormones that control a variety of important plant growth and developmental processes, including cell division/elongation, photomorphogenesis, and adaptation to biotic/abiotic stresses (Abraham et al., 2003; Clouse & Sasse, 1998; Krishna, 2003). BRs biosynthesis and signal transduction play an important role in the plant response to salt stress (Krishna,

2003; Steber & McCourt, 2001). BR-deficient *det2-1* and BR-insensitive *bin2-1* mutants displayed salt sensitive phenotype in seed germination and seedling growth compared with the wild-type plants (Zeng, Tang, & Hua, 2010). The exogenous application of BR significantly increases the salt stress tolerance during seed germination and in postgerminative growth stages (Kagale, Divi, Krochko, Keller, & Krishna, 2007; Ozdemir, Bor, Demiral, & Turkan, 2004). BRs signaling pathways were extensively studied in *Arabidopsis* (Clouse, 2011; Clouse & Sasse, 1998; T. W. Kim & Wang, 2010). BRI1 kinase inhibitor 1 (BKI1), a membrane-associated protein, functions as a negative regulator of the BR receptor, BR insensitive 1 (BRI1). In the presence of BR, BKI1 could be phosphorylated by BRI1 that results in delocalization of BKI1 from plasma membrane into the cytoplasm and release of BRI1 from the inhibition by BKI1 (X. Wang & Chory, 2006). The plasma membrane-dissociated and phosphorylated BKI1 also plays positive roles in BR signaling by interacting with a subset of 14-3-3 proteins. The cytosolic BKI1 antagonizes the 14-3-3 s and enhances the BRI1 EMS suppressor 1 (BES1)/brassinazole resistant 1 (BZR1) accumulation in the nucleus to activate the BR responsive gene expression (H. Wang et al., 2011).

In the present study, we reported that the *pub30-1* and its allelic mutant *pub30-3* are more tolerant to salinity stress in the seed germination stage. PUB30 plays an antagonistic role with its homologous protein PUB31 in seed germination although they share 81% identity. We showed that PUB30 possessed E3 ubiquitin ligase activity *in vitro* and interacted with BKI1 in the yeast two-hybrid assay. Pull-down and co-immunoprecipitation assays further confirmed the specificity of PUB30 over PUB31 for the interaction with BKI1. The accumulation of BKI1 protein was observed after treatment with the proteasome inhibitor MG132. BKI1 protein was ubiquitinated and degraded by the proteasome. We found that the cell-free degradation of BKI1 was slowed down in the *pub30-1* mutant compared with the wild type. The transgenic seedlings overexpressing *BKI1* showed salt-tolerance phenotype, whereas the *bki1* mutant displayed hypersensitivity to salt stress, suggesting that BKI1 is a positive regulator in salt stress tolerance mechanism. Overall, these results suggest that PUB30 negatively regulates salt tolerance through facilitating BKI1 degradation.

2 | MATERIALS AND METHODS

2.1 | Plant materials, growth conditions and stress treatments

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild type. T-DNA inserted mutants, *pub30-1* (Salk_012549), *pub30-3* (Salk_073739), and *pub31* (Salk_054774), were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH, USA). Seeds were sterilized with 20% bleach and 0.1% Triton-X100 for 10 min and washed 5 times with sterilized water, and then sown on plastic Petri plates containing half-strength Murashige-Skoog (MS) medium (Duchefa Biochemie, Haarlem, The Netherlands) including 1% sucrose and 0.3% phytoigel (Sigma-Aldrich, St Louis, MO, USA) with or without 150 mM NaCl. The seeds were laid in the refrigerator at 4 °C for 3 days and transferred to the incubator at 23 °C with continuous light with an intensity of 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

2.2 | Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from 10-day-old seedlings sown on 1/2 MS medium using the RNAPrep Pure Plant Kit (Tiangen, Beijing, China) following the manufacturer's instructions. Total RNA (2 μ g) was reverse-transcribed using Transcript All-in-one First-Strand cDNA Synthesis Supermix for qPCR Kit (Transgene, Beijing, China) and equal quantities of reverse-transcribed products were used for the real-time quantitative RT-PCR reactions. Quantitative RT-PCRs were performed using a SYBR Premix Ex Taq[™] RT-PCR kit (Takara, Shiga, Japan) following the manufacturer's instructions with the primers listed in Table S1.

2.3 | Yeast two-hybrid assay

In the initial library screening step, full-length PUB30 was used as a bait to screen a cDNA library made from 12-day-old *Arabidopsis* seedlings with the Matchmaker Library Construction & Screening Kit (Clontech). To confirm the interaction between PUB30 and BKI1, the full-length coding sequence of PUB30 and PUB31 were amplified and cloned into the *pGBKT7* (*BD-PUB30* and *BD-PUB31*) and BKI1 was inserted into *pGADT7* (*AD-BKI1*). The polymerase chain reaction (PCR) primers used for plasmid construction are listed in Table S2. The combinations of *BD-PUB30* and *AD-BKI1*, *BD-PUB31* and *AD-BKI1*, *BD-PUB30* and *AD*, *BD-PUB31* and *AD*, and *BD* and *AD-BKI1* were transformed into yeast strain AH109. Yeast synthetic drop-out media lacking leucine (Leu) and tryptophan (Trp; Coolaber, Beijing, China) was used to identify transformed colonies. Three days after transformation, selective colonies were grown in -Leu and -Trp liquid media for 24 h. Then, cell suspension was pipetted onto yeast synthetic drop-out media lacking histidine (His), Leu, and Trp (Coolaber, Beijing, China) with 2.5 mM 3-amino-1,2,4-triazole (3-AT, Sigma-Aldrich, St Louis, MO, USA).

2.4 | Pull-down assay

All of the PCR primers used for plasmid construction are listed in Table S2. In order to express the PUB30, PUB31, and BKI1 proteins in *Escherichia coli* DE3 cells, the coding sequence of PUB30, PUB31, and BKI1 were amplified and individually cloned into *pCold-MBP*, *pCold-MBP*, and *pCold-GST* vectors with infusion cloning kit (Takara, Shiga, Japan). The glutathione S-transferase (GST) and maltose binding protein (MBP) tags were fused to the N-terminus of target proteins (*pCold-MBP-PUB30*, *pCold-MBP-PUB31*, and *pCold-GST-BKI1*).

In pull-down assay, 2 μ g of GST-BKI1 protein was incubated with 10 μ g of MBP-PUB30 or MBP-PUB31 that was attached onto the agarose beads for 30 min at room temperature in 100 μ l of binding buffer (20 mM Tris-HCl, pH 7.2, 10 mM MgCl₂, 2 mM DTT, and 5% NP40). After 5 times washing with binding buffer, the beads were boiled with sodium dodecyl sulfate loading buffer, and samples were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and detected with 1:3,000 GST or 1:5,000 MBP antibody (CWBIO, Beijing, China). The horseradish peroxidase-conjugated anti-mouse IgG (H + L; 1:3,000 dilution, CWBIO, Beijing, China) was used as the second antibody. The western blots were

developed with enhanced chemiluminescence reagent (Coolaber, Beijing, China).

2.5 | Co-immunoprecipitation assay

The full-length coding sequence of PUB30, PUB31, and BKI1 were amplified and individually cloned into the vector *1307-FLAG* and *1307-Myc* (Zhao et al., 2016). The primers used for plasmid construction are listed in Table S2. The combinations of *1307-FLAG-PUB30* and *1307-Myc-BKI1* and *1307-FLAG-PUB31* and *1307-Myc-BKI1* were transformed to *Arabidopsis* mesophyll protoplast according to the standard protocols (Sheen, 2001). The transfected protoplasts were incubated overnight at 23 °C and homogenized in immunoprecipitation buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM DTT, 0.1% Triton, and 1% protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The sample was centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was incubated with agarose-conjugated anti-Myc antibody (Sigma-Aldrich, St Louis, MO, USA) for 3 hr at 4 °C. After 5 times washing with immunoprecipitation buffer, the protein were separated on 10% SDS-PAGE gel and subjected to immunoblotting using anti-Myc antibody (CWBIO, Beijing, China) and anti-FLAG antibody (CWBIO, Beijing, China).

2.6 | In vitro self-ubiquitination assay

In vitro self-ubiquitination assay was carried out according to the method reported by Xie et al. (2002). MBP-PUB30 (500 ng) was immobilized on amylose resin beads. UBA1, UBC8, and UBQ14 were individually cloned into the *pET28a* vector to express E1, E2, and ubiquitin. The primers used for plasmid construction are listed in Table S2. Ubiquitination assays were carried out by adding 200 ng UBA1 (E1), 100 ng UBC8 (E2), and 2 μ g ubiquitin in the buffer containing 50 mM Tris-HCl, pH 7.4, 2 mM ATP, 5 mM MgCl₂, and 2 mM DTT. The reaction mix (30 μ l) was incubated for 2 hr at 30 °C. The reaction products were separated on 6% SDS-PAGE gel and subjected to immunoblot using anti-MBP antibody (1:10,000 dilution, CWBIO, Beijing, China) and anti-ubiquitin (anti-Ub) antibody (1:3,000 dilution, Santa Cruz, CA, USA).

2.7 | In vivo ubiquitination assay

The *in vivo* ubiquitination assay was carried out according to the reported method (Irigoyen et al., 2014). Briefly, the wild-type and transgenic plants overexpressing *1307-Myc-BKI1* were grown on MS medium for 10 days and then treated with 50 μ M MG132 for 6 hr. Fresh seedlings (0.2 g) were grinded in liquid nitrogen, and the powder was solubilized in 600 μ l BI buffer containing 50 mM Tris-HCl, pH 7.5, 20 mM NaCl, 0.1% NP-40 and 5 mM ATP, 1 mM PMSF, 50 μ M MG132, 10 nM Ub aldehyde (Sigma-Aldrich, St Louis, MO, USA), and 10 mM N-ethylmaleimide (Coolaber, Beijing, China). After 10 min of centrifugation at 12,000 rpm at 4 °C, the supernatants were incubated with prewashed p62-agarose (Enzo Life Sciences, Farmingdale, USA) for 3 hr, and then the agarose was washed twice with BI buffer and once with BII buffer (supplemented with 200 mM NaCl in BI buffer). The proteins were separated on 10% SDS-PAGE gel and subjected to

immunoblotting using anti-Myc antibody (CW BIO, Beijing, China) and anti-Ub antibody (Santa Cruz, CA, USA).

2.8 | *In vitro* cell-free degradation assay

Ten-day-old seedlings of *pub30-1* and wild type were harvested and grinded into fine powder in liquid nitrogen. Total proteins were subsequently extracted in degradation buffer containing 25 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl₂, 4 mM PMSF, 5 mM DTT, and 10 mM ATP (F. Wang et al., 2009). After 10-min centrifugation at 17,000 g, the supernatants were adjusted to the equal concentration with degradation buffer. Purified GST-BK11 protein (100 ng) was incubated with 50 μ l total protein extracts at 23 °C. The products were subjected to western blotting with anti-GST antibody (CW BIO, Beijing, China).

2.9 | *In vivo* degradation assay

Ten-day-old transgenic seedlings overexpressing *Myc-BK11* were pretreated with MG132 for 16 hr and then washed 5 times before being transferred to liquid MS medium containing 200 mM cycloheximide (Sigma-Aldrich, St Louis, MO, USA) to block *de novo* protein synthesis. The samples were harvested after 0 min, 90 min, and 180 min, and the seedlings were grinded in liquid nitrogen and solubilized with extraction buffer (100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM EDTA, pH 8.0, 1% TrionX-100, 10% glycerol, and a protease inhibitor cocktail). After 10-min centrifugation at

12,000 rpm at 4 °C, the supernatant was incubated with 5 \times sodium dodecyl sulfate loading buffer and separated by 10% SDS-PAGE gel and analysed by immunoblotting with anti-Myc antibody at 1:3,000 dilution (CW BIO, Beijing, China).

3 | RESULTS

3.1 | The *pub30* mutants are more tolerant to salinity stress during seed germination stage

Hwang *et al.* (2015) reported that the *Arabidopsis pub30* mutant was more tolerant to salt stress in the germination stage compared with the wild type. However, little is known about the underlying mechanism for the salt-tolerance phenotype of the *pub30* mutant. Here, we aimed to elucidate the potential mechanism using two T-DNA insertion mutants, that is, *pub30-1* (Salk_012549) and *pub30-3* (Salk_073739; Figure 1a). Real-time PCR analysis showed that the full length transcript of *PUB30* could not be detected in *pub30-1* and *pub30-3* mutants, suggesting that *pub30-1* and *pub30-3* are knockout mutants (Figure 1b). To investigate the function of *PUB30* in salt stress responses, seeds of the wild type and the two *pub30* knockout mutants were germinated on 1/2 strength MS medium with or without 150 mM NaCl (Figure 1c). The untreated wild-type and mutants seeds grew normally with 100% germination rate (Figure 1c and 1d). The radicle emergence rate of both wild type and mutants decreased with 150 mM NaCl treatment. The wild-type seeds were affected severely

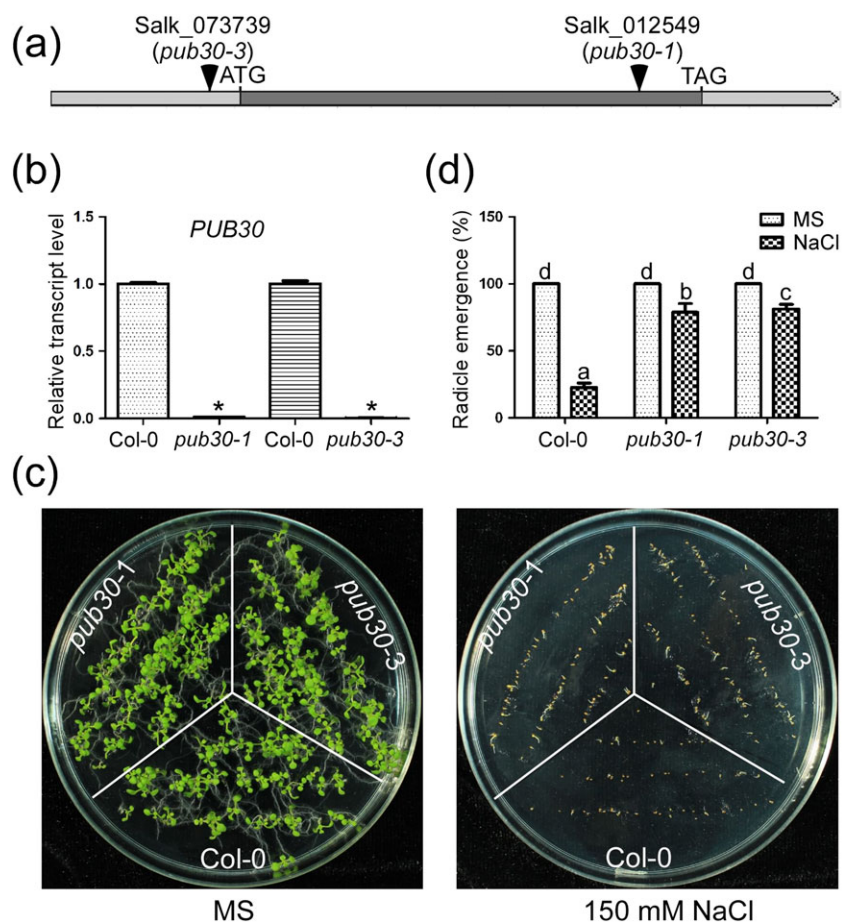


FIGURE 1 The *pub30* mutants are tolerant to salinity in seed germination stage. (a) Schematic structure of *PUB30*. Filled dark grey box, coding sequence; filled grey boxes, untranslated regions; and black arrow heads, T-DNA insertion sites. (b) The expression of *PUB30* in the wild-type and two *pub30* mutants. Real-time polymerase chain reaction analysis was used to detect the transcript level of *PUB30*. Actin was used as an internal control. Asterisks indicate significant differences ($p < .05$). (c) Comparison of salt tolerance between the wild-type and two *pub30* mutants in seed germination. Seeds were germinated and grew on the Murashige-Skoog (MS) medium containing 150 mM NaCl for 9 days. (d) Quantitative evaluation of radicle emergence for the wild-type and the *pub30* mutants in (c). Error bars represent standard deviation. Statistical significance was determined by a Student's *t*-test; significant differences ($p \leq .05$) are indicated by different lowercase letters [Colour figure can be viewed at wileyonlinelibrary.com]

with only 22.66% emergence rate, whereas the mutants *pub30-1* and *pub30-3* recorded 78.75% and 81.35% radicle emergence rate, respectively (Figure 1c and 1d). A similar trend was observed when the wild-type and *pub30-1* mutant seeds germinated on MS medium containing two other concentrations of NaCl (100 and 125 mM; Figure S1). These results suggest that the *pub30* mutants are more tolerant to salt stress in seed germination.

3.2 | Functional antagonism between two highly homologous proteins PUB30 and PUB31 in response to salt stress

According to homologous sequence alignment, we found PUB30 shares 81% identity with PUB31 (Figure 2a). Both proteins contain a U-box domain that is essential for E3 ubiquitin ligase activity at the N-terminal region and several Armadillo (ARM)-repeat motifs that mediate protein-protein interaction at the C-terminal region (Figure 2a). In order to determine whether PUB31 has functional redundancy with PUB30, we obtained the *pub31* mutant

(Salk_054774) from ABRC. Quantitative RT-PCR results showed that the full length transcript of *PUB31* could not be detected in the *pub31* mutant (Figure S2). The *pub30-1 pub31* double mutant was generated and used to observe the phenotype. There was no significant difference among the wild type, *pub30-1*, *pub31*, and *pub30-1 pub31* double mutant during seed germination when they grow on normal MS medium for 12 days. However, when they grow on the MS medium containing 150 mM NaCl for 12 days, the *pub31* mutant is a little sensitive to salt stress compared with the wild type, in contrast to the salt-tolerant phenotype of the *pub30-1* mutant. The double mutant *pub30-1 pub31* behaved in between the *pub30-1* and *pub31* single mutant for salt stress response and resembled the *pub31* mutant phenotype in radicle emergence rate (Figure 2b and 2c). In the presence of 150 mM NaCl, the radicle emergence rate of the wild type, *pub30-1*, *pub31*, and the *pub30-1 pub31* double mutant is 40.01%, 77.39%, 18.88%, and 24.80%, respectively (Figure 2c). These results suggest that *PUB31* has a functional antagonism with *PUB30* in salinity stress response.

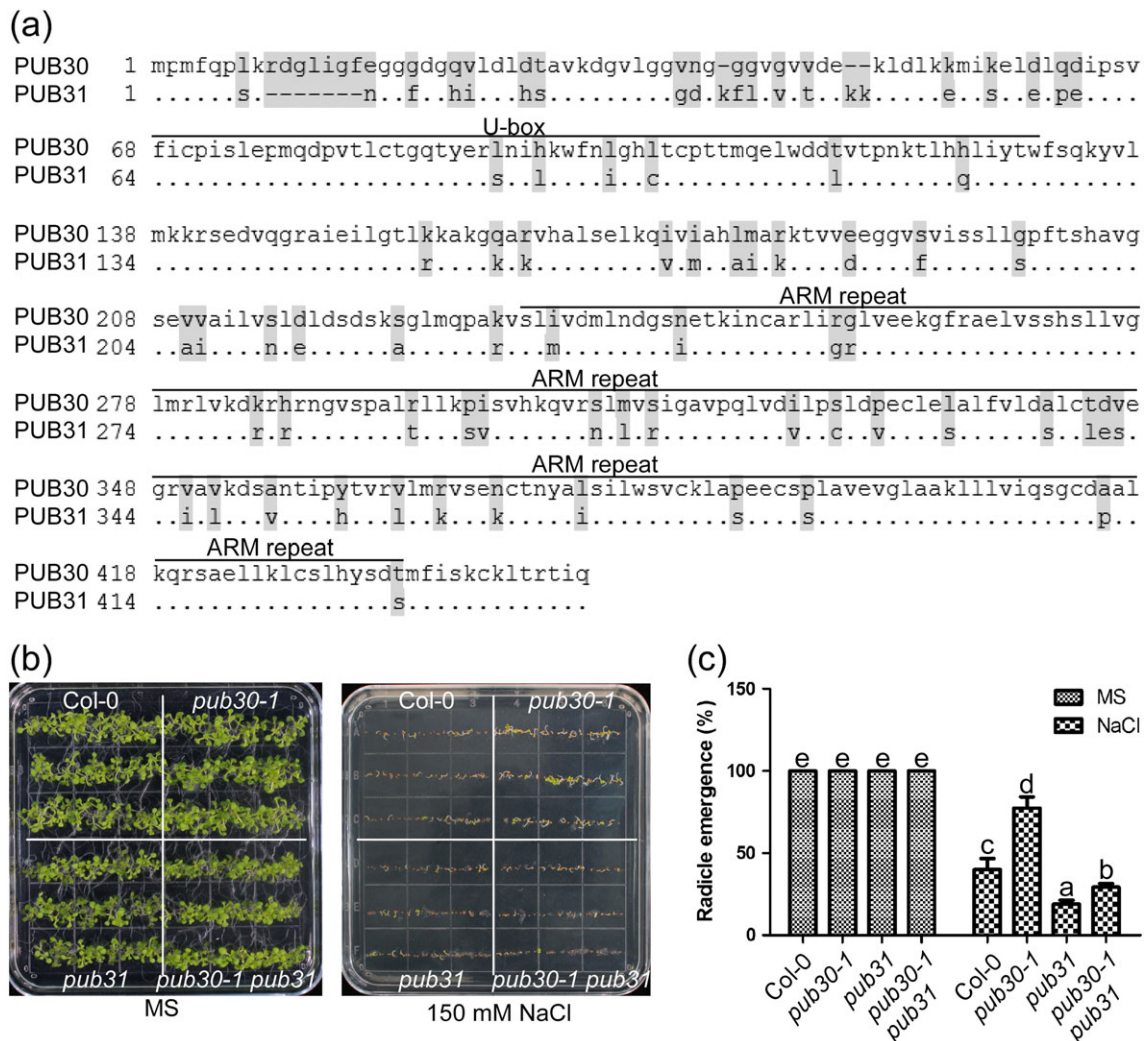


FIGURE 2 *PUB30* and its highly homologous protein *PUB31* play antagonistic role in salt tolerance in seed germination. (a) Homologous sequence alignment between *PUB30* and *PUB31*. (b) Seed germination of the wild-type, *pub30-1*, *pub31*, and *pub30-1 pub31* double mutant in Murashige-Skoog (MS) with or without 150 mM NaCl. (c) Quantitative evaluation of radicle emergence in (b). Error bars represent standard deviation. Statistical significance was determined by a Student's *t*-test; significant differences ($p \leq .05$) are indicated by different lowercase letters. ARM = Armadillo [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Expression pattern and subcellular localization of PUB30

To examine the expression pattern of *PUB30*, real-time quantitative RT-PCR was used to detect the expression of *PUB30* in different tissues, including root, stem, cauline leaf, rosette leaf, and silique. Relative higher expression level was found in root and stem, lower level in cauline and rosette leaves (Figure 3a). To explore the subcellular localization of the *PUB30* protein, we constructed two plant expression vectors. In the *GFP-PUB30* vector, green fluorescent protein (GFP) was fused to the N-terminus of *PUB30* genomic sequence driven by

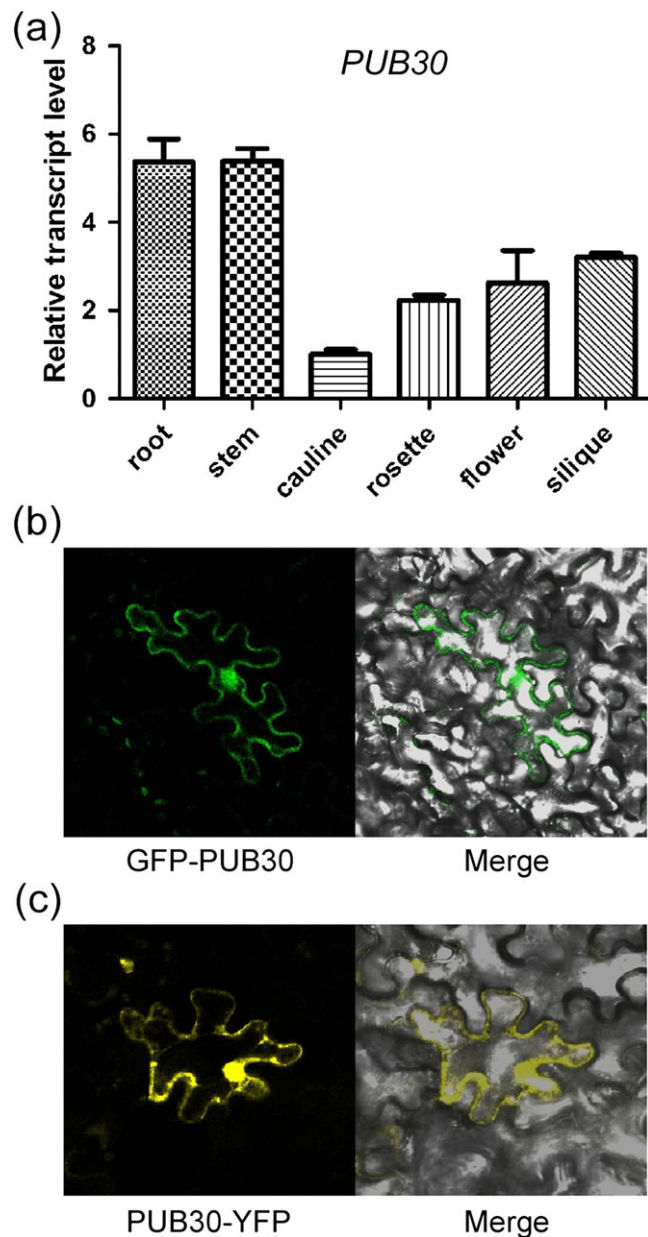


FIGURE 3 The expression pattern of *PUB30* and subcellular localization of *PUB30*. (a) Real-time quantitative reverse transcription polymerase chain reaction analysis of *PUB30* expression in different tissues (b) and (c) subcellular localization of *PUB30* in tobacco leaf cells. (b) Localization of *PUB30* when green fluorescent protein (GFP) was fused to the N-terminus of *PUB30*. (c) Localization of *PUB30* when yellow fluorescent protein (YFP) was fused to the C-terminus of *PUB30* [Colour figure can be viewed at wileyonlinelibrary.com]

the *PUB30* native promoter. In the *PUB30-YFP* vector, yellow fluorescent protein (YFP) was fused to the C-terminus of *PUB30* driven by a 35S promoter. These two vectors were transiently expressed in tobacco leaves. After incubation for 5 days, we found that these two vectors showed similar localization pattern where *PUB30* was extensively localized in the cytoplasm, nucleus, and plasma membrane (Figure 3b and 3c). Additionally, the transgenic seeds harboring *GFP-PUB30* in the *pub30-1* mutant background were used to detect the radicle emergence on the MS medium containing 150 mM NaCl. The results showed that radicle emergence rate of two independent transgenic lines was reduced compared with that of the *pub30-1* mutant, implying that the *GFP-PUB30* fusion protein is functional in complementing the salt-tolerant phenotype of the *pub30-1* mutant (Figure S3).

3.4 | PUB30 is a functional U-box E3 ubiquitin ligase

To examine whether *PUB30* is a functional E3 Ub ligase, we produced a full-length *Arabidopsis* *PUB30* protein fused with MBP in *E. coli* and subsequently affinity purified from the soluble fraction. *In vitro* self-ubiquitination assays were performed in the presence of *Arabidopsis* E1 (UBA1), *Arabidopsis* E2 (UBC8), and His-tagged Ub. Polyubiquitination was detected by anti-Ub or anti-MBP antibody only when E1, E2, and MBP-*PUB30* were all present. When any of them were omitted in the assay, polyubiquitination was not detected (Figure 4). This result suggests that *PUB30* possesses E3 Ub ligase activity *in vitro*.

3.5 | PUB30 interacts with BKI1 in vitro and in vivo

To investigate whether *PUB30* affects the salt stress response via ubiquitination of target protein, we performed yeast two-hybrid assay to screen its target using *PUB30* as bait. Some positive clones were

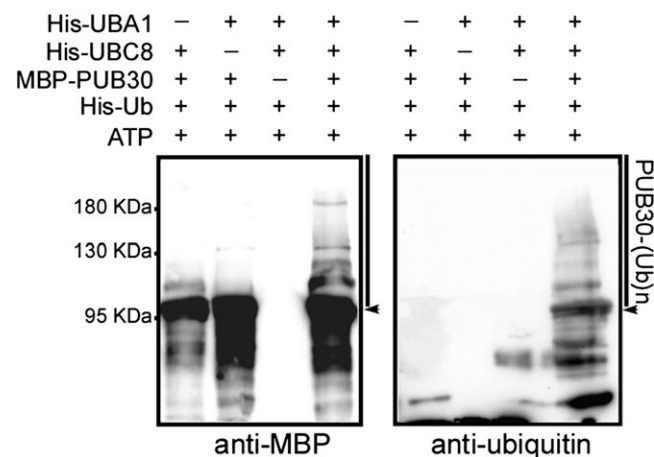


FIGURE 4 Ubiquitin ligase activity of *PUB30*. The maltose binding protein (MBP)-*PUB30* fusion protein was assayed for ubiquitin activity in the presence of E1 (UBA1), E2 (UBC8), and His-tagged ubiquitin. Samples were resolved by 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis and subjected to immunoblotting using anti-MBP and anti-ubiquitin antibodies. Arrow heads indicate MBP-*PUB30*. The smaller bands in the fourth lane of anti-ubiquitin blot may be the MBP-*PUB30* degradation products

isolated from the cDNA library made from 12-day-old *Arabidopsis* seedlings. One of these clones encoded the full length BK11 protein. The interaction between PUB30 and BK11 was confirmed by cotransformation of *AD-BK11* and *BD-PUB30* into the yeast cells (Figure 5a). Although PUB30 has highest identity (81%) with PUB31 in *Arabidopsis*, they have functional antagonism in response to salt stress (Figure 2). In order to investigate whether PUB31 also interacts with BK11, *AD-BK11* and *BD-PUB31* were cotransformed into the yeast cells. The result showed that PUB31 does not interact with BK11 although PUB30 does (Figure 5a). These results suggest that BK11 may be the target of PUB30 in salt tolerance mechanism.

To further confirm the interaction between PUB30 and BK11, we performed pull-down assay. *PUB30* and *PUB31* cDNA were cloned into *pCOLD-MBP* vector (Takara) containing a MBP tag, individually. MBP-PUB30 and MBP-PUB31 fusion proteins were purified from *E. coli* strain BL21 (DE3) with amylose resin (BioLabs). *BK11* cDNA was inserted into *pCOLD-GST* vector (Takara) with a GST tag. GST-BK11 was purified with Glutathione Sepharose™4B (GE Healthcare) and eluted with reduced GST. *In vitro* pull-down assays showed that GST-BK11 was pulled down by MBP-PUB30 but not by MBP-PUB31 (Figure 5b). This result further confirmed that PUB30 specifically interacts with BK11.

To confirm the interaction between PUB30 and BK11 *in vivo*, FLAG tag was translationally fused to the N-terminal region of PUB30 or PUB31 and 6 × Myc tag was translationally fused to the N-terminal region of BK11. These gene fusions were driven by a double 35S promoter. The resulting vector *Myc-BK11* was cotransfected with *FLAG-PUB30* or *FLAG-PUB31* constructs into the wild-type leaf protoplasts. Total extracted protein was used to detect the expression of Myc-BK11 and FLAG-PUB30 or Myc-BK11 and FLAG-PUB31 in each transformation with anti-Myc antibody or anti-FLAG antibody (Figure 5c). Myc-BK11 protein was then immunoprecipitated with anti-Myc affinity gel. The result showed that only FLAG-PUB30 is pulled-down by Myc-BK11 and FLAG-PUB31 is not (Figure 5c). This result suggests that PUB30 specifically interacts with BK11 *in vivo*.

3.6 | BK11 protein is ubiquitinated and degraded by the proteasome

U-box E3 Ub ligase specifically recognizes substrate proteins and target them for degradation by the 26S proteasome by attaching a polyubiquitin chain. Thus, we first examined whether BK11 is degraded by the 26S proteasome. The *Arabidopsis* seedlings expressing *Myc-BK11* were treated with 50 μM proteasome inhibitor MG132 for different time. The total protein was extracted to detect the protein level of Myc-BK11. The result showed that the level of Myc-BK11 protein increased gradually as the incubation time with MG132 was prolonged (Figure 6a). Serine hydroxymethyltransferase 1, which is degraded in a 26S proteasome-dependent manner, was used as a positive control of MG132 treatment (Zhou et al., 2012). This suggests that BK11 protein could be degraded by the 26S proteasome.

Then to check whether BK11 protein is ubiquitinated *in vivo*, we used a p62-agarose matrix that is capable of binding ubiquitinated proteins (Irigoyen et al., 2014) to enrich the ubiquitinated proteins from the transgenic plants overexpressing *Myc-BK11*. When the p62

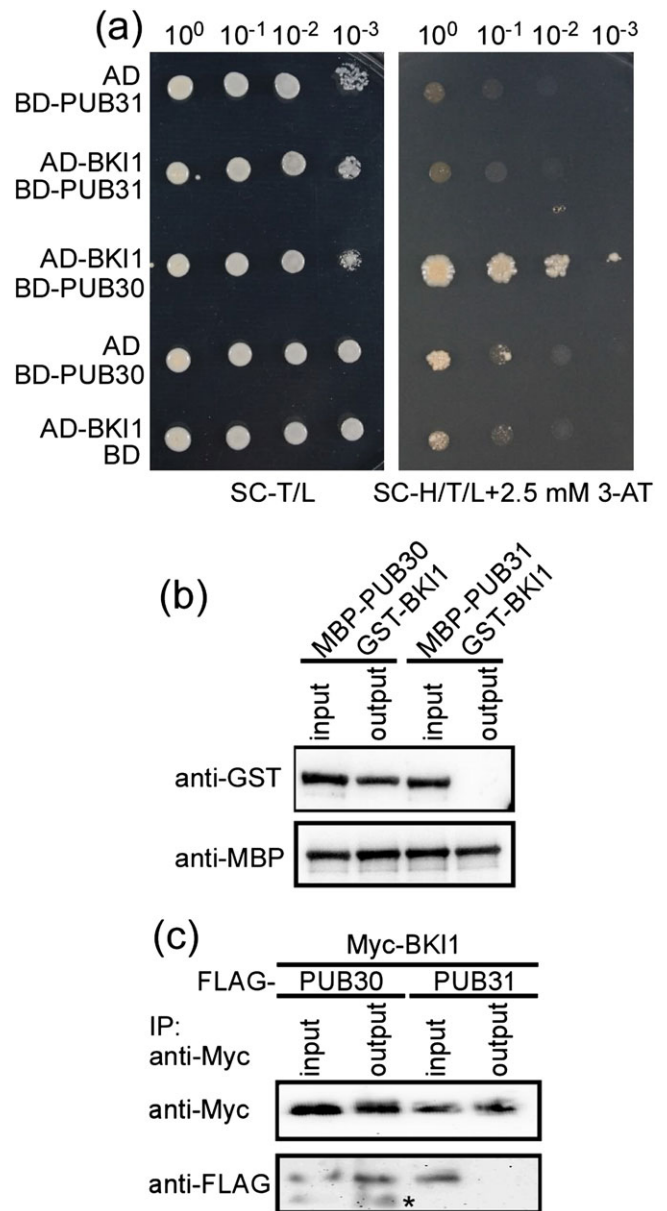


FIGURE 5 PUB30 specifically interacts with BK11 *in vitro* and *in vivo*. (a) PUB30 interacts with BK11 in yeast two-hybrid assays. Yeast strains expressing the indicated constructs were grown on synthetic complete medium without tryptophan and leucine (SC-T/L; left panel) and on synthetic complete medium without Trp, Leu, and His including 2.5 mM 3-amino-1,2,4-triazole (SC-H/T/L; right panel). Pictures were taken after 4 to 5 days of growth on the indicated medium. Panels show yeast serial decimal dilutions. (b) PUB30 interacts with BK11 in pull-down assay. For pull-down assay, 2 μg glutathione S-transferase (GST)-BK11 was incubated with 10 μg maltose binding protein (MBP)-PUB30 or MBP-PUB31 bound to the beads for 30 min at room temperature. Upper panel, immunoblotting with anti-GST antibody; lower panel, immunoblotting with anti-MBP antibody. (c) PUB30 interacts with BK11 in a co-IP assay. *FLAG-PUB30* and *Myc-BK11* or *FLAG-PUB31* and *Myc-BK11* plasmids were cotransfected into *Arabidopsis* mesophyll protoplasts. Co-IP was performed with anti-Myc agarose from total isolated proteins. Immunoblotting analysis was carried out with anti-FLAG and anti-Myc antibodies. Asterisk indicates truncated FLAG-PUB30 fusion detected by anti-FLAG [Colour figure can be viewed at wileyonlinelibrary.com]

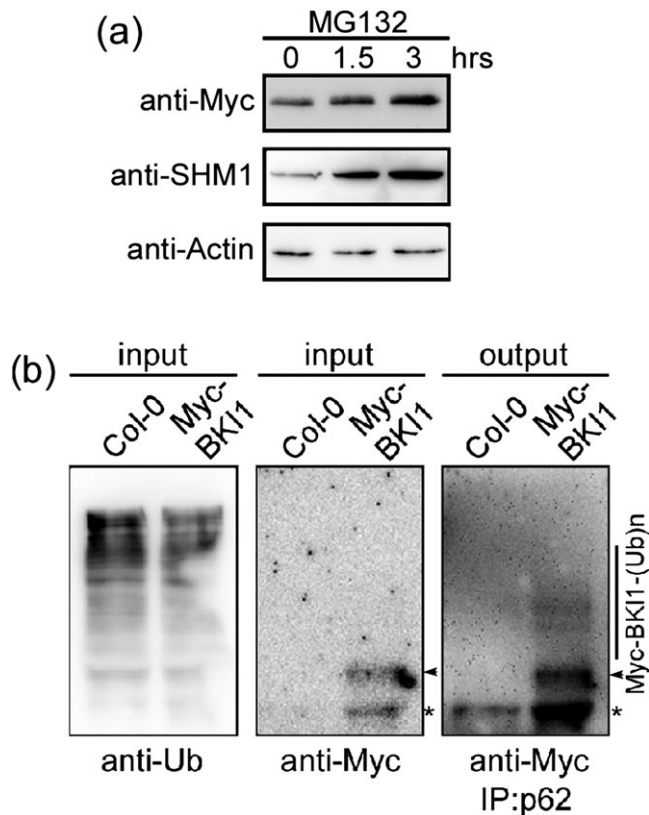


FIGURE 6 BRI1 kinase inhibitor 1 (BK11) protein is ubiquitinated and degraded by the 26S proteasome. (a) Myc-BK11 gradually accumulated when the transgenic plants harboring *Myc-BK11* were treated with 50 μ M MG132 at indicated time points. Anti-serine hydroxymethyltransferase 1 (SHM1) was used as a positive control of MG132 treatment and anti-actin as a loading control. (b) Affinity purification of polyubiquitinated Myc-BK11. The total protein extracted from the transgenic seedlings containing *Myc-BK11* or the wild type was incubated with ubiquitin-binding p62 resin. Anti-ubiquitin antibody was used in left panel to detect total ubiquitinated proteins. Anti-Myc was used to detect Myc-BK11 and ubiquitinated forms [Myc-BK11-Ub_(n)] in middle and right panel. Protein extracts from the wild type were used as immunoblot controls. Asterisks indicate nonspecific protein detected by anti-Myc. Arrow heads indicate Myc-BK11

resin-enriched protein fraction was detected using anti-Myc antibodies, multiple high molecular mass bands above the Myc-BK11 band were revealed in the transgenic plants but not in the wild type, indicating that Myc-BK11 is modified by polyubiquitin chains *in planta* (Figure 6b).

3.7 | PUB30 mediates BK11 protein degradation

To determine whether PUB30 is required for BK11 degradation, we used *in vitro* cell-free system to detect the degradation of GST-BK11 protein expressed in *E. coli*. The total protein was extracted from Col-0 and the *pub30-1* seedlings and incubated with GST-BK11 protein at 23 °C for 0, 20, 40, and 60 min. Subsequently, immunoblot assay was performed with anti-GST antibody. Initially, there was no difference in the protein level of GST-BK11 between the wild-type and the *pub30-1* mutant extract. However, the protein level of GST-BK11

was reduced more rapidly in the wild-type than in the *pub30-1* mutant extract as the incubation time was increased (Figure 7a and 7b).

To further confirm whether BK11 protein is targeted by PUB30 for ubiquitin-mediated degradation *in vivo* and to compare the BK11 protein dynamics in the wild-type and the *pub30-1* mutant, we performed an *in vivo* degradation assay. In this assay, the wild-type and the *pub30-1* mutants were transformed with *Myc-BK11*. Two transgenic lines showing comparable transcript levels of *Myc-BK11* were selected from both the wild-type and the *pub30-1* mutant genotypes (Figure 8a). To increase the starting level of the Myc-BK11 protein, these two transgenic lines were pretreated with MG132 for 16 hr as described previously (Jung et al., 2015) and then washed 5 times before being transferred to liquid MS medium containing 200 mM cycloheximide for 90 or 180 min. Total protein was isolated from the two transgenic lines and used to detect the degradation of Myc-BK11. The immunoblot assays showed that degradation of the Myc-BK11 protein was slowed down in the *pub30-1* mutant compared with the wild type (Figure 8b and 8c). All these results suggest that BK11 undergoes 26S proteasome-dependent degradation, and PUB30 is involved in this process.

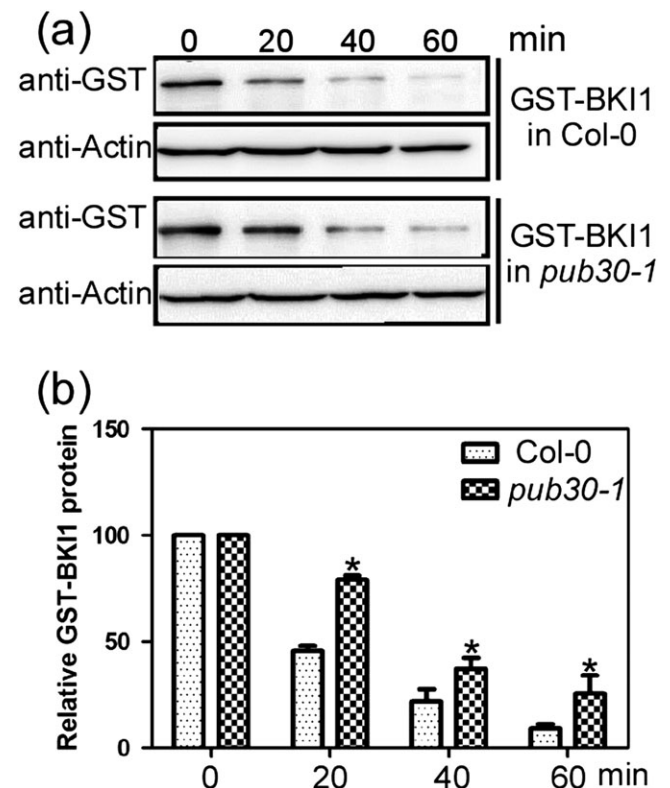


FIGURE 7 PUB30 regulates degradation of glutathione S-transferase (GST)-BRI1 kinase inhibitor 1 (BK11) in cell-free degradation assay. (a) GST-BK11 expressed in *Escherichia coli* DE3 cells was incubated with the protein extracts isolated from the wild type or the *pub30-1* mutant for the indicated time points. Anti-actin was used as a loading control. (b) Quantitative evaluation of GST-BK11 in the wild-type or the *pub30-1* mutants protein extract in (a). The signal intensity of GST-BK11 was first normalized to that of corresponding actin. The relative level of GST-BK11 at different time points was calculated as the percentage of normalized intensity to that at 0 min. Asterisks indicate significant difference ($p < .05$)

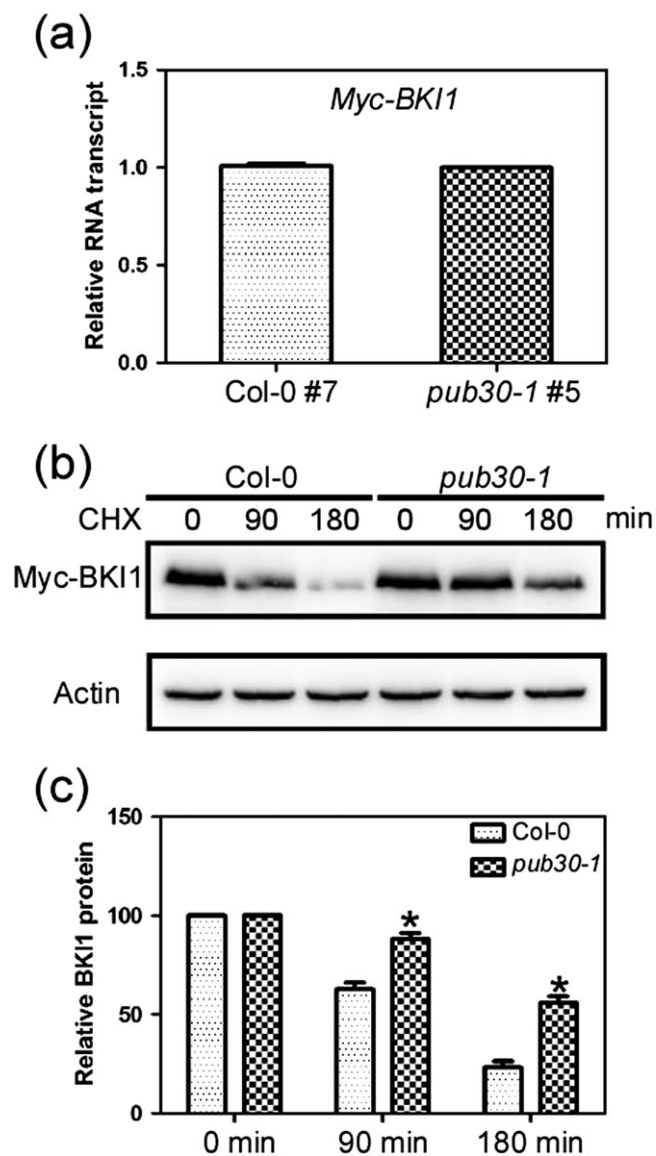


FIGURE 8 BK11 kinase inhibitor 1 (BK11) is stabilized in the *pub30-1* mutant. (a) Real-time reverse transcription polymerase chain reaction of *Myc-BK11* transcript levels in the transgenic seedlings harboring *Myc-BK11* in the wild-type background (Col-0 #7) and in the *pub30-1* background (*pub30-1* #5). Error bars represent standard deviation. (b) Degradation rate of *Myc-BK11* in the wild-type and *pub30-1* mutant. Ten-day-old transgenic seedlings with *Myc-BK11* in the wild-type or the *pub30-1* mutant background were incubated in liquid MS medium with 50 μ M MG132 for 16 h and washed for 5 times, then transferred to liquid Murashige–Skoog medium containing 200 mM cycloheximide (CHX) for different time points and detected with anti-Myc. Anti-actin was used as loading control. (c) Quantitative evaluation of *Myc-BK11* protein level in the wild-type or the *pub30-1* mutants in (b). The signal intensity of *Myc-BK11* was first normalized to that of corresponding actin. The relative level of *Myc-BK11* at different time points was calculated as the percentage of normalized intensity to that at 0 min. Asterisks indicate significant differences ($p < .05$)

3.8 | BK11 functions in salinity stress responses

In order to functionally characterize BK11 in seed germination under salt stress, the transgenic seeds harboring *Myc-BK11* were sown on 1/2 MS medium with or without 150 mM NaCl for 9 days. In contrast to the wild-type, transgenic plants overexpressing *BK11* showed

higher radicle emergence rate on MS medium containing 150 mM NaCl, that is, 34.42%, 87.58%, and 11.43% for transgenic line Col-0 #4, Col-0 #7, and the wild type, respectively (Figure 9a and 9c). The radicle emergence rate was positively correlated with the transcript level of *BK11* in transgenic plants Col-0 #7, Col-0 #4, and the wild type (Figure 9b).

In addition, we also used the *bki1* loss-of-function mutant (Jiang et al., 2015) to characterize its function in seed germination under salt stress. When the wild-type and the *bki1* mutant were grown on normal MS medium, no significant difference in seed germination was observed (Figure 9d). The radicle emergence of the wild-type and the *bki1* mutant grown on MS medium was almost 100% (Figure 9e). However, the *bki1* mutant displayed salt-hypersensitive phenotype when germinated on MS medium containing 100 mM NaCl (Figure 9 d). The fraction of seeds with radicle emergence was only 55% for the *bki1* mutant, compared with 85.8% for the wild type (Figure 9e). These results suggest that BK11 is a positive regulator of the salt-tolerance phenotype.

4 | DISCUSSION

In this study, we found that the *pub30-1* mutant is more tolerant to salt stress in seed germination stage compared with the wild type. The *PUB30* gene encodes a U-box E3 Ub ligase that possesses E3 Ub ligase activity *in vitro*. *PUB30* specifically interacts with BK11 *in vitro* and *in vivo*. *PUB30* regulates the stability of BK11 protein, and the transgenic plants overexpressing *Myc-BK11* showed salt tolerance phenotype similar to the *pub30-1* mutant phenotype during seed germination. Although *PUB30* has highly identical sequence with its homologous protein *PUB31* at the amino acid level, *PUB30* and *PUB31* have a reverse functionality in salinity stress tolerance in seed germination. The *pub30-1* mutant is tolerant to salt stress, whereas the *pub31* mutant is sensitive to salinity stress in germination stage. Phenotype of the *pub30-1 pub31* double mutant resembles the *pub31* mutant, suggesting the epistatic effect of *PUB31* in determining sensitivity to salt stress. It was previously reported that several highly homologous proteins can have reverse functionality. In *Arabidopsis*, two closely related RecQ helicases AtRECQ4A and 4B share nearly 70% identity at the protein level. However, knockout of these two genes leads to antagonistic phenotypes. RECQ4A mutants show sensitivity to DNA-damaging agents and partially suppress the lethal phenotype of an AtTOP3 α , a phenomenon that has previously been demonstrated for RecQ homologues of unicellular eukaryotes only. However, RECQ4B is not mutagen-sensitive and unable to suppress the induced lethality caused by loss of TOP3 α (Hartung, Suer, & Puchta, 2007). *Arabidopsis* trithorax-like protein 1 and *Arabidopsis* trithorax-like protein 2 share 65% identity and 75% similarity at the protein level. However, they have functional divergence in epigenetic modification owing to their different regulatory sequences (Saleh et al., 2008).

Armadillo-repeats in U-box E3 Ub ligase are motifs that mediate protein–protein interaction, which is responsible for interaction with the substrate proteins. The ARM-repeats of *PUB30* and *PUB31* share 84% identity at the protein level (Figure 2a). However, only *PUB30* can

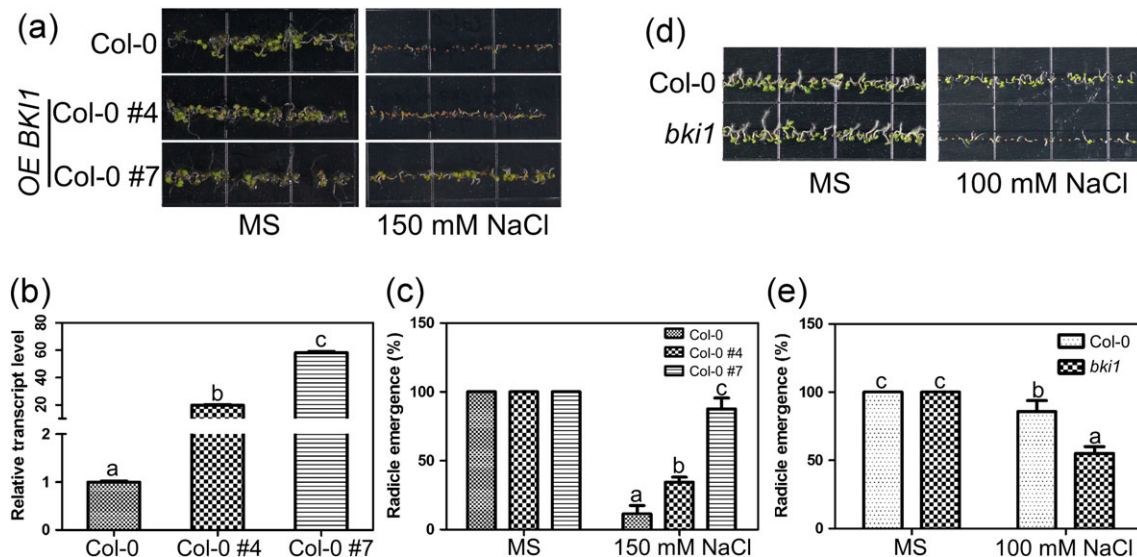


FIGURE 9 BKR1 kinase inhibitor 1 (*BKI1*) is involved in salt tolerance. (a) Germination of the wild-type and two transgenic lines overexpressing *Myc-BKI1* in the wild-type background. Seeds were sown and grown on the Murashige–Skoog (MS) medium with or without 150 mM NaCl for 9 days. (b) Real-time reverse transcription polymerase chain reaction of *BKI1* in the wild-type and two transgenic lines overexpressing *Myc-BKI1* in the wild-type background (Col-0 #4 and Col-0 #7). Actin was used as an internal control. Statistical significance was determined by a Student's *t*-test; significant differences ($p \leq .05$) are indicated by different lowercase letters. (c) Quantitative evaluation of radicle emergence in the wild type and two transgenic lines in (a). Statistical significance was determined by a Student's *t*-test; significant differences ($p \leq .05$) are indicated by different lowercase letters. (d) Germination of the wild-type and the *bki1* mutant on MS with or without 100 mM NaCl for 12 days. (e) Quantitative evaluation of radicle emergence in the wild-type and *bki1* mutant in (d). Statistical significance was determined by a Student's *t*-test; significant differences ($p \leq .05$) are indicated by different lowercase letters [Colour figure can be viewed at wileyonlinelibrary.com]

specifically interact with BKI1, suggesting that some amino acids in ARM-repeat are necessary for the interaction between PUB30 and BKI1. In order to elucidate the function of PUB30, identifying these amino acids in ARM-repeat would be necessary.

It is reported that the *pub30-1* mutant shows salt-tolerant phenotype only in seed germination stage, whereas there is no significant difference between the *pub30-1* mutant and the wild type in postgerminative growth in response to salt stress (Hwang et al., 2015). However, in our *in vitro* (Figure 7) and *in vivo* (Figure 8) degradation assay of BKI protein, 10-day-old seedlings of WT, *pub30-1*, and *MYC-BKI1* transgenic plants in both genotypes were used because it was very difficult to harvest enough plant sample for protein degradation assay at the seed germination stage. Yet defects in BKI1 destabilization could be observed in the seedling stage of the *pub30-1* mutant. These results suggest that the regulation of BKI1 stability by PUB30 still exists after the germination stage. However, the *pub30* mutant has no salt-tolerant phenotype at later stages. It is possible that functions of the PUB30 and BKI1 protein or their interaction may be affected by other biochemical processes, which hinder the *pub30* mutant to display salt tolerant phenotype at this stage.

The negative effect of PUB30 on salt stress tolerance seems to be limited to the seed germination stage (Hwang et al., 2015). However, BKI1 positively regulates salt tolerance response not only in the seed germination stage but also in seedling growth stage. We transferred the 5-day-old seedlings of wild type, the *bki1* mutant, and transgenic plants overexpressing *BKI1* to MS medium containing 0, 125, and 150 mM NaCl and kept them growing for 20 days. Without salt

treatment, the primary root length of the *bki1* mutant and transgenic seedlings overexpressing *BKI1* were indistinguishable from that of the wild type, although the fresh weight of the *bki1* mutant was less than other seedlings (Figure S5). Under salt stress treatment, the *bki1* mutant was more sensitive to salt than wild type as illustrated by the retarded growth of primary root, whereas transgenic lines overexpressing *Myc-BKI1* were more tolerant to salt as illustrated by the increased primary root length and fresh weight than wild type (Figure S5). These results suggest that BKI1 activity help plants to cope with high salinity at both seed germination and later stages during plant development.

It was reported that the transcript level of *BKI1* is induced by 150 mM salt (Hwang et al., 2015). In our study, we found that the transcript level of *BKI1* is much higher in the *pub30-1* mutant compared with the wild type grown without salt treatment. In addition, *BKI1* transcript level increased significantly in the *pub30-1* mutant when seedlings were treated with 150 mM NaCl (Figure S6). However, the protein level of BKI1 could not be induced by salt stress (Figure S7). We speculate that the U-box E3 Ub ligase PUB30 may be the crucial player in regulating the protein level of BKI1.

All these results indicated that BKI1 is the target of PUB30 in response to salt stress in seed germination stage. The loss-of-function mutant *bki1* was sensitive to salt while the transgenic plants overexpressing *BKI1* showed salt-tolerant phenotype just like the *pub30* mutants. However, the intrinsic molecular mechanism of BKI1 as a positive regulator in salt tolerance is unknown. BKI1 is a well-known component in BRs signal transduction. BKI1 has dual functions in BR signaling. BKI1 localized in the plasma membrane acts as a negative

regulator that interacts with BRI1 and inhibits the phosphorylation activity of BRI1 (Jailais et al., 2011; X. Wang & Chory, 2006). Once BKI1 dissociates from the plasma membrane, cytosolic BKI1 interacts with a subset of 14-3-3 proteins, which can also bind the phosphorylated BES1/BZR1. Phosphorylated BKI1 antagonized 14-3-3 proteins and enhance the accumulation of BZR1 and BES1 in the nucleus to regulate the down-stream genes expression (H. Wang et al., 2011). Earlier reports have proven that BR obviously enhances the tolerance of plants to abiotic stresses (Kagale et al., 2007; Ozdemir et al., 2004). In BR signaling, BR induces BKI1 protein to dissociate from the plasma membrane to cytoplasm. We hypothesize that salt can act as a signal similar to BR to induce BKI1 dissociation from plasma membrane to activate BR signal. In order to prove this hypothesis, the transgenic seedlings harboring *BKI1-YFP* were treated with 50 mM NaCl, instead of 150 mM NaCl, because the high concentration of salt results in plasmolysis in a short period of time, which hinders to observe the dissociation of BKI1 from the plasma membrane. As a positive control, we employed the most active form of BR (brassinolide, BL) to treat the transgenic seedlings and found that BKI1-YFP signal transfers to the cytoplasm to form vesicles compared with the mock (Figure S8). When 50 mM NaCl was used, we found that BKI1-YFP also dissociated from the plasma membrane and formed many vesicles in the cytoplasm (Figure S8). Therefore, similar to BL, salt can also induce the dissociation of BKI1 from plasma membrane. BKI1 protein accumulates in the *pub30-1* mutant in contrast to the wild type, which may enhance BR signaling in the *pub30* mutants, resulting in tolerance to salinity in seed germination stage. Additionally, we found that low concentration of BL obviously promotes the seed germination. The *pub30-1* mutant showed more vigorous early root growth than the wild type when exogenous BL was applied, suggesting that the intracellular BR signal maybe enhanced in the *pub30-1* mutant (Figure S9). Further studies are necessary to elucidate the BR signaling in the wild type and the *pub30* mutant under salt stress conditions and to understand the underlying molecular mechanism of *BKI1* and *PUB30* in response to salt stress.

According to a previous study (Hwang et al., 2015), the *pub30* mutant showed normal response to ABA at the seed germination stage. To determine whether the *bki1* mutant has altered responsiveness to ABA at the seed germination stage, we sowed the wild-type and the *bki1* mutant seeds on MS with or without ABA. The results showed that there was no significant difference between the wild-type and the *bki1* mutant in radicle emergence when grown on MS or MS containing different concentrations of ABA (Figure S4). These results suggest that *PUB30* and *BKI1* are not involved in response to ABA at seed germination stage.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. Germination of the wild-type and the *pub30-1* mutant seeds on MS medium containing 100 mM NaCl (a) and 125 mM NaCl (b). The seeds were vernalized for 3 days and grown on MS medium for 3 days (100 mM NaCl) or 4 days (125 mM NaCl).

Figure S2. Real-time RT-PCR of *PUB31* in the wild-type and the *pub31* (Salk_054774) mutant.

Figure S3. Analysis of the transgenic lines harboring *GFP-PUB30* in *pub30-1* background in response to salt.

(a) The wild-type, *pub30-1* and two independent lines were germinated on MS medium for 9 days.

(b) The wild-type, *pub30-1* and two independent lines were germinated on MS medium containing 150 mM NaCl.

(c) Quantitative evaluation of radicle emergence of the wild-type, *pub30-1* and two independent transgenic lines in (a) and (b).

Figure S4. Analysis of the wild-type and the *bki1* mutant in radicle emergence when grown on the medium containing different concentration of ABA for different time.

Figure S5. The response of the wild-type, the *bki1* mutant and two independent transgenic lines overexpressing *Myc-BK11* to salt in seedling growth.

(a) Five days old seedlings grown on the MS medium were transferred to MS medium containing different concentration of NaCl for 20 days. (b) and (c) Quantitative evaluation of seedling growth in (a). Fresh weight (b) and primary root length (c).

Figure S6. Real-time RT-PCR of *BK11* in the wild-type and the *pub30-1* mutant treated with or without 150 mM NaCl for 3 hours.

Figure S7. Protein level of *Myc-BK11* in the transgenic seedlings overexpressing *Myc-BK11* treated with 150 mM NaCl for indicated time points. Anti-actin was used as a loading control.

Figure S8. Subcellular localization and membrane dissociation of *BK11-YFP*. Seven-day-old transgenic seedlings expressing *BK11-YFP* were

treated with 1 μ M BL or 50 mM NaCl for 5 min. The right column was the larger version of white box in the left column. Bars = 100 μ m.

Figure S9. Analysis of the wild-type and the *pub30-1* mutant in response to BL. The seeds were grown on the MS medium containing two different concentrations (0.5 and 2 μ M) of BL for 2 days.

Table S1. Primers for real-time quantitative RT-PCR.

Table S2. Primers for plasmids construction.

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