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Research Article

U-box E3 ubiquitin ligase PUB8 attenuates abscisic acid responses during early seedling growth

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Abstract

ABSCISIC ACID-INSENSITIVE3 (ABI3) and ABI5 are 2 crucial transcription factors in abscisic acid (ABA) signaling, and their homeostasis at the protein level plays a decisive role in seed germination and subsequent seedling growth. Here, we found that PLANT U-BOX 8 (PUB8), a U-box E3 ubiquitin ligase, physically interacts with ABI3 and ABI5 and negatively regulates ABA responses during early Arabidopsis (*Arabidopsis thaliana*) seedling growth. Loss-of-function *pub8* mutants were hypersensitive to ABA-inhibited cotyledon greening, while lines overexpressing *PUB8* with low levels of ABI5 protein abundance were insensitive to ABA. Genetic analyses showed that ABI3 and ABI5 were required for the ABA-sensitive phenotype of *pub8*, indicating that PUB8 functions upstream of ABI3 and ABI5 to regulate ABA responses. Biochemical analyses showed that PUB8 can associate with ABI3 and ABI5 for degradation through the ubiquitin-mediated 26S proteasome pathway. Correspondingly, loss-of-function of *PUB8* led to enhanced ABI3 and ABI5 stability, while overexpression of *PUB8* impaired accumulation of ABI3 and ABI5 *in planta*. Further phenotypic analysis indicated that PUB8 compromised the function of ABI5 during early seedling growth. Taken together, our results reveal the regulatory role of PUB8 in modulating the early seedling growth by controlling the homeostasis of ABI3 and ABI5.

Introduction

Seed germination and post-germination growth are indispensable events during the early stages of plant growth and development, and they are precisely regulated by endogenous and environmental cues (Finkelstein et al. 2002, 2008; Finch-Savage and Leubner-Metzger 2006; Rajjou et al. 2012; Nonogaki 2017). As a key endogenous signal in plants, the phytohormone abscisic acid (ABA) is involved in promoting seed maturation and dormancy while inhibiting seed germination and early seedling establishment, as determined in studies on Arabidopsis (*Arabidopsis thaliana*; Finkelstein et al. 2002, 2008; Finch-Savage and Leubner-Metzger 2006; Rajjou et al. 2012; Nonogaki 2014, 2017). The ABA signal is perceived by its receptors PYRABACTIN RESISTANCE/REGULATORY COMPONENT OF ABSCISIC ACID RECEPTOR (Ma et al. 2009; Park et al. 2009). After ABA binds to PYR1-LIKEs, the ABA-bound receptors tightly interact with type 2C protein phosphatases to inhibit their dephosphorylation activity and dissociate from SNF1-RELATED KINASES 2 (SnRK2s). Subsequently, the released SnRK2s directly phosphorylate targeted transcription factors, such as ABSCISIC ACID INSENSITIVE (ABI5) as well as its homologs,

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to mediate ABA responses (Fujii et al. 2009; Nakashima and Yamaguchi-Shinozaki 2013; Yoshida et al. 2014; Vishwakarma et al. 2017).

In Arabidopsis, ABI3 and ABI5 are the core regulators of the ABA-signaling pathway that mediates multiple ABA responses, especially the regulation of seed germination and subsequent seedling establishment. The *ABI3* and *ABI5* genes were identified from the basis of ABA-insensitive germination. Mutations of *ABI3* and *ABI5* resulted in ABA-insensitivity during seed germination and early seedling development (Kornneef et al. 1984; Giraudat et al. 1992; Finkelstein 1994; Finkelstein and Lynch 2000). The ABI3 protein containing 3 basic domains B1, B2, and B3, along with the N-terminal A1 domain that is thought to be important for activation. The B1 and B2 domains are responsible for nuclear localization and association with other proteins, while the B3 domain is known to bind the RY motif of seed development genes (Suzuki et al. 1997; Ezcurra et al. 2000; Monke et al. 2004). ABI3 plays an essential role in the establishment of both primary seed dormancy and thermoinhibition, and is also required for ABA-dependent post-germinative growth arrest (Kornneef et al. 1984; Parcy et al. 1994; Lopez-Molina et al. 2002; Tamura et al. 2006). ABI5, which belongs to the subfamily of bZIP transcription factors, can bind to the ABA-responsive element within the promoter region of target genes, activating their expression to inhibit seed germination and subsequent seedling establishment (Giraudat et al. 1992; Shen et al. 1996; Hattori et al. 2002). ABI3 and ABI5 can either work alone or together with other factors to modulate ABA-dependent biological functions (Lopez-Molina et al. 2002; Hu and Yu 2014; Hu et al. 2019; Pan et al. 2020; Yang et al. 2021).

Recently, a growing body of research has demonstrated that precise regulation by post-translational modifications (PTMs) plays a decisive role during ABA signal transduction (Liu and Stone 2014; Yu et al. 2015, 2021). For instance, phosphorylation of ABI5 directly influences seed germination and subsequent seedling establishment (Stone et al. 2006; Lyzenga et al. 2013; Hu and Yu 2014; Zhou et al. 2015). In response to ABA, ABI5 is phosphorylated and stabilized by SnRK2s, resulting in the inhibition of seed germination (Kobayashi et al. 2005; Furihata et al. 2006; Fujii et al. 2007; Fujii and Zhu 2009; Nakashima et al. 2009). In addition, ABI5 is regulated by other modifications, such as sumoylation and ubiquitination. The level of ABI5 is stabilized by SAP AND MIZ1 DOMAIN-CONTAINING LIGASE 1 (SIZ1), a SUMO E3 ligase that directly sumoylates ABI5 (Miura et al. 2009). The RING-type E3 ligase KEEP ON GOING (KEG) ubiquitinates ABI5, thereby maintaining it at low level during seed germination (Liu and Stone 2010). The ABI3-INTERACTING PROTEIN 2 (AIP2) E3 ligase negatively regulates ABA signaling through promoting ABI3 degradation (Zhang et al. 2005). Although remarkable progress has been achieved in recent years, the precise mechanism of how ABA signaling is attenuated still need to be further explored.

In the present study, we found that a plant U-box E3 ligase, PLANT U-BOX8 (PUB8), targets ABI3 and ABI5, and negatively mediates ABA responses. Loss-of-function *pub8* mutants were hypersensitive to ABA treatment during cotyledon greening, while lines overexpressing *PUB8* were less sensitive to ABA. Genetic analyses showed the ABA-sensitive phenotype of *pub8* was dependent on ABI3 and ABI5, implying that PUB8 functions upstream of ABI3 and ABI5 to regulate ABA responses. Biochemical analyses showed that PUB8 can associate with ABI3 and ABI5 for degradation through the ubiquitin-mediated 26S proteasome pathway both in vivo and in vitro. Further phenotypic analyses showed that PUB8 dampens the function of ABI5 *in planta*. Disruption of *PUB8* enhanced the ABA-hypersensitive phenotype of the transgenic seedlings overexpressing *ABI5*, whereas the overexpression of *PUB8* attenuated the ABA-hypersensitive phenotype of the *ABI5*-overexpressing seedlings. Collectively, our results show that a PUB8-mediated ubiquitination proteasome pathway participates in regulating ABA signaling to precisely control early seedling establishment after seed germination.

Results

PUB8 physically interacts with ABI3 and ABI5

As mentioned above, ABA signal transduction plays a decisive role during seed germination and subsequent seedling establishment. To explore the precise regulation of ABA signaling, we performed yeast 2-hybrid (Y2H) screening to identify proteins that may interact with ABI3. In these assays, we fused ABI3 to the Gal4 DNA-binding domain (BD) and used it as bait (BD-ABI3) to screen a homogenized cDNA library of Arabidopsis. Sequencing revealed that the 5 positive clones contained the same insert sequence, encoding the N-terminal region of PUB8. To confirm the interaction between ABI3 and PUB8, we fused the full-length coding sequence (CDS) of *PUB8* with the Gal4 activation domain (AD) and conducted Y2H assays. As shown in Fig. 1A, ABI3 physically associated with PUB8 in the Y2H system, and the C-terminal amino acid residues 416–720 of ABI3 containing the B2 and B3 domains were required for the interaction with PUB8. Because ABI3 associates with ABI5 to mediate ABA signal during seed germination (Lopez-Molina et al. 2002; Pan et al. 2020), we speculated that PUB8 may also interact with ABI5. To test this hypothesis, we conducted a targeted Y2H assay between ABI5 and PUB8. As expected, ABI5 also associated with PUB8 in the Y2H system, and the N-terminal amino acid residues 1–164 of ABI5 were required for the physical interaction with PUB8 (Fig. 1B). To identify which region of PUB8 is essential for the interaction with ABI3 and ABI5, we fused 2 truncated PUB8 variants to the Gal4 AD and examined the interaction between these variants and ABI3 and ABI5. As shown in Supplemental Fig. S1, the C-terminal region of PUB8 was required for its interaction with ABI3 and ABI5.

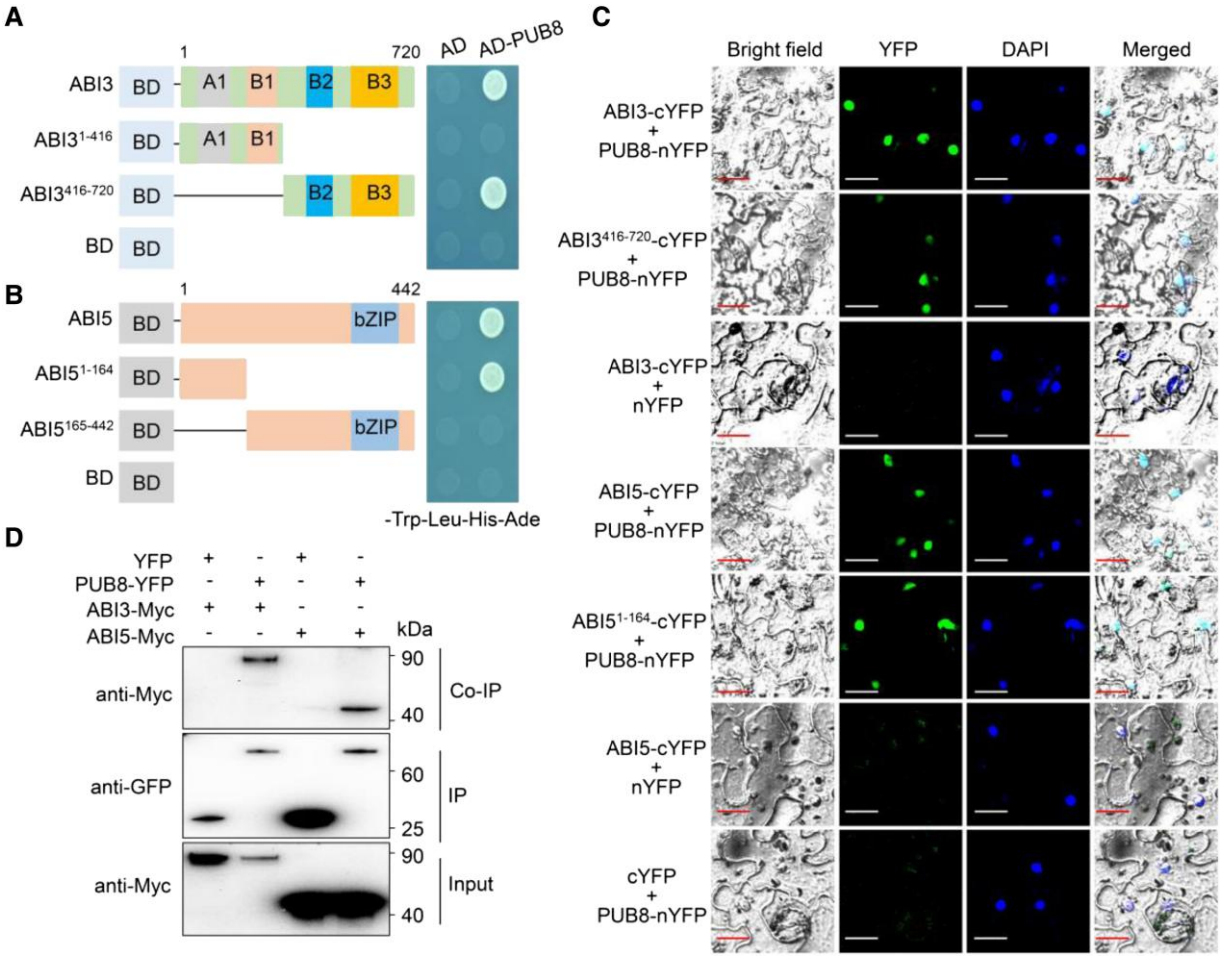

Figure 1. Physical interactions of PUB8 with ABI3 and ABI5. A, B) ABI3 and ABI5 interact with PUB8 in yeast. Interactions of PUB8 with full length and truncated ABI3 and ABI5 are indicated by the ability of yeast cells to grow on dropout medium lacking Leu, Trp, His, and Ade for 4 d after plating. pGBKT7 (BD) and pGADT7 (AD) were used as negative controls. C) BiFC assays. Fluorescence was observed in the nuclear compartment of transformed *N. benthamiana* cells, resulting from the complementation of PUB8-nYFP with ABI3-cYFP, PUB8-nYFP with ABI3$^{416–720}$-cYFP, PUB8-nYFP with ABI5-cYFP, or PUB8-nYFP with ABI5$^{1–164}$-cYFP. No signal was obtained for the negative controls in which PUB8-nYFP was coexpressed with cYFP, and n-YFP was coexpressed with ABI3-cYFP and ABI5-cYFP. Nuclei are indicated by DAPI staining. Scale = 20 mm. D) CoIP assay. Arabidopsis protoplast expressing combinations of PUB8-YFP and ABI3-Myc, YFP and ABI3-Myc, PUB8-YFP and ABI5-Myc, and YFP and ABI3-Myc were incubated in darkness for 16 h. Total proteins were extracted, and then immunoprecipitated with GFP-trap beads. The coimmunoprecipitated proteins were detected by anti-Myc antibody. Experiments in this figure were repeated at least 4 times with similar trends.

To further confirm the interaction of ABI3 and ABI5 with PUB8 in plant cells, we conducted bimolecular fluorescence complementation (BiFC) assays. The full-length and truncated CDSs of ABI3 and ABI5 were each ligated with the sequence encoding the C-terminal yellow fluorescent protein (YFP) fragment driven by the Cauliflower mosaic virus (CaMV) 35S promoter to generate ABI3-cYFP, ABI5-cYFP, ABI3$^{416–720}$-cYFP and ABI5$^{1–164}$-cYFP, respectively, while the full-length CDS of PUB8 was fused with the sequence encoding the N-terminal YFP fragment to produce PUB8-nYFP. When each pair (ABI3-cYFP and PUB8-nYFP, ABI5-cYFP and PUB8-nYFP, ABI3$^{416–720}$-cYFP and PUB8-nYFP, and ABI5$^{1–164}$-cYFP and PUB8-nYFP) was transiently coexpressed in leaf cells of *Nicotiana benthamiana*, strong YFP fluorescence was detected in the nucleus of the transformed cells, as indicated by the nuclear 4′,6-diamidino-2-phenylindole (DAPI) signal (Fig. 1C and Supplemental Fig. S2). As controls, the pairs ABI3-cYFP and nYFP, ABI5-cYFP and nYFP, PUB8-nYFP and cYFP were coexpressed transiently in leaf cells of *N. benthamiana*. As expected, no YFP fluorescence was detected in the nucleus of the cells expressing these pairs (Fig. 1C). Consistently, in a co-immunoprecipitation (CoIP) assay, both ABI3 and ABI5 were immunoprecipitated by anti-GFP agarose beads in Arabidopsis protoplasts co-expressing PUB8-YFP and ABI3-Myc or PUB8-YFP and ABI5-Myc, but not in those co-expressing YFP and ABI3-Myc or YFP and ABI5-Myc (Fig. 1D). These findings provided further evidence for the association between ABI3 and PUB8, and between ABI5 and PUB8. Together, these results demonstrated that both ABI3 and ABI5 physically interact

with PUB8, implying that the E3 ubiquitin ligase PUB8 may be involved in modulating ABA-mediated seed germination and postgerminative growth.

PUB8 is a negative regulator of ABA responses during early seedling establishment

Since ABA is the essential phytohormone inhibiting seed germination and subsequent seedling growth, we tested whether the transcript level of *PUB8* responded to ABA treatment during germination and subsequent early growth establishment. Interestingly, the transcript levels of *PUB8* were low in the first 2 d but increased from day 3 of the ABA treatment (Fig. 2A). Correspondingly, PUB8 protein accumulated from day 3 of the ABA treatment (Fig. 2B). These results suggested that PUB8 may not be involved in the very early stage of seed germination, but in the postgerminative growth stage.

To test this hypothesis, we conducted germination and cotyledon greening phenotype experiments. First, we evaluated the germination of the loss-of-function *pub8* mutants (Supplemental Fig. S3), *pub8-1* and *pub8-2*, on half-strength Murashige and Skoog (MS) medium supplemented with ABA at different concentrations. As shown in Supplemental Fig. S4, there was no significant difference in germination rate between Col-0 and the *pub8* mutants. Because early seedling establishment is also another key character of ABA responses, we turned our attention to cotyledon greening during postgerminative growth. As expected, the *pub8* mutants showed an ABA-hypersensitive phenotype with much lower greening percentages than that of Col-0 during ABA-mediated inhibition of cotyledon greening (Fig. 3, A and B). To support the phenotype of the *pub8* mutants in response to ABA, we determined the transcript levels of several well-characterized ABA-responsive genes, namely *LATE EMBRYOGENESIS ABUNDANT 1* (*EM1*), *EM6*, *RESPONSIVE TO DESICCATION 29B* (*RD29B*), and *RESPONSIVE TO ABA 18* (*RAB18*) in ABA-treated germinating seeds. The transcript levels of these 4 ABA-responsive genes increased slightly in response to ABA treatment in Col-0 seeds but increased to much higher levels in *pub8* seeds (Supplemental Fig. S5, A–D). These results indicated that ABA signal transduction is enhanced in *pub8* mutants and that PUB8 negatively modulates early seedling establishment.

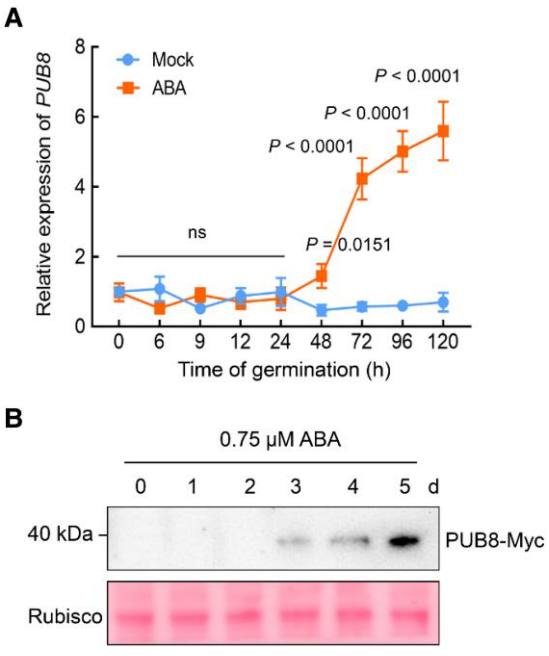

Figure 2. PUB8 protein and transcript accumulation is regulated by ABA. A) Transcriptional levels of *PUB8* responses to ABA during seed germination. RT-qPCR analysis conducted from Col-0 seedlings germinated on MS medium containing 0.75-μM ABA for indicated times. Data are mean ± s.e.m. ($n = 3$ biologically independent samples); analyzed by 2-way ANOVA with Tukey's test ($P < 0.05$, significant difference; ns, not significant). Experiments were repeated at least 4 times with similar trends. *P* values for additional comparisons are provided in Supplemental Data Set S1. B) Abundance of PUB8 protein induced by ABA during seed germination. Western blot analysis obtained from *PUB8-Myc-OE* seedlings germinated on MS medium containing 0.75-μM ABA for indicated times by anti-Myc primary antibody.

Given that ABI5 protein abundance determines the efficiency of the postgermination of ABA-dependent growth arrest (Lopez-Molina et al. 2001; Brocard et al. 2002), we detected the endogenous ABI5 protein levels in Col-0 and *pub8-1* before and after ABA treatment to clarify if the ABA hypersensitive phenotype of *pub8* mutants resulted from high ABI5 accumulation. Consistent with the previous studies, ABI5 protein was undetectable without ABA treatment, however, it accumulated and reached high levels after 2 d of ABA treatment, and then degraded gradually after 3 d in Col-0 (Lopez-Molina et al. 2001; Guan et al. 2014; Hu and Yu 2014; Zhao et al. 2016; Fig. 3C). However, the ABI5 protein accumulated high levels in *pub8-1* even up to 5 d of ABA treatment, indicating the ABA hypersensitive phenotype of *pub8* mutants during postgerminative stage was related to high endogenous ABI5 abundance (Fig. 3C).

To further confirm this conclusion, we generated transgenic plants *PUB8-OE-1* (*35S:YFP-PUB8*) and *PUB8-OE-2* (*35S:Myc-PUB8*) overexpressing *PUB8* under the control of the CaMV 35S promoter and then evaluated their greening on half-strength MS medium supplemented with ABA at different concentrations. Compared with Col-0, *PUB8-OE* seedlings showed significantly higher greening rates and an ABA-insensitive phenotype upon ABA treatment (Fig. 3, D and E). In contrast to *pub8* mutants, *PUB8-OE* seeds showed significantly decreased transcript levels of ABA-responsive genes and lower ABI5 protein levels in response to ABA treatment, compared with those in Col-0 (Fig. 3F and Supplemental Fig. S6, A–D). Thus, the overexpression of *PUB8* inhibited ABA responses during early seedling growth. These results further supported the conclusion that *PUB8* negatively mediates ABA signaling to promote early seedling establishment in Arabidopsis by inhibiting the accumulation of endogenous ABI5 protein.

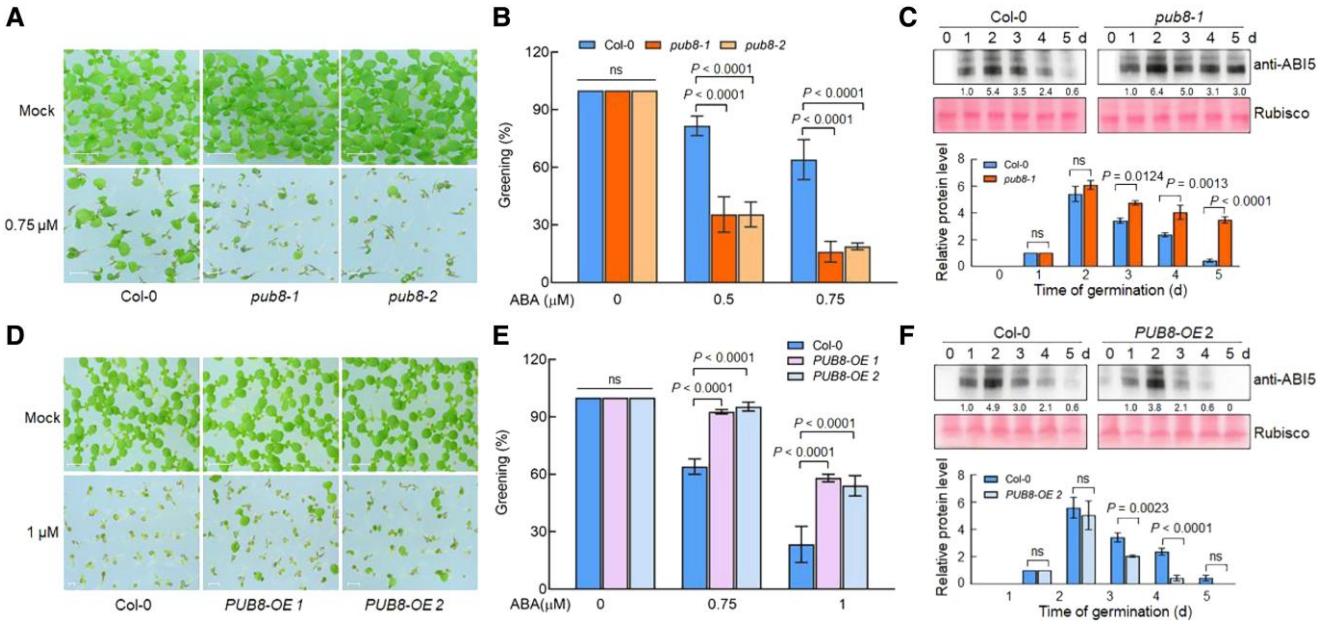

Figure 3. ABA responses of *pub8* mutants and *PUB8*-overexpressing seedlings during Cotyledon greening. A) Seedlings of Col-0, *pub8-1* and *pub8-2* mutants after germination on half-strength MS medium containing 0.75 μM ABA for 7 d. Scale bar represents 5 mm. B) Cotyledon greening rates of Col-0, *pub8-1* and *pub8-2* mutants. Cotyledon greening was scored 5 d after stratification on half-strength MS medium supplemented with different concentrations of ABA. Data are mean ± s.e.m. ($n = 5$ biologically independent samples); analyzed by 2-way ANOVA with Tukey's test ($P < 0.05$, significant difference; ns, not significant). Experiments were performed at least 6 times by analyzing different batches of seeds with similar trends. Each batch of seeds of Col-0, *pub8-1* and *pub8-2* mutants was pooled from more than 60 independent plants. C) Endogenous ABI5 protein accumulation in the Col-0 and *pub8-1* seedling. Protein samples were collected from Col-0 and *pub8-1* seedling with 0.75 μM ABA treatment following 3 d of imbibition. D) Seedlings of *PUB8*-overexpressing plants, *35S:PUB8-YFP* (*PUB8-OE-1*) and *35S:PUB8-Myc* (*PUB8-OE-2*), after germination on half-strength MS medium containing 1.0-μM ABA for 7 d. Scale bar represents 5 mm. E) Cotyledon greening rates of Col-0, *PUB8-OE-1* and *PUB8-OE-2*. Cotyledon greening was scored 5 d after stratification on half-strength MS medium supplemented with different concentrations of ABA. Data are mean ± s.e.m. ($n = 3$ biologically independent samples); analyzed by 2-way ANOVA with Tukey's test ($P < 0.05$, significant difference; ns, not significant). Experiments were performed at least 6 times by analyzing different batches of seeds with similar trends. Each batch of seeds of Col-0, *PUB8-OE-1* and *PUB8-OE-2* was pooled from more than 60 independent plants. F) Endogenous ABI5 protein accumulation in the Col-0 and *PUB8-OE 2* seedling. Protein samples were collected from Col-0 and *PUB8-OE* seedling with 0.75 μM ABA treatment following 3 d of imbibition. For (C) and (F), abundance of ABI5 was detected with anti-ABI5 (Solarbio, K900024P), and Ponceau S staining of Rubisco confirmed equal loading. Data are mean ± s.e.m. ($n = 3$ biologically independent samples); analyzed by 2-way ANOVA with Tukey's test ($P < 0.05$, significant difference; ns, not significant). Relative protein levels of ABI5 were quantified by Image J. Experiments in these figures were repeated at least 3 times with similar trends.

PUB8 exerts genetic effects upstream of ABI3 and ABI5

Because our results showed that PUB8 interacts with ABI3 and ABI5 and it negatively regulates ABA responses during early seedling growth, we wondered if PUB8 and ABI3/ABI5 have a clear up- or downstream regulatory relationship genetically. To test this possibility, we constructed the *pub8-1 abi3-8* double mutant by genetically crossing *pub8-1* with *abi3-8* (a loss-of-function mutant of *ABI3* in the Col-0 background). Unlike the *pub8-1* mutant, the *pub8-1 abi3-8* double mutant showed an ABA-insensitive phenotype similar with that of the *abi3-8* single mutant (Fig. 4A), with a much higher greening percentage than those of Col-0 and *pub8-1* seedlings (Fig. 4B). Correspondingly, high transcript levels of ABA-responsive genes in *pub8-1* seedlings were largely restored by *abi3-8* (Supplemental Fig. S7, A–D).

We also obtained the *pub8-1 abi5-8* double mutant by genetic crossing. The *pub8-1 abi5-8* double mutant displayed an ABA-insensitive phenotype like that of the *abi5-8* single mutant, with much higher percentages of greening and lower expression levels of ABA-responsive genes compared with those of the wild-type and *pub8* seedlings (Fig. 4, C and D and Supplemental Fig. S8, A–D). Collectively, these results demonstrated that the ABA hypersensitivity of *pub8* requires functional ABI3 and ABI5 transcription factors, and that PUB8 acts upstream of ABI3 and ABI5 to participate in the ABA-mediated regulation of early seedling establishment.

PUB8 mediates the ubiquitination of ABI3 and ABI5

Given that PUB8 encodes an E3 ubiquitin ligase and associates with ABI3 and ABI5 in Arabidopsis cells, we assessed whether PUB8 can ubiquitinate ABI3 and ABI5 by using purified recombinant His-PUB8 along with GST-ABI3 and GST-ABI5. In these analyses, the ubiquitination of ABI3 and ABI5 occurred in the presence of E1, E2, PUB8, and ubiquitin (Ub), but not when any of E1, E2, or PUB8 was missing

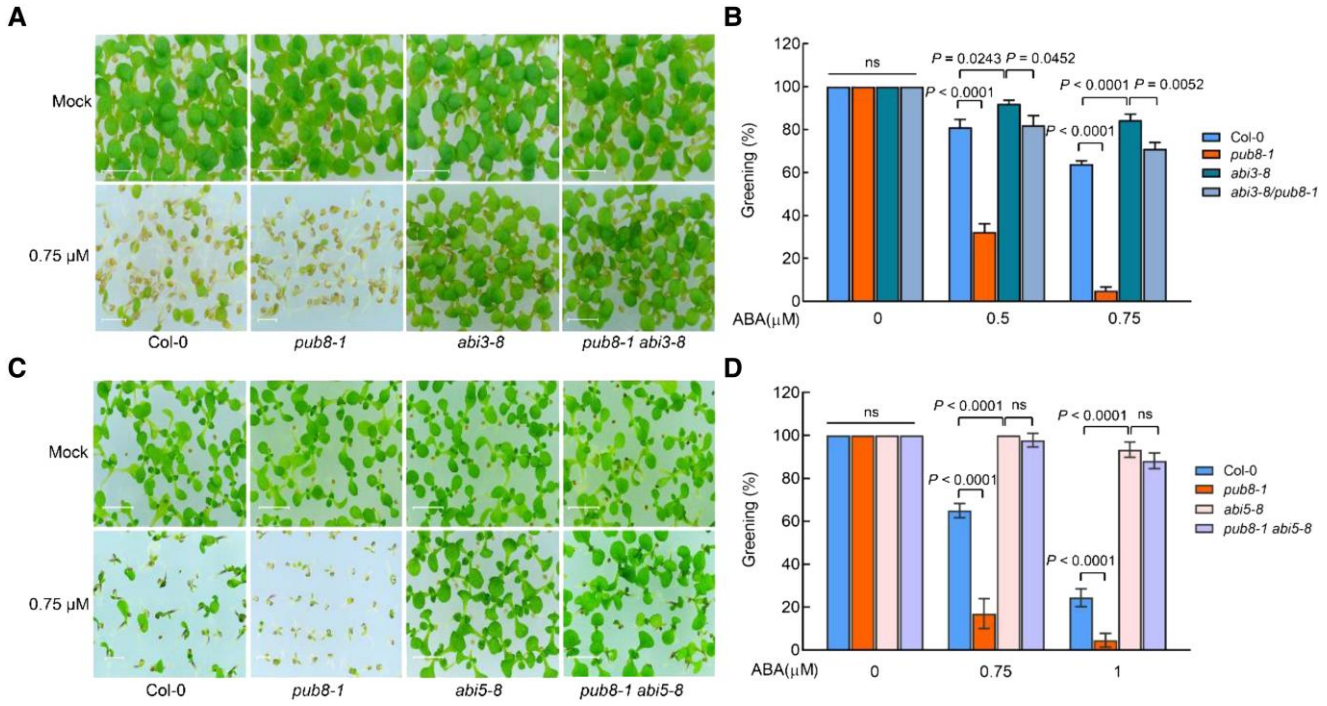

Figure 4. ABA hypersensitivity of *pub8* requires functional ABI3 and ABI5. A) Seedlings of Col-0, *pub8-1*, *abi3-8* and *pub8-1 abi3-8* double mutants 6 d after germination on half-strength MS medium containing 0.75 μM ABA. Scale bar represents 5 mm. B) Cotyledon greening of Col-0, *pub8-1*, *abi3-8* and *pub8-1 abi3-8* double mutants. Cotyledon greening was scored 5 d after stratification on half-strength MS medium supplemented with different concentrations of ABA. Data are mean ± s.e.m. ($n = 3$ biologically independent samples); analyzed by 2-way ANOVA with Tukey's test ($P < 0.05$, significant difference; ns, not significant). Experiments were performed at least 6 times by analyzing different batches of seeds with similar trends. Each batch of seeds of Col-0, *pub8-1*, *abi3-8* and *pub8-1 abi3-8* double mutants was pooled from more than 60 independent plants. C) Seedlings of Col-0, *pub8-1*, *abi5-8* and *pub8-1 abi5-8* double mutants 7 d after germination on half-strength MS medium containing 0.75 μM ABA. Scale bar represents 5 mm. D) Cotyledon greening of Col-0, *pub8-1*, *abi5-8* and *pub8-1 abi5-8* double mutants. Cotyledon greening was scored 5 d after stratification on half-strength MS medium supplemented with different concentrations of ABA. Data are mean ± s.e.m. ($n = 5$ biologically independent samples); analyzed by 2-way ANOVA with Tukey's test ($P < 0.05$, significant difference; ns, not significant). Experiments were performed at least 6 times by analyzing different batches of seeds with similar trends. Each batch of seeds of Col-0, *pub8-1*, *abi5-8* and *pub8-1 abi5-8* double mutants was pooled from more than 60 independent plants.

(Fig. 5, A and B). These results confirmed that PUB8 mediates the ubiquitination of ABI3 and ABI5 in vitro.

Next, we tested whether ABI3 and ABI5 are ubiquitinated by PUB8 in vivo. We co-transformed *N. benthamiana* leaves with either Myc-UBQ and ABI3-YFP along with PUB8-Myc, Myc-UBQ, and ABI5-YFP along with PUB8-Myc or Myc-UBQ and ABI3-YFP alone, or with Myc-UBQ and ABI5-YFP alone. After immunoprecipitation with GFP-trap beads, an anti-Myc antibody was used to detect polyubiquitinated ABI3-YFP and ABI5-YFP. Their abundance was increased in the presence of PUB8-Myc (Fig. 5, C and D). These results demonstrated that PUB8 targets ABI3 and ABI5 for polyubiquitination in vivo.

PUB8 promotes degradation of ABI3 and ABI5 *in planta*

To determine whether PUB8 mediates the degradation of ABI5 *in planta*, we generated transgenic plants overexpressing *ABI5-Myc* in Col-0 (*ABI5-Myc* Col-0), *pub8-1* (*ABI5-Myc pub8-1*), and *PUB8-OE* (*ABI5-Myc PUB8-YFP*) backgrounds and treated them with 50 μM ABA combined with 500 μM chlorhexidine (CHX), a protein synthesis inhibitor, to further examine the effect of PUB8 on ABI5 degradation. As shown in Fig. 6, A and B, the degradation of ABI5-Myc was reduced in the *pub8-1* mutant compared with Col-0 and was largely suppressed by the proteasome inhibitor, MG132. By contrast, the degradation of ABI5-Myc was significantly increased in the background of *PUB8-OE* (Fig. 6, C and D).

Because the protein level of ABI3 was undetectable in transgenic plants overexpressing *ABI3* driven by the 35S promoter, we transiently expressed *ABI3-Myc* in Col-0, *pub8-1*, and *PUB8-OE* protoplasts. At 16 h after transfection, *ABI3-Myc*-expressing protoplasts were treated with 25 μM ABA comminated with 200 μM CHX to evaluate the effect of PUB8 on ABI3 degradation. Compared with Col-0, the *pub8-1* mutant showed compromised degradation of ABI3-Myc (Fig. 6, E and F), whereas the degradation of ABI3-Myc was sharply induced in the *PUB8-OE* (Fig. 6, G and H). Taken together, these findings indicated that PUB8 targets both ABI3 and ABI5 for degradation by the 26S proteasome pathway *in planta*, which may release their inhibition for early seedling establishment.

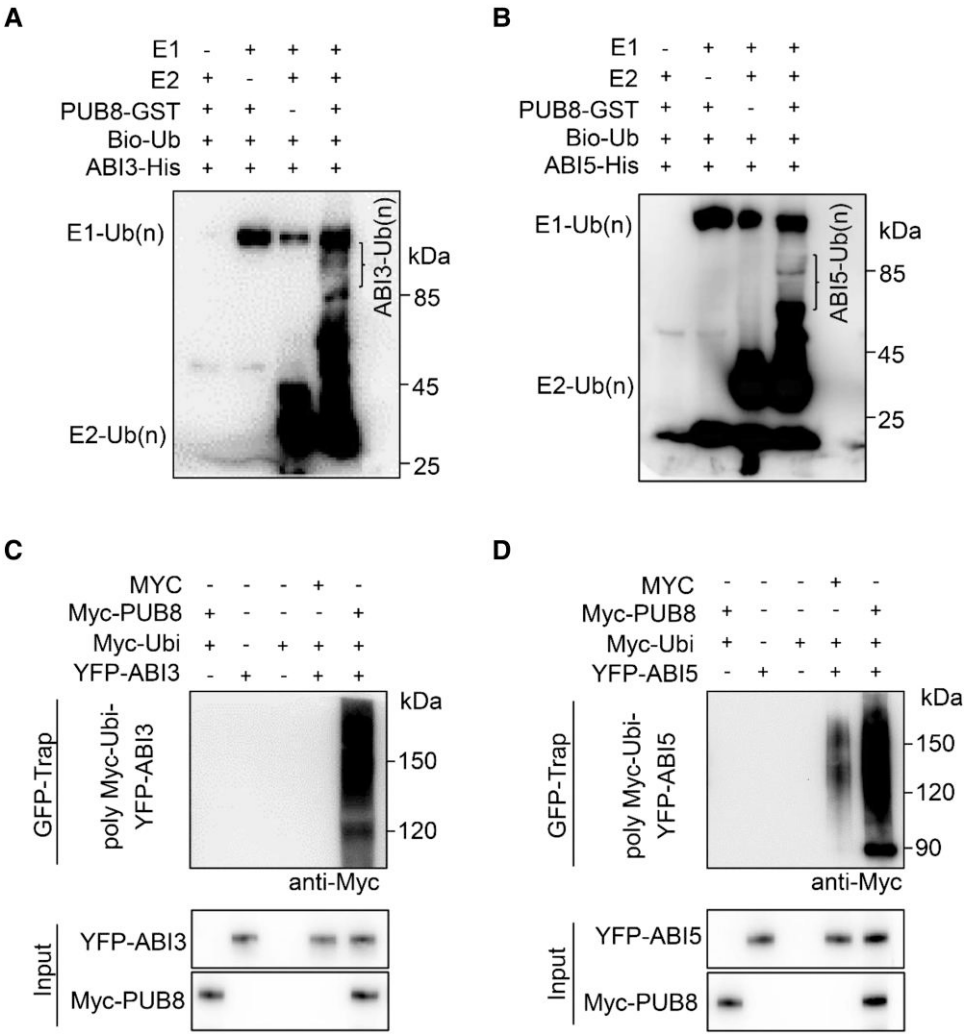

Figure 5. ABI3 and ABI5 are ubiquitinated by PUB8. A, B) PUB8 ubiquitinates ABI3 and ABI5 in vitro. *E. coli*-purified His-PUB8 and GST-ABI3 as well as His-PUB8 and GST-ABI5 were incubated with 1× ubiquitinylation buffer, 5 mM Mg-ATP solution, 1 mM DTT, 100 nM human E1, 2.5 mM E2 (ubiquitin conjugating enzyme UBCH8), 2.5 mM biotin-ubiquitin, and 1 unit of inorganic pyrophosphatase at 37°C for 4 h and subjected to in vitro ubiquitination assay. The ubiquitination of ABI3 and ABI5 were detected by an HRP-Streptavidin detection system. C, D) PUB8 ubiquitinates ABI3 and ABI5 in vivo. *N. benthamiana* leaves expressing combinations were incubated in darkness for 48 h in the presence of 50 μM MG132. Total proteins were extracted and immunoprecipitated with GFP-trap beads. The poly-ubiquitination of ABI3 and ABI5 were detected with anti-Myc antibody. Experiments in this figure were repeated at least 4 times with similar trends.

PUB8 compromises the function of ABI5 *in planta*

We expected that the ubiquitin-mediated degradation of ABI3 and ABI5 by PUB8 would restrict their functions during cotyledon greening. Given that ABI5 is the master regulator of ABA responses and acts downstream of ABI3 to regulate ABA-responsive genes during early seedling establishment, we selected ABI5 for further analyses of its function. For these analyses, we investigated the cotyledon greening phenotypes of *ABI5-Myc* in Col-0 and *pub8-1* backgrounds upon ABA treatment. As expected, the greening percentage of *pub8-1 ABI5-Myc* was significantly lower than that of *ABI5-Myc* and *pub8-1* seedlings (Fig. 7, A and B). Consistently, the transcript levels of ABA-responsive genes were sharply increased in *pub8-1 ABI5-Myc* and reached higher levels than those in *ABI5-Myc* and *pub8-1* seedlings (Supplemental Fig. S9, A–D). These results demonstrated that the loss of function of *PUB8* indeed enhances the inhibitory function of ABI5 during cotyledon greening.

To provide further evidence that PUB8 regulates the function of ABI5 *in planta*, we evaluated the performance of *PUB8-OE ABI5-Myc* double-overexpression seeds growing on half-strength MS medium containing ABA at different concentrations. As shown in Fig. 7, C and D, the sensitivity of *ABI5-Myc* seedlings to ABA was compromised by *PUB8-OE*. Correspondingly, the high transcript levels of ABA-responsive genes in *ABI5-Myc* were antagonized by *PUB8-OE* (Supplemental Fig. S10, A–D). Overall, the results showed that overexpression of *PUB8* can partially restore

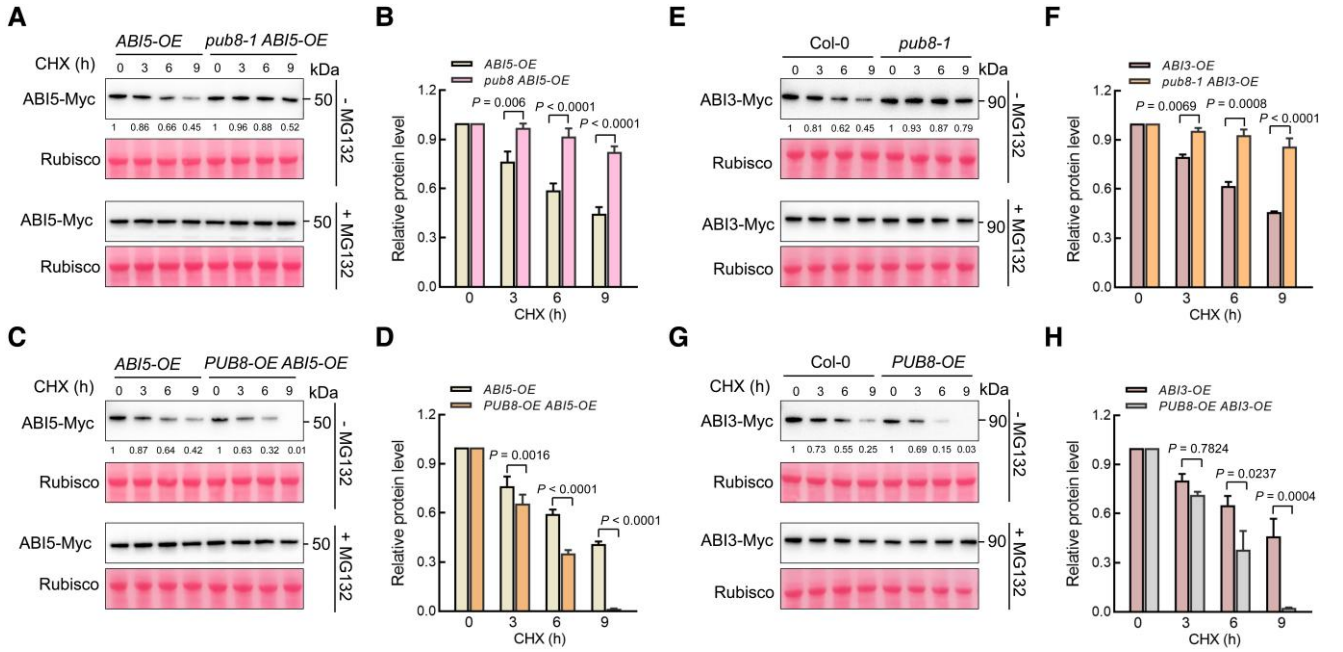

Figure 6. PUB8 are required for ABI3 and ABI5 degradation *in planta*. A–D) Stability and relative protein levels of ABI5 in Col-0, *pub8-1* mutant, and *PUB8-OE* plants background. Fourteen-day-old *ABI5-OE*, *pub8-1 ABI5-OE* and *PUB8-OE ABI5-OE* transgenic seedlings were treated with 500 μM CHX and 50 μM ABA for the indicated time with or without 100 μM MG132. ABI5-Myc was detected with anti-Myc antibody, and Ponceau S staining of Rubisco confirmed equal loading. Data are mean ± s.e.m. ($n = 3$ biologically independent samples); analyzed by 2-way ANOVA with Tukey's test ($P < 0.05$, significant difference; ns, not significant). Relative protein levels of ABI5 were quantified by Image J. E–H) Stability and relative protein levels of ABI3 in vivo. Protoplast of Col-0, *pub8-1* and *PUB8-YFP* expressing *ABI3-Myc* for 16 h in the presence of 5 μM MG132 then treated with 200 μM CHX and 25 μM ABA for the indicated time with or without 100 μM MG132. ABI3-Myc was detected with anti-Myc antibody, and Ponceau S staining of Rubisco confirmed equal loading. Data are mean ± s.e.m. ($n = 3$ biologically independent samples); analyzed by 2-way ANOVA with Tukey's test ($P < 0.05$, significant difference; ns, not significant). Relative protein levels of ABI3 were quantified by Image J. Experiments in this figure were repeated at least 3 times with similar trends.

the ABA-sensitive phenotype of *ABI5*-overexpressing plants and provided further evidence that PUB8 affects the function of ABI5 *in planta*, thus regulating its biological functions during cotyledon greening.

Discussion

In plants, ABA plays a vital role in repressing seed germination and subsequent early seedling establishment, and ABI3 and ABI5 are 2 essential regulators of these developmental processes (Lopez-Molina and Chua 2000; Lopez-Molina et al. 2001; Finkelstein et al. 2002; Lopez-Molina et al. 2002). Recent studies have provided several lines of evidence showing that PTMs of ABI3 and ABI5 affect ABA signal transduction and directly influence seed germination and seedling establishment (Zhang et al. 2005; Stone et al. 2006; Miura et al. 2009; Lee et al. 2010; Hu and Yu 2014; Jin et al. 2018). The findings of those studies and our study indicate that PTMs are indispensable for ABA responses. Identifying other crucial regulators of ABI3 and ABI5 and determining how their protein levels are precisely controlled increase our understanding of the regulation of seed germination and early developmental stages of seedlings. In the present study, we identified PUB8 as a regulatory factor that negatively modulates ABA responses by reducing the stability of ABI3 and ABI5 during early seedling growth. This protein was screened as prey of ABI3 from a homogenized cDNA library of Arabidopsis. Further analyses showed that PUB8 physically interacts not only with ABI3 but also ABI5 both in yeast and plant cells (Fig. 1), indicating that PUB8 participates in the regulation of ABA signaling during seed germination and postgerminative growth.

Seed germination is the first phase of the growth cycle and initiates with the uptake of water by the resting dry seed. The early stage of seedling establishment after germination involves the activation of major storage reserves and is characterized by cotyledon opening, cotyledon greening, hypocotyl growth, and radicle growth. This is the key developmental event during postgerminative growth (Bewley and Black 1985). Interestingly, in this study, we found that PUB8 plays a prominent role in the regulation of early seedling growth, but not in seed germination. One of the underlying reasons for this phenotype may be the low transcript and protein levels of PUB8 at the early stage of seed germination but both increased at the late ABA treatment stage—the phase of cotyledon greening (Fig. 2, A and B). Consistently, seedlings of *pub8* mutants showed an ABA-hypersensitive phenotype, while *PUB8-OE* seedlings displayed ABA-insensitive

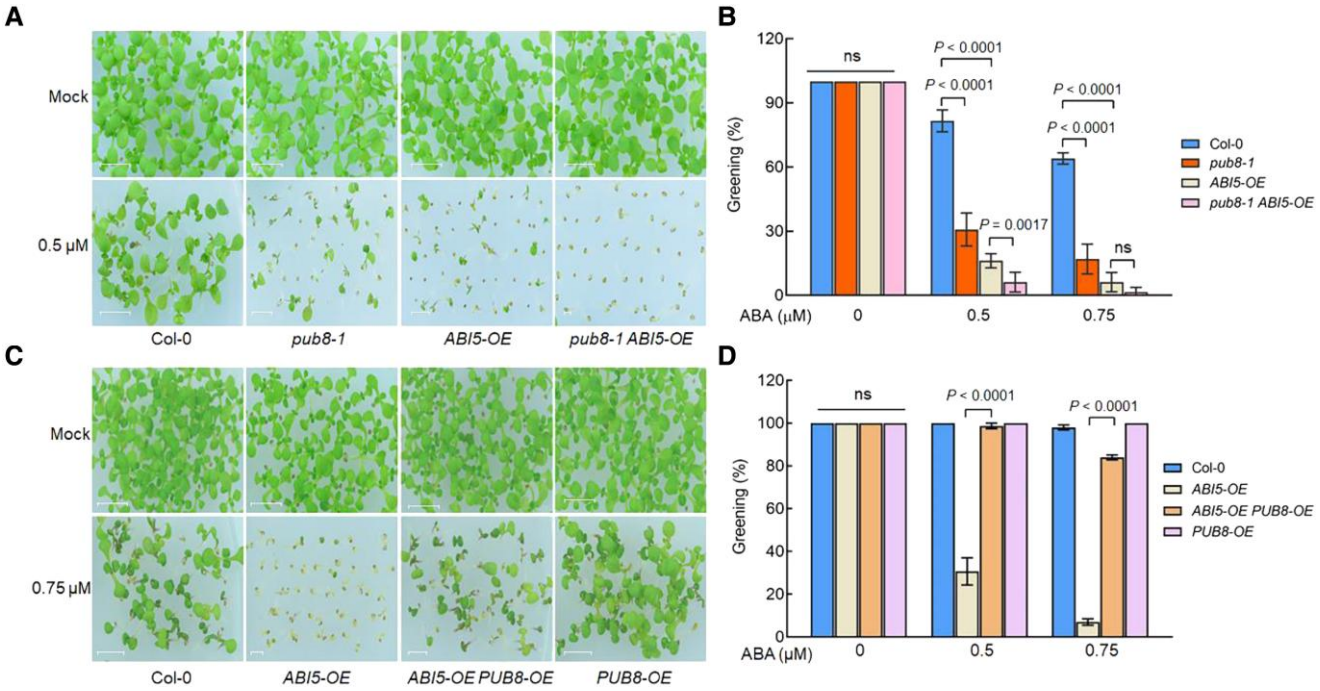

Figure 7. *PUB8* knock-down enhances the function of ABI5 in plants. A) Seedlings of Col-0, *ABI5-OE* (*35S:ABI5-4xMyc*), *pub8-1* and *pub8-1 ABI5-OE* plants after germination on half-strength MS medium containing 0.5 μM ABA for 8 d. Scale bar represents 5 mm. B) Cotyledon greening rates of Col-0, *ABI5-OE*, *pub8-1* and *pub8-1 ABI5-OE* plants. Cotyledon greening was scored 5 d after stratification on half-strength MS medium supplemented with different concentrations of ABA. Data are mean ± s.e.m. ($n = 5$ biologically independent samples); analyzed by 2-way ANOVA with Tukey's test ($P < 0.05$, significant difference; ns, not significant). Experiments were performed at least 6 times by analyzing different batches of seeds with similar trends. Each batch of seeds of Col-0, *ABI5-OE*, *pub8-1* and *pub8-1 ABI5-OE* was pooled from more than 60 independent plants. C) Seedlings of Col-0, *ABI5-OE*, *PUB8-OE*-1 and *PUB8-OE-1 ABI5-OE* double overexpressing plants after germination on half-strength MS medium containing 0.75-μM ABA for 9 d. Scale bar represents 5 mm. D) Cotyledon greening rates of the Col-0, *ABI5-OE*, *PUB8-OE*-1 and *PUB8-OE-1 ABI5-OE* double overexpressing plants. Cotyledon greening was scored 7 d after stratification on half-strength MS medium supplemented with different concentrations of ABA. Data are mean ± s.e.m. ($n = 3$ biologically independent samples); analyzed by 2-way ANOVA with Tukey's test ($P < 0.05$, significant difference; ns, not significant). Experiments were performed at least 6 times by analyzing different batches of seeds with similar trends. Each batch of seeds of Col-0, *ABI5-OE*, *PUB8-OE*-1 and *PUB8-OE-1 ABI5-OE* was pooled from more than 60 independent plants.

cotyledon greening (Fig. 3, A–F). These results suggest that PUB8 may function during a narrow time window to regulate early seedling establishment. These results are also similar with those of some other studies. For instance, SENSITIVE TO ABA 1 (SAB1) was found to regulate ABI5 mainly at the postgerminative growth stage because the transcript level of *SAB1* is very low during seed germination but high during postgerminative growth (Ji et al. 2019). Another study found that cytokinin specifically antagonizes the inhibitory effect of ABA during early seedling establishment but is not required for antagonizing the ABA effect during seed germination (Guan et al. 2014). Thus, our results provide further evidence that seed germination and postgerminative growth are linked, but are different processes during early plant development, and are under the tight control of genetic programs and environmental factors. Nevertheless, the precise regulatory mechanisms involving PUB8, SAB1, and cytokinin during early seedling growth remain to be characterized. Further studies should explore whether there is crosstalk among PUB8, SAB1, and cytokinin during postgerminative growth, especially between PUB8 and SAB1 as their encoding genes show the same expression patterns upon ABA treatment.

During their lifecycle, plants are constantly subjected to external biotic and abiotic stresses. To resist these stresses, plants have evolved a variety of complex physiological and molecular responses. Signals from both internal and external sources are received and transmitted through PTMs, such as ubiquitination (Yu et al. 2021). Recently, ABI3 and ABI5 have been reported to be targeted by different types of E3 ligases (Zhang et al. 2005; Stone et al. 2006; Liu and Stone 2010). The RING-type E3 ligase KEG is required to maintain a low level of ABI5, and ABI5 is a substrate of KEG for ubiquitination (Liu and Stone 2010, 2014). A protein complex of DWD (DDB1-BINDING WD40 PROTEIN) HYPERSENSITIVE TO ABA 1 (DWA1), DWA2, DAMAGED DNA BINDING PROTEIN 1 (DDB1), and CULLIN4 (CUL4) E3 ubiquitin ligases mediates the ubiquitination of ABI5, thereby promoting seed germination and the root growth of seedlings in response to ABA (Lee et al. 2010). Although several E3 ligases have been reported to mediate the abundance of ABI3 and ABI5, the importance of ABI3 and ABI5 as nodes of convergence of

signaling networks suggests that their activity might be tightly regulated. In this study, we found that PUB8 acts upstream of ABI3 and ABI5 (Fig. 4) and destabilizes them by ubiquitination (Figs. 5 and 6), thus compromising the function of ABI5 during postgerminative growth (Fig. 7). All these examples suggest that multiple E3 ligases target ABI3 and ABI5 for precise regulation of the initiation of life under the right conditions. However, the relationships among these E3 ligases remain to be further studied. Further research should explore how different E3 ligases coordinately modulate seed germination and/or seedling establishment, and whether these E3 ligases also directly regulate each other.

Both ABI3 and ABI5 are essential regulators of the ABA-signaling pathway, and they interact with many transcription factors that regulate their stability, promoter-binding activity, and transcription activity. For instance, the core circadian clock protein PSEUDO-RESPONSE REGULATOR5 activates ABA responses during seed germination through physically interacting with ABI5 and enhancing its transcriptional activity (Yang et al. 2021). INDUCER OF CBF EXPRESSION1 (ICE1) associates with ABI5 and DELLA and antagonizes their activity to maintain ABA responses at an appropriate level during seed germination (Hu et al. 2019). ABI5 and ELONGATED HYPOCOTYL 5 (HY5) form a stable and efficient transcriptional complex, and then associate with other chromatin complexes to activate ABA responses during seed germination (Wang et al. 2022). The results of all those studies illustrate that ABI3 and ABI5 can associate with diverse transcription factors to form transcriptional complexes that enhance the adaptive capacity of the plant under adverse growth conditions. There is also evidence that U-box family proteins control the protein stability of transcription factors. For example, MYC2, a homolog of ICE1, is targeted by PUB10 for degradation during the response to jasmonic acid. The stability of MYC2 was found to be enhanced in a *pub10* mutant compared with Col-0, and root growth of *pub10* seedlings was hypersensitive to methyl jasmonate, which phenocopied *35S:Myc* seedlings (Jung et al. 2015). The results of those studies suggest that PUB8 may also mediate the stability of transcription factors that interact with ABI3 and/or ABI5 to interfere with the inhibition of early seedling growth. Further studies should clarify how PUB8 interacts with other components of the transcriptional complex that regulates germination. The findings of such studies will shed light on the regulatory processes that precisely control seed germination and early seedling establishment.

The protein levels of ABI3 and ABI5 are precisely maintained by multiple internal phytohormone signals and external environmental cues. More specifically, in the presence of ABA, the protein kinase SnRK2 phosphorylates ABI5 and promotes its stability, thereby inhibiting seed germination (Kobayashi et al. 2005; Fujii et al. 2007; Nakashima et al. 2009). The critical repressor of brassinosteroid (BR) signaling, BRASSINOSTEROID-INSENSITIVE 2, phosphorylates and stabilizes ABI5 to regulate the antagonism of BR against the ABA response during seed germination (Hu and Yu 2014). Accumulation of RGA-LIKE 2 in the presence of a low

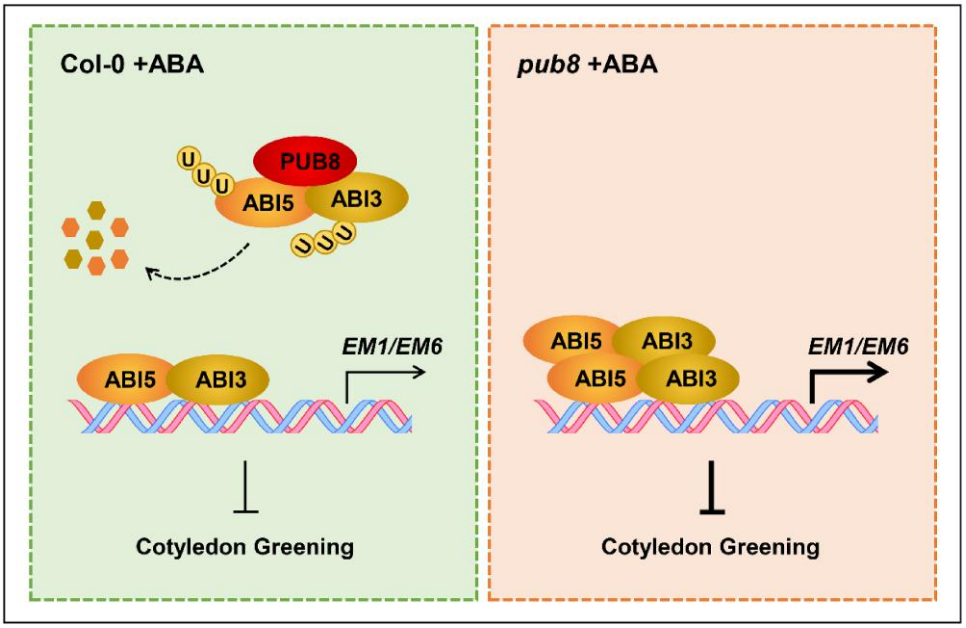

Figure 8. Model of PUB8 modulating ABA responses through targeting ABI3 and ABI5 for degradation during early growth. In the presence of ABA, PUB8 associates with ABI3 and ABI5 for degradation via 26S proteasome mediated ubiquitination in Col-0 Arabidopsis seedlings, which compromises the transcription levels of downstream ABA-responsive genes and promotes early seedling establishment. However, if *PUB8* function is absent, the degradation of ABI3 and ABI5 is weakened, leading to high accumulation of ABI3 and ABI5 and promoting the transcription levels of ABA-responsive genes to enhance the ABA inhibition of early growth, cotyledon greening.

gibberellin level leads to increased synthesis of ABA, which elevates ABI5 protein levels to inhibit seed germination (Piskurewicz et al. 2008). Cytokinin promotes the proteasomal degradation of ABI5, thereby antagonizing ABA-mediated inhibition of cotyledon greening in Arabidopsis (Guan et al. 2014). Recently, it was reported that CONSTITUTIVELY PHOTOMORPHOGENIC1 promotes the stability of ABI5 protein in the dark through associating with ABA-hypersensitive DDB1-CUL4 ASSOCIATED FACTOR 1, a substrate receptor of the CUL4-DDB1 E3 ligase that targets ABI5 for degradation (Peng et al. 2022). Because PUB8 also regulates the protein levels of ABI3 and ABI5 (Fig. 7), it is possible that these endogenous and exogenous signals control the stability of ABI3 and ABI5 via influencing PUB8. It is still unknown whether PUB8 functions in these signaling pathways to establish ABI3 and ABI5 homeostasis during seed germination through associating with their crucial components.

Based on the results of our study and other studies, we developed a simplified model for how PUB8 regulates ABA responses during early seedling growth in Arabidopsis (Fig. 8). In the presence of ABA, the abundance of ABI3 and ABI5 maintains at an appropriate level in Col-0 seedlings by E3 ligases, such as PUB8, and this restricts the transcription of downstream ABA-responsive genes and promotes early seedling establishment. However, in the absence of PUB8, the ubiquitination-mediated degradation of ABI3 and ABI5 is weakened, leading to accumulation of ABI3 and ABI5 and increased transcription of ABA-responsive genes to enhance the ABA-induced inhibition of cotyledon greening.

Materials and methods

Plant materials and growth conditions

The Arabidopsis (*A. thaliana*) mutants used in this study were in the Col-0 genetic background. Seeds of *abi5-8* (Salk_013163) were purchased from AraShare (www.arashare.cn). The same *abi3-8* mutant used in the study of Nambara et al. was used in this study. Seeds of *pub8-1* were kindly provided by the Sheng Yang He lab (Duke University, Durham, NC, USA). The *pub8-2* mutant was constructed by a CRISPR/Cas9-mediated method as described previously (Liang et al. 2016). Detailed information for *pub8-1* and *pub8-2* is provided in Supplemental Fig. S4. To generate *PUB8-OE* transgenic plants, the full-length CDS of *PUB8* was inserted into a donor vector, pDonor207, and then recombined into destination vectors to obtain *PUB8-YFP* and *PUB8-Myc* fusions driven by the 35S promoter. The transgenic line *35S:ABI5-4MYC* used in the study of Yang et al. (2021) was used in this study. The lines *pub8-1 abi3-8*, *pub8-1 abi5-8*, *pub8-1 ABI5-Myc*, *PUB8-OE ABI5-Myc* were constructed by genetic crossing and identified by PCR and sequencing. Arabidopsis plants were grown in an artificial growth chamber at 22°C under a 16-h-light (100-mE m^{-2} s^{-1}, white fluorescent bulbs, full-spectrum light), 8-h-dark photoperiod until maturity for seed collection.

Yeast two-hybrid assays

To screen for proteins that interact with ABI3, the full-length CDS of *ABI3* was fused to pGBKT7 (Clontech, Palo Alto, CA, USA) as bait, and then this construct was used to screen a cDNA library generated from ABA-treated Arabidopsis seeds. To confirm the protein interaction, the full-length CDS of PUB8 was inserted into pGADT7 (Clontech) and the full-length CDS of ABI5 was inserted into pGBKT7. To identify specific regions critical for the interactions, multiple truncated sequences of *ABI3* and *ABI5* were fused to pGBKT7, and the sequences encoding the 1–80 N-terminal residues of PUB8 and 81–375 C-terminal residues of PUB8 were cloned into pGADT7. The Y2H assays were performed as described previously (Jiang et al. 2014). The vector pairs were co-transformed into the yeast strain AH109, and physical interactions were indicated by the ability of cells to grow on dropout medium lacking Leu, Trp, His, and Ade for 4 d after plating. Experiments were repeated at least 4 times. The primers used for vector construction are listed in Supplemental Table S1.

Bimolecular fluorescence complementation assays

The cDNA sequences encoding the 64 amino acids of the C-terminal end of enhanced YFP (cYFP) and the 173 amino acids of the N-terminal end of YFP (nYFP) were PCR-amplified and individually inserted into tagging pFGC5941 plasmids to produce pFGC-cYFP and pFGC-nYFP, respectively (Kim et al. 2008). The full-length cDNA of *ABI3* and the sequences encoding the N-terminal residues of ABI3 and C-terminal residues of ABI3 were each cloned into pFGC-cYFP to produce different fusions with cYFP (ABI3-cYFP, $ABI3^{1-416}$-cYFP, $ABI3^{417-720}$-cYFP). The full-length cDNA of *ABI5* and the sequences encoding the 164 N-terminal residues of ABI5 and C-terminal residues of ABI5 were each cloned into pFGC-cYFP to produce different fusions with cYFP (ABI5-cYFP, $ABI5^{1-164}$-cYFP, $ABI5^{165-442}$-cYFP). The full-length sequence of *PUB8* and the sequences encoding the 80 N-terminal residues of PUB8 and C-terminal residues of PUB8 were each inserted into pFGC-nYFP to generate in-frame fusions with nYFP (PUB8-nYFP, $PUB8^{1-80}$-nYFP, $PUB8^{81-375}$-nYFP). The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101, and different pairs of *A. tumefaciens* were then transfected into *N. benthamiana* leaves by the hand infiltration method. At 48 h after infiltration, positive signals with YFP and DAPI fluorescence were detected under a confocal laser-scanning microscope (Olympus, Tokyo, Japan). YFP was detected using white light laser (WLL) at 40% intensity, and photomultiplier module (PMT) at 655% gain with excitation wavelength of 514 nm and emission at 524 to 570 nm, whereas DAPI was detected using WLL at 22% intensity and PMT detector at 668% gain with excitation wavelength of 360 nm and emission at 400 to 488 nm. The experiments were performed at least 4 times using different batches of *N. benthamiana* plants. For each experimental replicate, more than 12 *N. benthamiana* plants

were infiltrated, and more than 600 cells were analyzed. The statistical data for nuclei with positive signals are shown in Supplemental Fig. S2. The primers used for vector construction are listed in Supplemental Table S1.

Co-immunoprecipitation assays

To confirm the ABI3-PUB8 and ABI5-PUB8 interactions, Arabidopsis protoplasts were transformed with different combinations of plasmids, including *35S:YFP* and *35S:ABI3-Myc*, *35S:ABI3-Myc* and *35S:YFP-PUB8*, *35S:YFP* and *35S:ABI5-Myc*, and *35S:ABI5-Myc* and *35S:YFP-PUB8* with 5 μM MG132. Total protein was extracted with an IP buffer containing 50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.2% v/v Triton X-100, 50 μM MG132, 5 mM dithiothreitol, and 1× protease inhibitor cocktail. Immunoprecipitation experiments were performed with GFP-trap beads following the manufacturer's protocol. In brief, cell lysates were incubated with GFP-trap beads (catalog no. gta-100, ChromoTek, Munich, Germany) for 4 h at 4 °C. After incubation, the beads were washed 4 times with extraction buffer and the co-immunoprecipitated protein was then detected by immunoblotting using an anti-Myc antibody (catalog no. M20002M, Abmart, Berkeley Heights, NJ, USA; 1:10,000). Experiments were repeated at least 4 times. The primers used for the vector construction are listed in Supplemental Table S1.

Determination of germination and greening

The seed germination and greening of the Col-0 and mutant seeds were determined as described previously (Hu et al. 2019; Yang et al. 2021). Seeds were sown on medium and cold-stratified at 4°C in the dark for 4 d. Then, they were transferred to an artificial growth chamber to germinate at 22°C under a 16-h light/8-h dark photoperiod. To analyze the ABA sensitivity of germination and greening, seeds were plated on half-strength MS medium supplemented with ABA at different concentrations. Germination was determined based on the appearance of the embryonic axis (i.e. radicle protrusion) as observed under a microscope. Seedling greening was determined based on the appearance of green cotyledons on seedlings. More than 4 independent experiments were performed, and similar results were obtained.

RNA extraction and RT-qPCR analyses

Total RNA was extracted from germinating seeds with or without ABA treatment using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and RT-qPCR was performed as described previously (Han et al. 2020). Briefly, 1.0 μL DNase-treated RNA was reverse-transcribed in a 20 μL reaction volume with oligo (dT)18 primer using Moloney murine leukemia virus reverse transcriptase (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA). Then, 1.0 μL cDNA was used for RT-qPCR with the SYBR Premix Ex Taq kit (Takara, Dalian, China) on a Roche LightCycler 480 real-time PCR machine, according to the manufacturer's instructions. At least 4 biological replicates for each genotype were used for RT-qPCR analysis. The *At1g13320* gene, which encodes a subunit of Ser/Thr PP2A and is stably expressed in seeds during germination, was used as the control (Czechowski et al. 2005). All primers used for RT-qPCR are listed in Supplemental Table S2.

In vitro ubiquitination assay

Ubiquitination assays were carried out using a ubiquitinylation kit (ENZO Life Sciences, Farmingdale, NY, USA). We used a previously described protocol (Zhang et al. 2017) with some modifications. In brief, *Escherichia coli*-purified His-PUB8 (E3) and GST-ABI3 (substrate), and His-PUB8 (E3) and GST-ABI5 (substrate) were incubated with 1× ubiquitinylation buffer, 5 mM Mg-ATP solution, 1 mM dithiothreitol, 100 nM human E1, 2.5 mM human E2 (ubiquitin conjugating enzyme UBCH8), 2.5 mM biotin-ubiquitin, and 1 unit inorganic pyrophosphatase (New England Biolabs, Beverley, MA, USA) at 37°C for 4 h. Proteins were separated on a 4% to 20% (w/v) SDS–PAGE (catalog no. F11420Gel, ACE Biotechnology, Nanjing, China) under nonreducing conditions. Immunodetection was carried out using the HRP-Streptavidin detection system as described in the product manual. Experiments were repeated at least 4 times. The primers used for the vector construction are listed in Supplemental Table S1.

Ubiquitination assay in *N. benthamiana*

The ubiquitination assay was performed as described previously (Li et al. 2021) with some modifications. Briefly, the combinations *Pro35S:Myc/Pro35S:Myc-UBQ11/Pro35S:GFP-ABI3*, *Pro35S:Myc/Pro35S:Myc-UBQ11/Pro35S:GFP-ABI5*, *Pro35S:Myc-PUB8/Pro35S:Myc-UBQ11/Pro35S:GFP-ABI3*, and *Pro35S:Myc-PUB8/Pro35S:Myc-UBQ11/Pro35S:GFP-ABI5* were each infiltrated into leaves of *N. benthamiana* and then the plants were kept in the dark for 48 h. The OD_{600} value for each construct was 0.5. Then, proteins were extracted from 1 g infiltrated leaf tissue using IP buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 20% v/v glycerol, 0.2% v/v Triton-X100, 1× protease inhibitor cocktail, and 1× protease inhibitor cocktail [Roche, Mannheim, Germany]). Cell lysates were incubated with GFP-Trap agarose beads (catalog no. gta-100, ChromoTek, Planegg, Germany) for 4 h at 4°C. Agarose beads were collected after washing 4 times with IP buffer and then immunoblot analyses were conducted. Experiments were repeated at least 4 times. The primers used for vector construction are listed in Supplemental Table S1.

Statistical analysis

Statistical analysis was performed by analysis of variance. The results are shown in Supplemental Data Set S1.

Accession numbers

The genes discussed in this article can be found in the Arabidopsis Genome Initiative database as follows: *ABI5*, AT2G36270; *ABI3*, AT3G24650; *PUB8*, AT4G21350; *EM1*,

AT3G51810; *EM6*, AT2G40170; *RAB18*, AT1G43890; and *RD29B*, AT5G52300.

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Author contributions

Y.J., Z.L., and Y.H. designed this study and wrote the article; Z.L., S.L., D.J., Y.Y., Z.P., X.H., and Y.H. performed experiments or interpreted data; all authors approved the final article.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Identification of PUB8 motif required for interaction with ABI3 and ABI5.

Supplemental Figure S2. The statistical data for nuclei with positive signals in BiFC assay.

Supplemental Figure S3. Information of *pub8* mutants.

Supplemental Figure S4. Germination rates of *pub8* mutants seeds with different concentration of ABA treatment.

Supplemental Figure S5. Relative expression of *EM1*, *EM6*, *RD29B* and *RAB18* in *pub8* mutants.

Supplemental Figure S6. Relative expression of *EM1*, *EM6*, *RD29B*, and *RAB18* in *PUB8-OE* plants.

Supplemental Figure S7. Relative expression of *EM1*, *EM6*, *RD29B*, and *RAB18* in *abi3-8 pub8-1* double mutant plants.

Supplemental Figure S8. Relative expression of *EM1*, *EM6*, *RD29B*, and *RAB18* in *abi5-8 pub8-1* double mutant plants.

Supplemental Figure S9. Relative expression of *EM1*, *EM6*, *RD29B*, and *RAB18* in *pub8-1 ABI5-OE* plants.

Supplemental Figure S10. Relative expression of *EM1*, *EM6*, *RD29B*, and *RAB18* in *PUB8-OE ABI5-OE* plants.

Supplemental Table S1. Primers used for Y2H, BiFC, Co-IP, protein purification.

Supplemental Table S2. Primers used for RT-qPCR analysis.

Supplemental Data Set S1. Analysis of variance (ANOVA) tables.

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Conflict of interest statement. No conflict of interest is declared.

Data availability

All data can be found in the manuscript and in the supporting information.

References

Bewley JD, Black M. Seeds: physiology of development and germination. New York: Plenum Press; 1985

Brocard IM, Lynch TJ, Finkelstein RR. Regulation and role of the Arabidopsis *Abscisic Acid-Insensitive* 5 gene in abscisic acid, sugar, and stress response. Plant Physiol. 2002:**129**(4):1533–1543. https://doi.org/10.1104/pp.005793

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. 2005:**139**(1):5–17. https://doi.org/10.1104/pp.105.063743

Ezcurra I, Wycliffe P, Nehlin L, Ellerstrom M, Rask L. Transactivation of the *Brassica napus* napin promoter by ABI3 requires interaction of the conserved B2 and B3 domains of ABI3 with different *cis*-elements: B2 mediates activation through an ABRE, whereas B3 interacts with an RY/G-box. Plant J. 2000:**24**(1):57–66. https://doi.org/10.1046/j.1365-313x.2000.00857.x

Finkelstein RR. Mutations at two new Arabidopsis ABA response loci are similar to the *abi3* mutations. Plant J. 1994:**5**(6):765–771. https://doi.org/10.1046/j.1365-313X.1994.5060765.x

Finkelstein RR, Lynch TJ. Abscisic acid inhibition of radicle emergence but not seedling growth is suppressed by sugars. Plant Physiol. 2000:**122**(4):1179–1186. https://doi.org/10.1104/pp.122.4.1179

Finkelstein RR, Gampala SS, Rock CD. Abscisic acid signaling in seeds and seedlings. Plant Cell. 2002:**14**(suppl 1):S15–S45. https://doi.org/10.1105/tpc.010441

Finkelstein R, Reeves W, Ariizumi T, Steber C. Molecular aspects of seed dormancy. Annu Rev Plant Biol. 2008:**59**(1):387–415. https://doi.org/10.1146/annurev.arplant.59.032607.092740

Finch-Savage WE, Leubner-Metzger G. Seed dormancy and the control of germination. New Phytol. 2006:**171**(3):501–523. https://doi.org/10.1111/j.1469-8137.2006.01787.x

Fujii H, Verslues PE, Zhu JK. Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in Arabidopsis. Plant Cell. 2007:**19**(2):485–494. https://doi.org/10.1105/tpc.106.048538

Fujii H, Chinnusamy V, Rodrigues A, Rubio S, Antoni R, Park SY, Cutler SR, Sheen J, Rodriguez PL, Zhu JK. In vitro reconstitution of an abscisic acid signalling pathway. Nature. 2009:**462**(7273):660–664. https://doi.org/10.1038/nature08599

Fujii H, Zhu JK. Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. Proc Natl Acad Sci USA. 2009:**106**(20):8380–8385. https://doi.org/10.1073/pnas.0903144106

Furihata T, Maruyama K, Fujita Y, Umezawa T, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K. Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. Proc Natl Acad Sci USA. 2006:**103**(6):1988–1993. https://doi.org/10.1073/pnas.0505667103

Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM. Isolation of the Arabidopsis *ABI3* gene by positional cloning. Plant Cell. 1992:**4**(10):1251–1261. https://doi.org/10.1105/tpc.4.10.1251

Guan C, Wang X, Feng J, Hong S, Liang Y, Ren B, Zuo J. Cytokinin antagonizes abscisic acid-mediated inhibition of cotyledon greening by

promoting the degradation of abscisic acid insensitive5 protein in Arabidopsis. Plant Physiol. 2014:**164**(3):1515–1526. https://doi.org/10.1104/pp.113.234740

Han X, Zhang M, Yang M, Hu Y. Arabidopsis JAZ proteins interact with and suppress RHD6 transcription factor to regulate jasmonate-stimulated root hair development. Plant Cell. 2020:**32**(4): 1049–1062. https://doi.org/10.1105/tpc.19.00617

Hattori T, Totsuka M, Hobo T, Kagaya Y, Yamamoto-Toyoda A. Experimentally determined sequence requirement of ACGT-containing abscisic acid response element. Plant Cell Physiol. 2002:**43**(1): 136–140. https://doi.org/10.1093/pcp/pcf014

Hu Y, Yu D. BRASSINOSTEROID INSENSITIVE2 interacts with ABSCISIC ACID INSENSITIVE5 to mediate the antagonism of brassinosteroids to abscisic acid during seed germination in Arabidopsis. Plant Cell. 2014:**26**(11):4394–4408. https://doi.org/10.1105/tpc.114.130849

Hu Y, Han X, Yang M, Zhang M, Pan J, Yu D. The transcription factor INDUCER OF CBF EXPRESSION1 interacts with ABSCISIC ACID INSENSITIVE5 and DELLA proteins to fine-tune abscisic acid signaling during seed germination in Arabidopsis. Plant Cell. 2019:**31**(7): 1520–1538. https://doi.org/10.1105/tpc.18.00825

Ji H, Wang S, Cheng C, Li R, Wang Z, Jenkins GI, Kong F, Li X. The RCC1 family protein SAB1 negatively regulates ABI5 through multidimensional mechanisms during postgermination in Arabidopsis. New Phytol. 2019:**222**(2):907–922. https://doi.org/10.1111/nph.15653

Jiang YJ, Liang G, Yang SZ, Yu DQ. Arabidopsis WRKY57 functions as a node of convergence for Jasmonic acid- and auxin-mediated signaling in Jasmonic acid-induced leaf senescence. Plant Cell. 2014:**26**(1):230–245. https://doi.org/10.1105/tpc.113.117838

Jin D, Wu M, Li B, Bücker B, Keil P, Zhang S, Li J, Kang D, Liu J, Dong J, et al. The COP9 signalosome regulates seed germination by facilitating protein degradation of RGL2 and ABI5. PLoS Genet. 2018:**14**(2): e1007237. https://doi.org/10.1371/journal.pgen.1007237

Jung C, Zhao P, Seo JS, Mitsuda N, Deng S, Chua NH. PLANT U-BOX PROTEIN10 regulates MYC2 stability in Arabidopsis. Plant Cell. 2015:**27**(7) 2016–2031. https://doi.org/10.1105/tpc.15.00385

Kim KC, Lai Z, Fan B, Chen Z. Arabidopsis WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. Plant Cell. 2008:**20**(9):2357–2371. https://doi.org/10.1105/tpc.107.055566

Kobayashi Y, Murata M, Minami H, Yamamoto S, Kagaya Y, Hobo T, Yamamoto A, Hattori T. Abscisic acid-activated SNRK2 protein kinases function in the gene-regulation pathway of ABA signal transduction by phosphorylating ABA response element-binding factors. Plant J. 2005:**44**(6):939–949. https://doi.org/10.1111/j.1365-313X.2005.02583.x

Kornneef M, Reuling G, Karssen CM. The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. Physiol Plant. 1984:**61**(3):377–383. https://doi.org/10.1111/j.1399-3054.1984.tb06343.x

Lee JH, Yoon HJ, Terzaghi W, Martinez C, Dai M, Li J, Byun MO, Deng XW. DWA1 And DWA2, two Arabidopsis DWD protein components of CUL4-based E3 ligases, act together as negative regulators in ABA signal transduction. Plant Cell. 2010:**22**(6):1716–1732. https://doi.org/10.1105/tpc.109.073783

Li Y, Lu CK, Li CY, Lei RH, Pu MN, Zhao JH, Peng F, Ping HQ, Wang D, Liang G. IRON MAN interacts with BRUTUS to maintain iron homeostasis in Arabidopsis. Proc Natl Acad Sci USA. 2021:**118**(39): e2109063118. https://doi.org/10.1073/pnas.2109063118

Liang G, Zhang H, Lou D, Yu D. Selection of highly efficient sgRNAs for CRISPR/Cas9-based plant genome editing. Sci Rep. 2016:**6**(1):21451. https://doi.org/10.1038/srep21451

Liu H, Stone SL. Abscisic acid increases Arabidopsis ABI5 transcription factor levels by promoting KEG E3 ligase self-ubiquitination and proteasomal degradation. Plant Cell. 2010:**22**(8):2630–2641. https://doi.org/10.1105/tpc.110.076075

Liu H, Stone SL. Regulation of ABI5 turnover by reversible post-translational modifications. Plant Signal Behav. 2014:**9**(1):e27577. https://doi.org/10.4161/psb.27577

Lopez-Molina L, Chua NH. A null mutation in a bZIP factor confers ABA-insensitivity in *Arabidopsis thaliana*. Plant Cell Physiol. 2000:**41**(5):541–547. https://doi.org/10.1093/pcp/41.5.541

Lopez-Molina L, Mongrand S, Chua NH. A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. Proc Natl Acad Sci USA. 2001:**98**(8):4782–4787. https://doi.org/10.1073/pnas.081594298

Lopez-Molina L, Mongrand S, McLachlin DT, Chait BT, Chua NH. ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. Plant J. 2002:**32**(3):317–328. https://doi.org/10.1046/j.1365-313X.2002.01430.x

Lyzenga WJ, Liu H, Schofield A, Muise-Hennessey A, Stone SL. Arabidopsis CIPK26 interacts with KEG, components of the ABA signalling network and is degraded by the ubiquitin-proteasome system. J Exp Bot. 2013:**64**(10):2779–2791. https://doi.org/10.1093/jxb/ert123

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E. Regulators of PP2C phosphatase activity function as abscisic acid sensors. Science. 2009:**324**(5930):1064–1068. https://doi.org/10.1126/science.1172408

Miura K, Lee J, Jin JB, Yoo CY, Miura T, Hasegawa PM. Sumoylation of ABI5 by the Arabidopsis SUMO E3 ligase SIZ1 negatively regulates abscisic acid signaling. Proc Natl Acad Sci USA. 2009:**106**(13): 5418–5423. https://doi.org/10.1073/pnas.0811088106

Monke G, Altschmied L, Tewes A, Reidt W, Mock HP, Conrad U. Seed-specific transcription factors ABI3 and FUS3: molecular interaction with DNA. Planta. 2004:**219**(1):158–166. https://doi.org/10.1007/s00425-004-1206-9

Nakashima K, Fujita Y, Kanamori N, Katagiri T, Umezawa T, Kidokoro S, Maruyama K, Yoshida T, Ishiyama K, Kobayashi M, et al. Three Arabidopsis SnRK2 protein kinases, SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, involved in ABA signaling are essential for the control of seed development and dormancy. Plant Cell Physiol. 2009:**50**(7):1345–1363. https://doi.org/10.1093/pcp/pcp083

Nakashima K, Yamaguchi-Shinozaki K. ABA signaling in stress-response and seed development. Plant Cell Rep. 2013:**32**(7): 959–970. https://doi.org/10.1007/s00299-013-1418-1

Nonogaki H. Seed dormancy and germination-emerging mechanisms and new hypotheses. Front Plant Sci. 2014:**5**:233. https://doi.org/10.3389/fpls.2014.00233

Nonogaki H. Seed biology updates-highlights and new discoveries in seed dormancy and germination research. Front Plant Sci. 2017:**8**: 524. https://doi.org/10.3389/fpls.2017.00524

Pan J, Hu Y, Wang H, Guo Q, Chen Y, Howe GA, Yu D. Molecular mechanism underlying the synergetic effect of jasmo-nate on abscisic acid signaling during seed germination in Arabidopsis. Plant Cell. 2020:**32**(12):3846–3865. https://doi.org/10.1105/tpc.19.00838

Parcy F, Valon C, Raynal M, Gaubier-Comella P, Delseny M, Giraudat J. Regulation of gene expression programs during Arabidopsis seed development: roles of the ABI3 locus and of endogenous abscisic acid. Plant Cell. 1994:**6**(11):1567–1582. https://doi.org/10.1105/tpc.6.11.1567

Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TF, et al. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Science. 2009:**324**(5930):1068–1071. https://doi.org/10.1126/science.1173041

Peng J, Wang M, Wang X, Qi L, Guo C, Li H, Li C, Yan Y, Zhou Y, Terzaghi W, et al. COP1 positively regulates ABA signaling during Arabidopsis seedling growth in darkness by mediating ABA-induced ABI5 accumulation. Plant Cell. 2022:**34**(6):2286–2308. https://doi.org/10.1093/plcell/koac073

Piskurewicz U, Jikumaru Y, Kinoshita N, Nambara E, Kamiya Y, Lopez-Molina L. The gibberellic acid signaling repressor RGL2 inhibits Arabidopsis seed germination by stimulating abscisic acid synthesis and ABI5 activity. Plant Cell. 2008:**20**(10):2729–2745. https://doi.org/10.1105/tpc.108.061515

Rajjou L, Duval M, Gallardo K, Catusse J, Bally J, Job C, Job D. Seed germination and vigor. Annu Rev Plant Biol. 2012:**63**(1):507–533. https://doi.org/10.1146/annurev-arplant-042811-105550

Shen Q, Zhang P, Ho TH. Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient or ABA induction of gene expression in barley. Plant Cell. 1996:**8**(7):1107–1119. https://doi.org/10.1105/tpc.8.7.1107

Stone SL, Williams LA, Farmer LM, Vierstra RD, Callis J. KEEP ON GOING, a RING E3 ligase essential for Arabidopsis growth and development, is involved in abscisic acid signaling. Plant Cell. 2006:**18**(12):3415–3428. https://doi.org/10.1105/tpc.106.046532

Suzuki M, Kao CY, McCarty DR. The conserved B3 domain of VIVIPAROUS1 has a cooperative DNA binding activity. Plant Cell. 1997:**9**(5):799–807. https://doi.org/10.1105/tpc.9.5.799

Tamura N, Yoshida T, Tanaka A, Sasaki R, Bando A, Toh S, Lepiniec L, Kawakami N. Isolation and characterization of high temperature-resistant germination mutants of *Arabidopsis thaliana*. Plant Cell Physiol. 2006:**47**(8):1081–1094. https://doi.org/10.1093/pcp/pcj078

Vishwakarma K, Upadhyay N, Kumar N, Yadav G, Singh J, Mishra RK, Kumar V, Verma R, Upadhyay RG, Pandey M, et al. Abscisic acid signaling and abiotic stress tolerance in plants: a review on current knowledge and future prospects. Front Plant Sci. 2017:**8**:161. https://doi.org/10.3389/fpls.2017.00161

Wang Y, Wu Y, Zhong H, Chen S, Wong KB, Xia Y. Arabidopsis PUB2 and PUB4 connect signaling components of pattern-triggered immunity. New Phytol. 2022:**233**(5):2249–2265. https://doi.org/10.1111/nph.17922

Yang M, Han X, Yang J, Jiang Y, Hu Y. The Arabidopsis circadian clock protein PRR5 interacts with and stimulates ABI5 to modulate abscisic acid signaling during seed germination. Plant Cell. 2021:**33**(9):3022–3041. https://doi.org/10.1093/plcell/koab168

Yoshida T, Mogami J, Yamaguchi-Shinozaki K. ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. Curr Opin Plant Biol. 2014:**21**:133–139. https://doi.org/10.1016/j.pbi.2014.07.009

Yu F, Li M, He D, Yang P. Advances on post-translational modifications involved in seed germination. Front Plant Sci. 2021:**12**:642979. https://doi.org/10.3389/fpls.2021.642979

Yu F, Wu Y, Xie Q. Precise protein post-translational modifications modulate ABI5 activity. Trends Plant Sci. 2015:**20**(9):569–575. https://doi.org/10.1016/j.tplants.2015.05.004

Zhao WM, Guan CM, Feng J, Liang Y, Zhan N, Zuo JZ, Ren B. The Arabidopsis *CROWDED NUCLEI* genes regulate seed germination by modulating degradation of ABI5 protein. J Integr Plant Biol. 2016:**58**(7):229–678. https://doi.org/10.1111/jipb.12448

Zhang H, Li Y, Yao X, Liang G, Yu D. POSITIVE REGULATOR OF IRON HOMEOSTASIS1, OsPRI1, facilitates iron homeostasis. Plant Physiol. 2017:**175**(1):543–554. https://doi.org/10.1104/pp.17.00794

Zhang X, Garreton V, Chua NH. The AIP2 E3 ligase acts as a novel negative regulator of ABA signaling by promoting ABI3 degradation. Genes Dev. 2005:**19**(13):1532–1543. https://doi.org/10.1101/gad.1318705

Zhou J, Lu D, Xu G, Finlayson SA, He P, Shan L. The dominant negative ARM domain uncovers multiple functions of PUB13 in Arabidopsis immunity, flowering, and senescence. J Exp Bot. 2015:**66**(11):3353–3366. https://doi.org/10.1093/jxb/erv148