The Arabidopsis GTL1 Transcription Factor Regulates Water Use Efficiency and Drought Tolerance by Modulating Stomatal Density via Transrepression of SDD1

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A goal of modern agriculture is to improve plant drought tolerance and production per amount of water used, referred to as water use efficiency (WUE). Although stomatal density has been linked to WUE, the causal molecular mechanisms have yet to be determined. Arabidopsis thaliana GT-2 LIKE 1 (GTL1) loss-of-function mutations result in increased water deficit tolerance and higher integrated WUE by reducing daytime transpiration without a demonstrable reduction in biomass accumulation. gtl1 plants had higher instantaneous WUE that was attributable to ~25% lower transpiration and stomatal conductance but equivalent CO2 assimilation. Lower transpiration was associated with higher STOMATAL DENSITY AND DISTRIBUTION1 (SDD1) expression and an ~25% reduction in abaxial stomatal density. GTL1 expression occurred in abaxial epidermal cells where the protein was localized to the nucleus, and its expression was downregulated by water stress. Chromatin immunoprecipitation analysis indicated that GTL1 interacts with a region of the SDD1 promoter that contains a GT3 box. An electrophoretic mobility shift assay was used to determine that the GT3 box is necessary for the interaction between GTL1 and the SDD1 promoter. These results establish that GTL1 negatively regulates WUE by modulating stomatal density via transrepression of SDD1.

INTRODUCTION

Drought causes water deficit that limits plant growth and survival because root water uptake from the soil is insufficient to meet the transpirational requirements of the plant (Blum, 1996). Water deficit reduces leaf cell turgor, restricting cell expansion, canopy area development, and photosynthetic source size, thus negatively affecting biomass accumulation and yield (Chaves et al., 2003). An effective plant drought acclimation or adaptation strategy is used to reduce transpirational water loss, which conserves soil moisture and allows plants to maintain an adequate water status to sustain critical physiological and biochemical processes (Nobel, 1999; Chaves et al., 2003). However, a reduction in transpirational water loss often leads to a decline in biomass accumulation because carbon assimilation is also reduced (Sinclair et al., 1984; Udayakumar et al., 1998).

Transpiration and CO2 uptake occur primarily through stomata, the pores bordered by a pair of guard cells (Hetherington and Woodward, 2003). Conductance through these pores regulates transpirational flux and water use (Bacon, 2004; Chaerle et al., 2005; Morison et al., 2008; Huang et al., 2009; Song and Matsuoka, 2009) and is modulated by stomatal movements (opening and closing) and/or density (Hetherington and Woodward, 2003; Yoo et al., 2009; Casson and Hetherington, 2010; Kim et al., 2010). Alteration of stomatal aperture in response to the environment is a well-understood process that has been linked functionally to drought tolerance and water use efficiency (WUE; Chaerle et al., 2005; Nilson and Assmann, 2007; Kim et al., 2010). Likewise, there is substantial understanding of stomatal development determinants (Bergmann and Sack, 2007; Casson and Hetherington, 2010), and it is known that stomatal density is regulated by environmental factors such light, CO2, temperature, humidity, and drought (Lake et al., 2001; Bergmann, 2004; Casson and Gray, 2008; Casson and Hetherington, 2010). However, it is unclear how these environment factors regulate the developmental determinants and what the consequences of altered stomatal density are on drought tolerance and WUE.

Stomata develop predominantly in the leaf epidermis but do exist in other organs (Bergmann and Sack, 2007; Dong and Bergmann, 2010). Asymmetric division of a protodermal meristemoid mother cell forms a meristemoid and a larger stomatal lineage ground cell, which differentiates into an epidermal pavement cell or another meristemoid. The triangular-shaped meristemoid differentiates into a guard mother cell (GMC) that...
undergoes a symmetric division, forming a pair of guard cells. Then, morphogenesis of the stoma occurs (Bergmann and Sack, 2007; Dong and Bergmann, 2010). This basal pathway of stomatal lineage is regulated by well-characterized genetic determinants. BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE and SPEECHLESS (SPCH) are necessary for asymmetric division of a meristemoid mother cell to produce a meristemoid (MacAlister et al., 2007; Dong et al., 2008). MUTE facilitates meristemoid differentiation to a GMC (MacAlister et al., 2007; Pillitteri et al., 2007). FAMA then regulates differentiation of guard cells (Ohashi-Ito and Bergmann, 2006). INDUCER OF CBF EXPRESSION (also annotated as SCREAM) 1 and 2 physically interact with SPCH, MUTE, and FAMA and regulate the basal pathway (Kanaoka et al., 2008).

A current model for Arabidopsis thaliana stomatal development includes a signaling pathway that negatively regulates the basal pathway of stomatal lineage, which is necessary to achieve a balance between pavement and guard cells in the leaf epidermis (Bergmann and Sack, 2007; Casson and Hetherington, 2010). Determinants of this negative signal regulatory pathway include the leucine-rich repeat receptor-like protein TOO MANY MOUTHS (TMM) that is presumed to interact with the ERECTA (ER) family members of leucine-rich repeat receptor-like kinases (Shpak et al., 2005). STOMATAL DENSITY AND DISTRIBUTION 1 (SDD1) encodes a subtilisin-like Ser protease that likely processes propeptides into ligands that activate the TMM-ER complex (Berger and Altmann, 2000; von Groll et al., 2002). Ligand interaction with the receptor is presumed to activate a mitogen-activated protein kinase (MAPK) cascade that includes YODA (YDA); MAPKKK), MKK4/5 (MAPKK), and MPK3/6 (MAPK). Then, SPCH is phosphorylated by MPK3/6, which leads to its inactivation and repression of the basal pathway (Bergmann et al., 2004; Wang et al., 2007; Lampard et al., 2008). Recently, the secretory peptides EPIDERMAL PATTERNING FACTOR1 (EPF1), EPF2, and EPF-like 9 (also annotated as STOMAGEN) have been implicated as ligands of TMM that regulate the MAPK cascade, independently of SDD1 (Hara et al., 2007; Hunt and Gray, 2009; Hunt et al., 2010; Sugano et al., 2010).

The GT-2 transcription factor family proteins contain two trihelix DNA binding domains that interact with GT cis-acting elements (GT elements) during transcriptional regulation (Zhou, 1999). GT elements were identified initially in the promoters of light-regulated genes, such as pea (Pisum sativum) RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE SMALL SUBUNIT 3A and rice (Oryza sativa) PHYTOCHROME A (PHYA) (Green et al., 1987; Dehesh et al., 1990; Zhou, 1999). GT-2 transcription factor proteins have been implicated in other processes, including endoreduplication, petal development, and abiotic stress tolerance in Arabidopsis and soybean (Glycine max; Brewer et al., 2004; Breuer et al., 2009; Xie et al., 2009). In this report, evidence is presented that GT-2 LIKE 1 (GTL1) functions as a focal regulator of water stress tolerance and water use efficiency (WUE) through a mechanism that involves transcriptional repression of SDD1 and regulation of stomatal density and transpiration. GTL1 expression is downregulated by dehydration, establishing a potential paradigm for how the environment influences stomatal development to reduce transpiration under low water availability conditions.

RESULTS

GTL1 Is Involved in Plant Water Stress Responses

Two independent alleles harboring T-DNA insertions in the first exon and first intron of Arabidopsis GTL1 were annotated as gtl1-4 (SALK_005972) and gtl1-5 (SALK_044308) (Figure 1A), respectively, because three other T-DNA insertion alleles (gt1-1, gtl1-2, and gtl1-3) were described previously (Breuer et al., 2009). The gtl1-4 T-DNA insertion location is the same as gtl1-3, based on sequence data analysis (http://signal.salk.edu; Salk Institute Genomic Analysis Laboratory). Homozygous gtl1-4 and gtl1-5 plants were identified by PCR amplification using allele-specific primers (Figure 1B; see Supplemental Table 1 online). GTL1 transcript was undetectable in gtl1-4 plants but could be detected at very low abundance in gtl1-5 plants relative to the wild type, which suggests that these mutations may cause loss of function and reduced function of GTL1, respectively (Figure 1C). gtl1-4 and gtl1-5 plants were better able to survive low relative soil water content (SWC) than were wild-type plants (Figure 1D). More than 80% of gtl1-4 and gtl1-5 and <10% of wild-type plants survived SWC of 15% ± 1.4% (Figure 1E). Most of the wild-type plants wilted at 15% ± 1.4% SWC, while most gtl1-4 and gtl1-5 plants exhibited less severe leaf wilting symptoms (see Supplemental Figure 1A online). Increased water deficit survival of gtl1 plants was associated with the capacity to maintain higher leaf relative water content (RWC) than the wild type at 13% ± 1.0% SWC (Figure 1F).

GTL1 transcript was abundant in whole shoots of well-watered 4-week-old Columbia-0 (Col-0) plants but was less abundant in those of plants exposed to water deficit stress caused by withholding irrigation for 11 d (see Supplemental Figure 2A online). This treatment induced expression of the dehydration-responsive COR15a gene (see Supplemental Figure 2A online; Baker et al., 1994), indicating the plants were experiencing water deficit. Dehydration stress of detached shoots also caused a reduction in GTL1 expression (see Supplemental Figure 2B online). Results deposited in the Genevestigator database (https://www.genevestigator.com; Zimmermann et al., 2004; Perera et al., 2008) also indicate that GTL1 is downregulated in response to water deficit stress and was confirmed by the induction of DREB2A expression (see Supplemental Figure 2C online; Liu et al., 1998). These results indicate that GTL1 is expressed when plants have sufficient available water but is downregulated by water deficit.

gtl Mutations Improve WUE by Reducing Transpiration

To understand the physiological mechanisms by which gtl plants are more water stress tolerant through maintenance of higher leaf water status under low soil moisture conditions, transpiration rates were assessed by gravimetric analyses over diurnal light/dark periods. gtl plants exhibited lower light period (but not dark period) transpiration rates than wild-type plants when grown under both water-sufficient (Figure 2A) and water deficit (see Supplemental Figure 3 online) conditions. Lower transpiration rates resulted in reduced daily water loss from gtl plants (Figure 2B), which likely enhanced the capacity of gtl
plants to maintain higher leaf RWC (Figure 1F) and tolerate water deficit stress (Figures 1D and 1E). The significant reduction in transpiration (Figures 2A and 2B) was not associated with decreased shoot dry weight (Figure 2C) or total leaf area (see Supplemental Figures 4A and 4B online), suggesting that reduced stomatal conductance, which led to reduced water loss, did not result in a concomitant reduction in biomass accumulation. Consequently, gtl1 plants had higher integrated WUE (biomass/water use) than did the wild type (Figure 2D). These results indicate that GTL1 is a negative regulator of WUE.

Figure 1. gtl1 T-DNA Insertional Mutations Enhance Survival and Maintenance of Leaf RWC under Water Deficit Stress.

(A) The schematic illustrates T-DNA locations in the first exon (black bar) and the first intron (black line) in GTL1 for gtl1-4 and gtl1-5, respectively. Hatched bars indicate 5′- and 3′-untranslated regions. Arrows indicate the positions of primers used in (B).

(B) Homozygosity of the T-DNA insertion in gtl1-4 and gtl1-5 was determined by PCR analysis of the GTL1 genomic fragment using left primers (LP) and right primers (RP) and the T-DNA insertion using a T-DNA–specific left border primer (LB) with LP for gtl1-4 and LB with RP for gtl1-5. Col-0 is the wild type.

(C) GTL1 expression level in wild-type, gtl1-4, and gtl1-5 plants was determined by RT-PCR analysis with forward and reverse primers (GTL1F and GTL1R) and ACTIN2 (ACT2; reference standard).

(D) and (E) Plant water stress responses were analyzed in 3-week-old wild-type and gtl1 (gtl1-4 and gtl1-5) plants grown under a long-day photoperiod (16 h light/8 h dark, 30% relative humidity). Five containers of each genotype (20 plants/container) were evaluated in three independent experiments. Relative SWC is the soil water relative to the soil water at day 0 of withholding water and is the average of five containers (see Supplemental Figure 1B online). The photograph in (D) illustrates results of one replicate from one experiment. Plant survival (E) was determined 4 d after rewatering (mean ± SE, n = 5).

(F) In a separate experiment, 4-week-old wild-type and gtl1 plants were grown under a short-day photoperiod (8 h light/16 h dark, 60% relative humidity) and exposed to water deficit stress by withholding water. Leaf RWC (mean ± SE, n = 3 to 4) and relative SWC (see Supplemental Figure 1C online) were determined. In (E) and (F), mean values of gtl1-4 and gtl1-5 plants are significantly different from the wild type at *P < 0.05 and **P < 0.01.
To test the hypothesis that gtl1 mutations reduced transpiration to a greater extent than CO2 assimilation, gas exchange (water and CO2) of fully expanded leaves of wild-type and gtl1 plants was determined using an infrared gas analyzer. Leaf transpiration of gtl1 plants was 26.0% ± 1.9% lower than that of wild-type plants at saturating light levels (Figure 3A). Stomatal conductance of gtl1 plants was also lower than in the wild type (Figure 3B), indicating that the reduced transpiration of gtl1 plants was due to decreased water loss through stomata. Net CO2 assimilation rates of gtl1 plants and the wild type were not significantly different (Figure 3C). Consequently, gtl1 plants had higher instantaneous WUE (CO2 assimilation/transpiration) (Figure 3D), which was attributable to reduced transpiration (Figure 3A). Vapor pressure deficit (VPD) was similar for all measurements (Figure 3F), indicating that the lower transpiration and stomatal conductance in gtl1 plants were not due to different VPD.

Quantum efficiency of gtl1 and wild-type plants was similar (see Supplemental Figure 5A online), indicating there was no difference in the capacity to use photons to fix carbon. In addition, dark respiration and the light compensation point (light intensity at which CO2 assimilation is equal to respiration) of gtl1 plants and the wild type were similar (see Supplemental Figures 5B and 5C online). Internal CO2 concentration (ci) in leaves of gtl1 plants was lower than in those of the wild type (Figure 3E), possibly due to a reduced CO2 flux from the air to the substomatal cavity because of the lower stomatal conductance in in gtl1 plants. These results indicate that gtl1 and wild-type plants have equivalent CO2 assimilation and respiration rates. Furthermore, gtl1 plants have higher instantaneous WUE, due primarily to reduced transpiration without an appreciable reduction in net CO2 assimilation and biomass accumulation under our experimental conditions.

**GTL1 Regulates Stomatal Density but Does Not Affect Stomatal Aperture or Opening/Closing**

gtl1 mutations reduced leaf transpiration because of decreased stomatal conductance (Figures 3A and 3B), which may be caused by effects on stomatal aperture or stomatal density (Hetherington and Woodward, 2003; Yoo et al., 2009). There was no difference in leaf abaxial stomatal aperture of gtl1 and wild-type plants under water-sufficient conditions (see Supplemental Figure 6A online). Abscisic acid (ABA) treatment caused an equivalent reduction in transpiration rates of gtl1 and wild-type plants (see Supplemental Figures 6B and 6C online), indicating that both had equivalent stomatal responsiveness to ABA. Seed germination and seedling root growth of gtl1 plants and the wild type also responded to ABA similarly (see Supplemental Figures 6D and 6E online). These data indicate that GTL1 does not regulate ABA responsiveness, and the lower transpiration rates of gtl1 plants are not caused by differences in stomatal aperture or ABA-induced stomatal closure.
Fully expanded leaves of gtl1 plants had fewer stomata than did the wild type (Figure 4), which was determined on the same leaves used for gas exchange analyses (Figure 3). Leaf abaxial stomatal density was 24.1% ± 2.5% lower in gtl1 plants compared with the wild type (Figures 4A and 4B), which correlated with a 26.0% ± 1.9% lower transpiration rate at saturating light levels (Figure 3A). The larger leaf trichome phenotype associated with gtl1 plants (see Supplemental Figure 7 online; Breuer et al., 2009) is unlikely a principal cause of reduced transpiration because any effects on boundary layer conductance due to trichomes would be minimal for small leaves such as those of Arabidopsis plants (Jarvis and McNaughton, 1986). These results indicate that higher WUE of gtl1 plants is due to reduced transpiration that is attributable to a reduction in leaf abaxial stomatal density.

Figure 3. gtl1 Plants Exhibit Higher Instantaneous WUE Due to Lower Transpiration and Stomatal Conductance.

Leaf transpiration, stomatal conductance, net CO2 assimilation, instantaneous WUE, internal CO2 concentration, and VPD were determined on individual leaves of 8-week-old wild-type (Col-0) and gtl1 plants (8-h diurnal photoperiod) using a Li-Cor 6400 gas exchange system (mean ± SE, n = 4).

Implicate GTL1 as a regulator of stomatal and pavement cell development and stomatal density that affects transpiration.

GTL1 Is Expressed in the Abaxial Epidermis and Negatively Regulates SDD1 Expression

Stomatal development genes are expressed primarily in epidermal cells (Bergmann and Sack, 2007; Hunt and Gray, 2009). Analysis of plants transformed with a 2.9-kb GTL1 promoter-β-glucuronidase (GUS) reporter gene fusion (GTL1pro:GUS) indicated that the gene is expressed predominantly in guard cells, pavement cells, and meristemoids in the leaf abaxial epidermis (Figures 5A and 5B). GTL1 localization was determined by monitoring a C-terminal green fluorescence protein (GFP)–tagged fusion protein (GTL1pro:GTL1:GFP) (Figure 5C). GTL1-GFP expression, which was driven by the same GTL1 promoter that was used to drive GUS expression (Figures 5A and 5B), suppressed the reduced stomatal density phenotype of gtl1-4 (Figure 5D). The GTL1-GFP fusion protein was detected by protein gel blot analysis using anti-GFP (Figure 5E). GTL1 protein localized to nuclei in abaxial epidermal cells and was detected primarily in pavement cell nuclei (Figure 5C). These results indicate that GTL1 is expressed in guard cells throughout the abaxial epidermis, although GTL1 accumulates predominantly in pavement cell nuclei.
SDD1 activates the MAP kinase pathway that negatively regulates stomatal development (see Supplemental Figure 10 online; Bergmann and Sack, 2007; Casson and Hetherington, 2010). SDD1 transcript abundance in fully expanded leaves was much higher in gt1 compared with wild-type plants (Figure 5F). Similarly, MPK3 and MPK6 expression was higher in leaves of gt1 plants (Figure 5G). ER, TMM, and YDA expression was similar in gt1 and wild-type plants (see Supplemental Figure 8 online). Thus, GTL1 appears to function as a stomatal development determinant by negatively regulating SDD1, MPK3, and MPK6 expression.

GTL1 Binds to the GT Element in the SDD1 Promoter

GT-2 transcription factor family proteins bind to GT elements (GT1 box, GGTTAA; GT2 box, GGTAAAT; GT3 box, GGTTAAA) in the promoters of target genes to activate or repress transcription (Kuhn et al., 1993; Ni et al., 1996; Zhou, 1999). Initially, GTL1 DNA binding activity was assessed using the rice PHYA promoter fragment that has been reported to interact with Arabidopsis and rice GT2 proteins (Kuhn et al., 1993; Ni et al., 1996). Two partial fragments of GTL1 (Nt1 and GTL1N polypeptides) were fused with maltose binding protein (MBP) to enhance solubility during purification (Figure 6A). An electrophoretic mobility shift assay (EMSA) determined that only GTL1N interacted with the rice PHYA promoter fragment (Figure 6B). The N1 polypeptide that was predicted to have an incomplete DNA binding domain did not interact with the PHYA promoter (Figures 6A and 6B). Unlabeled probe was an effective competitor (Figure 6C), indicating that GTL1N specifically binds to the PHYA promoter. Furthermore, the GTL1 N-terminal DNA binding domain effectively bound all three types of GT elements (see Supplemental Figure 9 online).

We hypothesized that GTL1 binds directly to the GT3 box (GGTTAAA; −428 to −423) of the SDD1 promoter to repress SDD1 transcription. Migration of labeled SDD1 promoter fragment was shifted by the addition of GTL1N polypeptide. However, this gel shift was abolished by adding unlabeled SDD1 promoter (Figure 6D), indicating that GTL1 specifically binds to the SDD1 promoter. Two guanine residues in the GT3 box (GGTTAAA) are critical nucleotides in the interaction of GT elements with GT2 family proteins (Kuhn et al., 1993; Ni et al., 1996). We substituted CC for GG in the GT3 box (GGTTAAA → CCTAAA) in the SDD1 promoter fragment to determine specificity of the GT3 box for GTL1 DNA binding activity. The mutated SDD1 promoter was used as a noncompetitor (unlabeled mutated SDD1 promoter).
and did not inhibit the interaction between GTL1 and the SDD1 promoter (Figure 6D). Furthermore, GTL1N did not bind to the labeled mutated SDD1 promoter (GG→CC) (Figure 6E). The results of an in vitro EMSA indicate that GTL1 specifically binds to the GT3 box in the SDD1 promoter.

In vivo interaction of GTL1 with the SDD1 promoter was tested by chromatin immunoprecipitation (ChIP) analysis using transgenic plants harboring the GTL1pro:GTL1:GFP construct that suppressed the reduced stomatal density phenotype of gtl1-4 plants (Figures 5D and 5E). Chromatin associated with GTL1-GFP was immunoprecipitated with anti-GFP antibody and subjected to PCR analysis using primers specific for different regions of the SDD1 promoter (see Supplemental Table 1 online). Region 3 (−279 to −556) included the GT3 box in the SDD1 promoter.

**Figure 5.** GTL1 Is Expressed in the Abaxial Epidermis and Negatively Regulates SDD1 Expression.

(A) GUS activity in the abaxial epidermis of the first rosette leaf from 10-d-old seedlings harboring GTL1pro:GUS was analyzed by histochemical staining. Arrow indicates a triangle-shaped meristemoid.

(B) GUS activity was not detected in the adaxial epidermis of fully expanded rosette leaves from 4-week-old transgenic plants harboring GTL1pro:GUS.

(C) GTL1-GFP protein localization was analyzed in the leaf abaxial epidermis of 4-week-old transgenic plants harboring GTL1pro:GTL1:GFP (#12-1) by fluorescence microscopy.

(D) Stomatal density was measured in 6-week-old wild-type (Col-0), gtl1-4 (empty vector control line), and GTL1pro:GTL1:GFP complementation lines (#7-6 and #12-1) (mean ± SE, n = 4; * indicates significant difference from the wild type at P < 0.05).

(E) GTL1-GFP protein expression in 6-week-old complementation lines and GFP protein expression in 6-week-old empty vector control plants were detected by immunoblot using anti-GFP antibody.

(F) and (G) RT-PCR analysis of SDD1, MPK3, MPK6, or UBC was performed on total RNA from fully expanded leaves of 8-week-old wild-type and gtl1 plants.
Region 2 (−538 to −824) was adjacent to, but did not contain, the GT3 box. Region 1 (−1136 to −1400) was distal to the GT3 box. Region 3 primers resulted in the greatest amount of PCR product (Figure 6F). The amount of PCR amplification product was reduced with primers for region 2 and even more so with those for region 3 (Figure 6F). These results are consistent with the GTL1 fragment binding to the GT3 box in the SDD1 promoter determined by in vitro DNA binding (Figure 6D). Together, these results indicate that GTL1 binds to the SDD1 promoter to repress SDD1 expression, which regulates stomatal density.

DISCUSSION

The trihelix transcription factor GTL1, previously identified as a trichome development regulator (Breuer et al., 2009), is shown herein to regulate stomatal development. Furthermore, GTL1
loss-of-function mutations enhanced water deficit tolerance, which was associated with maintenance of leaf water status, even when plants were grown in media with low SWC (Figure 1). gtl1 mutations caused higher instantaneous and integrated plant WUE because of reduced transpiration that occurred without a difference in net CO₂ assimilation (Figures 2 and 3). Lower transpiration, drought tolerance, and higher WUE were associated with reduced stomatal density in the leaf abaxial epidermis and higher SDD1 expression (Figures 4 and 5), which suggests that gtl1 reduced stomatal density by increasing expression of SDD1, a negative regulator of stomatal development (Berger and Altmann, 2000; von Groll et al., 2002). GTL1 expression was downregulated by plant water deficit (see Supplemental Figure 2 online), and GTL1 localized to the nucleus in abaxial epidermal pavement cells (Figure 5C). EMSA indicated that GTL1 physically interacts with the GT3 box in the SDD1 promoter, and ChIP analysis confirmed that GTL1 was associated with the GT3 box of the SDD1 promoter (Figures 6D and 6F). Together, these results indicate that GTL1 regulates plant water use by modulating stomatal development through SDD1 transrepression (Figure 7).

GTL1 Affects Plant Water Use and CO₂ Assimilation through Regulation of Stomatal Development

Transpiration and CO₂ uptake occur primarily through stomatal pores, and conductance is dependent on stomatal density and pore aperture (Hetherington and Woodward, 2003; Nilson and Assmann, 2007). gtl1 caused a reduction in leaf abaxial surface stomatal density (Figure 4) and decreased transpiration (Figures 2 and 3), which resulted in enhanced water deficit tolerance (Figure 1D). Reduced stomatal density caused by gtl1 did not affect CO₂ assimilation and biomass accumulation and resulted in higher integrated and instantaneous plant WUE (Figures 2 to 4). For most C3 plants, net CO₂ assimilation rate saturates as stomatal conductance increases because of nonstomatal limitations, such as the regeneration of ribulose 1,5-bisphosphate (Farquhar and Sharkey, 1982). However, over a similar stomatal conductance range, transpiration will continue to increase linearly with stomatal conductance (Yoo et al., 2009). Therefore, a moderate decrease in stomatal density can reduce transpiration significantly without a concomitant effect on CO₂ assimilation and result in higher WUE (Yoo et al., 2009). It is plausible that a more substantial reduction in stomatal density would reduce CO₂ uptake substantially and lower both WUE and biomass accumulation. Consistent with this hypothesis, cauliflower mosaic virus 3SS-driven SDD1 expression in C24 plants resulted in an ~60% reduction in stomatal density that was linked to a 20% reduction in net CO₂ assimilation rate (Büssis et al., 2006).

Genetic variation for WUE is negatively correlated with transpiration in primary gene pools (Van den Boogaard et al., 1997; Impa et al., 2005; Yoo et al., 2009). ER, GPA1, CA1/4, and HDG11 are Arabidopsis genetic determinants known to regulate WUE through modulation of stomatal density (Masle et al., 2005; Yu et al., 2008; Hu et al., 2010; Nilson and Assmann, 2010). ER alleles were the cause of quantitative trait loci associated with natural variation in WUE between Col-4 and Landsberg erecta (Masle et al., 2005). ER mutations resulted in lower WUE, which was associated with a number of measurable phenotypes, including increased stomatal density and reduced photosynthetic capacity and mesophyll development (Masle et al., 2005). GPA1 (G protein α-subunit 1) loss of function resulted in reduced stomatal density and stomatal conductance but similar net CO₂ assimilation leading to higher WUE (Nilson and Assmann, 2010). Improved WUE of gpa1 plants was due primarily to reduced stomatal density rather than to insensitivity to ABA-induced stomatal closure or to inhibition of stomatal opening (Fan et al., 2008; Nilson and Assmann, 2010). CA1 and CA4 (carbonic anhydrase 1 and 4) have also been implicated in the regulation of WUE (Hu et al., 2010). cat4 double mutations increased stomatal density, whereas guard cell–targeted overexpression of CA1 or CA4 in ca1 cat4 plants decreased stomatal density and improved WUE (Hu et al., 2010). The target determinants and molecular mechanisms by which GPA1, CA1, and CA4 regulate stomatal development and WUE remain to be elucidated.

A forward genetic screen of a T-DNA activation-tagged population for drought tolerance resulted in the identification of enhanced drought tolerance1 (edt1). The T-DNA in edt1 was inserted in the 5′-untranslated region of HOMEODOMAIN GLABROUS 11 (HDG11) and resulted in overexpression of HDG11, reduced stomatal density, and higher WUE. Drought tolerance and higher WUE were attributable to reduced stomatal density and transpiration and to enhanced root growth, photosynthesis, and ABA and Pro biosynthesis (Yu et al., 2008). ER expression was greater in edt1, and HDG11 transactivated the ER promoter when cotransformed into onion cells, implying that HDG11 directly activates ER expression and may regulate stomatal development through the negative regulatory pathway as does GTL1. The recent characterization of these genetic determinants that affect WUE indicates that plants have the capacity to

Figure 7. Model of GTL1 Function as a Determinant of WUE by Regulating Stomatal Density through Transrepression of SDD1.

GTL1 binds to the GT element (GT3 box: GGTAAA) in the SDD1 promoter to transrepress SDD1, which is a negative regulator of stomatal development. GTL1 negatively regulates WUE by positively regulating stomatal density and transpiration through transrepression of SDD1. GTL1 is expressed in water-sufficient conditions to facilitate stomatal development and is downregulated under water deficit to reduce transpiration.
optimize water use and CO₂ assimilation by regulating stomatal development.

**GTL1 Is a Positive Regulator of Stomatal Development as a Transrepressor of SDD1**

SDD1 and MPK3/6 transcripts were misregulated in gt1 plants (Figures 5F and 5G), indicating that GTL1 functions upstream of these determinants in the negative regulatory pathway (see Supplemental Figure 10 online; Bergmann and Sack, 2007). GTL1 interacted with the GT3 box in an SDD1 promoter fragment in vitro (Figure 6D) and was associated with an SDD1 promoter fragment in chromatin that contained the GT3 box (Figure 6F). These data indicate that GTL1 is a transcriptional repressor of SDD1 and, through this function, positively regulates stomatal density (see Supplemental Figure 10 online; Shpak et al., 2005; Bergmann et al., 2007).

The developmental program that determines stomatal density is sufficiently plastic to enable plants to regulate CO₂ uptake and transpiration in response to changing environments (Casson and Gray, 2008). Climatic factors (e.g., light, CO₂, relative humidity, and water deficit) and hormones alter stomatal density (Lake et al., 2011; Bergmann, 2004; Casson and Gray, 2008; Xu and Zhou, 2008; Ahmed et al., 2010), although the mechanism(s) by which these effectors modulate stomatal development has not been identified (Casson and Hetherington, 2010). GTL1 transcript was abundant when plants were growing with adequate water supply but expression was downregulated by water stress or dehydration (see Supplemental Figure 1 online). This drought-responsive downregulation of GTL1 expression may indicate that the transcription factor is an integrative node that links stomatal development to environmental regulation of density and gas exchange (Figure 7). Further investigation is necessary to determine the mechanisms by which other environmental factors regulate stomatal development and to determine the adaptive significance of these as plants cope with climatic fluctuations.

GTL1 was expressed in both pavement and guard cells of the leaf abaxial epidermis, but expression was not detected in the adaxial epidermis (Figures 5A and B), which correlates with GTL1 regulation of abaxial stomatal density (Figure 4A). GTL1 accumulated in the nuclei of pavement cells (Figure 4C), where SDD1 is not expressed (von Groll et al., 2002), which is consistent with the notion that GTL1 transrepresses this negative regulator of stomatal development. These GTL1 expression and protein localization data, together with high expression of SDD1 in gt1 plants, indicate that SDD1 repression by GTL1 is necessary to allow formation of stomata during development and, in response to environmental changes, to optimize transpiration and CO₂ assimilation. Further research is needed to determine whether tissue- and cell type-specific regulation of SDD1 is mediated by GTL1 and environmental changes.

**GTL1 Fine-Tunes Stomatal Development to Regulate WUE**

ER and TMM are known receptors of the signal cascade that negatively regulates the basal stomatal development pathway (see Supplemental Figure 10 online; Shpak et al., 2005; Bergmann and Sack, 2007). SDD1 encodes a subtilisin-like protease that activates ER and TMM to repress stomatal development (von Groll et al., 2002; Shpak et al., 2005). These receptors are negatively regulated by EPF1/2 ligands and positively regulated by STOMAGEN, independent of SDD1 (von Groll et al., 2002; Hara et al., 2007; Hunt and Gray, 2008; Sugano et al., 2010). It is plausible that convergent functions of EPF1/2, STOMAGEN, and SDD1 on ER and TMM receptors are necessary for precise regulation of stomatal number and plant water use in different ecosystems and in response to developmental and environmental cues (Casson and Hetherington, 2010). We posit that GTL1 is a determinant that integrates the effects of plant water-deficit stress on stomatal development to regulate carbon assimilation and transpiration. It is possible that other members of the GT-2 family that are closely related to GTL1 may have similar functions as transcriptional repressors of SDD1 expression. The existence of multiple regulatory determinants to optimize gas exchange and improve WUE in different environments is analogous to fine-tuning of signal transduction that is necessary for amplification and/or maintenance of a signal and for reducing noise fluctuations of signals (Thattai and van Oudenaarden, 2001; Kashiwagi et al., 2006; Saez et al., 2006; Alon, 2007).

**METHODS**

**Plant Materials and Growth Conditions**

*Arabidopsis thaliana* genetic resources were wild type (Col-0 ecotype), gt1-4 (SALK_005972), and gt1-5 (SALK_044308). gt1 mutant seeds were obtained from the ABRC at Ohio State University. Transgenic plants harboring and expressing GTL1pro:GUS and GTL1pro:GTL1:GFP were generated using the Agrobacterium tumefaciens–mediated floral dip method (Zhang et al., 2006). Single-copy and homozygous T3 plants were identified by genetic segregation on agar medium containing hygromycin. Seeds were stratified for 3 d at 4°C in the dark and then sown onto medium (1× Murashige and Skoog basal salt mixture, 2% sucrose, 2.5 mM MES, pH 5.7, and 0.8% agar) or in a 2:1 mixture of ProMix PGX soilless media (Premier Horticulture) and Turface calcined clay (Product Profiles) in containers (200 mL, 1 liter, or 1.7 liter) or Falcon tubes (50 mL). Plants grown on agar were maintained in a growth room (temperature: 22°C [light]/18°C [dark]; photoperiod: 16 h [light]/8 h [dark]; light intensity: ~80 μmol quanta m⁻² s⁻¹ provided by fluorescent bulbs).

Plants grown in soilless media were maintained in a controlled environment growth chamber (temperature: 22°C [light]/18°C [dark]; light intensity: ~125 μmol quanta m⁻² s⁻¹ provided by fluorescent bulbs; relative humidity: ~60%). Photorperiods for soilless media-grown plants are described in the corresponding figure legends.

**Plasmid Construction**

GTL1 promoter (GTL1pro):GUS was constructed in the pCAMBIA1303 vector (CAMBIA). The 2973-bp GTL1 promoter was amplified by designated primers (GTL1p1-F-Xmal and GTL1p1-R-NcoI) (see Supplemental Table 1 online). After the 3SS promoter in pCAMBIA1303 was removed by Xmal and NcoI digestion, the GTL1 promoter was inserted into the Xmal/NcoI site. For GTL1pro:GTL1:GFP construction, the open reading frame of GTL1 was amplified by designated primers (GTL1p1-F-Xmal and GTL1p1-R-SPel) from cDNA. The amplified GTL1 open reading frame was inserted into the Xmal and Spel sites in the pCAMBIA1302 vector (CAMBIA) after the 3SS promoter was removed and was fused to GFP in frame (pCAMBIA1302-GTL1:GFP). Then, the GTL1 promoter, amplified by designated primers (GTL1p1-F-Xmal and GTL1p1-R-Xmal), was inserted...
into the Xma site in pCAMBIA1302-GTL1-GFP. MBP-GTL1 was constructed in the pMAL-C2 vector (Kim et al., 2007). Two GTL1 fragments (Nt1 and GTL1N) were amplified by designated primers (Nt1, Nt1-F-EcoRI and Nt1-R-PstI; GTL1N, Nt1-F-EcoRI and Nt2-R-PstI). The two amplified fragments were inserted into the EcoRI and PstI sites in the pMAL-C2 vector. Sequence information for all designated primers used for plasmid construction is provided in Supplemental Table 1 online.

Physiological Analyses
Water deficit stress was imposed by withholding water from containers (1 liter) with 288.7 ± 3.1 g (dry weight) of soilless media containing 20 plants (3 weeks old). Containers were irrigated with water to saturation and weighed at the start of water deficit stress treatment (initial weight) and then periodically throughout the treatment period. Relative SWC was calculated as (final fresh weight – dry weight)/(initial weight – dry weight) × 100. After 14 d, plants were watered and survival was assessed 4 d after watering. In a separate experiment, leaf RWC of fully expanded leaves from 4-week-old plants grown in 200-mL containers at different relative SWC was assessed. Leaves were removed and immediately weighed to obtain leaf FW. Leaves were then placed into vials filled with distilled water for 24 h, blotted to remove excess water, and then weighed to obtain leaf turgid weight (TW). Leaves were then dried to a constant weight at 65°C and reweighed to obtain leaf dry weight (DW). Leaf RWC was calculated as (FW – DW)/(TW – DW) × 100.

Whole-plant transpiration of 5- to 6-week-old plants was determined by a gravimetric method. Individual plants were grown in 200-mL containers. During the period that transpiration was measured, each container was covered with a polyethylene wrap to prevent evaporation from the soil surface. An individual plant was placed onto a balance, and the weight of each container was determined every 5 min. At the end of the experiment, total leaf area was determined from photographs of excised leaves using the ImageJ program (National Institutes of Health). Transpiration rate (mmol water m⁻² s⁻¹) was calculated based on gravimetric water loss rate and leaf area data. A cubic polynomial was then fit to the smoothed data separately for the day period. The equation for the light period transpiration curve was then differentiated and solved for zero to give the time at which maximum transpiration occurs. This time was then used to calculate maximum light period transpiration rate.

Integrated WUE was calculated as final shoot dry weight divided by total water loss over a period of 6 weeks. Individual containers were covered with plastic wrap containing a central hole through which three to four seeds for each genotype were sown. Seven days after germination, containers were thinned to one seedling per container. Individual containers were weighed before and after irrigating with water every 5 to 6 d to determine moisture loss. Water loss from control containers (without plants) was subtracted from treatment containers. Shoot dry weight was determined at the end of the experiment.

Leaf gas exchange of fully expanded leaves, including transpiration, stomatal conductance, and net CO₂ assimilation, was determined using a LI-6400XT infrared gas analyzer (LI-COR Biosciences). Plants were grown in 6-cm² Li-6400 chamber. Gas exchange was measured at PAR levels of 1500, 3000, and 6000 μmol m⁻² s⁻¹. A nonrectangular hyperbola curve was fit to the net CO₂ assimilation data (Lambers et al., 1998). Quantum efficiency is the initial slope of the curve, dark respiration is the point at which the curve crosses the y axis at PAR = 0, and the light compensation point is the point at which the curve crosses the x axis (Lambers et al., 1998). VPD during measurement was ~1 kPa. Instantaneous WUE was calculated as net CO₂ assimilation rates divided by transpiration rates.

Anatomical Analysis
Abaxial epidermal anatomy of fully expanded leaves from plants that were used for leaf gas exchange analyses was characterized. Transparent cellulose adhesive tape was attached to the middle portion of the adaxial lamina, and mesophyll layers of the leaf were removed by pulling the tape so that only the abaxial epidermis remained. The abaxial epidermis was placed on a slide and images were obtained under ×100 magnification using a Nikon-OptiPhot microscope. Stomatal density (stomatal number per area), stomatal index (ratio of stomata to total epidermal cells, including stomata, stomatal precursor cells, and pavement cells), the number of stomatal precursor cells, and pavement cell density (pavement cells per area) were obtained from a leaf area of 0.069 mm². In all calculations, stomata were considered to be a pair of guard cells. Stomatal precursor cells were identified as cells at the meristemoid or GMC stage. These were not counted as stomata. The OminiGraffe Pro program was used to transform microscopy images to simplified black and white images.

Stomatal aperture was analyzed in abaxial epidermal peels from fully expanded leaves (Li et al., 2006). The epidermis was incubated in buffer solution (20 mM KCl, 5 mM MES-KOH, and 1 mM CaCl₂, pH 6.15) under ~200 μmol m⁻² s⁻¹ for 4 h. Subsequently, the epidermis was placed onto a slide and images were photographed using a Nikon-OptiPhot2 microscope (×400 magnification). The aperture width of each stomatal pore was determined from the image.

Histochemical Assay for GUS Activity
Epidermal peels from T3 transgenic plants harboring GTL1pro:GUS were incubated in a fixation solution (0.3% formaldehyde, 10 mM MES, pH 5.6, and 300 mM mannitol) for 1 h, washed with 50 mM sodium phosphate buffer, and then incubated further in staining solution (50 mM sodium phosphate buffer, 2.5 mM potassium ferricyanide, 2.5 mM potassium ferrocyanide, 0.3% Triton X-100, and 1.9 mM 5-bromo-4-chloro-3-indolyl β-D-glucoronicide) for 12 h at 37°C. Tissues were then washed several times in 70% ethanol until chlorophyll was completely extracted.

Fluorescence Microscopy for GFP Imaging
Subcellular localization of GTL1-GFP fusion protein in the abaxial epidermis of 4-week-old transgenic plants harboring GTL1pro:GTL1-GFP was determined. A Nikon-OptiPho2 microscope with epifluorescence was used to detect GFP expression using a B1E filter (excitation 470 to 490 nm; dichroic mirror 515 nm; barrier filter 520 to 560 nm). Images were photographed with a digital camera.

Purification of MBP-GTL1 Fusion Proteins
MBP-GTL1 fusion protein was expressed in BL21 Escherichia coli cells after induction with 0.1 mM of isopropyl β-D-thiogalactopyranoside and incubation for 2 h at 30°C. The culture was harvested by centrifugation for 5 min at 5000g. The pellet was resuspended in 50 mL of ice-cold MBP resuspension buffer (10 mM Tris-Cl, pH 7.5, 30 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1× Protease inhibitor cocktail), sonicated with a 550 Sonic Dismembrator (Fisher Scientific), and then centrifuged for 20 min at 14,000g. The supernatant was incubated with prewashed amylase resin (NEB) for 2 h at 4°C and washed five times with buffer (10 mM Tris-Cl, pH 7.5, and 1 M NaCl). MBP-GTL1 protein was eluted with buffer (100 mM Tris-Cl, pH 7.5, and 10 mM maltose). The eluate was collected with a centrifugal filter (Amicon Ultra 50K; Millipore) for buffer exchange according to the manufacturer’s protocol. The fusion protein was suspended in buffer (100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1% Triton X-100, and 10% glycerol).
EMSA

MBP-GTL1 fusion proteins purified on amylose resin were used to determine DNA binding by EMSA. Single-stranded complementary oligonucleotide fragments corresponding to regions of Arabidopsis SDD1 or rice (Oryza sativa) PHYA promoters (see Supplemental Table 1 online) that included the GT elements were synthesized (Macrogen) and biotinylated using the Biotin 3’-end DNA labeling kit (Thermo Fisher Scientific). Biotinylated complementary oligonucleotide pairs were annealed to make double-stranded and biotin-labeled probes (100 ng µL⁻¹) by mixing in a buffer (10 mM Tris and 1 mM EDTA) boiling for 5 min, and cooling slowly overnight. Unlabeled complementary oligonucleotide pairs were also annealed to make double-stranded competitor probes (500 ng µL⁻¹). EMSA reaction solutions were prepared by adding the following components in order according to the manufacturer’s protocol (LightShift Chemiluminescent EMSA kit; Thermo Fisher Scientific): 1× binding buffer, 50 ng poly (di-dC), 2.5% glycerol, 0.06% Nonidet P-40, 5 mM MgCl₂, 19 µg BSA, proteins, competitor, noncompetitor, and biotin-labeled probes. Reaction solutions were incubated for 20 min at room temperature. The protein-probes mixture was separated in a 6% polyacrylamide native gel and transferred to a Biodyne B Nylon membrane (Thermo Fisher Scientific). Migration of biotin-labeled probes was detected on x-ray film using streptavidin-horseradish peroxidase conjugate (Thermo Fisher Scientific). The membrane was exposed to a chemiluminescent enhanced chemiluminescence Plus protein gel blotting detection system (GE Healthcare).

Genomic DNA Extraction, RNA Extraction, and RT-PCR Analysis

Genomic DNA from the leaves of 3-week-old plants was extracted by the cetyltrimethyl-ammonium bromide (CTAB) method (Richards et al., 2001) and used to determine homozygosity of T-DNA insertion in gtl1-4 and gtl1-5 by PCR analysis using GTL1-specific primers and a T-DNA–specific primer (see Supplemental Table 1 online). Total RNA was extracted from shoots of 4-week-old plants or fully expanded leaves of 8-week-old plants using TRIzol (Invitrogen) according to the manufacturer’s protocol. One microgram of total RNA was used to synthesize cDNA by the ThermoScript RT-PCR system for first-strand cDNA synthesis (Invitrogen). The same amount of cDNA was used for PCR analysis using primers for UBC (26 or 28 cycles) or ACT2 (28 cycles; internal control), GTL1 (35, 28, or 25 cycles for Figure 1C, Supplemental Figure 2A online, and Supplemental Figure 2B online, respectively), Cor15a (28 cycles), stomatal development genes including SDD1 (40 cycles), MPK3 (28 cycles), and MPK6 (28 cycles) (see Supplemental Table 1 online).

Statistical Analysis

All bar graphs were analyzed by Student’s t-test for pairwise comparison.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: GTL1 (At1g33240; NM_103052) and SDD1 (At1g04110; NM_100292).

Supplemental Data

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

Supplemental Figure 1. gtl1 Plants Were Less Wilted in Response to Water Deficit Stress.

Supplemental Figure 2. GTL1 Expression Is Downregulated by Water Stress and Dehydration.

Supplemental Figure 3. gtl1 Plants Have Reduced Light Period Transpiration Rates under Both Well-Watered and Water Deficit Stress Conditions.

Supplemental Figure 4. Leaf Morphology and Area of gtl1 Plants Are Similar to Those of Wild-Type Plants.

Supplemental Figure 5. Quantum Efficiency, Dark Respiration, and Light Compensation Point Were Similar Among Genotypes.

Supplemental Figure 6. gtl1 Plants Exhibited Similar Stomatal Aperture, and Transpiration, Germination, and Root Growth Were Similarly Affected by ABA in Wild-Type and gtl1 Plants.

Supplemental Figure 7. gtl1 Plants Have Increased Trichome Branch Length.

Supplemental Figure 8. GTL1 Does Not Regulate The Expression of TMM, ER, and YDA.

Supplemental Figure 9. The N-Terminal DNA Binding Domain of GTL1 Binds to Three Types of GT Elements.

Supplemental Figure 10. Stomatal Development Pathways Include a Basal Pathway and a Negative Regulatory Pathway for Fine-Tuning of Stomatal Development in Epidermal Cells.
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Supplemental Table 1. Sequence Information Used for Plasmid Construction, RT-PCR, ChiP Analyses, and EMSA.

Supplemental References.

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The *Arabidopsis* GTL1 Transcription Factor Regulates Water Use Efficiency and Drought Tolerance by Modulating Stomatal Density via Transrepression of *SDD1*

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