Roles of Four Arabidopsis U-Box E3 Ubiquitin Ligases in Negative Regulation of Abscisic Acid-Mediated Drought Stress Responses[1][C][W][OA]

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AtPUB18 and AtPUB19 are homologous U-box E3 ubiquitin ligases in Arabidopsis (*Arabidopsis thaliana*). AtPUB19 is a negative regulator of abscisic acid (ABA)-mediated drought responses, whereas the role of AtPUB18 in drought responses is unknown. Here, loss-of-function and overexpression tests identified AtPUB18 as a negative regulator in ABA-mediated stomatal closure and water stress responses. The *atpub18-2atpub19-3* double mutant line displayed more sensitivity to ABA and enhanced drought tolerance than each single mutant plant; therefore, AtPUB18 and AtPUB19 are agonistic. Stomatal closure of the *atpub18-2atpub19-3* mutant was hypersensitive to hydrogen peroxide (H_2O_2) but not to calcium, suggesting that AtPUB18 and AtPUB19 exert negative effects on the ABA signaling pathway downstream of H_2O_2 and upstream of calcium. AtPUB22 and AtPUB23 are other U-box E3 negative regulators of drought responses. Although *atpub22atpub23* was more tolerant to drought stress relative to wild-type plants, its ABA-mediated stomatal movements were highly similar to those of wild-type plants. The *atpub18-2atpub19-3atpub22atpub23* quadruple mutant exhibited enhanced tolerance to drought stress as compared with each *atpub18-2atpub19-3* and *atpub22atpub23* double mutant progeny; however, its stomatal behavior was almost identical to the *atpub18-2atpub19-3* double mutant in the presence of ABA, H_2O_2, and calcium. Overexpression of *AtPUB18* and *AtPUB19* in *atpub22atpub23* effectively hindered ABA-dependent stomatal closure, but overexpression of *AtPUB22* and *AtPUB23* in *atpub18-2atpub19-3* did not inhibit ABA-enhanced stomatal closure, highlighting their ABA-independent roles. Overall, these results suggest that AtPUB18 has a linked function with AtPUB19, but is independent from AtPUB22 and AtPUB23, in negative regulation of ABA-mediated drought stress responses.

Ubiquitin (Ub)-mediated posttranslational protein modifications have ubiquitous functions in eukaryotic cells (Dye and Schulman, 2007; Hunter, 2007). In higher plants, they occur as part of the ubiquitination pathway in physiological processes as diverse as cell cycle progressions, circadian rhythms, environmental stress responses, and hormone and light signaling (Moon et al., 2004; Smalle and Vierstra, 2004; Dreher and Callis, 2007; Vierstra, 2009; Lee and Kim, 2011; Park et al., 2011a). Approximately 6% of the Arabidopsis (*Arabidopsis thaliana*) proteome is associated with the Ub-26S proteasome system, and over 1,400 different E3 Ub ligase genes exist in the Arabidopsis genome (Smalle and Vierstra, 2004; Vierstra, 2009). E3 proteins possess distinct functional domains, such as those derived from the Homology to E6-AP Carboxyl Terminus, Really Interesting New Gene (RING), and U-box gene families.

Arabidopsis contains at least 64 U-box motif-containing E3 proteins (Mudgil et al., 2004; Wiborg et al., 2008; Yee and Goring, 2009; Lyzenga and Stone, 2012). They have various functions related to biotic and abiotic stress responses (Yang et al., 2006; Cho et al., 2008; Trujillo et al., 2008; Liu et al., 2011), hormonal responses (Luo et al., 2006; Raab et al., 2009), self-incompatibility (Liu et al., 2007; Samuel et al., 2008, 2009), and seed germination (Bergler and Hoth, 2011; Salt et al., 2011). U-box E3s also have cellular functions within a diverse group of monocot

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and dicot model crops, such as *Brassica* spp., *Medicago* spp., rice (*Oryza sativa*), tobacco (*Nicotiana tabacum*), and tomato (*Solanum lycopersicum*) plants (Gu et al., 1998; Stone et al., 1999, 2003; Zeng et al., 2004, 2008; González-Lamothe et al., 2006; Vega-Sánchez et al., 2008; Bos et al., 2010; Mbengue et al., 2010; Park et al., 2011b), suggesting that they are widely conserved in the plant kingdom.

The plant stress hormone abscisic acid (ABA) is a key mediator in drought stress response because it induces stomatal closing to minimize transpirational water loss (Yamaguchi-Shinozaki and Shinozaki, 2006; Cutler et al., 2010; Kim et al., 2010; Raghavendra et al., 2010). ABA turns on various genes necessary for triggering rapid and effective defense programs. ABA-induced genes are diverse and include U-box E3s (Hoth et al., 2002; Cho et al., 2006, 2008); therefore, U-box E3s may be functionally correlated with ABA-mediated stress responses.

Recent studies suggest that Arabidopsis U-box E3s are necessary to induce ABA-mediated responses. PUB44/SAUL1 U-box E3 prevents premature leaf senescence by down-regulating ABA level (Raab et al., 2009). PUB44/SAUL1 inhibits seed germination under stressful conditions, such as high levels of ABA, Glc, salt, or mannitol (Salt et al., 2011), and therefore is a negative regulator of ABA-mediated cell death and seed germination processes. Bergler and Hoth (2011) showed that AtPUB18 and AtPUB19 homologs had negative effects during ABA and salt inhibition of seed germination. AtPUB19 also negatively regulates ABA-mediated drought stress responses (Liu et al., 2011). In contrast, AtPUB22 and AtPUB23 are negative regulators of drought stress responses, but their expression is unaffected by ABA (Cho et al., 2008). AtPUB22 and AtPUB23, in addition to AtPUB24, are involved in the negative regulation of pathogen-associated molecular pattern-triggered immunity (Trujillo et al., 2008), suggesting that AtPUB22 and AtPUB23 play roles in both biotic and abiotic stress responses.

In this study, we investigated AtPUB18 during ABA-mediated drought stress responses. ABA-induced stomatal closure was strongly enhanced in loss-of-function *atpub18-2atpub19-3* double mutant plants relative to *atpub18-2* and *atpub19-3* single mutants and wild-type plants. The progeny of *atpub18-2atpub19-3* exhibited even greater drought tolerance than the single mutants. *AtPUB18* overexpressors showed inverse phenotypes to knockout plants in all categories examined. These results suggest that AtPUB18 and AtPUB19 have paired functions as negative regulators in ABA-dependent drought stress responses. The stomatal movements of *atpub18-2atpub19-3* mutant leaves were hypersensitive to hydrogen peroxide (H_2O_2) as compared with those of wild-type leaves. In contrast, *atpub18-2atpub19-3* displayed wild-type stomatal movement in response to calcium, suggesting that AtPUB18 and AtPUB19 act downstream of H_2O_2 and upstream of calcium in the ABA-mediated drought response. Our *atpub18-2atpub19-3atpub22atpub23* quadruple mutation and reciprocal complementation (*35S:AtPUB18/19-atpub22atpub23* and *35S:AtPUB22/23-atpub18-2atpub19-3*) tests suggest that AtPUB18 has a combined function with AtPUB19, but is independent from AtPUB22 and AtPUB23, during negative regulation of ABA-mediated drought stress responses.

RESULTS

AtPUB18 Is an ABA- and Drought-Inducible Gene

AtPUB18 and AtPUB19 are homologous U-box E3 Ub ligases (Supplemental Fig. S1). Hoth et al. (2002), in a previous genome-scale gene expression study, identified *AtPUB18* and *AtPUB19* as ABA-induced genes, and Liu et al. (2011) recently confirmed, via RNA gel-blot analysis, the expression of *AtPUB19* to be enhanced by ABA and abiotic stress. Here, real-time quantitative reverse transcription (qRT)-PCR showed that *AtPUB18* was induced in response to ABA and various abiotic stresses, including NaCl, drought, and coldness (Fig. 1A). Induction kinetics of *AtPUB18* were similar to those of both *AtPUB19* and marker genes (*Responsive to Desiccation29A* [*RD29A*] for drought and salt, *C-repeat Binding Factor1* [*CBF1*] for cold, and *Responsive to ABA18* [*RAB18*] for ABA), while the magnitude of *AtPUB18* induction was significantly lower than that of *AtPUB19*.

A promoter-GUS assay of T3 transgenic lines showed that *AtPUB18* and *AtPUB19* promoter activities (in the 1.0-kb upstream region) were barely detectable in 5-d-old (Fig. 1B) and 2-week-old (Fig. 1C) plants. This low basal activity was markedly heightened by ABA and drought treatments. In 5-d-old seedlings, drought and ABA inductions of *AtPUB18* were predominantly detected in the roots, whereas those of *AtPUB19* were found in leaves and upper roots (Fig. 1B). In 2-week-old plants, drought and ABA inductions of both genes were observed throughout the plant tissues, including guard cells (Fig. 1C). These results suggest that AtPUB18 is involved in ABA-mediated stress responses. Promoter activities of *AtPUB22* and *AtPUB23* were induced by drought but not by ABA treatment (Fig. 1, B and C). Their drought inductions also occurred in guard cells. Overall, gene expression studies indicated that *AtPUB18* and *AtPUB19* are ABA- and drought-inducible genes, but *AtPUB22* and *AtPUB23* are only induced by dehydration stress.

Expression of *AtPUB18* and *AtPUB19*, But Not *AtPUB22* and *AtPUB23*, Is Dependent on SnRK

abi1-1 is an ABA-insensitive dominant mutant (Hubbard et al., 2010; Kim et al., 2010; Raghavendra et al., 2010). Figure 2A shows that ABA failed to induce *AtPUB18*, *AtPUB19*, and the marker gene, *RAB18*, in *abi1-1*. This demonstrates that both *AtPUB18* and *AtPUB19* are ABA-induced genes. In contrast, *AtPUB22* and *AtPUB23* expression remained unchanged before and after ABA treatment in wild-type and *abi1-1* plants, confirming their ABA-independent expression (Fig. 2A).

SnRK protein kinases are key regulators in the ABA signaling pathway. They mediate ABA-dependent

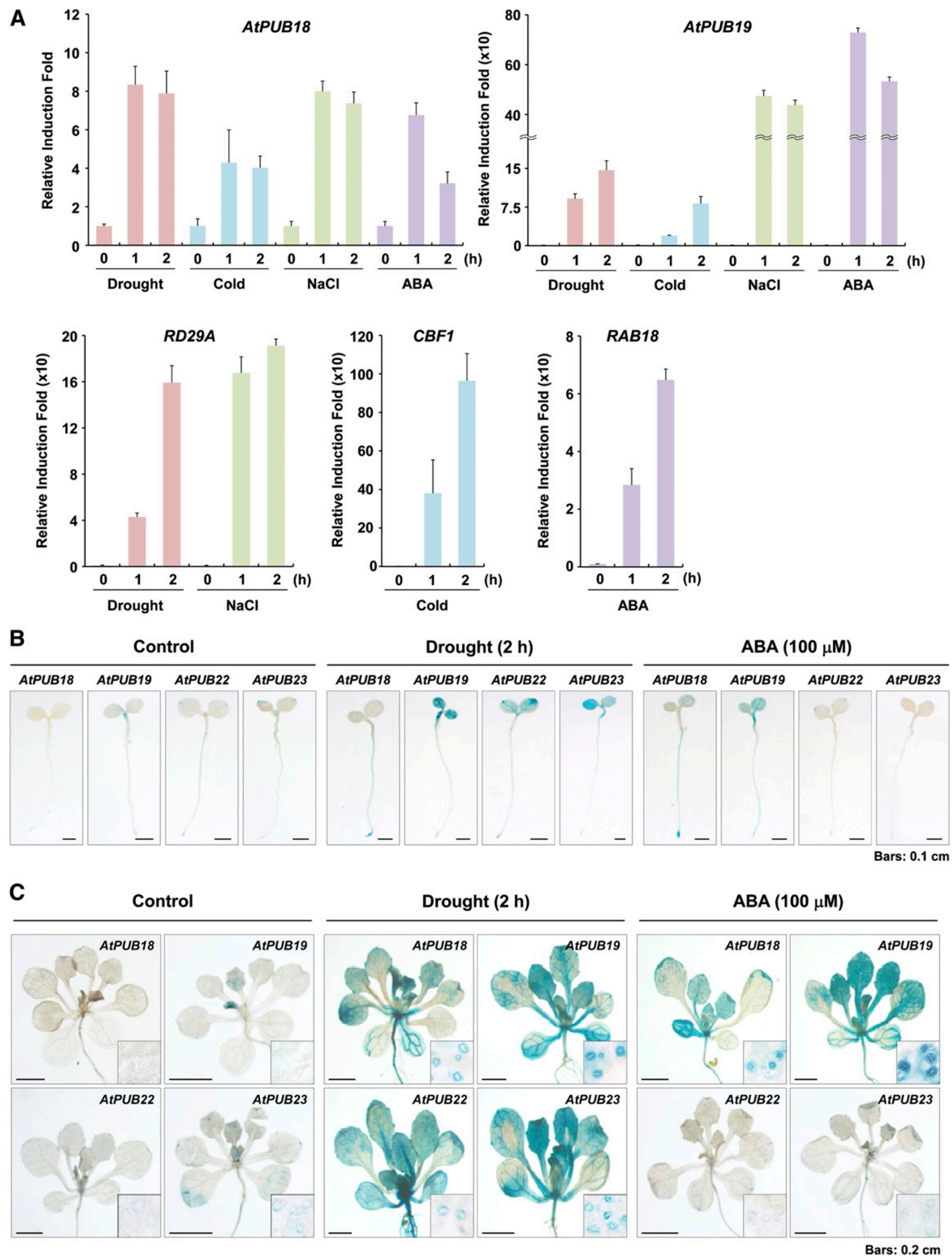

Figure 1. Induction patterns of *AtPUB18*, *AtPUB19*, *AtPUB22*, and *AtPUB23* in response to ABA and abiotic stresses, as determined by real-time qRT-PCR and histochemical GUS assays. A, Light-grown, 10-d-old Arabidopsis seedlings were treated with drought, cold, high salinity, and ABA. Total RNA was extracted from the stress-treated whole seedlings and used for qRT-PCR. *RD29A*, *CBF1*, and *RAB18* were positive controls for drought and high salt, cold, and ABA treatments, respectively. To calculate the relative expression levels of each gene, glyceraldehyde-3-phosphate dehydrogenase C subunit was used as an internal reference gene. Error bars represent SD from three independent experiments. B and C, Promoter (1.0-kb upstream region) activities of *AtPUB18*, *AtPUB19*, *AtPUB22*, and *AtPUB23* in response to ABA (100 μM) and dehydration (2 h). B, Histochemical GUS expression patterns in 5-d-old T3 *promoter:GUS* transgenic seedlings. Bars = 0.1 cm. C, GUS activities in aerial parts and guard cells of 2-week-old transgenic plants. Bars = 0.2 cm. [See online article for color version of this figure.]

reactive oxygen species production and water and osmotic stress responses (Mustilli et al., 2002; Fujita et al., 2009; Fujii et al., 2011). Furthermore, ABA- and dehydration-induced gene expressions are greatly impaired by triple knockout mutations of *SnRK2.2*, *SnRK2.3*, and *SnRK2.6* (Fujii and Zhu, 2009; Fujita et al., 2009). ABA induction of *AtPUB18* and *AtPUB19* in *snrk2.2*, *snrk2.3*, and *snrk2.6* single and *snrk2.2snrk2.3*, *snrk2.2snrk2.6*, and *snrk2.3snrk2.6* double knockout mutants was comparable to that in wild-type plants (Fig. 2, B and C). In contrast, ABA induction of both genes was completely abolished in *snrk2.2snrk2.3snrk2.6* triple loss-of-function mutant plants (Fig. 2D). Therefore, the redundant functions of SnRK members resulted in ABA induction of *AtPUB18* and *AtPUB19* in the single and double *snrk* mutant lines. Similar induction patterns were also observed for *RAB18*. These results indicate that SnRKs are necessary for ABA induction of *AtPUB18* and *AtPUB19*; however, *AtPUB22* and *AtPUB23* are not induced by ABA in either wild-type or *snkr* lines (Fig. 2).

Phenotypic Analyses of *atpub18* Single and *atpub18atpub19* Double Knockout Mutant Plants in Response to ABA and Drought

Liu et al. (2011) showed that suppression of *AtPUB19* increased ABA sensitivity with regard to stomatal movement. We examined ABA-dependent stomatal behaviors of wild-type, *atpub18-2* (SALK_001831) and *atpub19-3* (SALK_058791) single mutant, and *atpub18-2atpub19-3* double mutant plants (Supplemental Fig. S2). Stomatal apertures (the ratio of width to length) of wild-type and mutant plants were indistinguishable without ABA treatment (Fig. 3A). After treatment with ABA, however, stomatal movement in the *atpub18-2* mutant was more markedly enhanced, as compared with that in the wild type, in a dose-dependent manner. The average stomatal apertures of wild-type, *atpub18-2*, *atpub19-3*, and *atpub18-2atpub19-3* plants were 0.123 $\pm$ 0.002, 0.091 $\pm$ 0.015, 0.073 $\pm$ 0.007, and 0.059 $\pm$ 0.013, respectively, in response to 10 μM ABA (Fig. 3A). The *atpub18-2* plants, therefore, exhibited hypersensitive phenotypes, relative to the wild type, during ABA-mediated stomatal closure; and the *atpub18-2atpub19-3* double mutant was even more sensitive to ABA than each single mutant. In contrast, *AtPUB18*-overexpressing plants (*35S:AtPUB18* transgenic lines 1 and 7) showed hyposensitive stomatal responses toward ABA, which were comparable to those of *AtPUB19*-overexpressing plants (*35S:AtPUB19* lines 5 and 8; Fig. 3A). With 10 μM ABA, stomatal apertures of *35S:AtPUB18* and *35S:AtPUB19* were 0.149 $\pm$ 0.014 to 0.159 $\pm$ 0.014 and 0.158 $\pm$ 0.009 to 0.165 $\pm$ 0.010, respectively. Thus, the expression level of *AtPUB18* was inversely associated with ABA-regulated stomatal closure, suggesting that AtPUB18 participates in negative regulation of ABA-dependent stomatal movement.

AtPUB19 is negatively involved in drought stress responses (Liu et al., 2011); therefore, we next tested for AtPUB18 function in response to drought. Light-grown, 3-week-old, healthy wild-type, *atpub18-2*, *atpub19-3*, and *atpub18-2atpub19-3* plants were further grown for 12 d while withholding water and then irrigated for 3 d, at which time their survival rates were recorded, similar to Ryu et al. (2010). The results showed that 25.4% (18 of 71) of wild-type plants resumed their growth after drought stress, whereas survival rates of *atpub18-2*, *atpub19-3*, and *atpub18-2atpub19-3* plants were 66.2% (47 of 71), 70.0% (49 of 70), and 88.2% (60 of 68), respectively (Fig. 3B). The second alleles of *atpub18-1* and *atpub19-2* were similarly tolerant to drought stress (Supplemental Fig. S3). In contrast, both *AtPUB18*- and *AtPUB19*-overexpressing lines were more susceptible to mild dehydration conditions than wild-type plants. After 9 d of dehydration, the survival rate of wild-type plants was 58.7% (38 of 64; Fig. 3C); however, only 17.7% (11 of 62) to 29.5% (17 of 66) of *35S:AtPUB18* and 16.9% (11 of 66) to 22.2% (14 of 63) of *35S:AtPUB19* survived. Consistent with these results, rosette leaves detached from mutants lost water more slowly during dehydration, and overexpressing plants lost water more quickly, than wild-type leaves during dehydration (Fig. 3D). Finally, leaf chlorophyll contents were higher in mutant leaves than in overexpressing and wild-type leaves after drought (Fig. 3E). Overall, these results indicate that both AtPUB18 and AtPUB19 participate in negative regulation of ABA-dependent drought stress responses.

Stomatal Movement Analysis of *atpub18-2atpub19-3* and *atpub22atpub23* Double Knockout Mutant Plants in Response to H_2O_2 and Calcium

Our results (Figs. 1–3) and Cho et al. (2008) show that four Arabidopsis U-box E3 members, AtPUB18, AtPUB19, AtPUB22, and AtPUB23, participate in negative regulation of drought stress responses. Therefore, we investigated their functions in plant stomatal behavior in response to H_2O_2 and calcium, both of which are important regulators of ABA-dependent stomatal movement (Huang et al., 2009; Jammes et al., 2009; Kim et al., 2010). After H_2O_2 treatment, stomatal closure increased in *atpub18-2atpub19-3* mutants significantly more than in the wild type. In contrast, H_2O_2-mediated stomatal movement of *atpub22atpub23* mutants was almost identical to that of the wild type. With 100 μM H_2O_2, stomatal apertures of wild-type, *atpub18-2atpub19-3*, and *atpub22atpub23* plants were 0.153 $\pm$ 0.001, 0.121 $\pm$ 0.002, and 0.154 $\pm$ 0.004, respectively (Fig. 4A). With 200 μM H_2O_2, the stomatal aperture of *atpub18-2atpub19-3* leaves was 0.088 $\pm$ 0.002, which was approximately 1.5 times smaller than those of wild-type (0.128 $\pm$ 0.001) and *atpub22atpub23* (0.126 $\pm$ 0.002) plants. These results show that differences in stomatal apertures between *atpub18-2atpub19-3* and *atpub22atpub23* mutants are more prominent as H_2O_2 concentrations increase; therefore, knockout mutations of *AtPUB18* and *AtPUB19* enhance H_2O_2-mediated stomatal movement, whereas those of *AtPUB22* and

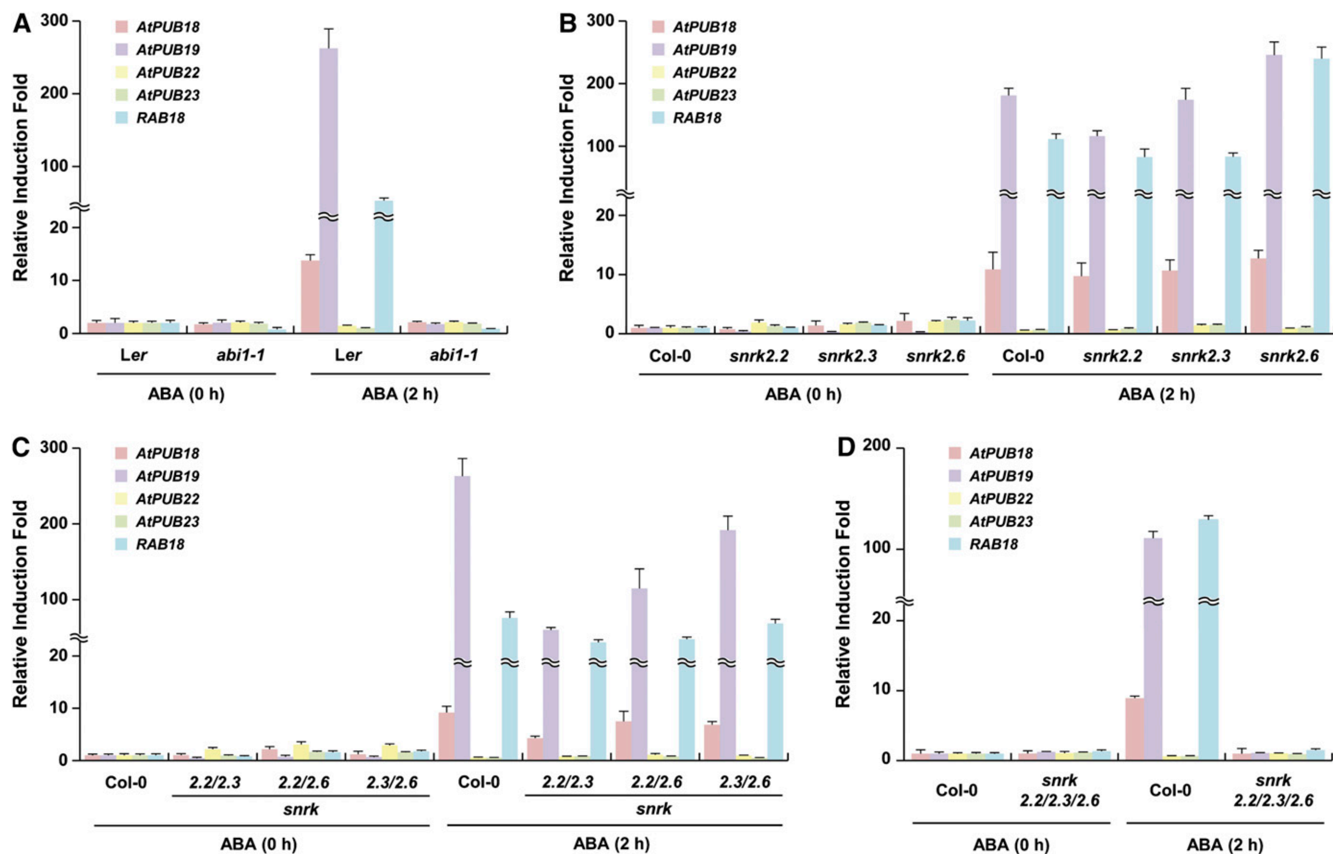

Figure 2. ABA induction profiles of *AtPUB18*, *AtPUB19*, *AtPUB22*, and *AtPUB23* in wild-type and *abi1-1* and *snrk2* mutant plants. Light-grown, 10-d-old wild-type and mutant seedlings were incubated with or without 100 μM ABA for 2 h. Total RNA was isolated and subjected to qRT-PCR. Glyceraldehyde-3-phosphate dehydrogenase C subunit was used as a reference gene for normalization. *RAB18* was a marker for the ABA-induced gene. Error bars represent SD from three independent experiments. A, ABA induction profiles of *AtPUB18*, *AtPUB19*, *AtPUB22*, and *AtPUB23* in wild-type (Landsberg *erecta* [L*er*]) and *abi1-1* mutant plants. B to D, ABA induction profiles of *AtPUB18*, *AtPUB19*, *AtPUB22*, and *AtPUB23* in wild-type (Columbia-0 [Col-0]) and various *snrk2* single (B), double (C), and triple (D) mutant plants. [See online article for color version of this figure.]

AtPUB23 result in wild-type stomatal behavior. Furthermore, *AtPUB18*- and *AtPUB19*-overexpressing plants displayed H_2O_2-insensitive stomatal responses relative to the wild type. In contrast, overexpression of *AtPUB22* and *AtPUB23* could not alter stomatal movement profiles in response to H_2O_2 treatments, indicating their ABA-independent functions (Fig. 4A). On the other hand, we found endogenous production of H_2O_2 in wild-type, mutant, and overexpressing plants to be highly similar (Supplemental Fig. S4), as determined by 3,3′-diaminobenzidine (DAB) staining (Cho et al., 2011; Zulfugarov et al., 2011).

Next, we examined calcium-dependent stomatal movements. Neither double mutations nor overexpression of *AtPUB18*/*AtPUB19* changed calcium-dependent stomatal behaviors relative to wild-type plants (Fig. 4B). Therefore, AtPUB18 and AtPUB19 likely acted upstream of calcium and downstream of H_2O_2 in the ABA-mediated drought responses. As expected, the *atpub22atpub23* mutant and *AtPUB22*/*AtPUB23*-overexpressing plants displayed wild-type stomatal movement in response to calcium (Fig. 4B).

Finally, stomatal movements of both *atpub18-2atpub19-3* and *atpub22atpub23* mutant leaves were similarly hypersensitive to mannitol treatment as compared with those of wild-type leaves. In contrast, all of the *AtPUB18*/*AtPUB19*- and *AtPUB22*/*AtPUB23*-overexpressing lines investigated were hyposensitive to mannitol, confirming their negative roles in drought stress responses (Fig. 4C).

The *atpub18-2atpub19-3atpub22atpub23* Quadruple Mutant Line Was Highly Tolerant to Drought Stress, But Their Stomatal Movements Were Similar to Those of *atpub18-2atpub19-3* Double Mutants

The *atpub18-2atpub19-3atpub22atpub23* quadruple mutant line was generated by crossing the *atpub22atpub23* and *atpub18-2atpub19-3* double mutant plants (Fig. 5A). The quadruple mutant was phenotypically normal, and their leaf stomatal density was indistinguishable from those of wild-type and double mutant plants (Fig. 5B). As shown in Figure 5C, stomatal movement profiles

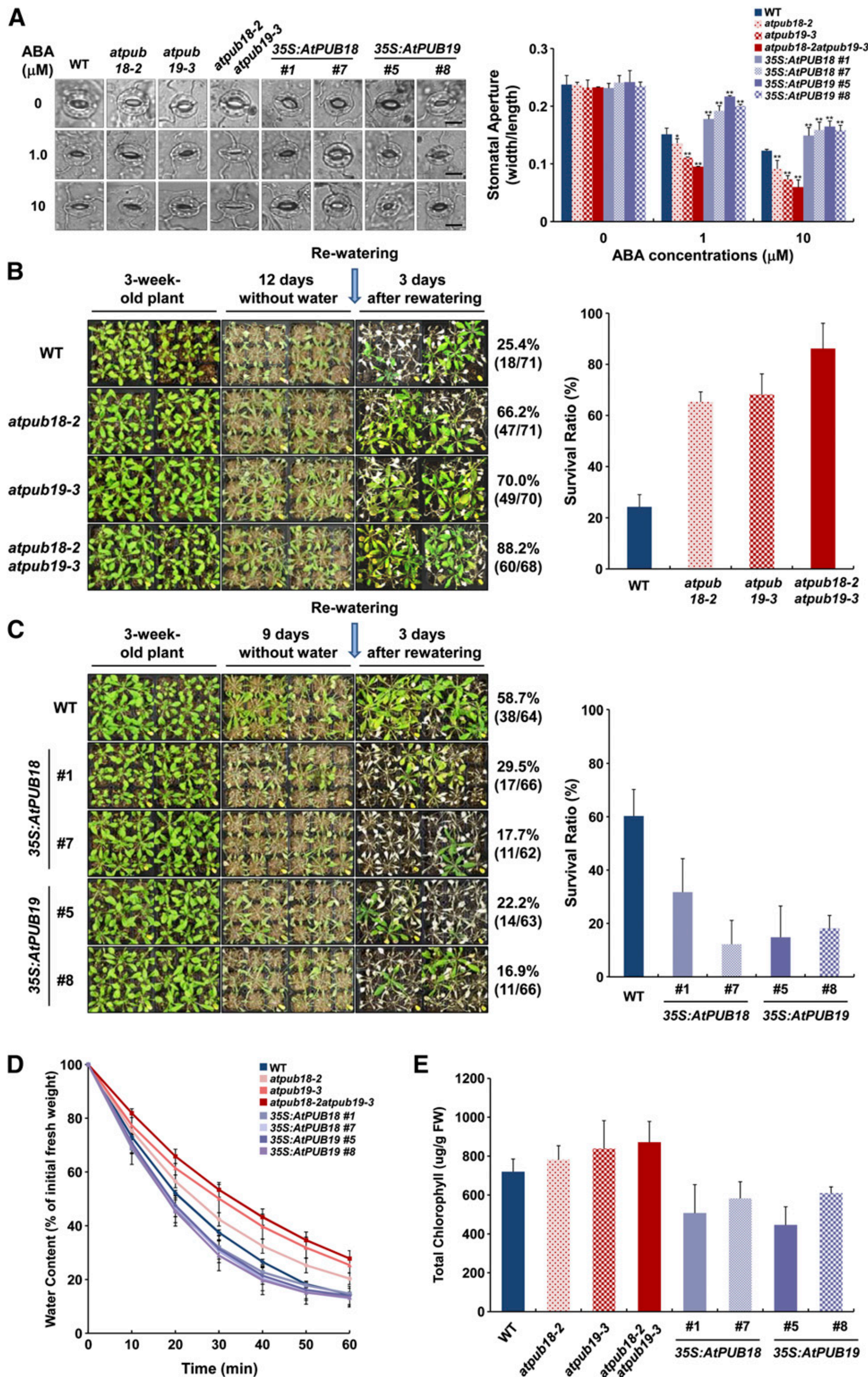

Figure 3. Phenotypic analyses of wild-type (WT), *atpub18-2*, *atpub19-3*, and *atpub18-2atpub19-3* knockout mutant, and *35S:AtPUB18*- and *35S:AtPUB19*-overexpressing transgenic plants in response to ABA and drought treatments. A, ABA-induced stomatal closure. Light-grown, fully expanded leaves were immersed in stomatal opening solution for 2 h and transferred into solution containing various concentrations (0, 1, and 10 μM) of ABA for 2 h. Stomata were photographed using bright-field microscopy. At least 30 stomatal apertures in epidermal peels were measured per replicate. Two replicates were performed for each experiment. Bars = 10 μm. Error bars represent SD ($n > 60$; $*P < 0.05$, $**P < 0.005$, Student's t test). B, Drought tolerance of wild-type, *atpub18-2*, *atpub19-3*, and *atpub18-2atpub19-3* plants. Wild-type and mutant plants were grown under normal growth conditions for 3 weeks and then exposed to drought stress by withholding water for 12 d. Surviving plants were counted 3 d after rewatering. C, Drought-sensitive phenotypes of *AtPUB18*- and *AtPUB19*-overexpressing plants, which, after 3 weeks, were subjected to water deficit conditions for 9 d. Survival rates were calculated after 3 d of irrigation. D, Water loss rates of detached mature leaves that were incubated at room temperature under dim light. Decreased fresh weights (FW) were recorded at specified time points. Water loss rates were estimated as the percentage of initial to final fresh weights. Data represent means ± SD ($n = 12$) from four replicates. E, Chlorophyll contents of whole seedlings dehydrated on dry filter paper for 45 min (drought stress). Chlorophyll contents were measured 2 d after rewatering as means ± SD of three replicates. [See online article for color version of this figure.]

of the *atpub18-2atpub19-3atpub22atpub23* quadruple mutant were highly similar to those of the *atpub18-2atpub19-3* double mutant in response to ABA, H_2O_2, and $CaCl_2$, which is consistent with the ABA-dependent functions of AtPUB18/AtPUB19 and the ABA-independent functions of AtPUB22/AtPUB23, respectively. In contrast, stomatal closure of the quadruple mutant line was even more sensitive than those of *atpub18-2atpub19-3* and *atpub22atpub23* double mutant plants in response to mannitol treatments (Fig. 5D). These results indicate that quadruple mutations of *AtPUB18*, *AtPUB19*, *AtPUB22*, and *AtPUB23* greatly increased tolerance toward drought stress. Consistently, the *atpub18-2atpub19-3atpub22atpub23* quadruple mutant was markedly more tolerant to

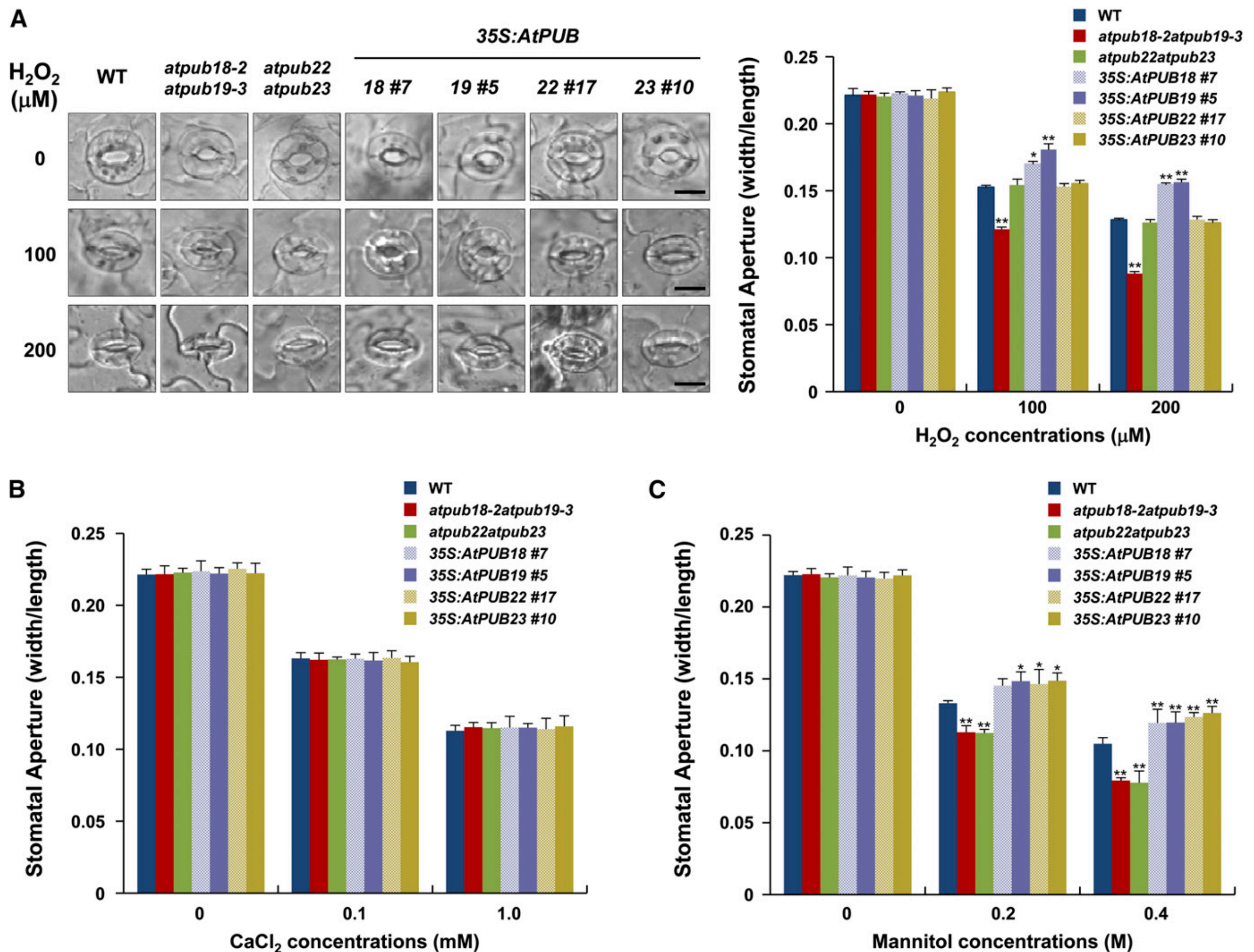

Figure 4. Stomatal closure of wild-type (WT), *atpub18-2atpub19-3* and *atpub22atpub23* mutant, and *35S*:*AtPUB18*-, *35S*:*AtPUB19*-, *35S*:*AtPUB22*-, and *35S*:*AtPUB23*-overexpressing transgenic plants in response to H_2O_2, $CaCl_2$, and mannitol. Mature leaves, pretreated with stomatal opening solution, were transferred to solutions containing various concentrations of H_2O_2 (A), $CaCl_2$ (B), or mannitol (C), each for 2 h. Epidermal peels were mounted on microscope slides, and stomata were observed with bright-field microscopy. Stomatal apertures were measured with three replicates. Error bars represent SD ($n > 87$; $*P < 0.05$, $**P < 0.005$, Student's t test). Bars = 10 μm. [See online article for color version of this figure.]

dehydration stress: after 12 d of drought treatment, 100% (32 of 32) of the *atpub18-2atpub19-3atpub22atpub23* line survived, whereas the survival rates of wild-type, *atpub18-2atpub19-3*, and *atpub22atpub23* plants were 25.0% (8 of 32), 84.4% (27 of 32), and 87.5% (28 of 32), respectively (Fig. 5E). Detached rosette leaves of the quadruple mutant lost their fresh weight more slowly than each double mutant in the dehydration process (Fig. 5F). These results are consistent with the results that the four U-box E3s participate in the drought stress responses as negative regulators.

Reciprocal Complementation Analysis of *AtPUB18/AtPUB19* and *AtPUB22/AtPUB23*

The functional relationships between AtPUB18/AtPUB19 and AtPUB22/AtPUB23 were further addressed by reciprocal complementation studies. *AtPUB18*/*AtPUB19* and *AtPUB22*/*AtPUB23* were overexpressed in *atpub22atpub23* and *atpub18-2atpub19-3* double mutant plants, respectively (Fig. 6A). Complementation transgenic plants (*35S*:*AtPUB18/19-atpub22atpub23* and *35S*:*AtPUB22/23-atpub18-2atpub19-3*) were used for the mannitol- and ABA-dependent stomatal movement tests. The results revealed that ectopic expression of *AtPUB18/AtPUB19* and *AtPUB22/AtPUB23* in *atpub22atpub23* and *atpub18-2atpub19-3*, respectively, partially inhibited mannitol-dependent stomatal closure, confirming their negative roles in drought stress responses (Fig. 6B). Overexpression of *AtPUB18/AtPUB19* in *atpub22atpub23* greatly hindered an ABA-mediated stomatal movement, whereas overexpression of *AtPUB22/AtPUB23* in *atpub18-2atpub19-3* failed to alter the ABA-mediated stomatal movement

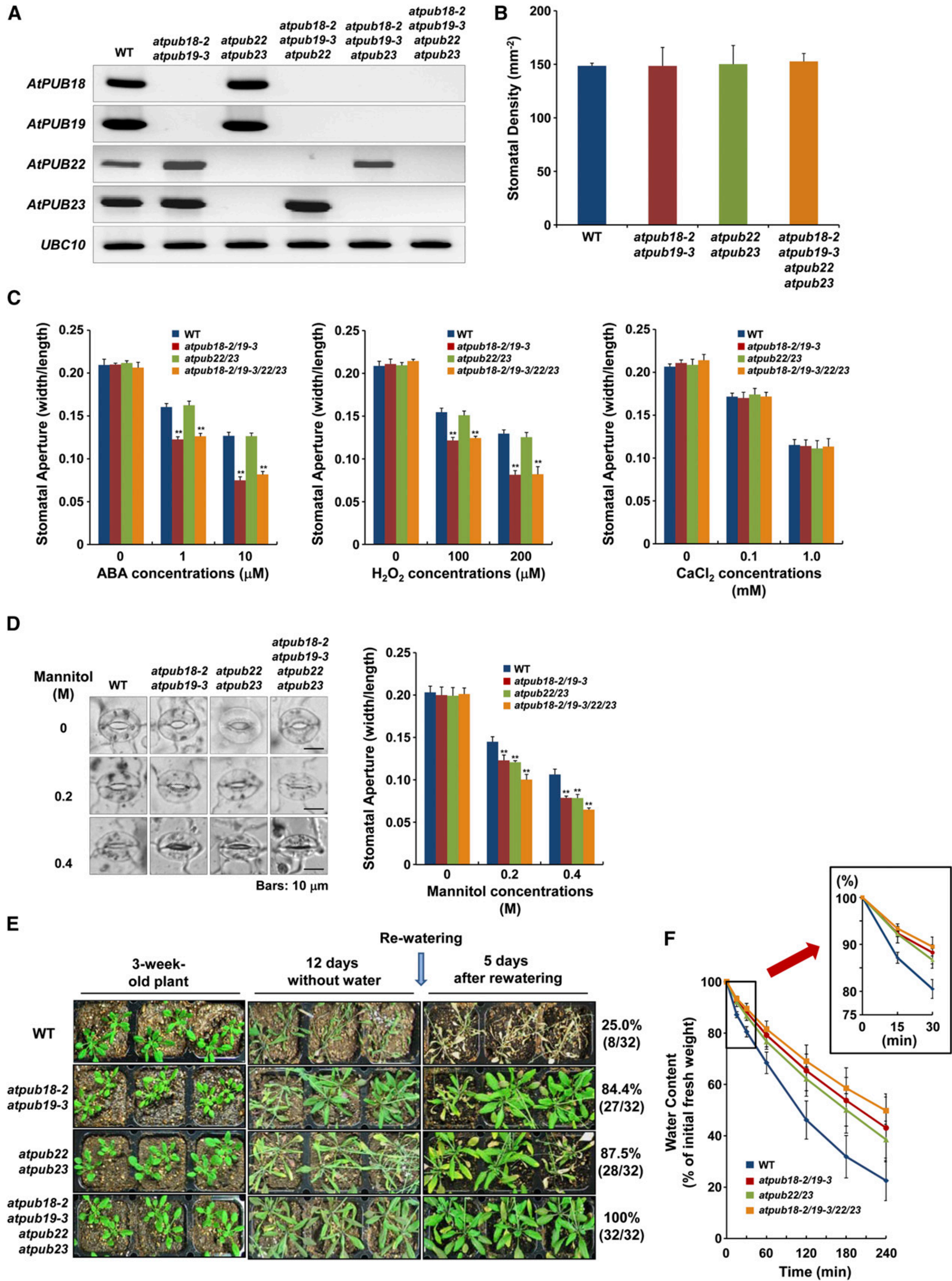

Figure 5. Molecular characterization and phenotype analyses of a series of double, triple, and quadruple homozygous mutant combinations containing *atpub18-2*, *atpub19-3*, *atpub22*, and *atpub23*. A, Generation of homozygous *atpub18-2atpub19-3atpub22*

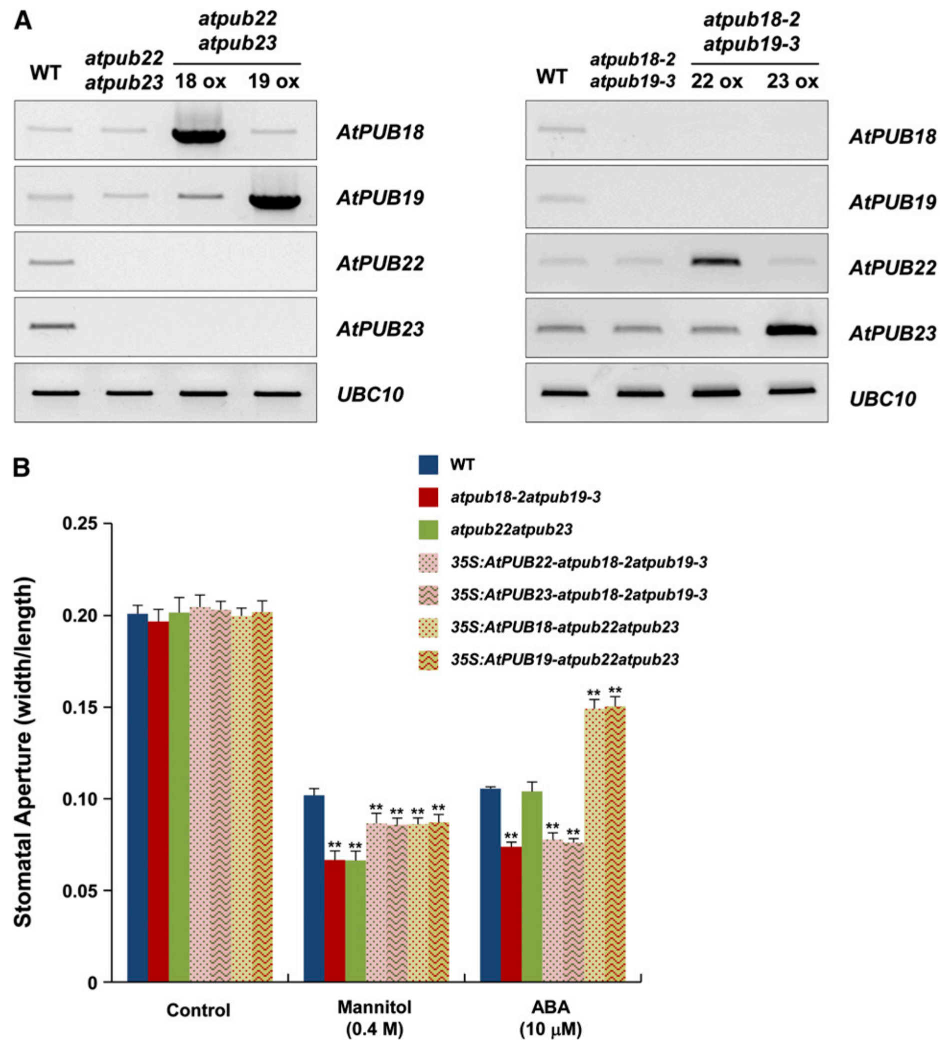

Figure 6. Identification and characterization of *35S:AtPUB18/AtPUB19-atpub22atpub23* and *35S:AtPUB22/AtPUB23-atpub18-2atpub19-3* complementation transgenic plants. A, Semi-quantitative RT-PCR analysis used to detect ectopic and reciprocal expression of *AtPUB18/AtPUB19* and *AtPUB22/AtPUB23* in *atpub22atpub23* and *atpub18-2atpub19-3*, respectively. *Ubiquitin Conjugating Enzyme10* (*UBC10*) was used as a loading control. B, ABA- and mannitol-dependent stomatal behaviors in T2 *35S:AtPUB18/AtPUB19-atpub22atpub23* and T1 *35S:AtPUB22/AtPUB23-atpub18-2atpub19-3* transgenic plants. Fully expanded, light-grown leaves were harvested, and their stomata were observed as described in Figure 3A. Three replicates were performed for each experiment. Error bars represent SD (n = 90; $^{**}P < 0.005$, Student's t test). WT, Wild type. [See online article for color version of this figure.]

of *atpub18-2atpub19-3* mutant lines (Fig. 6B). On the other hand, *AtPUB19* effectively complemented the mutant phenotypes of the *atpub18-2* line (Supplemental Fig. S5). Collectively, these results suggest that the U-box E3 Ub ligase AtPUB18 has a combined function with AtPUB19, but is independent from AtPUB22 and AtPUB23, in negative regulation of ABA-mediated drought stress responses.

DISCUSSION

AtPUB19 is a negative regulator of ABA-mediated seed germination and drought response (Bergler and Hoth, 2011; Liu et al., 2011). Although AtPUB18 was recently reported to participate negatively in the ABA- and salt-inhibited seed germination process (Bergler and Hoth, 2011), its role in drought responses was unknown. In this report, *AtPUB18* was identified as a

Figure 5. (*Continued.*)

and *atpub18-2atpub19-3atpub23* triple and *atpub18-2atpub19-3atpub22atpub23* quadruple knockout mutant plants. Semi-quantitative RT-PCR analyses were performed to confirm the absence of transcripts. B, Stomatal density (average number of stomata per mm^2) of wild-type (WT) and various mutant plants. Abaxial epidermal peels of fully expanded rosette leaves were harvested and observed with a light microscope. This experiment was repeated three times. Error bars represent SD. C, Stomatal movements of the *atpub18-2atpub19-3atpub22atpub23* quadruple mutant in response to ABA, H_2O_2, and $CaCl_2$. Error bars represent SD ($n > 87$; $^{**}P < 0.005$, Student's t test). D, Stomatal movements of the *atpub18-2atpub19-3atpub22atpub23* mutant in response to osmotic stress imposed by mannitol. Error bars represent SD ($n > 87$; $^{**}P < 0.005$, Student's t test). E, Drought-tolerant phenotypes of *atpub18-2atpub19-3*, *atpub22atpub23*, and *atpub18-2atpub19-3atpub22atpub23* plants. Wild-type and mutant plants were grown for 3 weeks with normal irrigation, then subjected to dehydration conditions by interrupting irrigation for 12 d, and supplied with sufficient water for 5 d, after which survival rates were calculated. F, Water loss rates of detached mature leaves, as determined by change in fresh weights (described in Fig. 3D). Data represent means ± SD (n = 5) from three replicates. [See online article for color version of this figure.]

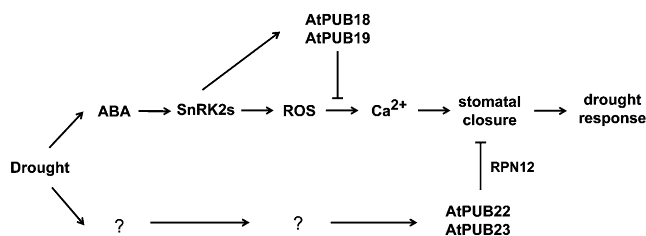

Figure 7. Negative regulation of AtPUB18, AtPUB19, AtPUB22, and AtPUB23 during drought stress responses. Modes of action of the four U-box E3s were divided into two different pathways: AtPUB18 and AtPUB19 were ABA dependent, and AtPUB22 and AtPUB23 were ABA independent. AtPUB18 and AtPUB19 exerted their negative effects on the ABA signaling pathway downstream of H_2O_2 and upstream of calcium. The cellular mechanism by which AtPUB22 and AtPUB23 regulate drought responses is not known. RPN12a, a non-ATPase subunit of the 19S regulatory particle in the 26S proteasome, is one of the targets of AtPUB22 and AtPUB23, while the possible substrates of AtPUB18 and AtPUB19 are currently unknown. ROS, Reactive oxygen species.

drought- and ABA-induced gene and its induction was SnRK dependent (Figs. 1 and 2). Our loss-of-function and overexpression tests revealed that AtPUB18 was a negative regulator in ABA-mediated stomatal closure and water stress responses. Moreover, the *atpub18-2atpub19-3* double mutant line displayed even greater sensitivity to ABA and more enhanced drought tolerance than did each single mutant plant (Fig. 3). Therefore, AtPUB18 and AtPUB19 have agonistic functions. Interestingly, the stomatal response of the *atpub18-2atpub19-3* mutant was hypersensitive to H_2O_2, while its response to calcium was comparable to that of wild-type leaves (Fig. 4), suggesting that AtPUB18 and AtPUB19 exert negative effects on the ABA signaling pathway downstream of H_2O_2 and upstream of calcium (Fig. 7).

In contrast to clear phenotypic properties in postgermination stages, ABA inhibition of seed germination is relatively weak in *atpub18-1atpub19-2* (Bergler and Hoth, 2011). In addition, ABA inhibition of early root growth was also very weak in the mutant lines (Liu et al., 2011; Supplemental Fig. S6); therefore, negative regulation of AtPUB18 and AtPUB19 is likely to mainly occur in postgermination stages. This raises the possibility that other U-box E3 negative regulators that modulate ABA-dependent seed germination processes may exist. On the other hand, a basic leucine zipper (bZIP) ABI5 transcription factor is mainly responsible for the expression of the genes for inhibition of germination and postgermination arrest (Lopez-Molina et al., 2001), whereas other bZIP transcription factors (ABFs/AREBs), which are more highly expressed in mature plants, are likely to function in mature plants (Uno et al., 2000). Therefore, it would be interesting to examine whether various bZIP transcription factors, including ABI5, differentially (negatively or positively) affect the ABA induction of *AtPUB18* and *AtPUB19* and/or their E3 ligase enzyme activities.

While yeast and humans (*Homo sapiens*) contain two and eight U-box E3 Ub ligases, respectively, higher plants have more: Arabidopsis has 64 and rice has 77 (Yee and Goring, 2009; Lyzenga and Stone, 2012). Therefore, we speculate that U-box E3s have plant-specific roles and that their cellular functions are coordinated and interconnected as effective defense webs and for concomitant metabolic reprogramming. This is in agreement with other findings that plants have multicombinatorial defensive programs that work coordinately in response to abiotic stresses (Ahuja et al., 2010; Tardieu et al., 2011). In this context, we hypothesize that U-box E3 isoforms also work in a combinatorial pattern to efficiently cope with dehydration stress.

AtPUB22 and AtPUB23 are also negative regulators of drought responses (Cho et al., 2008); therefore, we wanted to elucidate the functional relationships among the four U-box E3s with regard to ABA-dependent drought stress responses. Stomatal behaviors of the *atpub18-2atpub19-3atpub22atpub23* quadruple mutant line were highly similar to those of *atpub18-2atpub19-3* double mutant plants in response to ABA, H_2O_2, and calcium (Fig. 5). In contrast, the quadruple mutant exhibited a hypersensitive stomatal response to mannitol and markedly enhanced tolerance to severe drought stress as compared with each *atpub18-2atpub19-3* and *atpub22atpub23* double mutant plant progeny (Fig. 5). These results strongly suggest that the modes of action of the four U-box E3 negative regulators are divided into two different pathways: AtPUB18 and AtPUB19 are ABA dependent, and AtPUB22 and AtPUB23 are ABA independent. This assumption was further supported by the results of reciprocal complementation studies. Overexpression of *AtPUB18* and *AtPUB19* in *atpub22atpub23* progeny effectively hindered ABA-dependent stomatal closure (Fig. 6). In contrast, overexpression of *AtPUB22* and *AtPUB23* in the *atpub18-2atpub19-3* line failed to inhibit ABA-enhanced stomatal closure. Overall, it is concluded that action mechanisms of AtPUB18 and AtPUB19 are overlapped but separated with those of AtPUB22 and AtPUB23 in ABA-mediated drought stress responses (Fig. 7). These two different drought-responsive pathways, however, are somehow interconnected by an as yet unknown mechanism, since reciprocal complementation partially restored the mannitol-responsive stomatal profiles of *atpub18-2atpub19-3* and *atpub22atpub23* double mutants (Fig. 6). Mudgil et al. (2004) showed that AtPUB22, AtPUB23, AtPUB18, and AtPUB19 are all related based on the presence of the C-terminal ARM domains in addition to the U-box but that AtPUB18 and AtPUB19 are larger proteins with the additional N-terminal UND domain, indicating different structural relationships between AtPUB18/AtPUB19 versus AtPUB22/AtPUB23 proteins (Supplemental Fig. S1). This is consistent with the possible differences between AtPUB18/AtPUB19 and AtPUB22/AtPUB23 with respect to their functions in drought stress responses.

RPN12a, a non-ATPase subunit of the 19S regulatory particle in the 26S proteasome, is one of the targets

of AtPUB22 and AtPUB23, and it was proposed that AtPUB22 and AtPUB23 control a drought signaling pathway by ubiquitinating cytosolic RPN12a (Cho et al., 2008). Ubiquitination and subsequent conformational changes in the 26S proteasome complex may signal negative regulation of drought responses. However, a more detailed mechanism, by which AtPUB22 and AtPUB23 regulate drought responses, remains to be elucidated (Fig. 7). The possible substrates of AtPUB18 and AtPUB19 are currently unknown. Therefore, identification of their target proteins are required to understand how these four U-box E3 negative regulators coordinately mediate a drought stress response in Arabidopsis. Several RING E3 Ub ligases (e.g. AtAIRP1, AtAIRP2, AtRDUF1, and AtRDUF2) positively regulate ABA-dependent drought stress responses (Ryu et al., 2010; Cho et al., 2011; Kim et al., 2012). Thus, functional balances between positive and negative regulators help plants fine-tune their cellular response to dehydration stress, one of the most common environmental stresses of crops. In conclusion, our results suggest that the ubiquitination pathway is critically involved, as a positive and a negative factor, in either ABA-dependent or ABA-independent defensive mechanisms against drought stress in Arabidopsis.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Stress Treatments

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 was used as the wild type in this study, except for *abi1-1*, which is in the Landsberg *erecta* background. The transferred DNA insertion mutants *atpub18-1* (SAIL_634_G01; Bergler and Hoth, 2011), *atpub18-2* (SALK_001831), *atpub19-1* (SALK_035871), *atpub19-2* (SALK_152677; Liu et al., 2011), *atpub19-3* (SALK_058791), *abi1-1* (CS22), *snrk2.2* (GABI-Kat 087G04), *snrk2.3* (SALK_107315), and *snrk2.6* (SALK_008608) were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org). Multiple knockout mutant plants were generated from genetic crosses (Cho et al., 2008). Full-length complementary DNAs (cDNAs) of *AtPUB18* and *AtPUB19* were cloned downstream of the *35S* promoter in the binary vector pBI121 (Arabidopsis Biological Resource Center stock no. CD3-338). The *AtPUB22* and *AtPUB23* genes were cloned and inserted into a modified pENTR vector (Invitrogen). *AtPUB22* and *AtPUB23* clones were transferred to pEarlyGate100 destination vectors with a Gateway cloning kit (Invitrogen) and transformed into the Arabidopsis genome using the floral dip method (Ryu et al., 2010). Methods for seed sterilization, growth conditions, and various stress treatments are described by Ryu et al. (2010). All primers used in reverse transcription (RT)-PCR, qRT-PCR, genotyping PCR, and plasmid construction are listed in Supplemental Tables S1 and S2.

RT-PCR and Real-Time qRT-PCR Analyses

Total RNA from ABA- and abiotic stress-treated tissues was extracted using the Easy-Spin IIp plant RNA extraction kit according to the manufacturer's instructions (Intron Biotechnology). Two micrograms of DNase I-treated total RNA was used for cDNA synthesis using the TOPscript cDNA Synthesis kit (Enzynomics). RT-PCR and real-time qRT-PCR were carried out as described by Cho et al. (2011). Real-time qRT-PCR analyses were performed using an IQ5 light cycler (Bio-Rad). The SYBR Premix Ex Taq II (Takara) was used for the reactions.

Histochemical GUS Assay

For the GUS analysis, mock-, drought-, and ABA-treated 5-d-old seedlings or 2-week-old plants were harvested and fixed with 90% prechilled acetone. After washing with rinsing solution (GUS staining solution without 5-bromo-4-chloro-3-indolyl-β-glucuronic acid A), GUS staining and destaining were performed as described by Cho et al. (2011).

Stomatal Aperture Measurement

Light-grown mature rosette leaves from 4- to 6-week-old plants were immersed in stomatal opening solution (30 mM KCl, 100 μM $CaCl_2$, and 10 mM MES, pH 6.15) for 2 h at 22°C (Kwak et al., 2001). Treated leaves were transferred to a stomatal opening solution containing various concentrations of mannitol (0, 0.2, and 0.4 M), ABA (0, 1, and 10 μM), H_2O_2 (0, 100, and 200 μM), or $CaCl_2$ (0, 0.1, and 1.0 mM). After incubation for 2 h in white light, 30 stomatal apertures in each epidermal peel were measured and analyzed as described by Ryu et al. (2010). Excel data files of stomatal apertures that were obtained in the experiments are provided in Supplemental Table S3. To measure endogenous leaf H_2O_2 level, light-grown, 2-week-old seedlings were treated with or without 100 mM ABA for 2 h and transferred to solution containing 100 μg mL^{-1} DAB as described previously (Cho et al., 2011; Zulfugarov et al., 2011). Chlorophylls of DAB-stained seedlings were removed by boiling in 95% (v/v) ethanol. Reactive oxygen species levels were visualized as a dark brown color.

Drought Phenotype Analysis

Light-grown, 3-week-old wild-type and mutant plants were exposed to water deficit conditions, 9 to 12 d, by withholding irrigation. Three to 5 d after rewatering, survival ratios were calculated as described by Kim et al. (2012). To investigate the water loss rate, aerial parts of 2-week-old seedlings grown on Murashige and Skoog agar plates, or fully expanded rosette leaves detached from soil-grown plants, were placed on glass slides at room temperature. Water loss rate was calculated as the percentage of final to initial fresh weight (Yamaguchi et al., 2007).

Chlorophyll Content Measurement

Light-grown, 2-week-old whole seedlings were collected from Murashige and Skoog agar plates and placed on dry filter papers for 45 min. After dehydration treatment, seedlings were incubated in water for 2 d under white light conditions (Yamaguchi et al., 2007). To extract pigment, each sample was ground in liquid nitrogen and subjected to 80% acetone. Spectrometric analysis of chlorophyll was performed using a DU 800 spectrophotometer (Beckman-Coulter). Chlorophyll contents were determined as described by Fujita et al. (2012).

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *AtPUB18* (At1g10560), *AtPUB19* (At1g60190), *AtPUB22* (At3g52450), *AtPUB23* (At2g35930), *AtPUB8* (At4g21350), *AtPUB10* (At1g71020), *AtPUB12* (At2g28830), *AtPUB13* (At3g46510), *AtPUB14* (At3g54850), *AtPUB15* (At5g42340), *AtPUB16* (At5g01830), *AtPUB17* (At1g29340), *AtPUB20* (At1g66160), *AtPUB21* (At5g37490), *AtPUB24* (At3g11840), *AtPUB25* (At3g19380), *AtPUB26* (At1g49780), *AtPUB27* (At5g64660), *AtPUB28* (At5g09800), *AtPUB29* (At3g18710), and *AtPUB31* (At5g65920).

Supplemental Data

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

Supplemental Figure S1. Sequence analysis of AtPUB18 and AtPUB19.

Supplemental Figure S2. Characterization of *atpub18*, *atpub19*, and *atpub18atpub19* knockout mutant and *35S*:*AtPUB18* and *35S*:*AtPUB19* overexpressing transgenic plants.

Supplemental Figure S3. Drought-tolerant phenotypes of *atpub18-1* and *atpub19-1* second allele mutant plants.

Supplemental Figure S4. ROS accumulation in wild-type, *atpub18*, *atpub19*, and *atpub18atpub19* knockout mutant, and *35S*:*AtPUB18* and *35S*:*AtPUB19* overexpressing transgenic plants in response to ABA.

Supplemental Figure S5. Characterization of *35S*:*AtPUB19-atpub18* complementation transgenic plants and their stomatal movements in response to mannitol and ABA treatments.

Supplemental Figure S6. ABA-induced root growth inhibition of wild-type, *atpub18*, *atpub19*, and *atpub18atpub19* knockout mutant, and *35S:AtPUB18* and *35S:AtPUB19* overexpressing transgenic plants.

Supplemental Table S1. Primer sequences used for RT-PCR and qRT-PCR.

Supplemental Table S2. Primer sequences used for genotyping PCR and constructions.

Supplemental Table S3. Excel data files of stomatal apertures that were obtained in experiments.

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