The deubiquitinases UBP12 and UBP13 integrate with the E3 ubiquitin ligase XBAT35.2 to modulate VPS23A stability in ABA signaling

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Ubiquitination-mediated protein degradation in both the 26S proteasome and vacuole is an important process in abscisic acid (ABA) signaling. However, the role of deubiquitination in this process remains elusive. Here, we demonstrate that two deubiquitinating enzymes (DUBs), ubiquitin-specific protease 12 (UBP12) and UBP13, modulate ABA signaling and drought tolerance by deubiquitinating and stabilizing the endosomal sorting complex required for transport-I (ESCRT-I) component vacuolar protein sorting 23A (VPS23A) and thereby affect the stability of ABA receptors in Arabidopsis thaliana. Genetic analysis showed that VPS23A overexpression could rescue the ABA hypersensitive and drought tolerance phenotypes of ubp12-2w or ubp13-1. In addition to the direct regulation of VPS23A, we found that UBP12 and UBP13 also stabilized the E3 ligase XB3 ortholog 5 in A. thaliana (XBAT35.2) in response to ABA treatment. Hence, we demonstrated that UBP12 and UBP13 are previously unidentified rheostatic regulators of ABA signaling and revealed a mechanism by which deubiquitination precisely monitors the XBAT35/VPS23A ubiquitination module in the ABA response.

INTRODUCTION
As sessile organisms, plants are facing a constantly changing environment, including drought, high salinity, and pathogen challenge (1–3). It should be noted that crop yield reduction by drought stress alone is equal to the loss caused by all pathogens (4). To cope with drought stress, plants have evolved various signaling pathways and coping mechanisms. Activation of abscisic acid (ABA) signaling is one critical way for plants to relay information relevant to and counteract drought stress. It should be noted that crop yield reduction by drought stress is a serious threat to food security. To combat drought stress, plants have evolved various strategies and mechanisms for coping with this stress. One of these mechanisms is the activation of the abscisic acid (ABA) signaling pathway, which plays a critical role in regulating various physiological processes in plants, including water conservation, stomatal closure, and seed dormancy (5–7).

ABA signaling is a complex process involving multiple components, including receptors, signal transducers, and transcription factors. The ABA receptors, including PYR1/PYL and PYL families, are responsible for detecting ABA and initiating signaling. Activated ABA receptors then activate heterotrimeric G proteins, leading to the activation of protein kinase C (PKC) and cAMP-dependent protein kinase A (PKA) (8–10). Activated PKC and PKA subsequently phosphorylate and activate downstream effectors, such as the transcription factor ABA insensitive 5 (ABI5) and ABA-responsive element-binding factors (ABFs) (11, 12). In addition, several group A PP2Cs are ubiquitinated by their corresponding E3 ligases, including PP2C1 (5) and PP2C2 (6). This process involves the covalent attachment of ubiquitin (Ub) to target proteins via an isopeptide bond. Conversely, deubiquitination enzymes (DUBs) catalyze the hydrolysis of the isopeptide bond between the Ub chain and proteinaceous substrate, which can allow Ub recycling. Ubiquitination can influence substrate subcellular localization, stabilization, and protein interactions (13–18). Recently, it was found that ubiquitination affects the stability of key components of ABA signaling through the 26S proteasome pathway. The substrate adaptors de-etiolated 1–, damage-specific DNA binding protein 1–associated 1 from the Cullin–RING–based E3 ligase (CRL) complex and RING-between-RING–type E3 ligase RING finger of seed longevity (RSLs) recognize and ubiquitinate PYR1/PYLs and accelerate their degradation via the 26S proteasome (19–22). In addition, several group A PP2Cs are ubiquitinated by their corresponding E3 ligases, including RING domain ligase 1.5 (RGL1), plant U-box 12/13 (PUB12/13), broad complex, tram track, bric-a-brac (BTB)/POX virus and zinc finger (POZ) and meprin and tumor necrosis factor receptor-associated factor homology (MATH) domain proteins, constitutively photomorphogenic 1, and Arabidopsis ABA-insensitive RING protein 3 (23–27).

Ubiquitination of a substrate protein is a conserved and reversible posttranslational modification (16, 17). A cascade of enzymatic reactions carried out by ubiquitin (Ub)–activating enzymes (E1s), Ub conjugation enzymes (E2s), and Ub ligase enzymes (E3s) leads to the covalent attachment of Ub chain to substrate through an isopeptide bond. Conversely, deubiquitination enzymes (DUBs) catalyze the hydrolysis of the isopeptide bond between the Ub chain and proteinaceous substrate, which can allow Ub recycling. Ubiquitination can influence substrate subcellular localization, stabilization, and protein interactions (18–20). Recently, it was found that ubiquitination affects the stability of key components of ABA signaling through the 26S proteasome pathway. The substrate adaptors de-etiolated 1–, damage-specific DNA binding protein 1–associated 1 from the Cullin–RING–based E3 ligase (CRL) complex and RING-between-RING–type E3 ligase RING finger of seed longevity (RSLs) recognize and ubiquitinate PYR1/PYLs and accelerate their degradation via the 26S proteasome (21, 22). In addition, several group A PP2Cs are ubiquitinated by their corresponding E3 ligases, including RING domain ligase 1.5 (RGL1), plant U-box 12/13 (PUB12/13), broad complex, tram track, bric-a-brac (BTB)/POX virus and zinc finger (POZ) and meprin and tumor necrosis factor receptor-associated factor homology (MATH) domain proteins, constitutively photomorphogenic 1, and Arabidopsis ABA-insensitive RING protein 3 (23–27).

Furthermore, two clade-III SnRK2s, SnRK2.3 and SnRK2.6, were reported to be ubiquitinated by SKP1/Cullin/F-box E3 Ub lige complex and high osmotic stress 15 to attenuate ABA signaling (28, 29), respectively. Moreover, ABI5 is targeted by the CRL-type E3 ligase CRL4 and RING-type E3 ligase KEEP ON GOING and degraded through the 26S proteasome pathway (30, 31).

Aside from the 26S proteasome pathway, ubiquitination-mediated vacuolar degradation also contributes to the regulation of ABA signaling. In response to environmental changes, membrane-integrated proteins or membrane-bound proteins are internalized into intraluminal vesicles of the multivesicular body (MVB) for fusion with the vacuole or are recycled back to the membrane (32). This process requires the function of endosomal sorting complexes required for trafficking (ESCRTs), which contain five subcomplexes, including ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III, and ESCRT-III–related complexes. Two main components of ESCRT-I, vacuolar protein sorting 23A (VPS23A) and FYVE1/FYVE domain protein required

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VPS23A as bait. We obtained 31 potential interacting proteins of performed a yeast two-hybrid (Y2H) screen assay with full-lengthponent of the ESCRT-I complex, recognizes and traffics the ubiqui-
Our previous work demonstrated that VPS23A, an essential com-
RESULTS
Fig. 1. UBP12 and UBP13 interact with VPS23A. (A) Y2H assays confirmed that UBP12/UBP13 interact with VPS23A. UBP12/UBP13 was fused to the GAL4 activation domain (AD) as the prey, and VPS23A was fused to the GAL4 binding domain (BD) as bait. The interaction of the two proteins was indicated by yeast growth on selective media. SD-L-W, synthetic dropout medium minus Trp and Leu; SD-L-W-H (2 mM 3-AT), synthetic dropout medium minus Trp, Leu, and His but with the addition of 2 mM 3-AT (3-aminotriazole). (B) Luciferase (LUC) complementation imaging (LCI) assays indicating the interaction between UBP12/UBP13 and VPS23A. UBP12-Nluc and UBP12-Nluc, UBP12 fused with Nluc; UBP13-Nluc, UBP13 fused with Nluc; Cluc-VPS23A, VPS23A fused with Cluc; UBP14-Nluc, UBP14 fused with Nluc as a negative control. Different pairs of constructs were transiently coexpressed in N. benthamiana leaves via A. tumefaciens–mediated infiltration. LUC activity was measured 3 days after infiltration. (C) Coimmunoprecipitation (Co-IP) of UBP12 and VPS23A. Proteins extracted from 10-day-old GFP-HA::Col-0 and GFP-VPS23A::Col-0 transgenic plants were incubated with anti–green fluorescent protein (GFP) magnetic beads, and immunoblotting assays were conducted using anti-GFP and anti-UBP12 antibodies. (D) Pull-down assays for analysis of the interaction between UBP12/UBP13 and VPS23A. An equal amount of His-UBP12 or His-UBP13 was incubated with immobilized glutathione S-transferase (GST) and GST-VPS23A separately, and bound proteins were detected by immunoblotting using the corresponding antibodies.

RESULTS
UBP12 and UBP13 interact with VPS23A in vivo and in vitro
Our previous work demonstrated that VPS23A, an essential com-
the LCI assays, proteins were transiently coexpressed in leaves of Nicotiana benthamiana through Agrobacterium tumefaciens–mediated infiltration. The results showed that LUC activity was restored when only both Cluc-VPS23A and UBP12/UBP13-Nluc were coexpressed, rather than in other controls that failed to produce reconstituted LUC, including Cluc-VPS23A and UBP14-Nluc (Fig. 1B). Hence, these results proved that UBP12 and UBP13 interact with VPS23A in planta. Total protein extracted from GFP-VPS23A and GFP-HA stable transgenic Arabidopsis seedlings in the Col-0 background and anti–green fluorescent protein (GFP) antibody was used for Co-IP assays. UBP12 was coimmunoprecipitated by GFP-VPS23A but not by GFP-hemagglutinin (HA), proving that VPS23A interacts with UBP12 in Arabidopsis (Fig. 1C). For BiFC assays, VPS23A and UBP12/UBP13 were fused with the N- and C-terminal portions of yellow fluorescent protein (YFP) to produce YFPVPS23A and YFPUBP12/UBP13 constructs, respectively. N. benthamiana leaves were coinfected with A. tumefaciens harboring different pairs of constructs. The physical interaction between UBP12/UBP13 and
VPS23A was revealed by reconstitution of YFP fluorescence in cells coinfiterated with constructs corresponding to YFP-UBP12/UBP13 and YFP-VPS23A, whereas the expression of other pairs of constructs did not restore YFP fluorescence. In addition, the restored YFP fluorescence signal colocalized with the signal from the MVB marker mCherry-ARA7, proving that the interaction between UBP12/UBP13 and VPS23A occurs in MVBs (fig. S1). To determine whether UBP12 and UBP13 directly interact with VPS23A in vitro, we performed pull-down assays. The results showed that glutathione S-transferase (GST)–VPS23A rather than GST alone pulled down HIS-UBP12 and HIS-UBP13, which demonstrated that VPS23A directly interacted with UBP12 and UBP13 in vitro (Fig. 1D). Together, these data consistently showed that VPS23A interacts with both UBP12 and UBP13 in vivo and in vitro.

**UBP12 and UBP13 regulate the stability of VPS23A**

Given that VPS23A is ubiquitinated by the E3 Ub ligase XBAT35.2 and SINATs en route to 26S proteasome degradation (35, 36), we wondered whether the DUBs UBP12 and UBP13 could affect the protein turnover of VPS23A. To answer this question, A. tumefaciens harboring UBP12-Nluc and GFP-VPS23A constructs was coinfiterated into tobacco leaves with red fluorescent protein (RFP) as an internal control. As Fig. 2A shows, an equal amount of GFP-VPS23A constructs produced a similar amount of VPS23A transcripts, whereas an increasing amount of UBP12-Nluc constructs yielded increased protein levels of UBPNluc. As the protein level of UBP12-Nluc increased, the VPS23A protein level also increased (Fig. 2A and fig. S2A). We obtained similar results when similar experiments were performed with UBP13 and VPS23A (Fig. 2B and fig. S2B). Together, both UBP12 and UBP13 were found to promote the protein stability of VPS23A in the tobacco transient expression system.

To determine whether UBP12 and UBP13 stabilize VPS23A in Arabidopsis, we generated stable transgenic lines of VPS23A-Flag in the Col-0 (VPS23A-Flag:Col-0) and UBP12 mutant (VPS23A-Flag::ubp12-2w) backgrounds and stable transgenic lines of GFP-VPS23A in the Col-0 (GFP-VPS23A::Col-0) and UBP13 mutant (GFP-VPS23A::ubp13-1) backgrounds for cycloheximide (CHX)–chase assays to assess the stability of VPS23A. ubp12-2w(CS2103163) is a transferred DNA (T-DNA) insertion mutant that causes a reduction in both UBP12 and UBP13 transcripts; hence, it is a weak double mutant of both UBP12 and UBP13. ubp13-1 (SALK_128312) is a ubp13 null allele (44). Our results showed that compared with Col-0, VPS23A protein decay was accelerated in both the ubp12-2w and ubp13-1 backgrounds (Fig. 2, C and D). Thus, UBP12 and UBP13 are required for the stabilization of VPS23A in Arabidopsis, consistent with the notion that UBP12 or UBP13 overexpression promotes the stability of VPS23A, suggesting that UBP12 and UBP13 maintain the stability of VPS23A with respect to protein turnover.

To further verify that UBP12 and UBP13 promote the stability of VPS23A, we used a β-estradiol inducible expression system to generate inducible HA-UBP12 (XVE:HA-UBP12/VPS23A $^{OE}$, two independent T$_2$ lines #1 and #2) and HA-UBP13 expression lines (XVE:HA-UBP13/VPS23A $^{OE}$, two independent T$_2$ lines #1 and #3) in the stable Flag-VPS23A overexpression background. Flag-VPS23A protein levels were elevated upon β-estradiol induction of either HA-UBP12 or HA-UBP13 (Fig. 2, E and F, and fig. S2C), but not VPS23A transcription levels (fig. S2, D and E), suggesting that UBP12 and UBP13 stabilize VPS23A. To address whether UBP12 and UBP13 play an important role in ABA signaling, we checked the effect of induced HA-UBP12 or HA-UBP13 on the stability of VPS23A and found that Flag-VPS23A protein levels were elevated upon β-estradiol induction of either HA-UBP12 or HA-UBP13 with the application of ABA (Fig. 2G and fig. S2F). β-Estradiol induction had no effects on the transcription level of VPS23A (fig. S2, G and H). Together, we found that the presence of UBP12 and UBP13 is important for the protein stability of VPS23A.

**Ubiquinated VPS23A is the substrate of UBP12 and UBP13**

Thus far, our data indicate that the deubiquitinas UBP12 and UBP13 stabilize VPS23A, which prompted us to study whether UBP12 and UBP13 can deubiquitinate ubiquitinated VPS23A to protect against the degradation of VPS23A. To this end, we first generated ubiquitinated VPS23A by performing in vitro ubiquitination of VPS23A with its E3 ligase XBAT35.2 as previously reported (36), and we detected the ubiquitination of VPS23A. With the further addition of either UBP12 or UBP13 in the reaction mix, these ubiquitinated VPS23A bands vanished (fig. S2I). The results implied that UBP12 and UBP13 had deubiquitination activity on a VPS23A substrate. To exclude the possibility that UBP12 and UBP13 affect the activity of E1, E2, or E3 in the assay, we purified ubiquitinated VPS23A following the substrate ubiquitination reaction of VPS23A and then performed a deubiquitination reaction with a DUB. Consistently, UBP12 and UBP13 deubiquitinated VPS23A, while the deubiquitination activity of UBP12 and UBP13 to ubiquitinated VPS23A was destroyed when the active cysteine site was mutated to serine in UBP12 or UBP13 (Fig. 2H and fig. S2J). The mutated forms of UBP12 and UBP13 were designated as UBP12 (m) and UBP13 (m), which contained an amino acid substitution of C208S or C207S, respectively.

To further determine that UBP12 and UBP13 conferred deubiquitination activity on plant endogenous ubiquitinated VPS23A, we immunoprecipitated ubiquitinated VPS23A-Flag with p62 matrixes and then subjected it to deubiquitination. We found that a high-molecular weight band collected with anti-Flag antibody was detected in VPS23A-Flag::Col-0 transgenic seedlings, indicating that VPS23A was ubiquitinated in vivo (Fig. 2, I and J). Ubiquitinated VPS23A was then assayed with HIS-UBP12 or HIS-UBP13 (m) for 1 hour in our deubiquitination system. The amount of ubiquitinated forms of VPS23A-Flag decreased substantial with HIS-UBP12 but not with HIS-UBP12 (m), indicating that UBP12 confers deubiquitination activity on ubiquitinated VPS23A isolated from Arabidopsis (Fig. 2I). For the deubiquitination activity of UBP13 to VPS23A, we attained a similar result (Fig. 2J). Consistently, IP assays with VPS23A-Flag in both Col-0 and ubp12-2w seelings showed that the ubiquitination level of VPS23A-Flag in ubp12-2w seedlings was increased compared with that in wild-type Col-0 seedlings with or without ABA treatment (fig. S2K). Similarly, the ubiquitination level of GFP-VPS23A also increased in ubp13-1 compared with Col-0 with or without ABA treatment (fig. S2K). Together, both UBP12 and UBP13 have deubiquitination activity capable of hydrolyzing the Ub chains of ubiquitinated VPS23A.

**UBP12 and UBP13 are negative regulators of ABA signaling**

VPS23A was shown to be a negative regulator in the ABA signaling pathway by promoting the degradation of ABA receptors (34). Here, we demonstrated that both UBP12 and UBP13 can stabilize VPS23A through deubiquitination. Hence, we suspected that UBP12...
Fig. 2. UBP12 and UBP13 regulate the stability of VPS23A. (A and B) UBP12 and UBP13 stabilize VPS23A. Equal amounts of A. tumefaciens (GFP-VPS23A) were coinfiltrated with increasing amounts of UBP12-Nluc or UBP13-Nluc strains (the ratio is indicated at the top of the panel). Epitope-tagged proteins were detected. RFP was used as an internal control. The VPS23A RNA level was analyzed with ACTIN1 as a control. (C and D) In vivo degradation assays. Ten-day-old seedlings were incubated with 100 μM cycloheximide (CHX). Samples were collected at the indicated time points after CHX treatment for immunoblot analysis using an anti-Flag antibody. 20S proteasome α-subunit G1 (PAG1) was used as a loading control. Right: Quantitative evaluation of the relative amount of VPS23A protein compared with PAG1. Values are average ± SD (n = 3). P values at each time point were determined by Student’s t test, *P < 0.05 and **P < 0.01. (E and F) VPS23A protein level accumulated after β-estradiol induction of either UBP12 (E) or UBP13 (F). Seven-day-old seedlings were treated with or without 25 μM β-estradiol for 16 hours. Two independent lines were used in (E) and (F) separately. PAG1 was used as a loading control. (G) VPS23A protein level accumulated after β-estradiol induction of either UBP12 or UBP13 under ABA treatment. Seven-day-old seedlings were treated with 50 μM ABA or 50 μM ABA plus 25 μM β-estradiol. Plant materials and protein loading control are the same as (E) and (F). (H) In vitro deubiquitination activity of UBP12 to VPS23A. The native and ubiquitinated forms were detected with anti-GST and Ub antibodies. (I and J) Ubiquitinated VPS23A purified from plant material using a p62 matrix was incubated with different forms of UBP for 1 hour. Anti-Flag and anti-HIS antibodies were used.
and UBP13 might regulate ABA signaling. Previous reports showed that double null mutants of UBP12 and UBP13 could not be obtained or led to severe developmental defects (43, 44). Hence, we used single mutants for ABA response analysis. Two independent T-DNA insertion lines of either UBP12 or UBP13 were obtained; ubp12-2w and ubp13-1 have been described above. ubp12 (CS854763) and ubp13-2 (SALK_024054) are null alleles for UBP12 and UBP13, respectively (43, 44). All of these mutant lines of UBP12 and UBP13 showed increased ABA sensitivity phenotypes in terms of both root length and seedling establishment compared with Col-0 (Fig. 3, A and B, and fig. S3, A to D). Furthermore, we detected the protein levels of ABI5, a downstream transcription factor in ABA signaling, in 7-day-old Col-0 and ubp12-2w seedlings with or without the treatment of 50 μM ABA. We barely detected ABI5 without ABA, whereas ABI5 protein accumulated much more in both ubp12 and ubp13 mutants relative to Col-0 upon ABA treatment (Fig. 3C and fig. S3E). To further confirm the function of UBP12 and UBP13 in the ABA response, transgenic plants overexpressing UBP12 or UBP13 were generated. All the independent transgenic lines of UBP12 and UBP13 showed ABA-insensitive phenotypes in terms of seedling establishment compared with Col-0 (fig. S3, F to K). These results indicated that UBP12 and UBP13 are negative regulators of ABA responses.

VPS23A acts in ABA signaling by recognizing the ABA receptor PYL4 and committing it to endosomal degradation (34). Given that UBP12 and UBP13 affect the protein stability of VPS23A, we then investigated whether the protein level of PYL4 was altered in mutants of either UBP12 or UBP13 compared with Col-0. Our results showed that PYL4 was slightly more accumulated in ubp12-2w, ubp12, ubp13-1, and ubp13-2 mutants compared with Col-0 without ABA treatment, while considerable amounts of PYL4 accumulated in these mutants compared with Col-0 under ABA treatment (Fig. 3, D and E, and fig. S3, L and M). However, the transcription level of PYL4 was comparable in ubp12-2w, ubp12, ubp13-1, and ubp13-2 mutants compared with Col-0 with or without ABA treatment (fig. S3, N and O). These results suggested that both UBP12 and UBP13 might negatively regulate ABA signaling by modulating the protein level of ABA receptors.

As ABA plays an essential role in plant drought response by altering stomatal aperture and water loss (3, 51), we measured the stomatal conductance of ubp12-2w and ubp13-1, and we found that the stomatal conductance of both ubp12-2w and ubp13-1 was significantly lower than that of Col-0 following ABA treatment (Fig. 3F), suggesting that ABA-induced reduction of stomatal conductance is enhanced in ubp12-2w and ubp13-1 mutant plants. Consistently, ubp12-2w and ubp13-1 mutants showed higher leaf temperature (fig. S4, A and B) and lower water loss rate (Fig. 3G and fig. S4, C and D) than Col-0 under drought treatment. Considering the function of UBP12 and UBP13 in ABA signaling, these results demonstrated that ABA-mediated stomatal regulation contributes to the enhanced drought tolerance of ubp12-2w and ubp13-1 plants.

To further verify that UBP12 and UBP13 regulate ABA signaling through suppression of ABA receptors, we generated ubp12-2w pyr1 pyl1/2/4 and ubp13-1 pyr1 pyl1/2/4 quintuple mutants by crossing ubp12-2w or ubp13-1 with pyr1 pyl1/2/4. The pyr1 pyl1/2/4 quadruple mutant of ABA receptors shows a hyposensitive response phenotype compared with Col-0. Introduction of the pyl1 pyl1/2/4 alleles to either ubp12-2w or ubp13-1 completely suppressed the ABA hypersensitivity phenotype of ubp12-2w or ubp13-1 (fig. S5), indicating that ABA receptor-coding genes are epistatic to UBP12 and UBP13 in ABA signaling. Thus, UBP12 and UBP13 might participate in the regulation of ABA signaling via VPS23A-mediated degradation of ABA receptors.

UBP12 and UBP13 interact with VPS23A to regulate ABA responses
To verify whether UBP12 and UBP13 regulate ABA responses through VPS23A, we overexpressed VPS23A in the ubp12-2w or ubp13-1 mutant background under the control of 3SS promoter to generate and obtain VPS23A::GFP-ubp12-2w (two independent lines 2-1 and 7-1) and GFP-VPS23A::ubp13-1 (two independent lines 1-3 and 2-7) T1 homozygous transgenic plants, respectively (fig. S6, A and B). As expected, enhanced primary root inhibition and lower cotyledon greening rate of ubp12-2w and ubp13-1 compared with Col-0 were partially rescued by overexpression of VPS23A (Fig. 4, A and B, and fig. S6, C to F). Moreover, the expression levels of the downstream ABA signaling genes ABI5 and ABF3 were consistent with these phenotypes in the presence or absence of ABA treatment (fig. S6, G and H). In addition, VPS23A overexpression significantly decreased the higher accumulation of PYL4 protein in ubp12-2w or ubp13-1 mutant plants without affecting the PYL4 transcription level (Fig. 4C and fig. S6, I and J). These results implied that UBP12 and UBP13 participate in the regulation of ABA signaling through a VPS23A-mediated pathway. To further determine that UBP12 and UBP13 interact genetically with VPS23A, ubp12-2w was crossed with vps23a to generate ubp12-2w/vps23a double mutant. In the absence of ABA, both mutants showed no significant differences in cotyledon greening compared with that of Col-0. In the presence of 0.3 μM ABA, the cotyledon greening rate of the ubp12-2w/vps23a double mutant was similar to that of the vps23a single mutant, proving that UBP12 interacts genetically with VPS23A (fig. S7, A and B). In the case of UBP13, as genomic loci of UBP13 and VPS23A are so close that we cannot obtain double mutants by genetic cross, we used the CRISPR-Cas9 system to knock out VPS23A in the ubp13-1 mutant background to produce ubp13-1/VPS23AKO double mutants. Phenotype analysis also proved that UBP13 interacts genetically with VPS23A (fig. S7, C to E).

Furthermore, the genetic interaction between VPS23A and UBP12 or UBP13 was also analyzed under drought stress conditions. As expected, the lower stomatal conductance of either ubp12-2w or ubp13-1 compared with Col-0 after ABA treatment was partially rescued by overexpression of VPS23A (Fig. 4, D and E). Consistently, the lower water loss and higher leaf temperature of ubp12-2w or ubp13-1 compared with Col-0 were also partially rescued by overexpression of VPS23A (Fig. 4, F and G, and fig. S8). Together, the overexpression of VPS23A partially rescued the ABA hypersensitive and drought tolerance phenotypes of ubp12-2w and ubp13-1 mutant plants. Thus, UBP12 and UBP13 participate in ABA signaling by modulating VPS23A-mediated degradation of the ABA receptor to affect ABA responses.

UBP12 and UBP13 act in a feed-forward loop mechanism in the regulation of ABA signaling via VPS23A and its E3 ligase XBAT35.2
In the VPS23A-mediated ABA signaling pathway, VPS23A is negatively modulated by the Ub ligase XBAT35.2, whereas it is protected by the deubiquitinases UBP12 and UBP13. How this kind of antagonistic effect is modulated by environmental stresses is of interest.
Since multiple stresses promote the high accumulation of ABA in plants, we designed an experiment to analyze the regulation of VPS23A upon ABA stimulation. ABA treatment promoted the protein degradation of VPS23A (36), which thereby enhanced ABA signaling. To reveal how UBP12 and UBP13 respond to ABA, their protein levels were also detected under ABA treatment. Unexpectedly, we found that ABA promoted the protein accumulation of UBP12 and UBP13 without transcript changes (Fig. 5), likely promoting the deubiquitination and stabilization of VPS23A, which seems to contradict with the promoted degradation of VPS23A under ABA stimulation.
Considering that VPS23A stability is modulated by the E3 ligase XBAT35.2, a previous study reported that ABA treatment also stabilizes XBAT35.2 \( (52) \). Hence, we surmised whether the increased amount of UBP12 and UBP13 could also deubiquitinate and thus stabilize XBAT35.2 to reduce the amount of VPS23A in response to ABA. We first performed in vivo LCI assays and in vitro pull-down assays to detect their interaction, both of which showed that UBP12 and UBP13 interact with XBAT35.2 (Fig. 6, A and B). Next, we investigated whether UBP12 and UBP13 deubiquitinate XBAT35.2 via in vitro deubiquitination assays. Treatment of
that ABA promotes the stabilization of XBAT35.2 by both UBP12 (Fig. 6G). These results demonstrated results in Col-0 and ubp13-1 Col-0 after ABA treatment (Fig. 6F). Similarly, we obtained similar while they were increased in the tentatively, the in vivo ubiquitination level of XBAT35.2-Flag in Col-0 after ABA treatment (Fig. 6D and fig. S9B). In the case of was comparable. By contrast, the XBAT35.2-Flag protein was comparable. In a previous report (52) nor UBP13 (m) had any effect on ubiquitinated XBAT35.2 (Fig. 6C). These results suggested that UBP12 and UBP13 might significantly decrease of ubiquitinated XBAT35.2 (Fig. 6C) but did not affect ubiquitinated SDR1 (fig. S9A), an E3 ligase reported to function in ABA signaling (53). Moreover, neither mutated UBPI2 (m) nor UBPI3 (m) had any effect on ubiquitinated XBAT35.2 (Fig. 6C). These results suggested that UBPI2 and UBPI3 might specifically deubiquitinate the E3 ligase XBAT35.2. Furthermore, to verify that UBPI2 stabilizes XBAT35.2 through its deubiquitination, we detected the degradation rate of XBAT35.2 in ubp12-2w and wild-type Col-0 seedlings with or without ABA treatment. XBAT35.2 protein stability increased after treatment with ABA compared with a mock treatment in the Col-0 background, which is consistent with a previous report (52). In the absence of ABA treatment, the degradation velocity of XBAT35.2-Flag in Col-0 and ubp12-2w was comparable. By contrast, the XBAT35.2-Flag protein was markedly degraded in the ubp12-2w mutant compared with Col-0 after ABA treatment (Fig. 6D and fig. S9B). In the case of ubp13-1, we obtained similar results (Fig. 6E and fig. S9C). Consistently, the in vivo ubiquitination level of XBAT35.2-Flag in Col-0 and ubp12-2w was comparable in the absence of ABA treatment, while they were increased in the ubp12-2w mutant compared with Col-0 after ABA treatment (Fig. 6F). Similarly, we obtained similar results in Col-0 and ubp13-1 (Fig. 6G). These results demonstrated that ABA promotes the stabilization of XBAT35.2 by both UBPI2 and UBPI3. As both VPS23A and XBAT35.2 could interact with UBPI2/UBPI3, we asked whether XBAT35.2 could compete with VPS23A to interact with UBPI2/UBPI3. Pull-down assays showed that increasing amounts of MBP-XBAT35.2 rather than the maltose binding protein (MBP) control could attenuate the interaction between UBPI2/UBPI3 and VPS23A (Fig. 7, A and B). In vivo LCI assays also showed that XBAT35.2-Flag inhibited the interaction between UBPI2/UBPI3 and VPS23A (Fig. 7C), while the protein levels of UBPI2/UBPI3 and VPS23A were not significantly different (Fig. 7D). Hence, XBAT35.2 could compete with VPS23A for interacting with UBPI2/UBPI3. This result might indicate that the competition between VPS23A and XBAT35.2 for UBPI2/UBPI3 plays a regulatory role in ABA signaling. Together, ABA could promote the accumulation of deubiquitinases UBPI2 and UBPI3 to stabilize the E3 ligase XBAT35.2 of VPS23A, and the accumulated XBAT35.2 further attenuates the protection of UBPI2/UBPI3 on VPS23A, thus accelerating the degradation of VPS23A in ABA signaling.

**DISCUSSION**

Previous work indicated that the ubiquitination-dependent 26S proteasomal degradation pathway plays an essential role in ABA signaling (14). Emerging evidence indicates that ubiquitination-dependent non-26S proteasomal degradation (i.e., the endocytic pathway) is also indispensable for ABA signaling. VPS23A functions in the ESCRT-I complex for proper PYL4 recognition, sorting, and endosomal degradation (34). In addition, another important component in ESCRT-I, FYVE1/FREE1, is a Ub- and phosphatidylinositol 3-phosphate–binding protein that was also reported to be indispensable for the degradation of the ubiquitinated substrate PYL4 (33).

Despite the importance of ESCRT-I, its regulatory mechanisms remain poorly understood. Here, we found that the deubiquitinases UBPI2 and UBPI3 are integrated into ABA signaling through interaction with and deubiquitination of VPS23A, which was supported by the following evidence. First, molecular and biochemical assays indicated that deubiquitination of VPS23A by UBPI2/UBPI3 contributed to the stability of VPS23A. Second, mutation of ABA receptors rescued the ABA-sensitive phenotype of either ubp12-2w or ubp13-1, and VPS23A was epistatic to either UBPI2 or UBPI3. Last, our data suggested that a feed-forward loop mechanism of UBPI2 and UBPI3 regulates ABA signaling via VPS23A and its E3 ligase XBAT35.2. Hence, we proposed that UBPI2 and UBPI3 function as rheostatic factors to regulate plant ABA responses, and this might be largely executed through the regulation of the stability of a main component in the ESCRT-I complex, VPS23A, to manipulate ABA signaling.

Most research on abiotic stress focuses on how Ub conjugates to its substrates; therefore, a considerable number of E3 Ub ligases have been characterized for specific substrates (16, 54). Although ubiquitination is reversible, how Ub is deconjugated from substrates remains elusive. We found that UBPI2 and UBPI3 participate in plant stress responses in Arabidopsis by modulating ABA receptor abundance in a VPS23A-dependent manner, which advances our knowledge of the role conferred by deubiquitinases in ABA signaling. Previously, deletion of UBPI4 decreased the activity of ABA coreceptor PP2Cs, and UBPI4 acted genetically upstream of ABI2, indicating that UBPI4 is a negative regulator of ABA signaling (55). Although UBPI4 is reported as the first deubiquitinating enzyme that functions in ABA signaling, the deubiquitinated
target of UBP24 has not yet been verified. Here, the substrate identification of UBP12 and UBP13 in ABA signaling strengthened our understanding of the detailed mechanism of how deubiquitinating enzymes influence ABA signaling and its related plant stress responses.

Recent studies found that UBP12 and UBP13 are versatile deubiquitinases that participate in various signaling pathways by stabilizing specific substrates (43, 45, 47–50). They were first recognized as negative regulators of plant immunity (46). Subsequently, they were revealed to regulate photoperiodic flowering by tuning clock gene expression (44). Furthermore, UBP12 and UBP13 were demonstrated to stabilize a multitude of substrates, for instance, RGFR1, which is important for root growth signaling (43), and MYC2 in the jasmonic acid (JA) signaling pathway (47). However, to our knowledge, a common feature among the substrates of UBP12 and UBP13 is obscure. Hence, it is important to identify the mechanism by which UBP12 and UBP13 recognize their substrates. In

Fig. 6. ABA promotes the degradation of VPS23A through UBP12- and UBP13-mediated stabilization of the E3 ligase XBAT35.2. (A) LCI assays demonstrated the interaction between UBP12/UBP13 and XBAT35.2. Cluc-XBAT35.2, XBAT35.2 fused with Cluc. (B) Pull-down assays for analysis of the interaction between UBP12/UBP13 and XBAT35.2. An equal amount of HIS-UBP12 or HIS-UBP13 was incubated with immobilized myelin basic protein (MBP) and MBP-XBAT35.2 separately, and then the bound proteins were detected by Western blotting using anti-HIS and anti-MBP antibodies. (C) XBAT35.2 was the deubiquitinated substrate of both UBP12 and UBP13. Polyubiquitinated MBP-XBAT35.2 produced by autoubiquitination in the presence of AtE1, UBCh5b, and Ub was incubated with HIS-UBP12, HIS-UBP12 (m), HIS-UBP13, or HIS-UBP13 (m) for 1 or 2 hours. Ubiquitinated MBP-XBAT35.2 was detected by anti-MBP and anti-Ub antibodies. HIS-UBP12 and HIS-UBP13 (m) were detected with anti-HIS antibody. (D and E) UBP12 and UBP13 stabilized XBAT35.2. Ten-day-old seedlings of XBAT35.2-Flag::Col-0, XBAT35.2-Flag::ubp12-2w, or Flag-XBAT35.2::ubp13-1 background were treated with 100 μM CHX or 100 μM CHX plus 50 μM ABA for 0, 2, and 4 hours. (F and G) In vivo ubiquitination level of XBAT35.2 in Col-0 and ubp12-2w or ubp13-1 backgrounds. The plants used were XBAT35.2-Flag::Col-0, XBAT35.2-Flag::ubp12-2w, Flag-XBAT35.2::Col-0, and Flag-XBAT35.2::ubp13-1. Crude proteins extracted from 10-day-old transgenic plants with or without 50 μM ABA treatment were used for incubation with anti-Flag beads, and immunoblotting assays were conducted using anti-Flag (middle) and anti-Ub antibodies (top). PAG1 was used as a loading control.
mammalian biology, USP7, the homolog of UBP12 and UBP13, has a MATH domain that is responsible for substrate recognition (56). Hence, it will be intriguing to explore whether the MATH domain-containing deubiquitinases UBP12 and UBP13 also confer the ability to bind their substrates in plants. In addition, because of the increased interest in UBP12 and UBP13, they might be good models to perform analysis on the specificity of deubiquitinase family members and their substrate(s) in plants.

As key and complicated conserved regulators, how UBP12 and UBP13 distinguish various signals to manage many plant biological processes by recognizing different ubiquitinated substrates is another outstanding question. UBP12 and UBP13 themselves might respond differently to various biological signals. We found that the protein levels of UBP12 and UBP13 increased after ABA treatment, and accumulated UBP12 and UBP13 stabilized XBAT35.2, the E3 ligase of VPS23A, to mediate the degradation of the ABA negative regulator VPS23A to thereby activate ABA signaling (36). This kind of functional module suggests a feed-forward loop with UBP12 or UBP13 as a rheostat. Since UBP12/UBP13 plays a protective role against both the negative factor VPS23A and the positive factor XBAT35.2 in ABA signaling, UBP12/UBP13 might play a buffer role in the fine regulation of VPS23A ubiquitination and stability. Under ABA treatment, the increased amounts of XBAT35.2 by UBP12/UBP13 compete with VPS23A to bind UBP12/UBP13 to increase the activity of XBAT35.2 and attenuate the protective role of UBP13/UBP13 on VPS23A, resulting in an enhanced ABA response in plants. In addition, some cofactors might exist to manage the function of UBP12 and UBP13 in response to different stimuli. Under light conditions, UBP12 and UBP13 interact with ZEITLUPE (ZTL; a photoreceptor with E3 Ub ligase activity) in a clock element GIGANTEA (GI)-dependent manner, whereas, in the dark, GI dissociates from ZTL and causes the dissociation of UBP12/UBP13 from ZTL (48, 57). Hence, we surmise that there might be cofactor(s) important for the interaction between XBAT35.2 and UBP12/UBP13 upon ABA treatment.

Last, the significance of reversible ubiquitination seems to be essential in ABA signaling and in establishing plant circadian rhythms, in which stimuli-derived signals counterbalance the role of Ub conjugation via an E3 ligase with Ub deconjugation via UBP12 and UBP13. Similarly, USP7 is homologous to plant UBP12 and UBP13 and was also reported to pair with E3 ligases, including mouse double minute 2 (MDM2) and tripartite motif-containing 27 (TRIM27).
experiment. At least three independent experiments were performed. Replicates (at least six plants per genotype) were performed in each experiment. The mean temperature ± SD of plants for each genotype is shown. Three NS9500 Standard was used to analyze the thermographic images. An InfReC Analyzer Avio InfRec R500 as reported previously (60). An InfReC Analyzer Avio InfRec R500 as reported previously (60). Thermal imaging

Seven-day-old seedlings of Col-0 and the corresponding mutants were transferred to soil within the same pot under a 16-hour light/8-hour dark photoperiod in the greenhouse. After fertilization with Gamborg B5 medium for approximately 2 weeks, the plants were subjected to drought stress for 2 weeks. The leaf temperature was tracked by Avio InfRec R500 as reported previously (61). An InfReC Analyzer NS9500 Standard was used to analyze the thermographic images. For quantification, the leaf temperature of each plant was measured. The mean temperature ± SD of plants for each genotype is shown. Three replicates (at least six plants per genotype) were performed in each experiment. At least three independent experiments were performed.

MATERIALS AND METHODS

Plant materials and growth conditions

*Arabidopsis thaliana* (Col-0 accession) seeds were plated on half-strength Murashige-Skoog (½ MS) medium containing 1% (w/v) sucrose and 0.8% (w/v) agar. Seedlings were transferred to soil and grown under a long-day (16-hour light and 8-hour dark) photoperiod in a growth room at 22°C. The growth conditions of *N. benthamiana* were as same as described previously (60). The vps23a (CS878714), ubp13-1 (SALK_128312), and ubp13-2 (SALK_024054) mutants were ordered from Arabidopsis Biological Resource Center (The Ohio State University, Columbus, OH, USA); ubp12-2w (CS2103163) and ubp12 (CS854763) were provided by X. Cui (Chinese Academy of Agricultural Sciences, Beijing, China) and W. Tang (Hebei Normal University, Shijiazhuang, China), respectively; and their properties were previously described. The ecotype of all the *Arabidopsis* mutants used in this study was Col-0. The primers used for verification of mutants are listed in table S1.

*Arabidopsis* seeds were surface sterilized with 10% NaClO for 20 min and stratified for 4 days at 4°C. After stratification, seeds were plated on ½ MS medium and grown at 22°C under a 16-hour light/8-hour dark photoperiod. For ABA phenotypic observation, seeds were germinated and grown on ½ MS medium with or without ABA from the time of sowing.

Root growth and seedling establishment assays

Seeds of different genotypes were grown on vertically plated ½ MS plates with or without ABA for 6 days. Afterward, the root length of seedlings was analyzed with National Institutes of Health (NIH) ImageJ. For each genotype, at least 18 plants per experiment were analyzed, experiments were performed with three replications, and representative results are shown.

For seedling establishment assays, at least 90 seeds of each genotype were sown. Seedling establishment was evaluated as the percentage of seeds with green cotyledons on the fourth or sixth day.

Stomatal conductance analysis

Stomatal conductance analysis was conducted according to previously reported methods (62). Briefly, 7-day-old seedlings were transferred to soil for another 7 weeks under a 10-hour light/14-hour dark photoperiod. For each genotype, the fifth true leaf with a common growth condition was chosen for detection. First, petioles were cut from seedlings and immediately emerge in Milli-Q water. Second, a razor blade was used to cut petioles again underwater. Third, the stomatal conductance of leaves was measured by LI-6400XT (LI-COR) under the following conditions: Gas exchange temperature was 21°C, the light-emitting diode light source was 150 μmol m⁻² s⁻¹, the relative humidity in the chamber was kept at approximately 70%, the CO₂ concentration was set at 400 parts per million, and the airflow was 500 μm. The steady-state stomatal conductance was recorded for 10 min before the addition of 2 μM ABA. Three replicates (three plants per genotype) were performed in each experiment. At least three independent experiments were performed.

Water loss assays

A time-resolved water loss experiment was performed following a previously reported method (61). Briefly, seedlings of each genotype were grown for 4 weeks under a 10-hour light/14-hour dark photoperiod light, with a light density of approximately 100 μmol m⁻² s⁻¹ and temperature of approximately 22°C. For each genetic background, at least three pots with nine seedlings per pot were planted, and each pot was adjusted to the same initial weight. Water loss was measured by weighing each pot on electronic balances (DroughtSpotter, Phenospec) every 30 min for 7 days. The data were recorded, and time-resolved water loss was calculated for each day. The experiments were performed with three replications.

Constructs

The full-length coding DNA sequence (CDS) of *UBP12* and *UBP13* without stop codon was cloned into pCAMBIA1300-Nluc, pET28a, pCAMBIA1300-Flag, and pSPYCE; the full-length CDS of *UBP12* (m) and *UBP13* (m) without stop codons was generated by overlapping polymerase chain reaction (PCR) and cloned into pET28a; and the full-length CDS of *UBP12* and *UBP13* was cloned into pGADT7 and pER10-N-HA. Similarly, the full-length CDS of *UBP14* was cloned into pCAMBIA1300-Nluc and the full-length CDS of *VPS23A* was cloned into pSPYNE. The full-length CDS of *XBAT35.2* and *VPS23A* without stop codons was cloned into pCanG-3Flag, and the full-length CDS of *XBAT35.2* was cloned into pCAMBIA1300-Cluc. All constructs described above were generated using a Seamless Infusion Cloning kit according to the manufacturer’s instructions (TRANSGENE). For pGBKTK7-VPS23A, the full-length CDS of *VPS23A* was cloned into the pGBKTK7 vector by restriction digestion and ligation. For CRISPR-Cas9–mediated VPS23A knockout, the guide RNA sequence of VPS23A was cloned into pCAMBIA1300-pYAO::Cas9 to generate a CRISPR-VPS23A construct. All clones were verified by PCR and subsequently DNA sequenced. The primers used are listed in table S1. GFP-VPS23A, Cluc-VPS23A, GST-VPS23A, MBP-XBAT35.2, Flag-VPS23A, and Flag-XBAT35.2 were generated as previously reported (34, 36).

Generation of transgenic plants

To generate constitutive overexpression transgenic plants, the GFP-VPS23A, VPS23A-Flag, pER10-N-HA-UBP12, pER10-N-HA-UBP13, UBP12-Flag, UBP13-Flag, CRISPR-VPS23A, and XBAT35.2-Flag
constructs were transformed into A. tumefaciens strain GV3101 for the generation of transgenic plants using the floral dip method as reported previously (63). Transgenic seedlings of the T2 or T3 generation were used for further study. The primers used are listed in table S1.

**Expression and purification of recombinant protein**  
Constructs of HIS-UBP12 or HIS-UBP13 (m), HIS-UBP13 or HIS-UBP13 (m), GST, GST-VPS23A, MBP, and MBP-XBAT35.2 were introduced into and expressed in Escherichia coli BL21 (DE3, TRANSGENE) for purification following the manufacturer’s instructions.

**Yeast two-hybrid assay**  
To confirm the interaction between UBP12/UBP13 and VPS23A, the full-length CDS of VPS23A or UBP12/UBP13 was separately amplified and cloned into the pGBKT7 (binding domain) and pGADT7 (activation domain) vectors. Sequence-verified clones were cotransformed into the yeast strain AH109. Transformed yeast cells were grown on selective media at 30°C for 4 to 5 days (64).

**Firefly LCI assay**  
LCI assays were performed according to a previously reported method (65). Briefly, the full-length CDS of UBP12/UBP13 and VPS23A or XBAT35.2 was fused to the N terminus and C terminus of LUC, respectively. The full-length CDS of UBP14 without a stop codon was also fused to the N-terminal segment of LUC and used as a negative control. The correct clones were transformed into Agrobacterium strain GV3101 and subjected to coinfiltration into N. benthamiana leaves for transient expression. The LUC signal was captured via a charge-coupled device (NightOWL LB 983) camera to indicate the interaction intensity. To determine the effect of XBAT35.2 on the interaction between UBP12/UBP13 and VPS23A, 36 of MBP and MBP-XBAT35.2 protein bound to amylase resin New England Biolabs (NEB) were used for incubation with the same amount of HIS-UBP12 or HIS-UBP13, respectively. Pulled-down proteins were detected by immunoblotting with their corresponding antibodies. To determine the effect of XBAT35.2 on VPS23A and UBP12/UBP13 interactions, equal amounts of GST and GST-VPS23A protein were incubated with equal amounts of HIS-UBP12 or HIS-UBP13 in the presence of increasing amounts of MBP or MBP-XBAT35.2.

**BiFC assay**  
The full-length CDS of VPS23A and UBP12/UBP13 was fused to the N-terminal and C-terminal portions of YFP to produce YFPN-VPS23A and YFPN-C-UBP12/UBP13, respectively. YFPN and YFPN were used as negative controls. Different constructs together with mCherry-ARA7 were transformed into Agrobacterium strain GV3101 and subjected to coinfiltrate into N. benthamiana leaves for transient expression. Fluorescence was visualized in epidermal cells of leaves 3 days after infiltration by LSM 710 confocal microscopy. The primers used for this assay are listed in table S1.

**Estradiol induction assays**  
Seven-day-old seedlings of XVE:HA-UBP12/VPS23A C or XVE:HA-UBP13/VPS23A C were pretreated with ½ MS medium for 12 hours and then treated with or without 25 μM β-estradiol for 16 hours.

**IP assays**  
IP assays were performed according to a previously reported method (36). Briefly, 10-day-old transgenic plants with or without 50 μM ABA treatment were subjected to IP with anti-Flag or anti-GFP magnetic beads. The bead-bound proteins were detected with anti-Flag (Sigma-Aldrich), anti-GFP, and anti-Ub (36) antibodies.

**In vivo CHX-chase degradation assays**  
CHX-chase assays in Arabidopsis were conducted according to a reported method (47). Briefly, transgenic seedlings were pretreated with 100 μM MG132 for 16 hours and then washed at least five times before treatment with 100 μM CHX. Samples were harvested at the indicated time points and subjected to immunoblotting against the corresponding antibodies.

**Protein transient expression assays**  
Agrobacterial strains carrying UBP12-Nluc or UBP13-Nluc, GFP-VPS23A, and internal control RFP constructs were coinfiltrated in N. benthamiana at different ratios. Samples were harvested for analysis 3 days after coinfiltration.

**p62 matrix immunoprecipitation of ubiquitinated proteins**  
Ubiquitinated VPS23A-Flag was purified as reported previously (25). Briefly, total proteins were extracted from VPS23A-Flag transgenic seedlings and Col-0 seedlings (negative control) for incubation with p62-agarose beads (Enzo Life Sciences, catalog no. BML-UW9010-0500) at 4°C. After incubation for 4 hours, p62-agarose beads were washed three times with wash buffer as previously reported, and the bound ubiquitinated proteins were detected by immunoblotting.

**In vitro ubiquitination and deubiquitination assays**  
The in vitro ubiquitination assay of GST-VPS23A was conducted following a previously published protocol (36). Briefly, 2 μg of Ub,
50 ng of AtE1, 50 ng of human UBCh5b, 1 μg of MBP-XBAT35.2, and 500 ng of GST-VPS23A were added to ubiquitination buffer [50 mM tris (pH 7.4), 2 mM adenosine 5′-triphosphate, 5 mM MgCl₂, and 2 mM dithiothreitol]. For in vitro deubiquitination, purified HIS-UBP12 or HIS-UBP13 was added along with E1, E2, and E3. Alternatively, after incubation with E1, E2, and E3 for 2 hours at 30°C, GST-VPS23A was purified with glutathione sepharose 4B beads. Polyubiquitinated GST-VPS23A was added to deubiquitination buffer (47) treated with HIS-UBP12, HIS-UBP13, or an active site–mutated HIS-UBP12 (m) or HIS-UBP13 (m) and incubated at 30°C for 1 or 2 hours. The deubiquitination reaction was tested by immunoblotting with anti-GST antibody. Similarly, autoubiquitination of XBAT35.2 was performed with the addition of 2 μg of Ub, 50 ng of AtE1, 50 ng of human UBCh5b, and 1 μg of MBP-XBAT35.2. Then, ubiquitinated MBP-XBAT35.2 was purified for a deubiquitination assay after treatment with HIS-UBP12, HIS-UBP13, or an active site–mutated HIS-UBP12 (m) or HIS-UBP13 (m) for 1 or 2 hours at 30°C.

**In vitro deubiquitination assays of VPS23A purified by p62 matrix**

First, the p62 matrix was used to purify ubiquitinated VPS23A-Flag, which was then added to deubiquitination buffer (47) with HIS-UBP12, HIS-UBP13, or active site–mutated HIS-UBP12 (m) or HIS-UBP13 (m) and incubated at 30°C for 1 hour. The deubiquitination reaction was terminated with the addition of 4× SDS loading buffer and then subjected to immunoblotting against the anti-Flag antibody.

**RNA extraction and real-time quantitative RT-PCR**

The seedlings harvested after the indicated treatments were subjected to RNA extraction by an Ultrapure RNA kit (CWBio) according to the manufacturer’s instructions. Two micrograms of total RNA was used as the template for reverse transcription (RT) with the FastQuant RT Kit with gDNase (TIANGEN). cDNAs were diluted 20 times with ddH₂O, and 3 μl was used for quantitative RT-PCR (qRT-PCR) with SYBR mix (TIANGEN). ACTIN2 was used as an internal control. The primers used for qRT-PCR are listed in table S1.

**Quantification of Western blotting densitometry band**

For each Western blotting result, at least three replications were chosen for quantitative analysis with NIH ImageJ following the published protocol (67).

**Data statistical analysis**

Values are averages obtained from three independent experiments ± SD. Significant differences (P value) were determined by using Student’s t test (*P < 0.05 and **P < 0.01) and one-way analysis of variance (ANOVA) or two-way ANOVA followed by Tukey’s multiple comparison test.

**Antibodies resources**

Anti-Myc, anti-HIS, anti-GFP, and anti-RFP antibodies were purchased from Easy Bio System Inc.; anti-LUC was purchased from Sigma-Aldrich; anti-HA was purchased from CoWin Biosciences; anti-AB15 was purchased from Abcam; anti-Flag, anti-GST, and anti-MBP antibodies were purchased from ProteinTech; anti-UBP12 was gifted by X. Cui from the Chinese Agricultural Academy of Sciences; and anti-Ub and anti-PYL4 antibodies were generated and purified from the stock of our laboratory.

**Accession numbers**

Arabidopsis Genome Initiative locus identifiers for the genes mentioned here are as follows: VPS23A (AT3G12400), UBIP2 (AT5G06600), UBIP13 (AT3G11910), PYR1 (AT4G7870), PYL1 (AT5G46790), PYL2 (AT2G26040), PYL4 (AT2G38310), ABI5 (AT2G36270), PAG1 (AT2G27020), and ABF3 (AT4G34000).

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at https://science.sciencemag.org/content/10.1126/sciadv.abi5765.

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