Drought and Salt Stress Tolerance of an Arabidopsis Glutathione *S*-Transferase U17 Knockout Mutant Are Attributed to the Combined Effect of Glutathione and Abscisic Acid[1][C][W][OA]

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Although glutathione *S*-transferases (GSTs) are thought to play major roles in oxidative stress metabolism, little is known about the regulatory functions of GSTs. We have reported that Arabidopsis (*Arabidopsis thaliana*) *GLUTATHIONE S-TRANSFERASE U17* (*AtGSTU17*; At1g10370) participates in light signaling and might modulate various aspects of development by affecting glutathione (GSH) pools via a coordinated regulation with phytochrome A. Here, we provide further evidence to support a negative role of *AtGSTU17* in drought and salt stress tolerance. When *AtGSTU17* was mutated, plants were more tolerant to drought and salt stresses compared with wild-type plants. In addition, *atgstu17* accumulated higher levels of GSH and abscisic acid (ABA) and exhibited hyposensitivity to ABA during seed germination, smaller stomatal apertures, a lower transpiration rate, better development of primary and lateral root systems, and longer vegetative growth. To explore how *atgstu17* accumulated higher ABA content, we grew wild-type plants in the solution containing GSH and found that they accumulated ABA to a higher extent than plants grown in the absence of GSH, and they also exhibited the *atgstu17* phenotypes. Wild-type plants treated with GSH also demonstrated more tolerance to drought and salt stresses. Furthermore, the effect of GSH on root patterning and drought tolerance was confirmed by growing the *atgstu17* in solution containing L-buthionine-(*S*,*R*)-sulfoximine, a specific inhibitor of GSH biosynthesis. In conclusion, the *atgstu17* phenotype can be explained by the combined effect of GSH and ABA. We propose a role of AtGSTU17 in adaptive responses to drought and salt stresses by functioning as a negative component of stress-mediated signal transduction pathways.

In both animals and plants, glutathione *S*-transferases (GSTs; EC 2.5.1.18) are induced by diverse environmental stimuli, with increased GST levels used to maintain cell redox homeostasis and protect organisms against oxidative stress. GSTs were proposed to afford protection under various stress conditions by detoxifying endogenous plant toxins that accumulate as a consequence of increased oxidative stress (Marrs, 1996). In plants, *GST* expression is induced by phytohormones, such as salicylic acid, ethylene, cytokinin, auxin, abscisic acid (ABA; Marrs, 1996), methyl jasmonate (Moons, 2003), and brassinosteroid (Deng et al., 2007). It is obvious that GSTs are also stimulated by various stresses, such as pathogen infection, herbicide application, hydrogen peroxide, ozone, 2,4-dichlorophenoxyacetic acid, heavy metals, dehydration, senescence, and wounding (Marrs, 1996), hypoxic stress and salt (Moons, 2003), as well as different qualities of light (Loyall et al., 2000; Tepperman et al., 2001; Chen et al., 2007). Glutathione (GSH) is an essential thiol antioxidant as well as a scavenger of reactive electrophilic compounds, functioning with GSTs to detoxify a range of herbicides (Marrs and Walbot, 1997; Edwards et al., 2000) by tagging electrophilic compounds for removal during oxidative stress. Theoretically, GST activities catalyze the conjugation of electrophilic compounds to GSH and target them for storage in vacuoles or apoplast (Marrs, 1996). Some plant GSTs play direct roles in reducing oxidative damage (Cummins et al., 1999; Roxas et al., 2000) and enhancing tolerance to stresses (Edwards and Dixon, 2005).

The GST family of Arabidopsis (*Arabidopsis thaliana*) contains 54 members belonging to seven distinct classes (Dixon et al., 2009). The plant-specific φ (GSTF) and τ (GSTU) classes are the largest, with 13 and 28 members, respectively. By applying a range of stress stimuli, with a focus on early changes in gene expres-

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sion, Sappl et al. (2009) indicated that individual GST genes have highly specific induction patterns, and they linked individual GSTs to particular stress stimuli. However, an analysis of metabolite pools of lines in which GSTF genes were silenced showed involvement in protecting plants against oxidation of the primary metabolism. There appears to be a high degree of functional redundancy within the GST family for protecting against oxidative stresses. So far, no known specific functions have been identified in plant development for most of the members in the large GST gene family, indicating a challenge when studying the functions of individual genes in response to a stress.

In addition to this well-documented catalytic function, GSTs also function as noncatalytic carrier proteins (Sheehan et al., 2001). Only limited reports revealed the endogenous function of GSTs. For example, At5g17200 (AtGSTF12; also referred to as TRANSPARENT TESTA19) is required for the vacuolar uptake of anthocyanins (Kitamura et al., 2004). Also, GSTs can serve as signaling molecules and are involved in regulating chalcone synthase following exposure to UV light (Loyall et al., 2000), possibly due to redox-modulated mechanisms. Recently, At1g78730 (AtGSTU20) was demonstrated to physically interact with Far-Red Insensitive219 (FIN219) in response to light and to play a crucial signaling role in cell elongation and plant development (Chen et al., 2007).

Very little information is available on the involvement of GSTs in response to drought and salt stresses, although changes in the GSH pool and glutathione reductase and glutathione peroxidase activities in dehydrated plants were described (Loggini et al., 1999; Gallé et al., 2009). Tobacco (*Nicotiana tabacum*) seedlings but not mature plants overexpressing a tobacco τ GST gene were more tolerant to low- and high-temperature stresses and salt stress (Roxas et al., 2000). Tobacco plants overexpressing a τ class of the GST gene, *GsGST* from *Glycine soja*, exhibited enhanced dehydration tolerance (Ji et al., 2010). However, no further study was provided to support the hypothesis of the regulatory role of this gene in drought-exposed plants.

AtGSTU17 came to our attention because it was regulated by FIN219/JAR1 and rapidly increased with far-red light irradiation but was inhibited by a *phytochrome A* (*phyA*) mutation (Tepperman et al., 2001; Jiang et al., 2010). The full-length cDNA of *AtGSTU17* encodes a 227-amino acid protein. The recombinant proteins generated from an *Escherichia coli* expression system showed enzymatic activities to the substrates GSH and 1-chloro-2,4-dinitrobenzene, which indicates that AtGSTU17 has high affinity to both substrates (Dixon et al., 2009; Jiang et al., 2010). Moreover, we found that AtGSTU17 participates in phyA-mediated photomorphogenesis and integrates with various phytohormones to modulate GSH homeostasis in regulating Arabidopsis development (Jiang et al., 2010). Surprisingly, *atgstu17* plants exhibited robust root system development, especially under stress conditions and lack of sensitivity to ABA-mediated inhibition of lateral root elongation. Our data here clearly elucidate AtGSTU17 functions in an undiscovered negative role of adaptation to drought and salt stresses, and the underlying mechanism of the *atgstu17* phenotypes can be explained by the synergic action of GSH and ABA, which accumulated to much higher levels than in wild-type plants. Therefore, GSH in addition to ABA in the protection of plants under drought and salt stress are important for the survival and growth of eukaryotic organisms.

RESULTS

AtGSTU17 Affects Arabidopsis Development

Two independent T-DNA insertions of *AtGSTU17*, *atgstu17-1* (SALK_139615) and *atgstu17-2* (SALK_025503), are located in the second exon and first intron of *AtGSTU17* (Supplemental Fig. S1A). Reverse transcription (RT)-PCR analyses indicated that *atgstu17-1* and *atgstu17-2* were null mutants (Supplemental Fig. S1B). The *atgstu17* mutants (*atgstu17-1* and *atgstu17-2*) exhibited a smooth elliptical leaf shape and a profusely growing root system (Fig. 1A; Supplemental Fig. S1) as well as delayed flowering time, which produced a leaf number of 27 in contrast to 17 for the wild-type (ecotype Columbia [Col-0]) plants (Fig. 1B). In contrast, the ectopic expression of *AtGSTU17* under the control of the cauliflower mosaic virus 35S promoter in the wild type, *GSTUOE-1*, and *GSTUOE-2*, confirmed by RNA-gel blotting (Supplemental Fig. S1C), resulted in an uneven leaf surface and root system similar to wild-type plants but with an earlier flowering time and a leaf number of only 12 (Fig. 1, A and B). To confirm that the mutated *AtGSTU17* was the cause of these phenotypes, the *atgstu17s* were transformed with a cDNA of *AtGSTU17* driven by the 35S promoter. These 35S:*AtGSTU17*/*atgstu17* transgenic plants showed that the leaf morphology, bolting time, and sensitivity to drought stress were similar to those of wild-type plants (Supplemental Fig. S2), verifying the role of AtGSTU17 in plant growth and development.

Tolerance to Drought and Salt Stresses of the *atgstu17* Mutants

Since *AtGSTU17* was induced by exogenous ABA (Jiang et al., 2010), we investigated the response to prolonged periods of drought and found that *atgstu17* plants (*atgstu17-1* and *atgstu17-2*) had improved resistance to water deficits (Fig. 1, C and D). Nearly all of the *atgstu17* plants had recovered and begun to grow again, while only 40% of the wild-type plants could resume growth. *GSTU17OE* plants (*GSTUOE-1* and *GSTUOE-2*) did not exhibit any difference in water deficit compared with wild-type plants (Fig. 1E). Enhanced salt stress tolerance of the *atgstu17* plants was also observed (Fig. 1, F and G). However, *GSTU17OE*

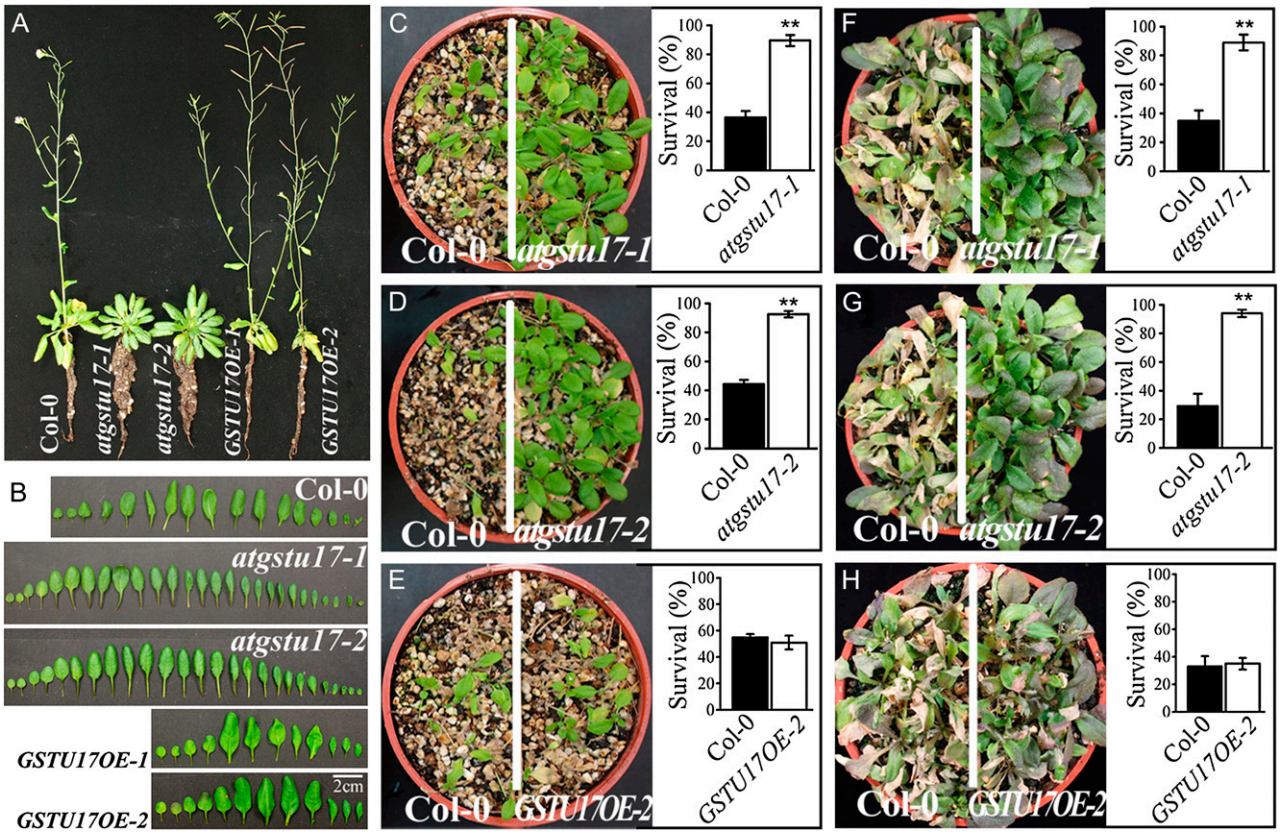

Figure 1. Phenotypes and tolerance to drought and salt stresses of *AtGSTU17* mutant lines. A, Eight-week-old plants growing under 12-h-light/12-h-dark conditions of the wild type, two knockout mutants, *atgstu17-1* and *atgstu17-2*, and two overexpressing lines, *GSTU17OE-1* and *GSTU17OE-2*. B, Leaf sizes and numbers beginning from the oldest one on the right side for different lines of 6-week-old plants under 12-h-light/12-h-dark conditions. C and D, Plants (wild type and *atgstu17s*) were grown in a single pot under 16-h-light/8-h-dark conditions. Watering of 3-week-old plants was withdrawn for 10 to 12 d and then resumed. The photographs and figure showing the differences in the reactions of the plants to the short-term drought were taken after 5 d of rewatering. E, Same as C but for the wild type and *GSTU17OE-2*. F and G, Three-week-old plants (wild type and *atgstu17s*) were watered for 12 d at 4-d intervals with increasing concentrations of NaCl of 100, 200, and 300 mM. The photographs and figure were taken 18 d after the salt treatments. Only the plant having the inflorescence base remaining green was considered a survivor. Only *atgstu17-2* is presented. H, Same as E but for the wild type and *GSTU17OE-2*. In E to H, survival rates (percentage) were calculated from the numbers of surviving plants per total plants tested. Data are presented as means ± SD. Five independent experiments were performed for C to F with similar results. **Significantly different from the Col-0 ($P < 0.01$) by Student's *t* test.

plants did not exhibit a difference in salt tolerance compared with wild-type plants (Fig. 1H).

The *atgstu17* Mutants Exhibit a Reduced Water Loss and Smaller Stomatal Aperture

As a reduction in stomatal pore aperture size mediated by ABA is a critical aspect of the response of plants to drought stress, we speculated that the enhanced drought resistance of the *atgstu17* plants might be correlated with an altered response to water scarcity. Indeed, the rate of water loss from *atgstu17s* was lower than that from wild-type plants, as measured by the fresh weight loss of detached leaves (Fig. 2A).

We found that stomata of *atgstu17* plants had a constitutively reduced aperture (Fig. 2, B and C). ABA treatment reduced the stomatal aperture to a similar extent in mutant and wild-type plants when the smaller initial aperture of *atgstu17* was taken into account. This constitutively smaller aperture size of stomata may explain the observed lower water loss rate of detached leaves by the *atgstu17* plants (Fig. 2A). The stomatal aperture response to ABA treatment in *GSTU17OE* was similar to that of wild-type plants (data not shown).

The *atgstu17* Mutants Show Altered Physiological Responses Regulated by ABA

Since the expression of *AtGSTU17* was induced by ABA, we speculated that the germination of mutant seeds in response to ABA might be altered. At a concentration of 2 μM ABA, the germination rate of

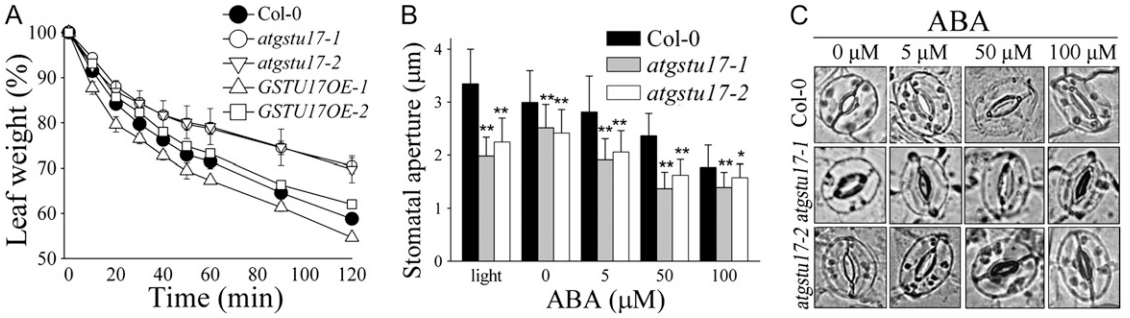

Figure 2. Effects of the *AtGSTU17* mutation on water loss rates and ABA-mediated stomatal closure. A, Progressive water loss from detached leaves as a function of time in 5-week-old wild-type, *atgstu17*, and *AtGSTU17* plants. Detached leaves were placed on weighing dishes and allowed to slowly dry on a laboratory bench (at 25°C and 60% relative humidity). Weights of the samples were recorded at regular intervals. Error bars represent the SD. Data are presented as mean water loss percentage ± SD. Three independent experiments were performed with the same trends. B, Effect of the *AtGSTU17* mutation on ABA inhibition of light-induced stomatal opening. Stomata were preopened in the light for 2.5 h and then incubated in the indicated concentrations of ABA for 2.5 h in the light. Stomatal apertures were measured on epidermal peels. Values are means ± SD ($n > 60$). * Significantly different from the wild type ($P < 0.05$), by Student's *t* test. **$P < 0.01$. These blind experiments were repeated at least three times. C, Micrographs representing the dynamics of ABA-mediated stomatal closure in wild-type and *atgstu17* plants.

atgstu17 mutants was 50% compared with no germination in wild-type seeds (Fig. 3A). In contrast, germination of the *GSTU17OE* seeds was similar to that of wild-type plants. This indicates that the germination of *atgstu17* seeds was less sensitive to ABA.

Root development is also sensitive to ABA (Sharp and LeNoble, 2002; De Smet et al., 2006), and this was further studied in *atgstu17* mutants. Under white light and unstressed conditions for 2 weeks, root lengths among wild-type, *atgstu17*, and *GSTU17OE* plants were similar (Fig. 3B). However, the *atgstu17* mutant was significantly less sensitive to ABA suppression of primary root and lateral root elongation (Fig. 3C), while *GSTU17OE* plants appeared to be more sensitive compared with wild-type plants (Fig. 3D).

To more systematically evaluate the effects of ABA, mutant plants were grown on vertical half-strength Murashige and Skoog (MS) agar plates supplemented with various concentrations of ABA (0–5 μM). Whereas primary root lengths were reduced by 50% in the presence of 5 μM ABA in 2-week-old *atgstu17* plants, they were reduced by 70% in wild-type plants (Fig. 3E). The number of lateral roots longer than 0.5 cm cm^{-1} primary root was significantly suppressed in *atgstu17* compared with the wild type under control and ABA treatment conditions (Fig. 3F). The average lateral root length was much greater in the *atgstu17* plant than in wild-type plants in the control and in the presence of ABA (Fig. 3G).

In contrast, primary root lengths of *GSTU17OE* were similar to the wild-type plants in the control condition but were reduced in the presence of 5 μM ABA (Fig. 3, D and H). The lateral root number per cm of primary root of *GSTU17OE* was significantly greater than in the wild-type plants only in the 5 μM ABA treatment, but the average lateral root length of *GSTU17OE* did not differ from the wild-type plants (Fig. 3, I and J).

Alteration in Stress-Related Gene Expression Confers Stress Tolerance in *atgstu17*

We were interested in determining if the altered gene expression conferred stress tolerance and in assigning a function to AtGSTU17 as a negative component of the stress response. A microarray assay using an Agilent Arabidopsis V4 Oligo 4×44K Microarray (http://www.agilent.com) was employed for *atgstu17-2* and wild-type plants under white light conditions, and expression profiles of genes with known function are presented in Supplemental Table S1. Both ABA-dependent and ABA-independent genes were found. For the validation of genes up-regulated or down-regulated in *atgstu17*, we performed real-time PCR analysis. Drought- or stress-related genes such as *XERICO*, *AREB1*, *Bax inhibitor-1*, *AnnAt1*, *COR15b*, and *AtMYB88* were all up-regulated in the *atgstu17-1* and *atgstu17-2* lines, similar to the microarray data (Supplemental Fig. S3). These genes maintained similar expression levels as wild-type plants in *GSTU17OE-1* and *GSTU17OE-2*. Expression levels of four down-regulated genes also agreed with the array data set (Supplemental Fig. S3). Again, these genes in overexpressors maintained similar expression levels as wild-type plants. Overall, the real-time PCR results agreed with the microarray analysis in showing that *atgstu17-2* affected the transcription of many genes. In the data set, we did not find any gene related to ABA metabolism; however, the *GLUTATHIONE SYNTHETASE2* (*GSH2*) gene (AT5G27380), catalyzing the ligation of Gly to the Cys residue of γ-glutamyl-Cys to form GSH, was up 1.68-fold, and the *DHAR2* gene (for dehydroascorbate reductase; AT1G75270), involved in the production of ascorbate, also increased 1.97-fold (Supplemental Table S1). The expression of *GSH2* and *DHAR2* genes was also confirmed by real-time PCR (Supplemental Fig. S3).

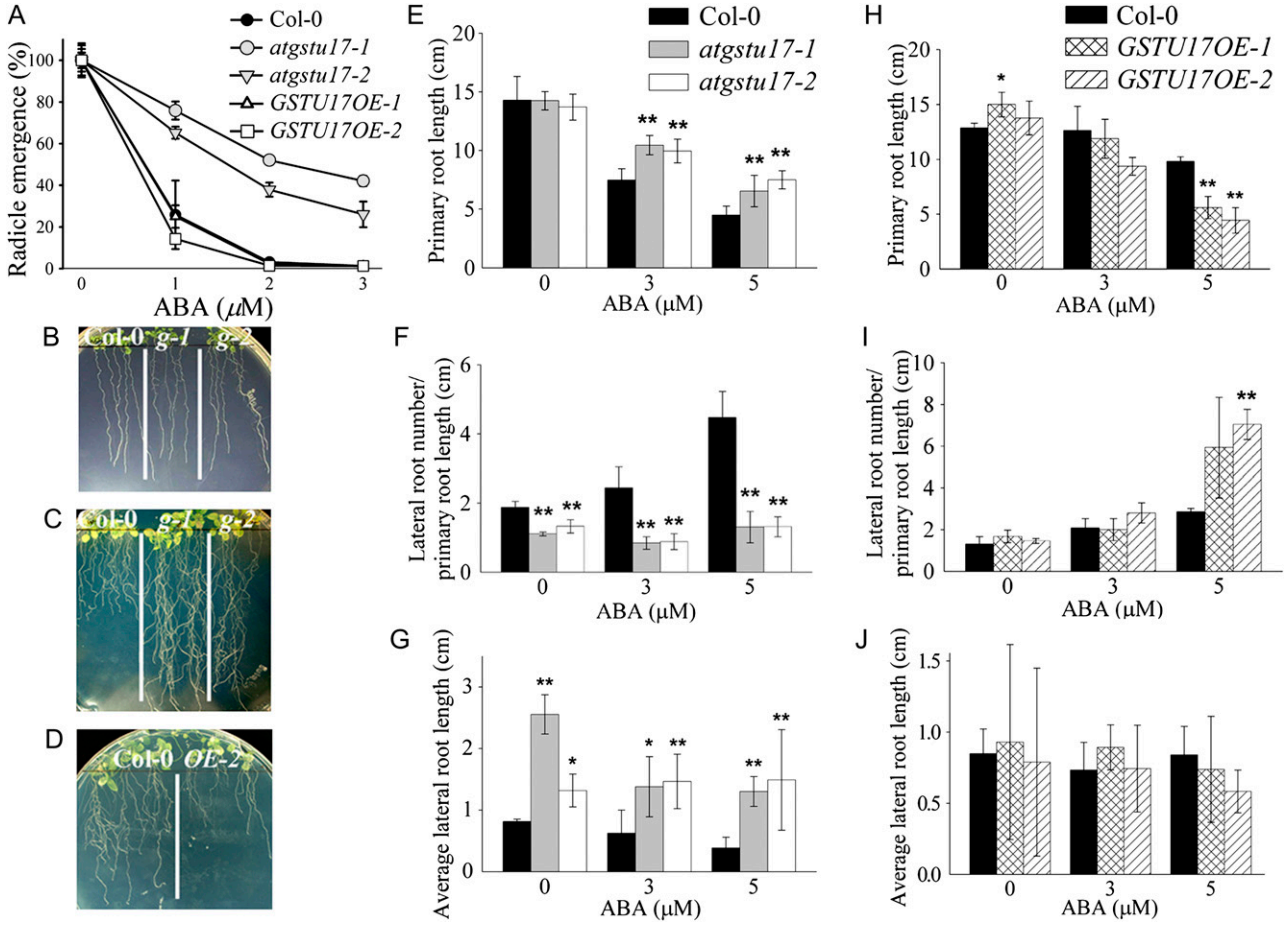

Figure 3. AtGSTU17 regulates seed germination and lateral root growth in response to ABA. A, Germination percentage of wild-type and mutant lines. Data are presented as means ± SD. Three independent experiments were performed with the same trend. B, Seedlings were germinated and grown on half-strength MS agar plates without ABA for 3 d and then transferred to the same MS medium without ABA for 1 week. C, Same as B, but the medium was supplemented with 3 μM ABA for 2 weeks. D, Same as B, but the medium was supplemented with 5 μM ABA for 3 weeks. E to J, Comparison of lengths of root systems (E and H), numbers of lateral roots greater than 0.5 cm cm^{-1} primary root length (F and I), and average lateral root lengths (G and J) of *atgstu17* (E–G), *GSTU17OE* (H–J), and wild-type plants grown on vertical half-strength MS agar plates supplemented with ABA concentrations as indicated for 2 weeks (E–G) or 3 weeks (H–J). Thirty plants at each ABA concentration were counted and averaged for E and H. Ten plants at each ABA concentration were counted and averaged for F, G, I, and J. Three independent experiments were performed with the same trends. Error bars represent the SD (*t* test: * $P < 0.05$, ** $P < 0.01$). [See online article for color version of this figure.]

atgstu17 Has Higher GSH and ABA Contents Compared with the Wild-Type Plant

Using the model xenobiotic substrate 1-chloro-2,4-dinitrobenzene as well as benzylisothiocyanate to measure GSH-conjugating activities, AtGSTU17 exhibited high specific activity when compared with other GSTs (Dixon et al., 2009). To investigate whether *AtGSTU17* mutations affected GSH levels during vegetative growth, we measured GSH in leaves and roots of wild-type plants and all mutant plants under normal growth conditions. GSH levels in the *atgstu17s* were significantly higher than in wild-type plants (Fig. 4C). GSH contents in roots of *atgstu17s* were also greater than those of wild-type and *GSTU17OE* plants (Fig. 4C). In our previous report, the GSH-oxidized glutathione (GSSG) ratio was significantly higher in *atgstu17* than in wild-type plants under normal growth conditions (Jiang et al., 2010). Taken together, loss of function of *AtGSTU17* contributed to an increased level of GSH and redox potential in plants growing in normal growth conditions.

Because of the stress-tolerant phenotype of *atgstu17*, we suspected that ABA content might be altered in the *atgstu17* mutant plants. Surprisingly, the ABA content in leaves was 2- and 2.3-fold significantly higher in *atgstu17-1* and *atgstu17-2*, respectively, than in wild-type plants, whereas it was lower in the overexpressors than in wild-type plants (Fig. 4A). ABA-deficient mutant *aba2* (GLUCOSE INSENSITIVE1), which con-

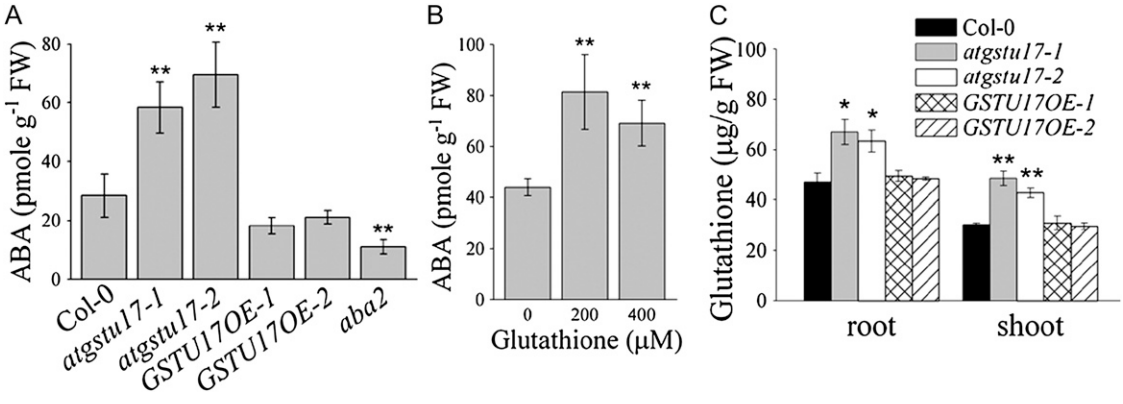

Figure 4. ABA and GSH contents in wild-type and *AtGSTU17* mutant plants. A, Determination of ABA levels under nonstressed conditions in 3-week-old wild-type and *AtGSTU17* mutant plants. Plants were grown in a growth chamber at 22°C under 16-h-light/8-h-dark conditions. Values represent means ± SD from three independent sets of samples. Three independent experiments were performed with similar results. *aba2* was used as a reference. B, Effect of exogenous GSH on ABA accumulation in leaf tissues. Wild-type plants were grown in water containing GSH (200 and 400 μM) in a growth chamber at 22°C under 16-h-light/8-h-dark conditions. ABA levels were determined using 3-week-old plants. Values represent means ± SD from three samples. Three independent experiments were performed with similar results. C, Determination of GSH levels in shoot and root under nonstressed conditions in wild-type and *AtGSTU17* mutant plants. Values are presented as means ± SD from five samples for each time point. Two independent experiments were performed with similar results. Three-week-old plants grown in a growth chamber at 22°C under 16-h-light/8-h-dark conditions were used in this study. FW, Fresh weight. *$P < 0.05$; **$P < 0.01$.

tains low levels of ABA, was used as a reference and had approximately one-third of the wild-type level of ABA.

Exogenous GSH Induced ABA Accumulation in Planta

We hypothesized that the accumulation of ABA in the *atgstu17* lines resulted from higher GSH content. To test this hypothesis, wild-type plants were grown in the solution with or without GSH for 2 weeks, and the leaf ABA content was measured. The plants grown in solution containing 200 μM GSH accumulated an ABA level that was 1.8-fold greater than the level detected in plants without exogenous GSH treatment (Fig. 4B). Plants treated with 400 μM GSH showed 1.4-fold higher ABA content. Our results support that ABA accumulation can be promoted by exogenous GSH treatment.

Effects of Exogenous GSH and ABA on Seed Germination, Stomata Aperture Size, Root Architecture, and Stress Tolerance

Knowing that GSH and ABA accumulated to higher extents in the *atgstu17*, we investigated the effect of these two chemicals separately and in combination on Arabidopsis. We found that GSH was able to suppress the germination inhibition caused by ABA. When treated with both GSH and ABA, seeds had a higher germination rate than seeds treated with ABA alone (Fig. 5A). The effect of GSH on the intrinsic stomata aperture size was not known. Figure 5, B and C, shows that stomatal apertures were significantly smaller in plants grown in the GSH-containing solutions.

To evaluate the effect of GSH and ABA on root architecture, wild-type seedlings were grown on vertical half-strength MS agar plates supplemented with various concentrations of GSH and/or ABA for 2 weeks. Whereas primary root lengths were reduced by 33% in the presence of 3 μM ABA in 2-week-old wild-type plants, they were increased by 62% in plants growing in 25 and 50 μM GSH (Fig. 6, A and B). However, higher GSH concentrations repressed primary root growth. The number of lateral roots longer than 0.5 cm cm^{-1} primary root was significantly increased in various concentrations of GSH (Fig. 6, A and C). The combinations of 3 μM ABA and GSHs slightly suppressed the lateral root number (Fig. 6C). The average lateral root length was much greater in wild-type seedlings receiving 3 μM ABA and combinations of 3 μM ABA and GSHs (Fig. 6, A and D). GSH treatment alone had no effect on lateral root length. We concluded that GSH and ABA have different effects on root growth and development.

To assess if exogenous ABA and/or GSH could confer drought tolerance, we grew wild-type plants in water containing GSH or ABA or combinations of GSH and ABA. We found that plants growing in water containing 400 μM GSH in the presence or absence of ABA recovered and resumed growth from the drought stress test, while no plants could resume growth in water only (Fig. 7, top panel). Exogenous GSH gave better protection than ABA in drought conditions. For the salt tolerance test, all of the wild-type plants receiving ABA and/or GSH exhibited enhanced salt stress tolerance and much-delayed leaf chlorosis (Fig. 7, bottom panel). All of the plants growing in water containing 3 μM ABA or a combination of ABA and

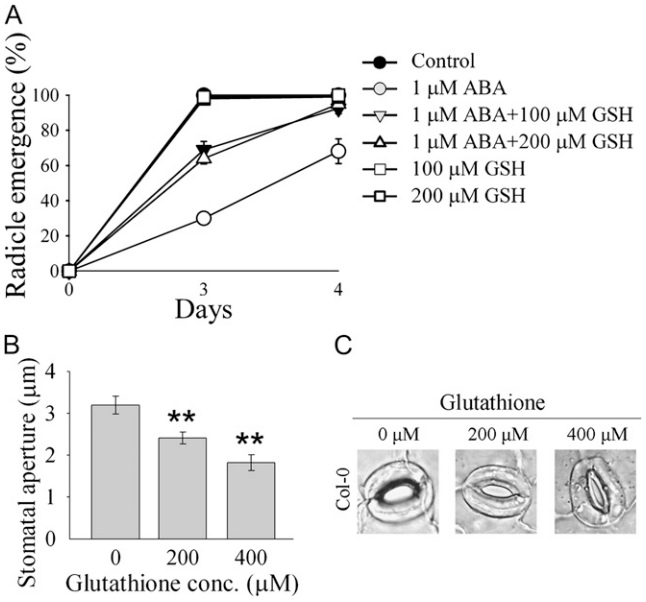

Figure 5. Effects of exogenous GSH and ABA on seed germination and stomata aperture. A, GSH reduced seed germination sensitivity to ABA inhibition. Wild-type seeds were germinated and grown on half-strength MS agar plates containing ABA or GSH for 4 d. Data are presented as means ± SD. Five independent experiments were performed with similar results. B, GSH reduced the intrinsic stomata aperture size. Leaves of 5-week-old wild-type plants growing in the water solution containing the indicated concentrations of GSH were peeled and floated on water under light for 2.5 h, and stomatal apertures were measured. Values are means ± SD ($n > 60$). ** Significantly different from the wild-type ($P < 0.01$), by Student's *t* test. These experiments were repeated at least three times. C, Micrographs representing the dynamics of GSH-mediated stomatal closure in B.

GSH resumed growth. Three micromolar ABA gave better protection than GSH in saline conditions.

atgstu17 Phenotypes Were Abolished by a GSH Synthesis Inhibitor

To further elucidate the effect of reduced levels of GSH in *atgstu17* on the phenotypes, we grew *atgstu17* mutants on vertical half-strength MS agar plates containing 3 μM L-buthionine-(*S*,*R*)-sulfoximine (BSO). BSO is a highly specific inhibitor of the first enzyme of GSH biosynthesis, and its application results in the depletion of cellular GSH (Vernoux et al., 2000). The GSH level in BSO-treated leaves was reduced compared with the mutants without BSO treatment (Fig. 6F). The *atgstu17* seedlings exhibited root development similar to wild-type plants by 2 weeks after germination on medium containing 3 μM BSO (Fig. 6, E and G–I), especially in lateral root development. These observations support that the root architecture of *atgstu17* to some extent is attributed to GSH content.

From the experiment in Figure 7, we learned that an increased GSH level confers drought and salt tolerance to wild-type plants. To test this observation using a different approach, we grew the *atgstu17s* in water containing 20 or 50 μM BSO for 2 weeks and found that they exhibited reduced drought tolerance compared

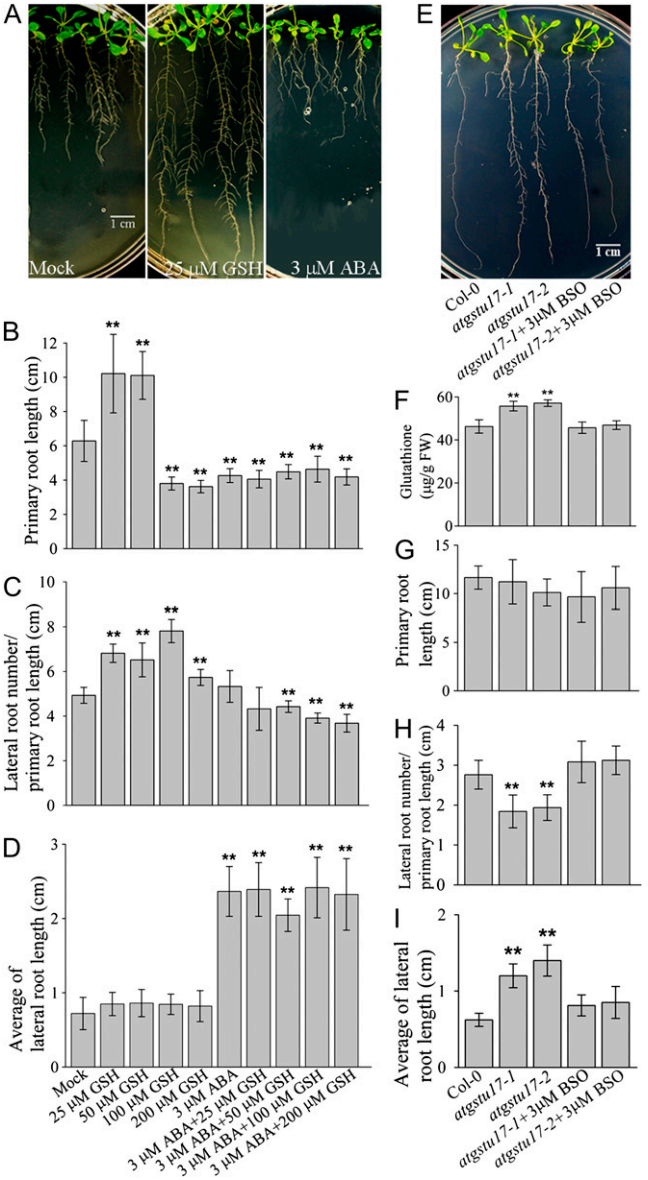

Figure 6. Effects of exogenous GSH, ABA, and BSO on root architecture. A, Seedlings of 2-week-old wild-type plants growing on a half-strength MS agar plate (left) or supplemented with 25 μM GSH (middle) or supplemented with 3 μM ABA (right). B, Comparison of the primary root length. Thirty plants at each condition were counted and averaged. C, Lateral root number per cm of primary root. Only lateral roots longer than 0.5 cm were used for the calculation. D, Average lateral root length. Ten plants at each condition were counted and averaged for C and D. E, Same as A, but plants were grown on a half-strength MS agar plate with or without 3 μM BSO. For photographic purposes, plants from separate plates were arranged side by side. F, BSO treatment reduces GSH content of *atgstu17* leaves. Plants were grown under 16-h-light/8-h-dark conditions with or without BSO, and leaves of 2-week old plants were used for the GSH assay. These experiments were repeated twice and gave comparable results. G, Same as B. H, Same as C. I, Same as D. Error bars represent SD (*t* test: ** $P < 0.01$). FW, Fresh weight.

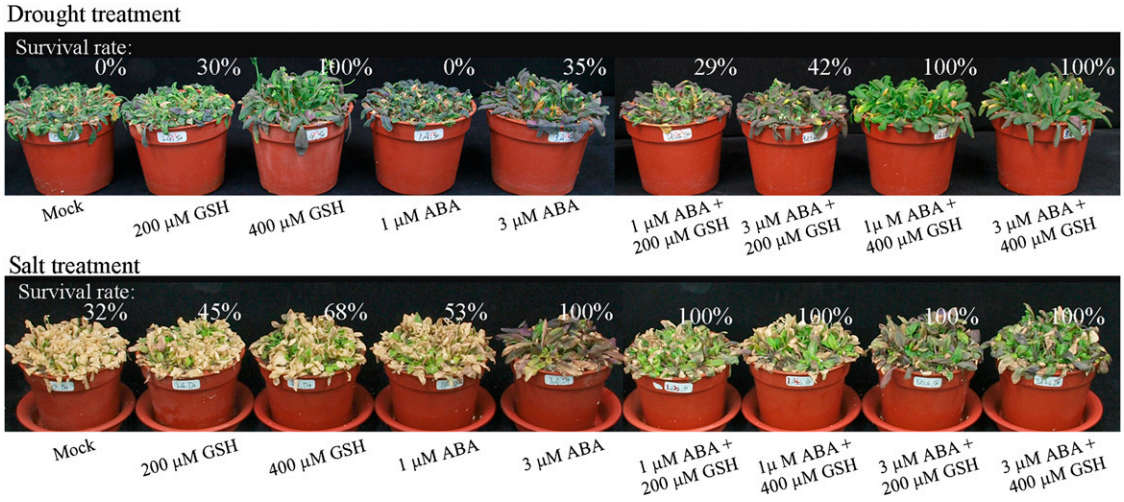

Figure 7. Effects of exogenous GSH and ABA on drought and salt tolerance. In the top panel, 3-week-old wild-type plants were incubated in water solution containing ABA, GSH, or different combinations of ABA and GSH at 22°C under 16-h-light/8-h-dark conditions. Newly prepared solution was supplied every 2 d. Mock indicates plants growing in water solution only. Each treatment consisted of three pots with 20 plants in each pot. Five independent experiments were performed with similar results. Watering was stopped for 10 d and then resumed. The photographs were taken before rewatering. The survival rates after rewatering for 5 d are indicated. In the bottom panel, plants were watered for 12 d at 4-d intervals with increasing concentrations of NaCl of 100, 200, and 300 mM along with ABA and GSH as indicated. The photographs were taken at 18 d of incubation. The survivors were quantified as described for Figure 1E.

with control mutants (Fig. 8A). BSO treatment also reduced the bolting time of the *atgstu17s* (Fig. 8B). However, treatment with or without BSO of wild-type plants did not change the drought tolerance and bolting time (Fig. 8). This experiment confirms the direct link between the stress phenotype of the *atgstu17s* and the accumulation of GSH.

DISCUSSION

Recent studies have shown that *AtGSTU17* transcripts were induced by far-red light irradiation and regulated by different photoreceptors, especially phyA. Its loss-of-function mutants resulted in a long-hypocotyl phenotype under far-red light and delayed flowering under long-day conditions (Jiang et al., 2010). In this study, we extend the function of *AtGSTU17*, the first member of the large GST family in Arabidopsis, as playing a negative role in drought and salt stress tolerance. The basic observation of an accumulation of GSH in shoot and root in *atgstu17* agrees with AtGSTU17 having high activity among GSTs in Arabidopsis when tested with different substrates (Dixon et al., 2009). Without GST mediation, GSH cannot be utilized as the substrate for xenobiotic detoxification (Rouhier et al., 2008). As there are no xenobiotics in the system, the turnover of GSH by GST may be a contributing function, but the cosubstrate is unknown. The higher GSH content also could be contributed by the increased expression of *GSH2* in the *atgstu17* mutants (Supplemental Table S1).

AtGSTU17 Plays a Negative Role in Drought and Salt Stress Tolerance

The stress-tolerant phenotype of *atgstu17* can be well explained by the greater GSH and ABA accumulation and gene expression patterns. According to the microarray data set of *atgstu17* plants, among up-regulated genes, *AREB1* and several other drought stress-tolerant genes, including *XERICO*, *RAP2.4*, *ENH1*, and *AnnAt1*, were induced. The expression of *XERICO*, *RAP2.4*, *ENH1*, and *MYB88* is ABA independent according to the Arabidopsis eFP Browser. XERICO overexpression exhibited a marked increase in drought tolerance (Ko et al., 2006). *RAP2.4* was up-regulated by drought and salt treatment and enhanced drought tolerance when overexpressed (Lin et al., 2008). *AnnAt1*, induced by ABA and NaCl treatment when overexpressed, was more drought tolerant than wild-type plants (Konopka-Postupolska et al., 2009). ENH1 functions in detoxification resulting from salt stress by participating in a salt-tolerant pathway (Zhu et al., 2007). In conclusion, ABA-dependent and ABA-independent stress-tolerant transcription factors and other genes are activated in *atgstu17* plants, leading to drought- and salt-tolerant phenotypes.

When a gene is repressed and can confer stress tolerance, this gene is usually considered to be a negative regulator of the stress response. Just a few examples, knockout mutants of *ABI1*, *ABI2*, *Nuclear protein X1* (*NPX1*), and *Altered expression of APX2 8* (*ALX8*), were more stress tolerant than wild-type plants and are considered to be negative regulators (Merlot et al., 2001; Kim et al., 2009; Wilson et al., 2009).

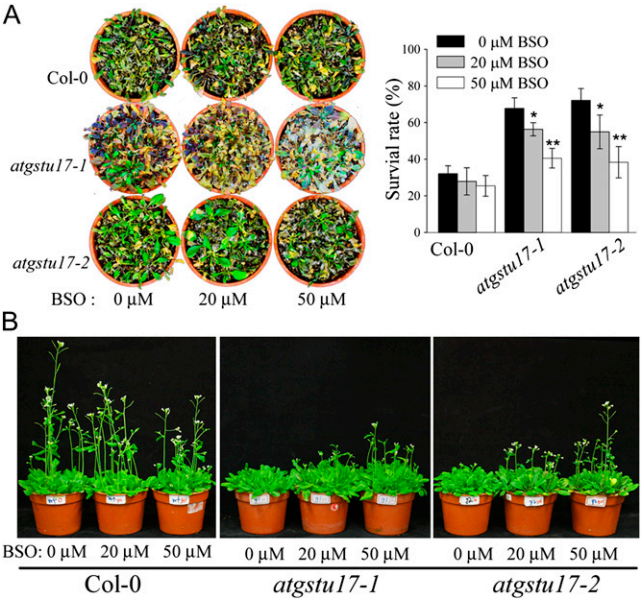

Figure 8. Drought tolerance test and flowering time of *atgstu17* and wild-type plants grown in the solution with or without BSO. A, BSO treatment confers reduced tolerance of the *atgstu17s* to short-term drought. Plants were grown under 16-h-light/8-h-dark conditions with or without BSO. Watering of 2-week-old plants was withdrawn for 10 to 12 d and then resumed. The photographs showing the differences in the reactions of plants to drought were taken after 5 d of rewatering. The survival rates (percentage) were calculated from the numbers of surviving plants per total plants tested. Data are presented as means of three pots for each treatment. Three independent experiments were performed with similar results. $*P < 0.05$; $**P < 0.01$. B, BSO treatment reduces the bolting time of the *atgstu17s*. The growth condition is the same as in A. These experiments were repeated three times and gave comparable results.

In this paper, we show that *atgstu17* exhibited altered transcriptome, metabolites, and morphology of the rosette and enhanced abiotic stress tolerance. Thus, AtGSTU17 is a negative regulator of drought and salt stress responses.

The Roles of GSH and ABA in Enhancing Drought and Salt Tolerance

An unexpected observation in this study is that exogenous GSH treatment is associated with the accumulation of ABA in Arabidopsis (Fig. 4B). It is surprising that, despite the widely assumed involvement of GSH in abiotic stress signaling in plants, no GSH-mediated drought and salt stress tolerance in plants have been reported in the literature. These facts underline the link between the loss of function of the *AtGSTU17* gene and all the phenotypes we have found in the *atgstu17* mutants.

GSH is a determinant of the cellular redox balance and a major cellular antioxidant. In addition, GSH is an important cellular signaling compound that influences many fundamental cellular processes (Foyer and Noctor, 2005). Genetic and other evidence shows that GSH concentration is important in many physiological responses (Foyer and Noctor, 2009, and refs. therein). Exposure to drought and salt stress caused an increased formation of reactive oxygen species and thus oxidative stress. The plant GSH and glutaredoxins are implicated in the response to oxidative stress and are involved in both the detoxification of reactive oxygen species and the transmission of the redox signal (Meyer, 2008). The GSH pools in *atgstu17* were 35% higher compared with wild-type plants (Fig. 4C), and the GSH-GSSG ratio in *atgstu17* was also significantly higher than in wild-type plants (Jiang et al., 2010). The redox potential difference might be the cause of the tolerant phenotype of *atgstu17* under conditions of stress.

When GSH is depleted, plants frequently exhibit decreased sensitivity to oxidative stress (Kushnir et al., 1995; Grant et al., 1996). Several transgenic plants with elevated levels of GSH have been shown to be resistant to oxidative stress (Foyer et al., 1995; Wellburn et al., 1998). In addition, elevated GSH synthesis by increasing γ-glutamyl-Cys synthetase activity was shown to correlate with cadmium resistance in cultured tomato (*Solanum lycopersicum*) cells (Chen and Goldsbrough, 1994) and chilling tolerance (Kocsy et al., 2000, 2001). During the growing stage, an exogenous supply of GSH alone at 400 μM had an obvious effect of enhancing the drought tolerance of wild-type plants. The effect of enhancing salt tolerance was also evident even at 200 μM GSH, because the chlorosis of leaf tissues was much delayed compared with wild-type

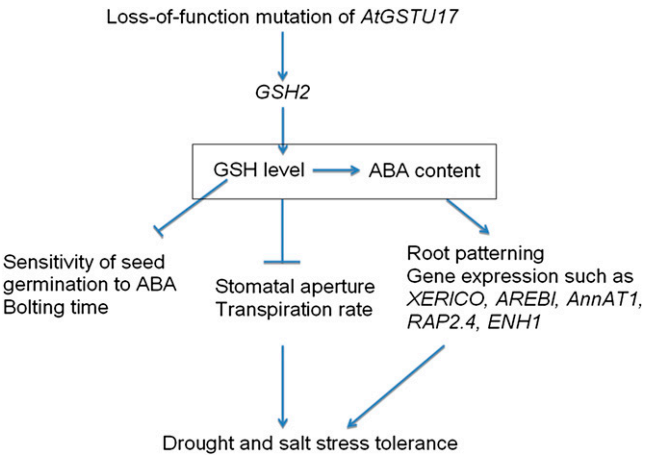

Figure 9. A model for the *atgstu17* modulation of drought and salt stress tolerance and other phenotypes. The underlying mechanism linking loss-of-function mutation of *ATGSTU17* with phenotypes is mainly the accumulation of GSH in the *atgstu17* mutants, which might associate with increased levels of ABA in planta. Some of the phenotypes (i.e. sensitivity of seed germination to ABA and delayed flowering) are specific to the GSH accumulation. Other phenotypes (i.e. stomatal aperture, root architecture, gene expression, and drought and salt stress tolerance) result from the combined action of GSH and ABA. [See online article for color version of this figure.]

plants without GSH treatment. Furthermore, if wild-type plants were exposed to a combination of ABA and GSH, the fitness increased dramatically (Fig. 6), much better than the performance produced by each chemical independently against drought and salt stresses.

An activation of ABA accumulation in Arabidopsis by exogenous GSH provides a link between GSH and drought and salt stress tolerance. ABA accumulation was correlated with increased drought tolerance (Thompson et al., 2007) and conferred drought tolerance in mutants like *enhanced drought tolerance1* (a homeodomain-START transcription factor), *poly(ADP-ribose) polymerase*, *npx1*, *isopentenyltransferase*, *xerico* (a RING-H2 gene), and *alx8* (Ko et al., 2006; Rivero et al., 2007; Vanderauwera et al., 2007; Yu et al., 2008; Kim et al., 2009; Wilson et al., 2009). In *atgstu17* plants, the higher ABA levels compared with wild-type plants under nonstressed conditions are consistent with levels of stress tolerance in these mutant plants. That exogenous ABA increased the drought tolerance compared with plants without ABA treatment was demonstrated previously (Huang et al., 2008).

GSH's Effects on the Stomata Aperture Size and Root Patterning

It was interesting to find that the constitutive stomatal aperture was smaller when the wild-type plants were grown in a solution containing GSH, a situation similar to smaller stomatal aperture in *atgstu17* mutants. This probably is an effect of ABA, because plants growing in the GSH solution exhibited greater ABA content.

The astonishing root architecture might have been due to higher levels of GSH accumulation in the loss-of-function *atgstu17* plants, which is consistent with high levels of endogenous GSH enhancing cell division in the root meristematic region, leading to root elongation (Vernoux et al., 2000), and exogenous GSH on auxin-induced in vitro root formation (Imin et al., 2007). Low concentrations of GSH (less than 50 μM) enhanced primary root elongation, but high concentrations suppressed root growth (Fig. 6). Our observation indicates the effect of an exogenous GSH in modulating the root growth pattern of wild-type plants, which can mimic the phenotype in the *atgstu17* plants. The effect of ABA on the root architecture differed from that of GSH. When applied exogenously in the well-watered condition, ABA acts as a growth inhibitor to suppress primary root growth (Sharp et al., 2004; Bai et al., 2009) while drought stress inhibits lateral root development of soil-grown plants (Xiong et al., 2006). ABA treatment reduced primary root length but encouraged lateral root growth (Fig. 6). It is apparent that the root systems in the *atgstu17* plants reflected the effects of different combinations of GSH and ABA.

Our findings support the model summarized in Figure 9 whereby repressed *AtGSTU17* expression is linked to the accumulation of GSH, which associates with the accumulation of ABA. The phenotypes of *atgstu17* can be mostly attributed to the combined effects of GSH and ABA, which explain the molecular mechanism of the repression of *AtGSTU17* expression in modulating the ABA sensitivity of seed germination, stomatal aperture, root architecture, and drought and salt stress tolerance. There must be some mechanism that regulates or represses the expression of *AtGSTU17* in stressful conditions. If this did not occur in the Col-0 wild-type background, *AtGSTU17* would be a target of natural selection in order for plants to adapt to adverse environments. The link between genotypes and the expression of specific GST genes was found in wheat (*Triticum aestivum*), which differed in drought tolerance (Gallé et al., 2009). Thus, in the whole plant, the repression of *AtGSTU17* may play a role of fine-tuning GSH homeostasis, redox status, and stress-responsive genes in adaptation to changes in environmental signals.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) wild-type plants, transgenic plants, and T-DNA-tagged mutant plants used in this work were of the Col-0 ecotype. Mutant seeds with a T-DNA insertion in the *AtGSTU17* gene (SALK_139615 for *atgstu17-1* and SALK_025503 for *atgstu17-2*) were obtained from the Arabidopsis Biological Resource Center. Vector construction and plant transformation for generating *AtGSTU17*-overexpressing lines were described previously (Jiang et al., 2010). For creating complementary lines, *35S:GSTU17OE-5/atgstu17-1* and *35S:GSTU17OE-3/atgstu17-2*, the plasmid originally used in the paper of Jiang et al. (2010) was applied.

Seeds were sown in a 2:2:1 mixture of vermiculite:perlite:peat moss. Plants were placed at 4°C for 3 d in the dark for stratification and then transferred to normal growth conditions. Plants were grown at 22°C under long-day conditions (a 16-h-light/8-h-dark cycle). For in vitro culture, seeds were surface sterilized by treatment with 70% ethanol for 5 min, followed by commercial bleach (0.5% sodium hypochlorite) containing 0.05% Triton X-100 for 20 min, followed by four washes with sterile distilled water. Seeds were stratified in the dark at 4°C for 3 d. Then, seeds were sown on half-strength MS medium composed of MS basal salts, 1% agar, and 1% Suc. The pH was adjusted to 5.7 with potassium hydroxide before autoclaving. Plates were sealed and incubated in a growth chamber at 22°C under a 16-h-light/8-h-dark photoperiod.

Stress Tolerance Tests, Water Loss Measurements, and Feeding Experiments

For the drought tolerance test, plants were initially grown in soil under a normal watering regime for 3 weeks. Watering was then halted, and observations were made after a further 10 to 12 d without water. When wild-type plants exhibited lethal effects of dehydration, watering was resumed and the plants were allowed to grow for a subsequent 5 d. For the salt tolerance test, 3-week-old plants were watered for 12 d at 4-d intervals with increasing concentrations of NaCl of 100, 200, and 300 mM. The survivor was recognized by examining the inflorescence base if it still remained green.

For transpiration (water loss) measurements, detached leaves from 5-week-old plants were exposed to room temperature (25°C). Leaves were weighed at various time intervals, and the loss of fresh weight (percentage) was used to indicate water loss.

Exogenous GSH has been used in feeding experiments because it could be taken up by the plant root system (Lappartient and Touraine, 1997; Tausz et al., 2004). For the feeding experiment, we germinated Arabidopsis seeds in a petri dish for 7 d and transferred the seedlings onto half-strength MS agar plates supplemented with GSH or ABA in regular growth conditions (22°C under a 16-h-light/8-h-dark cycle) for 2 weeks. We analyzed the stability of

GSH in the growth medium and found that GSH is stable for 2 weeks (Supplemental Fig. S4), or the seedlings were transferred into soil medium in regular growth conditions supplemented with GSH or ABA or combinations of GSH and ABA for another 2 weeks. To prevent the degradation or oxidation of GSH and ABA, the water solution was replaced every 2 d with newly prepared chemicals.

Seed Germination and Stomatal Aperture Measurements

Imbibed seeds were cold treated at 4°C in the dark for 3 d and moved to 22°C with a 16/8-h light/dark photoperiod. Germination was defined as 1-mm protrusion of the radicle. Epidermal peels were stripped from fully expanded leaves of 5-week-old plants and floated in a solution of 30 mM KCl and 10 mM MES-KOH, pH 6.15, in petri dishes. After incubation for 2.5 h under white light at 22°C to induce stomatal opening, different concentrations of ABA were added. Stomatal apertures were recorded with an Olympus BX51 system microscope and were analyzed using DP-PSW software. Measurements were performed using the free software IMAGEJ 1.36b (Broken Symmetry Software; http://brokensymmetry.com).

Quantification of the GSH and ABA Contents

Leaf tissues of 200 mg were ground with mortar and pestle in liquid nitrogen. Subsequently, 2 mL of 1 mM EDTA and 6% (v/v) metaphosphoric acid, pH 2.8, were added and mixed and then centrifuged at 15,000*g* for 20 min. Supernatant was neutralized with 0.2 M NaOH. The final pH of the neutralized acid extracts was between 5 and 6. The methods used to measure the total level of glutathione (GSH + GSSG) were as described by Griffith (1980). The GSSG in the supernatant was reduced to glutathione by glutathione reductase. Glutathione was determined in a kinetic assay in which the reduction of 5,5-dithiobis(2-nitrobenzoic acid) was spectrophotometrically measured at 412 nm.

Endogenous ABA contents in aerial parts were extracted, purified, and analyzed with a Phytodetek ABA measurement kit (Agdia) following the manufacturer's instructions.

RNA Isolation and Quantitative Real-Time RT-PCR Analysis

Total RNA samples were isolated from various plant tissues with the Rezol C&T reagent (PROtech). For RT-PCR, SuperScript III Moloney murine leukemia virus reverse transcriptase (Invitrogen; http://www.invitrogen.com) was used, following the manufacturer's instructions. The resulting single-stranded cDNA was then used as the template in a quantitative real-time RT-PCR. Quantitative real-time RT-PCR was carried out with gene-specific primers designed using Vector NTI 9.0 software. For the quantitative real-time RT-PCR experiments, KAPA SYBR Premix ExTaq was used according to the manufacturer's instructions (KAPA Biosystems) using Bio-Rad MyiQ. Quantitative real-time RT-PCR experiments were carried out in three separate biological replicates. Primers used for the quantitative real-time RT-PCR are listed in Supplemental Table S2.

Microarray Analysis

For details of microarray analysis, see Supplemental Materials and Methods.

Supplemental Data

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

Supplemental Figure S1. Locations of T-DNA insertions in the *AtGSTU17* gene, and analysis of *AtGSTU17* knockout and overexpressed transgenic lines.

Supplemental Figure S2. Complementation experiment of *AtGSTU17* in *atgstu17-1* and *atgstu17-2* plants.

Supplemental Figure S3. Expression of selected genes from the microarray data set in the *AtGSTU17* mutant lines and wild-type plants.

Supplemental Figure S4. The stability of exogenous GSH in the MS medium.

Supplemental Table S1. Selected genes up- or down-regulated by at least 1.5-fold in *AtGSTU17* knockout mutant leaves identified by GeneChip analysis.

Supplemental Table S2. Primers used in the quantitative real-time RT-PCR experiments.

Supplemental Materials and Methods. Supplementary experimental procedures.

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