The Arabidopsis Tetratricopeptide Repeat-Containing Protein TTL1 Is Required for Osmotic Stress Responses and Abscisic Acid Sensitivity[1][W]

Abel Rosado, Arnaldo L. Schapire, Ray A. Bressan, Antoine L. Harfouche, Paul M. Hasegawa, Victoriano Valpuesta, and Miguel A. Botella*

Departamento de Biología Molecular y Bioquímica Universidad de Málaga, 29010 Málaga, Spain (A.R., A.L.S., V.V., M.A.B.); and the Center for Plant Environmental Stress Physiology, Purdue University, West Lafayette, Indiana 47907–2010 (R.A.B., A.L.H., P.M.H.)

Mutations in the Arabidopsis (*Arabidopsis thaliana*) *TETRATRICOPEPTIDE-REPEAT THIOREDOXIN-LIKE 1* (*TTL1*) cause reduced tolerance to NaCl and osmotic stress that is characterized by reduced root elongation, disorganization of the root meristem, and impaired osmotic responses during germination and seedling development. Expression analyses of genes involved in abscisic acid (ABA) biosynthesis and catabolism suggest that *TTL1* is not involved in the regulation of ABA levels but is required for ABA-regulated responses. *TTL1* regulates the transcript levels of several dehydration-responsive genes, such as the transcription factor *DREB2A*, and genes encoding dehydration response proteins, such as *ERD1* (early response to dehydration 1), *ERD3*, and *COR15a*. The *TTL1* gene encodes a novel plant protein with tetratricopeptide repeats and a region with homology to thioredoxin proteins. Based on homology searches, there are four *TTL* members in the Arabidopsis genome with similar intron-exon structure and conserved amino acid domains. Proteins containing tetratricopeptide repeat motifs act as scaffold-forming multiprotein complexes and are emerging as essential elements for plant hormonal responses (such as gibberellin responses and ethylene biosynthesis). In this report, we identify *TTL1* as a positive regulator of ABA signaling during germination and seedling development under stress.

Drought and salinity are the two most substantial adverse environmental factors encountered by land plants (Boyer, 1982; Bohnert et al., 1995). Water deficit caused by drought and high salinity has been a major selective force in plant evolution as well as a constraint to crop productivity, limiting food production (Zhu, 2002). To cope with these environmental stresses, plants respond by initiating a number of physiological and metabolic adaptive processes where abscisic acid (ABA) is a key regulatory determinant (for review, see Hasegawa et al., 2000; Chinnusamy et al., 2004; Botella et al., 2005; Riera et al., 2005; Verslues and Zhu, 2005). In addition to functioning as a mediator of stress responses, ABA regulates other processes, such as seed maturation and germination (Finkelstein et al., 2002), stomatal conductance (Schroeder et al., 2001), plant growth (Sharp and LeNoble, 2002), osmolyte accumulation (Verslues and Bray, 2006), and gene expression (Bray, 2002; Shinozaki et al., 2003). Although the regulatory pathways that modulate these different processes share proteins and signaling intermediates, such as phospholipases, cADP-Rib, inositol 1,4,5-trisphosphate, and calcium ions (for review, see Himmelbach et al., 2003), our knowledge of separate yet overlapping ABA and stress signal transduction pathways is fragmentary (Verslues and Zhu, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006). In this context, the mechanism by which ABA regulates multiple plant stress responses is beginning to be revealed through genetic and physiological analyses in Arabidopsis (*Arabidopsis thaliana*; Chinnusamy et al., 2004; Verslues and Zhu, 2005). Genetic screens for ABA-hypersensitive mutants have led to the understanding that processes including inositol 1,4,5-triphosphate dephosphorylation (*fry1*; Xiong et al., 2001a), farnesylation (*era1*; Cutler et al., 1996), and RNA metabolism (*hyl1*: Lu and Fedoroff, 2000; *abh1*: Hugouvieux et al., 2001; *sad1*: Xiong et al., 2001b; *cpl1* and *cpl2*: Koiwa et al., 2002) are required to modulate ABA signaling. However, only a few recessive mutations in nontranscription factor genes have been identified to cause ABA insensitivity, known as *GCA2* for guard cell associated (Himmelbach et al., 1998), *GPA1* for the G-protein α-subunit (Wang et al., 2001), *RCN1* for protein phosphatase type 2A subunit (Kwak et al., 2002), *OST1/SnRK2E* for protein kinase (Mustilli et al., 2002), *AtRBOHD/F* for NADPH oxidase (Kwak et al., 2003), *RPK1* for receptor-like kinase (Osakabe et al., 2005), and *ABI8*, a protein without known biochemical function (Brocard-Gifford et al., 2004), suggesting that several positive regulators of the ABA signaling

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* Corresponding author; e-mail mabotella@uma.es; fax 34–952–13–42–67.

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network are still not identified, probably due to genetic redundancy (Kwak et al., 2003; Kuhn et al., 2006).

Proteins containing tetratricopeptide repeat (TPR) motifs have been identified in all kingdoms and mediate specific interactions with partner proteins, either forming active multiprotein complexes or acting as cochaperones involved in the folding of a growing set of substrates (D'Andrea and Regan, 2003; Davies and Sánchez, 2005). Recently, TPR proteins have been found to be involved in plant hormonal regulation, such as ethylene biosynthesis (Yoshida et al., 2005) and GA and cytokinin responses (Greenboim-Wainberg et al., 2005). In Arabidopsis, ETHYLENE-OVERPRODUCER1 (ETO1), a novel plant-specific BTB/TPR protein, negatively regulates ethylene biosynthesis in seedlings through direct interaction of its TPR domains with a 1-aminocyclopropane-1-carboxylate synthase (Wang et al., 2004; Yoshida et al., 2005). The *spindly* (*spy*) mutant was selected because of the capacity to germinate in the presence of paclobutrazol (PAC), an inhibitor of GA biosynthesis (Jacobsen and Olszewski, 1993). The SPY sequence contains TPR domains in its N terminus, and the C-terminus sequence shows high homology to Ser/Thr *O*-linked GlcNAc transferases from animals (Jacobsen et al., 1996; Roos and Hanover, 2000). The TPR domains of SPY physically interact with two transcription factors to form complexes that act as negative regulators in the signaling pathway for GA response (Robertson, 2004).

In this report, we present evidence that a novel TPR-containing protein, TETRATRICOPEPTIDE-REPEAT THIOREDOXIN-LIKE 1 (TTL1), functions in the regulation of ABA and dehydration signaling pathways and also in several salt/osmotic stress responses in both seeds and seedlings. The function of *TTL1* is likely based on multiprotein complexes implicated in the regulation of ABA signaling and abiotic stress responses.

RESULTS

Identification of the Salt-Sensitive *ttl1-1* Mutant in a C24 T-DNA-Insertion Population

A forward genetic screen of more than 96,500 independent T_2 seedlings identified novel salt tolerance determinants (Wu et al., 1996). To select mutations with altered ionic and/or osmotic stress responses, the root gravitropic bending assay was modified by increasing the NaCl concentration of the medium to 160 mM (Koiwa et al., 2006). As a result, we identified the mutant *ttl1-1* that exhibited NaCl hypersensitivity in root elongation but similar growth to that of the wild type in conditions without stress (Fig. 1A).

NaCl-Hypersensitive Phenotype Is Due to the Loss of Function of the Tetratricopeptide Thioredoxin-Like Gene *TTL1*

The *tt11-1* mutant was backcrossed to wild type (*ttl1-1* × C24), and F_1 progeny (*n* approximately 45) exhibited wild-type response to NaCl stress. Analysis of F_2 seedlings (1,051 from 10 F_1 parental lines) revealed that *ttl1-1* is a recessive mutation in a single nuclear locus; 780:271, wild type:mutant, $\chi^2 = 13.1$, $P < 0.001$ for a 3:1 ratio (Table I). Bialaphos resistance also segregated as a single dominant locus; 840 F_2 seedlings; 616:224, resistant:sensitive, $\chi^2 = 4.2$, $P < 0.005$ (Table I).

The genomic DNA flanking the left border of the T-DNA in *ttl1-1* was amplified by thermal asymmetric interlaced PCR, as described previously (Liu and Whittier, 1995; Koiwa et al., 2002). As a result, we identified an insertion located at nucleotide 80,319 in the bacterial artificial chromosome F12M16 of Arabidopsis chromosome 1. Thus, in the *ttl1-1* allele, the T-DNA is located 1,305 bp downstream of the ATG translation start site in the fourth exon of *At1g53300* (Fig. 2A). Forty-eight randomly selected NaCl-sensitive F_2 seedlings were all homozygous for the T-DNA insertion in *TTL1* based on genotypic analysis (Table I), indicating a tight genetic linkage between the NaCl-sensitive phenotype and *ttl1-1*. Two additional T-DNA alleles (*ttl1-2* and *ttl1-3*) were identified in SALK_063943 and SALK_075089 populations that are in the Columbia-0 ecotype (Col-0) genetic background (Fig. 2A). Homozygous *ttl1-2* and *ttl1-3* seedlings exhibited similar NaCl-sensitive root phenotypes to that of *ttl1-1* (Fig. 1, A and B). However, in contrast to *ttl1-1*, salt-stressed *ttl1-2* and *ttl1-3* seedlings accumulated more anthocyanins in the shoots than Col-0 (wild-type) seedlings, a characteristic stress symptom commonly observed in salt-sensitive mutants in the Col-0 background (Liu and Zhu, 1997; Rus et al., 2001; Miura et al., 2005). Using primers corresponding to sequences flanking the T-DNA insertions, *TTL1* transcripts were detected by reverse transcription (RT)-PCR in wild type but not in *ttl1-1*, *ttl-2*, or *ttl1-3* seedlings (Fig. 2C). These results confirm that salt sensitivity is linked to dysfunctional *ttl1* alleles, indicating that the protein encoded by *At1g53300* is involved in the plant response to NaCl.

TTL Family Proteins Are Specific to the Plant Kingdom

At1g53300 full-length cDNA encodes a 699-amino acid protein that is predicted to be basic, cytosolically localized, and with no transmembrane domains. The encoded protein is a member of a novel protein family unique to plants. It contains six predicted TPR motifs arranged in two TPR domains and a motif in the C terminus with homology to thioredoxins (TRXL for thioredoxin-like; Fig. 2B). In addition to *TTL1*, bioinformatic analysis predicts three other *TTL* genes in Arabidopsis, *At3g14950* (*TTL2*), *At2g42580* (*TTL3*), and *At3g58620* (*TTL4*), which display 62%, 53%, and 50% amino acid sequence identity, respectively, to TTL1.

The ClustalW sequence alignment (Thompson et al., 1997) followed by the PHYLIP algorithm (Felsenstein, 2005) generate a phylogram that groups the Arabidopsis TTL family members in the same cluster (Fig. 3). The phylogenetic analyses indicate that TTL1 and

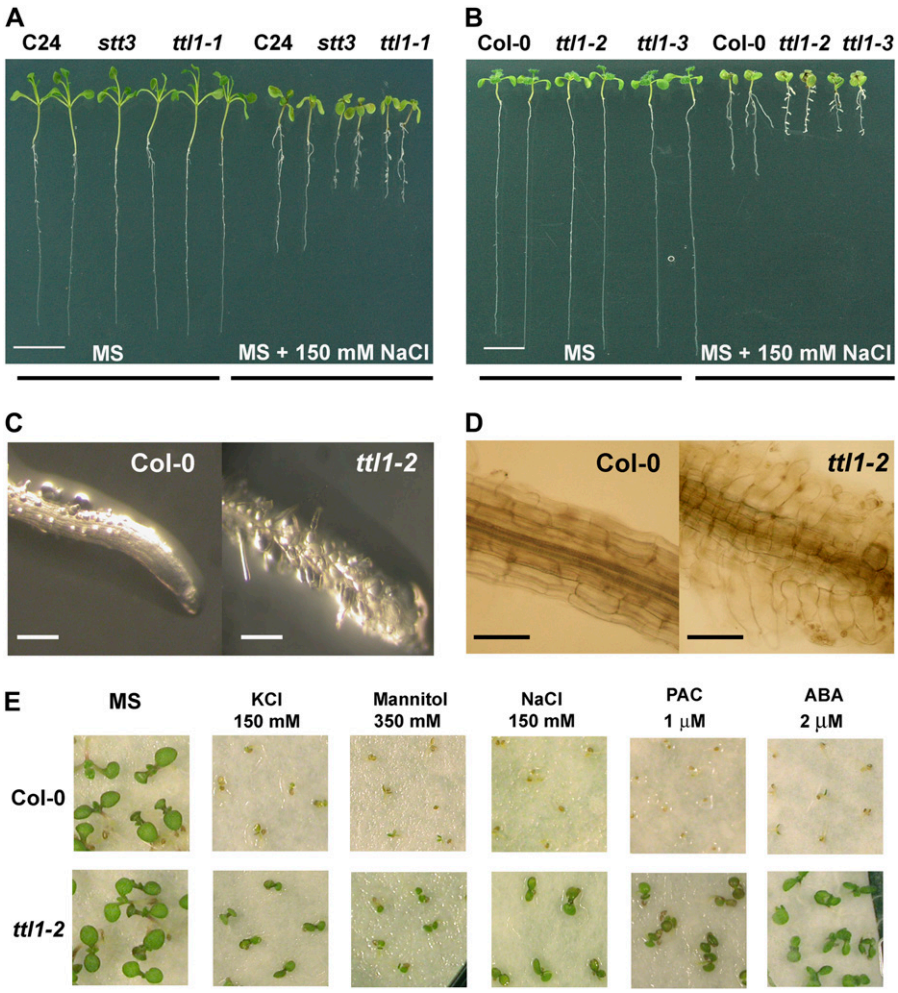

Figure 1. *ttl1* alleles shows hypersensitivity to NaCl in root elongation and increased germination rates under osmotic stress and exogenous ABA treatments. A, Photographs of seedlings that were grown on MS agar medium for 1 week and then transferred to MS agar medium for 8 additional days without (left) or with (right) 150 mM NaCl: wild type (C24), *stt3a* (positive control), and *ttl1-1*. B, Seedlings of *ttl1-2* and *ttl1-3* also show NaCl hypersensitivity compared to their respective wild-type control Col-0. Bar = 10 mm. C and D, Root tip morphology of wild-type and *ttl1* 1-week-old seedlings after treatment with 300 mM mannitol during 72 h (C) and the phenotype observed using Nomarski (D). Bars are 200 and 40 μm, respectively. E, Germination phenotypes of wild-type Col-0 and *ttl1-2* seeds sown on paper soaked with liquid MS media supplemented with NaCl (150 mM), KCl (150 mM), mannitol (350 mM), PAC (2 μM), or ABA (2 μM). Plates were placed under long-day photoperiod in a growth chamber for a minimum of 20 d. Two replicates were made for each treatment with similar results (n = 100). Photographs were taken 10 d after sowing.

TTL2 belong to a different clade than TTL3 and TTL4, and each clade evolved from a common ancestor. The TTL family is clustered most closely to four hypothetical proteins in rice (*Oryza sativa*) that contain both TPR and TRLX motifs (Fig. 3). Three related clusters from Arabidopsis, yeast (*Saccharomyces cerevisiae*), and animals (the latter with known chaperone function) are included in the phylogram (Fig. 3) and correspond to TPR-containing proteins without the TRXL motif. The closest related protein to TTL1 with established function is TPR2 from *Drosophila melanogaster* (CG4599), a co-chaperone involved in the suppression of polyglutamine toxicity (Kazemi-Esfarjani and Benzer, 2002). Finally, INTERPRO analyses (http://www.ebi.ac.uk/interpro) indicate that all the Arabidopsis proteins included in the cladogram are related with the mitochondrial protein translocase (MPT) family in yeast. The MPT complex in yeast is composed of chaperones that facilitate translocation of nuclear encoded preproteins into mitochondria (Wiedemann et al.,

Table I. *Genetic analysis of the ttl1 mutant*

Cross	Phenotype						Genotype[a]		
	Generation	Plant Tested	Wild Type[b]	Mutant[b]	Resistant[c]	Sensitive[c]	Homo	Hetero	Wild Type
ttl1 × C24	F_1	45	45	0					
	F_2	1,051	780	271					
		840[d]			616	224			
		48[e]	0	48	48	0	48	0	0
		48[e]	48	0	30	18	0	30	18

[a]Genotypes were determined by diagnostic PCR. [b]Mutant and wild-type phenotypes were determined using the root elongation assay with 160 mM NaCl. [c]Resistance or sensitivity was determined by either spraying the plants with bialaphos solution or with bialaphos treatments in vitro. [d]These seedlings were scored only for bialaphos resistance in vitro to estimate the T-DNA copy number. [e]These plants were selected from a root elongation assay with 160 mM NaCl, transferred to soil, and sprayed with bialaphos solution.

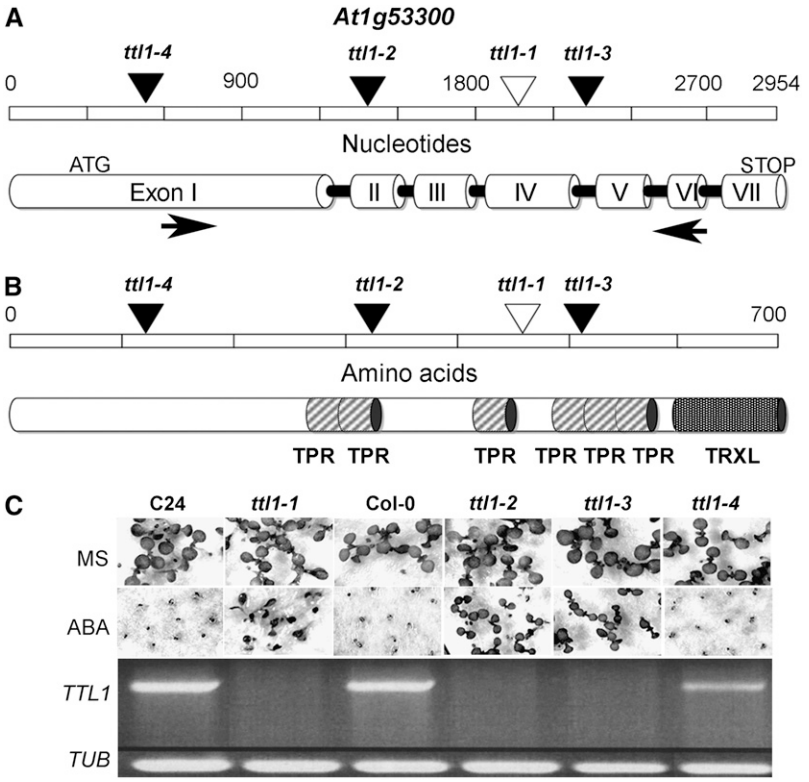

Figure 2. Localization of the T-DNA insertions and motif prediction within the *At1g53300* locus. A, Exon-intron organization and schematic representation of T-DNA insertions in the *At1g53300* gene. White and black boxes correspond to exons and introns, respectively. The white triangle corresponds to the insertion in C24 background and black triangles correspond to insertions in Col-0 background. B, Motif predictions using PROSITE and PFAM in the deduced amino acidic sequence of *At1g53300*. The light gray and the dark gray correspond to TPR and TRXL motifs, respectively. C, *TTL1* transcripts are detected in wild type (Col-0 and C24 backgrounds) and *ttl1-4* but not in *ttl1-1*, *ttl1-2*, and *ttl1-3* seedlings using RT-PCR. The position of the oligonucleotides used for the RT-PCR is indicated with black arrows in A. Pictures show the germination phenotypes of the different alleles in media containing 2 μM ABA. Photographs were taken 10 d after sowing.

2003). However, nothing is known about the function of the TTLs and the other MPT-related proteins in Arabidopsis.

Loss of Function of *TTL1* Causes Osmotic Stress Sensitivity in Seedling Root Elongation But Confers Resistance in Germination

The *ttl1-2* (from now *ttl1*) seedling hypersensitivity to ionic or osmotic stress was assessed by quantifying root growth in different media. The *ttl1* seedlings' root growth was sensitive to both NaCl and KCl (Fig. 4, A and B) but not to LiCl (Fig. 4C). Li^+ is a more toxic analog of Na^+ that at the concentrations assayed does not contribute to a significant increase in medium osmotic potential (Borsani et al., 2001; Rubio et al., 2004). The *ttl1* seedlings were also hypersensitive to mannitol (Fig. 4D) and Suc (Fig. 4E) at concentrations being near iso-osmotic to those of NaCl and KCl that caused hypersensitivity. Similar results were obtained with *ttl1-1* seedlings (data not shown), indicating that *TTL1* regulates osmotic but not ionic stress responses.

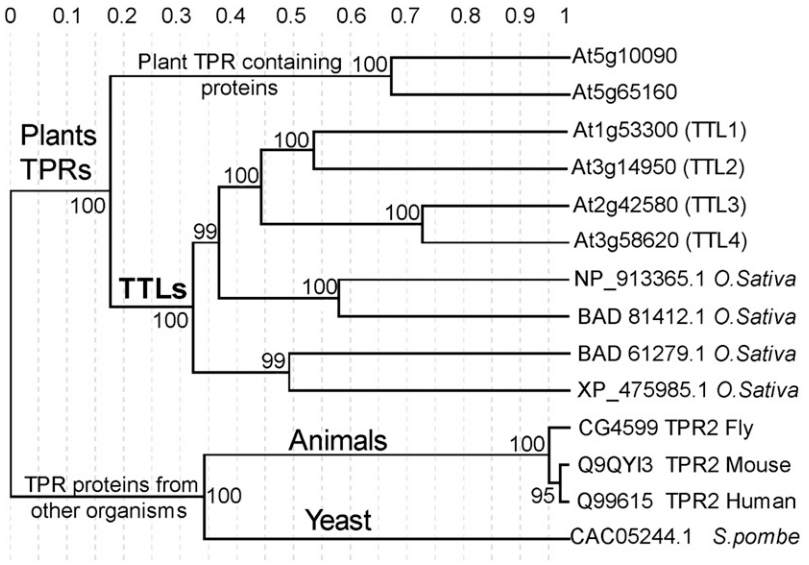

Figure 3. TTL1 belongs to a novel family of proteins composed of four members in the Arabidopsis genome. The phylogram was generated using the PHYLIP algorithm and shows the phylogenetic relationships between TTL1 and closely related proteins from different organisms. TTL1 is grouped in a cluster formed by four Arabidopsis proteins with unknown function, which are closely related with a second cluster formed by four putative orthologous in rice. AGI accession numbers for Arabidopsis sequences and GenBank accession numbers for the rest of the sequences are indicated. Bootstrap values are indicated in each node.

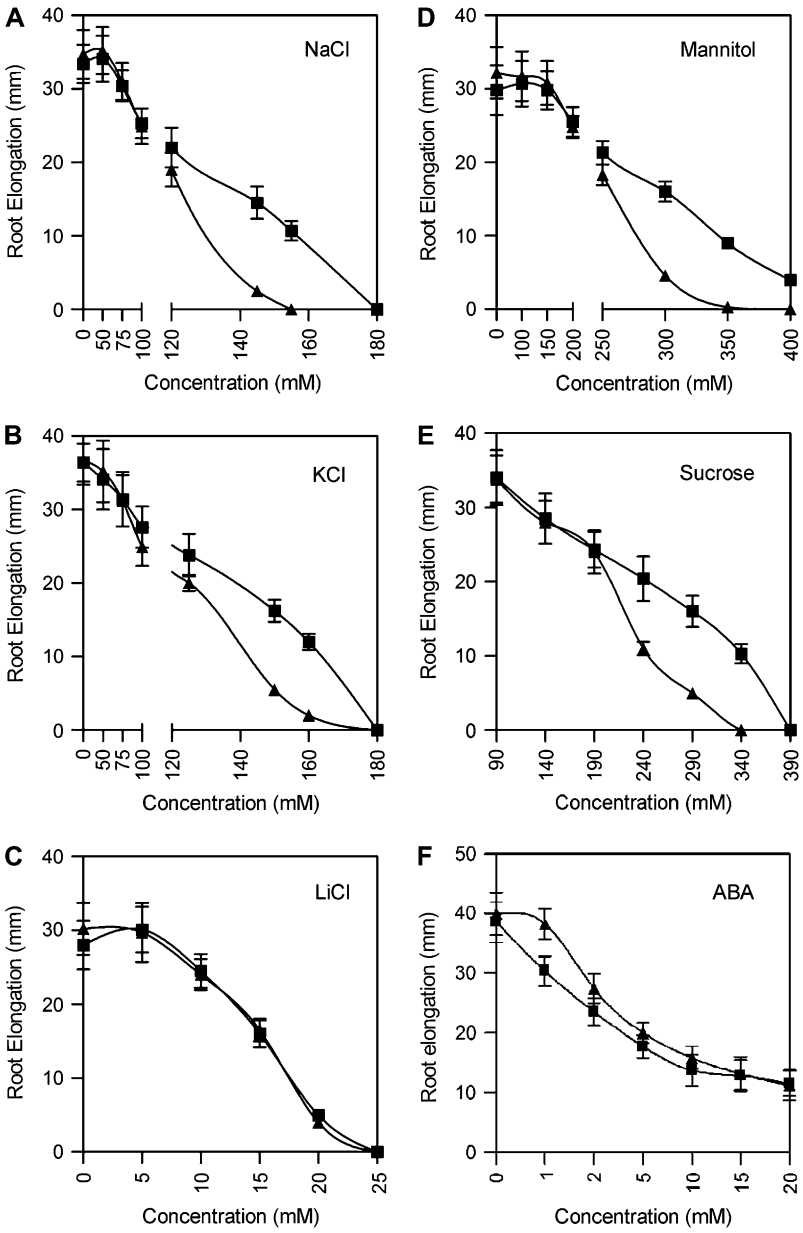

Figure 4. *ttl1* seedlings show root elongation inhibition under osmotic stress. Root elongation of wild type and *ttl1* was measured to quantify their sensitivities to several stress agents. Seedlings were grown on MS agar medium for 1 week and then transferred to MS medium with or without various concentrations of NaCl (A), KCl (B), LiCl (C), mannitol (D), Suc (E), or ABA (F). Root elongation (i.e. increase in length after transfer) was determined after 7 d in C24 or after 5 d in Col-0. Error bars indicate SES (n = 15). The experiments were repeated at least three times with similar results. Graphs correspond to one representative experiment.

Plants harboring *ttl1-1*, *ttl1-2*, or *ttl1-3* alleles were indistinguishable from the respective wild types throughout the plant life cycle when growing under standard conditions (150 μmol m^{-2} s^{-1}, 23°C, and 60% relative humidity) either in short or long days (data not shown).

Other phenotypic features, including enhanced lateral root development and root tip swelling, were associated with salt/osmotic sensitivity of *ttl1* seedlings (Fig. 1, A–D). The absence of primary root elongation after transfer to fresh medium indicated that the *ttl1*-terminal meristem is dysfunctional and not just arrested, which may contribute to lateral root development. Microscopic analysis of *ttl1* swollen root tips indicated that the terminal meristem is disorganized. The cell number in the elongation zone of *ttl1* roots is similar to that of the control, but the cells are more expanded and irregular in shape (Fig. 1D). Root tip swelling of *ttl1* seedlings induced by osmotic stress resembles that caused by irregular cell expansion of *sos5* seedlings (Shi et al., 2003) or differential mitotic activity of *stt3a* seedlings in response to NaCl stress (Koiwa et al., 2003).

Because plants at different stages of development show different degrees of sensitivity to environmental stresses (Wu et al., 1996), we determined whether germination of *ttl1* was also altered in response to different solutes (Fig. 1E). Interestingly, the *ttl1* seed germination rate and seedling development were higher than wild type at high KCl and NaCl concentrations and iso-osmotic concentration of mannitol (Fig. 1E). Cotyledon expansion and chlorophyll synthesis were completely abolished for wild type but not

for *ttl1* seedlings in response to hyperosmolarity (Fig. 1E). The germination and seedling growth results indicate that *ttl1* is impaired in osmotic stress responses at early stages of plant development.

TTL1 Specifically Regulates ABA Responses

ABA is a key hormone in the regulation of osmotic stress responses (Yamaguchi-Shinozaki and Shinozaki, 2006). Next, we determined whether germination, cotyledon expansion, and root elongation of *ttl1* are altered by ABA (Figs. 1E, 4F, and 5A). Radicle emergence of wild-type seeds was observed 5 d after sowing in medium containing 2 μM ABA. However, cotyledons did not develop in medium supplemented with ABA (Fig. 1E). In contrast, *ttl1* seedlings showed radicle emergence and expanded green cotyledons in the presence of ABA (Figs. 1E and 5A). This result suggests that the impaired responses of *ttl1* to osmotic stress at early stages of development could be due to an altered response to ABA in this mutant. Interestingly, despite the clear differences in ABA sensitivity during germination observed for *ttl1*, only slight differences were found in root growth when exogenous ABA was applied (Fig. 4F).

GA, an antagonist of ABA signaling in many respects, promotes seed germination. Therefore, mutants that show altered seed germination in response to ABA are often affected in GA sensitivity (Koornneef et al., 1998; Steber et al., 1998). The *ttl1* seed germination and seedling development exhibited reduced sensitivity to PAC, an inhibitor of GA biosynthesis (Figs. 1E and 5B).

We determined whether the responses of *ttl1* to other hormones were also affected. The *ttl1* mutant did not show altered plant responses to naphthaleneacetic acid (NAA), benzyladenine, epibrassinolide (BR), or the

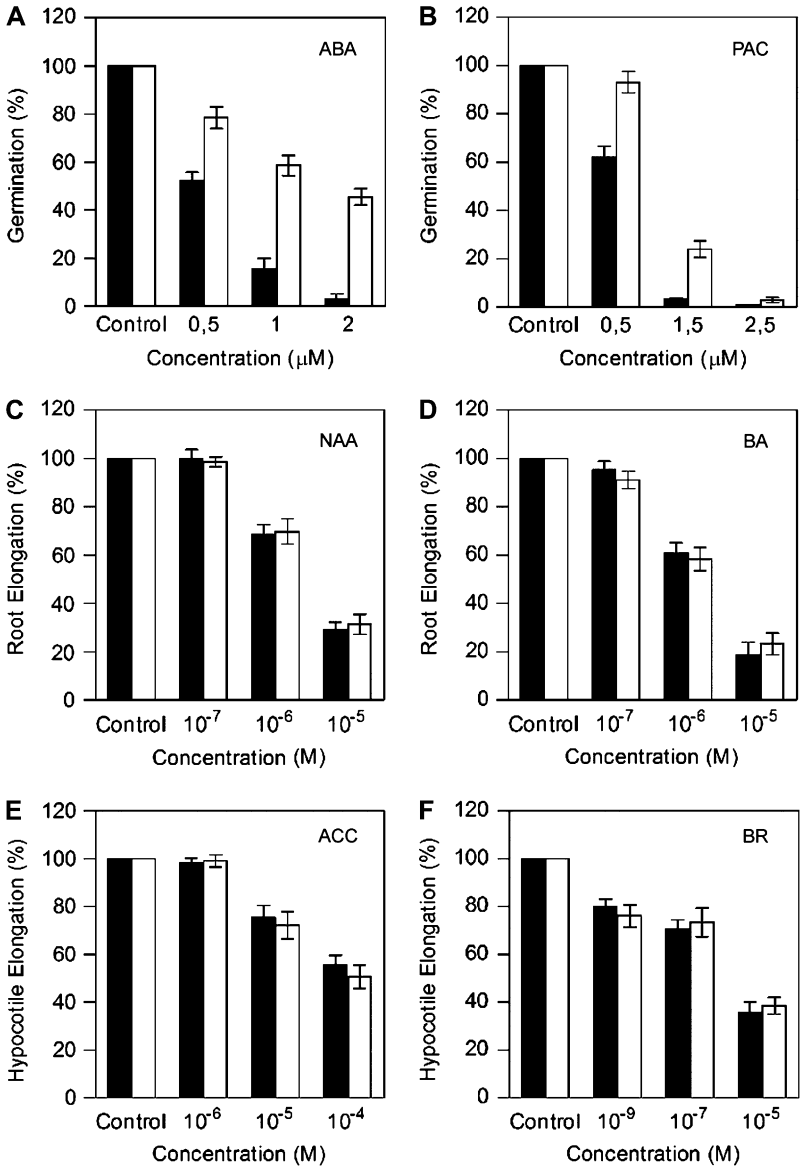

Figure 5. *ttl1* is specifically affected in ABA responses. Growth and germination responses of wild type (black bars) and *ttl1* (white bars) were analyzed for different plant hormones. The hormones analyzed were ABA (A), GA_3 using the GA biosynthesis inhibitor PAC (B), auxin (NAA; C), cytokinin (BA; D), ethylene (using its precursor ACC; E), and BR (F). Inhibition of root and hypocotyl growth is expressed relative to the mean growth without hormones. Error bars indicate SES (n = 15 for root and hypocotyl elongation, n = 100 for germination). Data was collected 5 d after treatments were applied. Two replicates were made for each treatment with similar results and graphs correspond to one representative experiment.

ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC; Fig. 5, C–F). These analyses suggest that *TTL1* is not involved in the auxin, cytokinin, brassinosteroid, or ethylene responses and are consistent with a specific role in ABA signaling.

TTL1 Is Expressed in Developing Tissues and Its Expression Is Regulated by Stress

We studied the expression profile of *TTL1* by histochemical β-glucuronidase (GUS) staining of Arabidopsis plants transformed with the *GUS* reporter gene fused to the *TTL1* promoter (Fig. 6). The AGRIS program (Davuluri et al., 2003) predicted that ABA and dehydration regulatory elements were located within the 2-kb *TTL1* promoter fragment used for the analysis (data not shown). *GUS* expression analysis of five independent T_3 homozygous promoter *TTL1*::*GUS* transgenic lines indicated that *TTL1* expression was predominant in the root elongation zone, stele, and root cap (Fig. 6, A–D), embryo vascular system (Fig. 6F), leaf axilar buds (Fig. 6H), silique abscission zone (Fig. 6I), and guard cells (Fig. 6J). GUS expression was greater in developing than in mature tissues and organs (Fig. 6, A–F). This expression pattern is consistent with the microarray tissue expression data of *At1g53300* provided by GENEVESTIGATOR (Zimmermann et al., 2004) and compiled in the electronic fluorescent protein representations based on the map of Arabidopsis development (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Schmid et al., 2005).

Because the *ttl1* mutant showed root elongation inhibition under osmotic stress, GUS expression of 2-week-old transgenic seedlings was assessed in response to mannitol. *TTL1* transcript levels were greater in root but not in shoot when compared with untreated seedlings (data not shown). Then, we analyzed the level of *TTL1* transcripts in 2-week-old wild-type seedlings after NaCl and ABA treatments using semiquantitative RT-PCR (Fig. 6K). *TTL1* transcripts appear in control conditions, and *TTL1* transcript levels are increased in 2-week-old seedlings 3 h after treatment with NaCl or ABA (Fig. 6K). This also points to a possible involvement of ABA in the induction of *TTL1* expression after osmotic stress.

ABA is indispensable for the maintenance of plant water status triggering stomatal pore closure in response to water deficit (Leung and Giraudat, 1998; Schroeder et al., 2001; Mishra et al., 2006). As shown previously, *TTL1* expression is up-regulated by osmotic stress and ABA and is substantial in guard cells (Fig. 6, J and K). In addition, *ttl1* root growth was sensitive to osmotic stress (Fig. 4) and insensitive to ABA in germination (Fig. 1E). Therefore, we determined whether *ttl1* exhibited differences in water loss in detached 4-week-old shoots compared to wild-type plants grown side by side. No difference between wild-type and *ttl1* plants was observed (Supplemental Fig. S1A). When water loss was evaluated after spraying with 100 μM ABA, it was equally reduced in

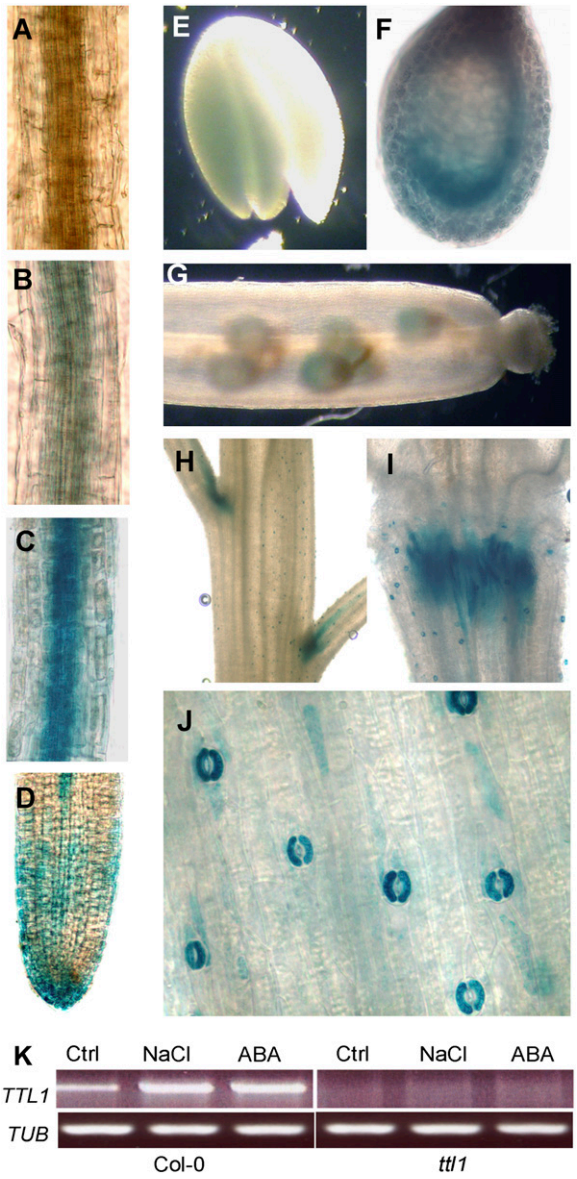

Figure 6. *TTL1* promoter-GUS expression is high in developing tissues, and *TTL1* transcript abundance is regulated by ABA and osmotic stress. A, Histochemical localization of GUS activity in transgenic plants carrying the *TTL1-GUS-GFP*. Five independent T_3 homozygous lines for the construct were grown on MS agar medium for 1 week for seedling staining or grown in soil in a cabinet under long days for adult plant staining. GUS expression was analyzed in three independent plants per line with very similar expression patterns. A to J, Root (5 cm from the apex; A), root (3 cm from the apex; B), elongation zone of the root (1 cm from the apex; C), root tip (D), embryo (E), developing seed (F), silique (G), leaf axil buds (H), silique abscission zone (I), and stem showing GUS staining in guard cells (J). K, Transcript levels of *TTL1* analyzed by semiquantitative RT-PCR. Total RNA was isolated from 10-d-old wild-type (Col-0) and *ttl1* seedlings treated with water, 100 μM ABA, or 300 mM NaCl for 3 h. *β-Tubuline* (*TUB*) expression was used as a control. Two replicates were made for each treatment with similar results.

wild-type and *ttl1* plants (Supplemental Fig. S1B) as indicated by the increased t_{50} (time to lose 50% of the fresh weight). These results indicate that guard cells of both wild-type and *ttl1* plants responded similarly to ABA-mediated stomatal closure. This means that either *TTL1* is not in the ABA signaling pathway for stomatal closure or that redundancy of other *TTL* family members substitute for *TTL1* function in the mutant.

TTL1 Regulates the Expression of a Subset of Dehydration-Responsive Genes

To gain insight into the role of *TTL1* in signaling pathways in response to osmotic stress and ABA, the expression of genes encoding enzymes for ABA biosynthesis and catabolism was analyzed in wild type and *ttl1* (Nambara and Marion-Poll, 2005). The biosynthetic genes assayed were those encoding for zeaxanthin epoxidase, which catalyzes the first committed step in ABA biosynthesis (zeaxanthin to violaxanthin); NCED3 (9-cis-epoxycarotenoid dioxygenase), which cleaves cis-xanthophylls, an intermediate step in ABA biosynthesis; and abscisic aldehyde oxidase, which catalyzes the abscisic aldehyde oxidation to ABA, the last step in biosynthesis. The catabolic gene was that encoding for CYP707A4 (cytochrome P450 monooxygenase), responsible for ABA 8′-hydroxylation, which is the predicted first step in the predominant pathway for ABA catabolism (Cutler and Krochko, 1999). Our studies by RT-PCR showed no differences between wild type and *ttl1* in the expression of these genes after ABA treatment (Fig. 7A). This result suggests that *TTL1* is not involved in the regulation of ABA biosynthesis or catabolism.

We further analyzed the expression of other genes regulated by ABA and abiotic stress. We selected several genes induced by different stresses (summarized in Supplemental Table S1), and transcript levels were also analyzed using semiquantitative RT-PCR, both before and after 3 h of ABA treatment. No clear differences were observed between wild type and *ttl1* in the expression of either general stress-responsive genes, such as *RD29A*, *RD22*, *ABF1*, or *ICK1* (Fig. 7C), or *ABI* genes (Fig. 7B). This indicates that *TTL1*, if located in any of the *ABI* pathways, is likely to act downstream of these genes. In contrast, ABA-induced expression was different in *ttl1* seedlings and wild type for *ERD1* (early response to dehydration 1) that encodes a chloroplast-targeted chaperonin possibly involved in proteolysis (Weaver et al., 1998); *COR15A*, which encodes a chloroplast-targeted LEA protein (Artus et al., 1996); *DREB2A*, which encodes a transcription factor activated in the early stages of the osmotic stress response; and *ERD3*, which encodes a protein with unknown function (Kiyosue et al., 1994). After ABA treatment, *ERD1*, *ERD3*, and *DREB2A* were up-regulated in the wild-type plants, whereas in *ttl1* plants remained unchanged (Fig. 7D). The induction of *COR15A* expression was higher in *ttl1* than in wild type after ABA treatment (Fig. 7D). The deregulation of different dehydration-responsive genes together with the osmotic stress sensitivity in *ttl1* could indicate that TTL1 is a node in a dehydration-ABA signaling pathway.

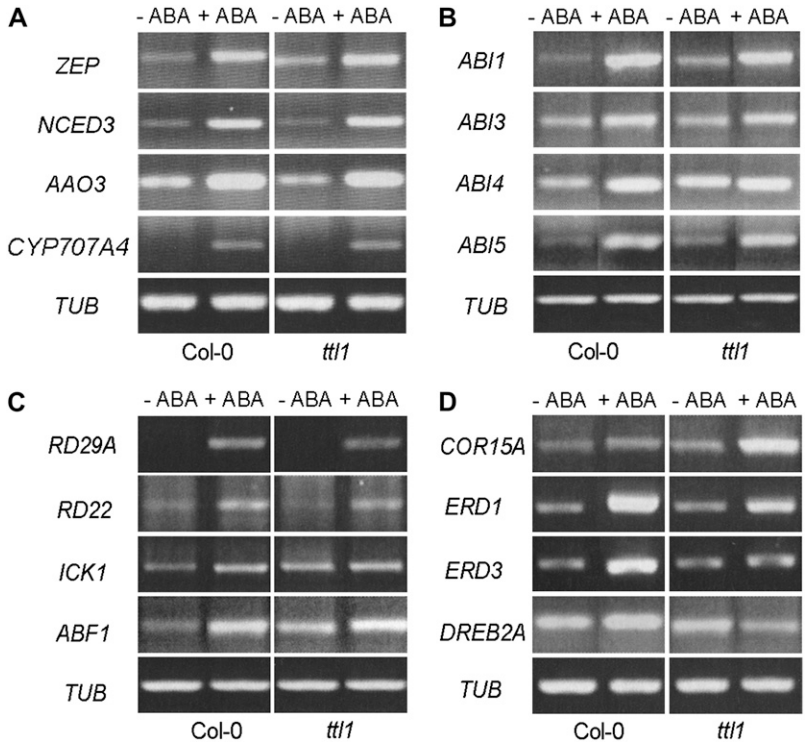

Figure 7. *ttl1* shows altered expression of several dehydration-responsive genes after ABA treatment. The expression level of several genes encoding enzymes involved in ABA biosynthesis and catabolism (A), ABA signaling (B), and general stress responses (C and D) was analyzed by semiquantitative RT-PCR. Total RNA was isolated from 10-d-old wild-type Col-0 and *ttl1* seedlings treated with water or a solution of 100 μM ABA for 3 h. In all experiments, the expression of the constitutive *β-tubuline* (*TUB*) gene was used as a control. Two replicates were made for each treatment with similar results.

The *ttl1-4* Allele Displays Wild-Type Phenotypes during Germination

An interesting result concerning TTL1 function was provided by the mutant allele *ttl1-4*. This allele did not show the phenotypes exhibited by *ttl1-1*, *ttl1-2*, and *ttl1-3* in germination after ABA treatment, thus behaving as a wild type (Fig. 2C). The T-DNA insertion in *ttl1-4* is in the 5′ region of the gene, upstream of the sequences encoding for the TPRs and TRXL motifs (Fig. 2, A and B). RT-PCR with primers annealing downstream of the T-DNA insertion showed the presence of *TTL1* transcripts in the *ttl1-4* mutant, in contrast to the other *ttl1* mutants (Fig. 2C). The absence of phenotype in this mutant (Supplemental Fig. S2, A–C) might be indicative of the presence of a truncated protein, with conserved TPRs and TRXL motifs, which is still functional in its response to osmotic and ABA treatments.

DISCUSSION

Proteins containing TPRs are essential mediators of plant hormone signaling, such as signaling of GA and ethylene, which act to form multiprotein complexes (Jacobsen et al., 1996; Wang et al., 2004). Here, we provide evidence that TTL1, a protein containing TPRs, mediates responses to osmotic stress and ABA during germination and seedling development in Arabidopsis plants. Further analysis of the remaining *TTL* family members in Arabidopsis could provide new insights into plant hormonal regulation mediated by proteins containing TPRs.

TTL1 Is Required for Osmotic Stress Tolerance and ABA Sensitivity

The role of ABA during seed maturation and germination, as well as the involvement of this plant hormone in osmotic stress responses and vegetative development, has been extensively described in the literature (for review, see Finkelstein et al., 2002; Nambara and Marion-Poll, 2003; Riera et al., 2005; Verslues and Zhu, 2005). Plants affected either in ABA metabolism or sensitivity have altered germination rate under osmotic stress (Werner and Finkelstein, 1995; Quesada et al., 2000; González-Guzmán et al., 2002). Thus, mutants that are ABA insensitive (*abi1–abi5*) or ABA deficient (*aba2* and *aba3*) exhibit increased germination rates under osmotic stress (Finkelstein, 1994; Quesada et al., 2000; Finkelstein et al., 2002; González-Guzmán et al., 2002), whereas the ABA hypersensitive mutants (*ahg1–ahg4*) have decreased germination rates under osmotic stress (Nishimura et al., 2004, 2005). As we expected, germination of *ttl1* was greater than wild type under osmotic stress because this mutant showed a diminished sensitivity to ABA.

The inhibitory effect of ABA on root growth involves crosstalk between several hormonal pathways, such as ethylene, auxin, brassinosteroid, jasmonic acid, and sugars (Beaudoin et al., 2000; Finkelstein and Lynch, 2000; Ghassemian et al., 2000), and the relationship between ABA and osmotic sensitivity is complex. Thus, in tomato (*Lycopersicon esculentum*), it has been shown that osmotic stress hypersensitivity in root elongation was associated with ABA hypersensitivity in the *tss2* mutant (Borsani et al., 2001; Rosado et al., 2006) and with ABA insensitivity in the *tos1* mutant (Borsani et al., 2002), indicating that proper ABA perception and signaling is essential for osmotic stress sensitivity.

Similar to *tos1*, *ttl1* seems to be specifically affected in ABA signaling and exhibits hypersensitivity to osmotic stress but slight insensitivity to exogenous ABA in root elongation. In both *tos1* and *ttl1*, the osmotic hypersensitivity in root elongation is not due to reduced levels of ABA but more likely to an inadequate ABA-dependent signaling pathway necessary for osmotic tolerance (Borsani et al., 2002). However, it is very unlikely that *TTL1* and *TOS1* encode for orthologous proteins. While *tos1* displays conditional growth defects, impaired stomatal responses, and ABA overaccumulation under osmotic stress, *ttl1* is indistinguishable from the wild type and is not affected in its stomatal responses (M. Botella, unpublished data). In addition, expression analysis of genes involved in ABA biosynthesis and degradation as well as preliminary ABA measurements indicate that *ttl1* has wild-type ABA content after osmotic stress treatments. Our results suggest that TTL1 acts downstream, or in an independent pathway, from the ABIs and that, unexpectedly, TTL1 is a positive regulator of a subset of dehydration-responsive genes, such as *ERD1*, *ERD3*, and *DREB2A*. *ERD1* and *DREB2A* act in the ABA-independent pathway and encode important proteins for dehydration signaling pathways and for osmotic stress protection. Based on the ABA-insensitive phenotype, it was expected that genes with altered expression in *ttl1* would be those regulated by ABA. For that, the fact that *ttl1* affects in a different way the expression of genes that are regulated by ABA-dependent pathways, such as *COR15a*, and ABA-independent pathways, such as *ERD1*, *ERD3*, and *DREB2A*, illustrates the complexity of the ABA and dehydration responses.

The Role of a TPR Protein in the ABA Signaling Pathway

The 34-amino acid TPR motif is conserved in all organisms studied and is present in proteins involved in numerous cellular processes. Typically, TPR motifs are arranged in tandem repeats of three to 16, although individual TPRs can be dispersed throughout the protein. Alignment of TPR motifs reveals a consensus sequence defined by a pattern of small and large amino acids forming an all-helical secondary structure involved in a plethora of cellular processes (Blatch and Lässle, 1999; D'Andrea and Regan, 2003). The basic function of TPR domains is to mediate protein-protein interactions that, among many other functions, affect

proper folding of proteins and mRNA stability, processing, and translation (Prodromou et al., 1999; Gounalaki et al., 2000; Fedoroff, 2002; Yang et al., 2005). Some of these interactions are related to hormonal regulation of different physiological processes (Jacobsen et al., 1996; Wang et al., 2004).

In Arabidopsis, 79 TPR-containing proteins have been identified (D'Andrea and Regan, 2003). However, the precise biochemical functions of these proteins remain elusive because only a few specific interacting partners have been identified. Although the yeast two-hybrid system has been successfully used to identify interacting partners of TPR-containing proteins in several organisms (Tseng et al., 2004; for review, see D'Andrea and Regan, 2003), we have been unable to find interacting proteins using this system with the full-length *TTL1* cDNA of Arabidopsis. This result could indicate, in the case of TTL1, the requirement of an additional component (or cofactor) for the correct interactions, but the possibility that TTL1 partners were not effectively expressed under the conditions in which the two-yeast hybrid library was made cannot be excluded.

Because TTL1 is involved in ABA response, two distinct mechanisms of action for this protein can be hypothesized based on the two best functionally characterized TPR proteins in plant hormonal regulation, i.e. the negative regulator of GA responses SPY (Jacobsen and Olszewski, 1993; Jacobsen et al., 1996) and the regulator of ethylene biosynthesis ETO1 (Wang et al., 2004). The SPY protein has 10 TPRs at the N terminus, and several *spy* alleles are affected in the TPR region, suggesting that these domains are important for proper GA signaling (Tseng et al., 2001; Lyer and Hart, 2003). Yeast two-hybrid screening using the *Hordeum vulgare* SPY homolog has shown that it physically interacts and regulates the activity of two transcription factors (*HSImyb* and *HSINAC*) that are negative regulators of the GA signaling pathway (Robertson, 2004). Based on this mode of action, TTL1 could regulate ABA signaling pathways by direct interaction with ABA-specific transcription factors such *ABI3*, *ABI4*, or *ABI5*, despite the fact that the expression of these genes is not affected in *ttl1*. On the other hand, ETO1 is a member of a novel plant-specific protein family with three distinct protein-protein interaction domains, namely, the BTB domain in its N terminus, the TPR, and the coiled-coil domains in its C terminus. The C-terminal TPR domain interacts with AtACS5, whereas the N-terminal BTB domain interacts with AtCUL3, a constituent of E3 ubiquitin ligase complexes. Thus, ETO1 is proposed to serve as a substrate-specific adaptor protein inhibiting the enzyme activity of AtACS5 and targeting this protein for degradation to the proteasome (Wang et al., 2004; Yoshida et al., 2005). It is tempting to speculate that TTL1 might be involved in protein complex formation for ABA regulation, and, as occurred for ETO1 BTB domain, the TRLX motif could act as an independent module interacting with a different partner than the TPR motifs.

Is There Functional Redundancy in the *TTL* Gene Family?

The *ttl1* alleles cause ABA insensitivity in germination but do not affect the general morphology of adult plants. In the analysis of other ABA-insensitive mutants, it has been shown that ABA responsiveness and signaling are dependent on the stage of development or specific tissues. For example, using electrophysiology, it has been shown that mesophyll cells and guard cells use distinct and different receptor types and/or signal transduction pathways in ABA regulation, at least for potassium channel regulation (Sutton et al., 2000). A defect in the *OST1/SRK2E* gene causes ABA insensitivity of guard cells in stomatal closure but has no apparent effect on seed germination (Yoshida et al., 2002). The function of the transcription factors ABI3, ABI4, and ABI5, as well as TTL1, are important in germination and seedling development, but their functions at adult stages are rather limited (Finkelstein, 1994). Therefore, it is difficult to reconcile the high expression of *TTL1* in metabolically active tissues and guard cells if its function is restricted to seeds and seedling roots. *TTL1* belongs to a family of four members in the Arabidopsis genome with still-unknown functions. Analysis of the *TTL* family expression using the microarray data available shows that not only the expression levels but also the tissue expression patterns are very different for each member of the *TTL* family. *TTL1* expression is ubiquitous, being greater in seedling roots. *TTL2* expression levels are very low in general and restricted to stamen and stages 4 to 8 of seed maturation. *TTL3* and *TTL4* expression are ubiquitous, and their relative expression is 3- to 5-fold greater than *TTL1*. The high similarity in the structure of proteins encoded by the four members of the family and their differential expression patterns suggest that *TTL* genes could have similar functions but in different tissues or developmental stages. In this scenario, their functions could be redundant in stomata regulation and not redundant during seed germination and seedling root growth under NaCl stress. Examples of functional redundancy have recently been reported for key proteins involved in hormonal responses, like the cytokinin or the auxin receptors (Higuchi et al., 2004; Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Furthermore, it has been proposed that the relatively low number of recessive ABA-insensitive mutants is most likely due to redundancy in genes encoding ABA transducers, requiring analyses of double or multiple mutations in (partially) redundant genes (Kwak et al., 2003; Kuhn et al., 2006). Therefore, future studies using double, triple, or even quadruple mutants in the *TTL* family will help to determine the role of the different members of the family in ABA and stress responses.

In conclusion, the novel TPR-containing protein TTL1 modulates plant responses to ABA in seeds and seedlings. Because TPR proteins are key regulators in the ethylene and GA hormonal pathways, the finding of a novel TPR protein involved in ABA signaling led us

to propose that the formation of protein complexes mediated by TPRs could be a common mechanism of hormonal regulation in plants. Thus, TTL1 could provide a new mechanism for manipulating the ABA responsiveness of crop plants during stress.

MATERIALS AND METHODS

Plant Materials

The Arabidopsis (*Arabidopsis thaliana*) ecotype $C24_{RD29a\text{-}LUC}$ T-DNA insertion lines (Ishitani et al., 1997) were provided by Professor J.K. Zhu (University of California, Riverside, CA). The Arabidopsis Col-0 T-DNA insertion mutants were identified using the SIGnAL Web site at http://signal.salk.edu and were obtained from the Arabidopsis Biological Resource Center stock center (Columbus, OH).

Isolation of Salt-Hypersensitive Mutants and Root Elongation Measurements

Preparation of the T-DNA-tagged Arabidopsis ecotype $C24_{RD29a\text{-}LUC}$ population and root-bending assay identification of salt-sensitive mutants were described previously (Zhu et al., 2002; Koiwa et al., 2006). Briefly, Arabidopsis seeds were sown onto Murashige and Skoog (MS) agar medium (Murashige and Skoog, 1962; 1× MS salts, 30 g/L Suc, and 12 g/L agar, pH 5.7), stratified for 3 d, and then incubated at 23°C for 1 week. Seedlings were then transferred to basal medium supplemented with 160 mM NaCl, and root growth was scored 8 d later.

For root elongation measurements, 15 seeds were used per replicate, and three replicates were made for each treatment. Five-day-old seedlings with 1- to 1.5-cm-long roots were transferred from vertical agar plates containing MS medium onto a second agar medium that was supplemented with different concentrations of salts and osmotic stress generator. Increases in root length were measured after 3 d of treatment.

Seed Germination Assays

Wild-type and *ttl1* seeds (>100 seeds for each replicate) were surface sterilized and kept for 3 d at 4°C in the dark to break dormancy. The seeds were sown directly on the surface of filter paper soaked with either different salts and osmotic stress generator solutions or solutions containing various levels of ABA and PAC and incubated at 23°C with a 16-h-light photoperiod. The number of germinated seeds was expressed as a percentage of the total number of seeds plated, and germination was recorded as the capacity to expand green and fully developed cotyledons after 10 d. Three replicate plates were used for each treatment.

Hormonal Treatments

Plants were grown side-by-side in growth chambers: 16-h-light/8-h-dark cycle, temperature of 23°C, and photon fluency rate of 150 μmol m^{-2} s^{-1}. To test the sensitivity of *ttl1* seedlings, these seedlings were grown on vertical plates containing 1× MS medium (Murashige and Skoog.,1962), supplemented with 1% Suc and the indicated hormones for 8 d. Sensitivity to NAA (Sigma) or 6-benzylamino purine (Sigma) was determined by measuring root growth inhibition (Cary et al., 1995). Sensitivity to brassinosteroid using BR (Sigma) and ethylene using the precursor ACC (Sigma) was studied by measuring the inhibition of hypocotyl growth of seedlings grown in the dark (Ephritikhine et al., 1999). The hormone ABA (Sigma) and the GA biosynthesis inhibitor PAC (Duchefa) were used to investigate the effect of ABA and GA on seed germination as described above.

Molecular Genetic Analysis of *ttl1* T-DNA Insertion Alleles

DNA flanking the left border of the inserted T-DNA in *ttl1* plants was isolated by thermal asymmetric interlaced PCR (Liu and Whittier, 1995; Koiwa et al., 2002) and subcloned into cloning vector pBluescript SK+ (Stratagene) and pGEM-T Easy Vector system (Promega) according to the manufacturer's instructions. The entire isolated fragment was sequenced. The primers used in the thermal asymmetric interlaced PCR were specific for the T-DNA left border (LB1, 5′-ATA CGA CGG ATC GTA ATT TGT C-3′; LB2, 5′-TAA TAA CGC TGC GGA CAT CTA C-3′; and LB3, 5′-TTG ACC ATC ATA CTC ATT GCT G-3′), and degenerate primers (DEG1, 5′-WGC NAG TNA GWA NAA-3′; and DEG2, 5′-AWG CAN GNC WGA NAT A-3′).

Genetic cosegregation analysis was performed using an F_2 population after backcrossing *ttl1-1* to wild-type C24. F_2 seedlings exhibiting salt/osmotic stress sensitivity (such as *ttl1-1*) were genotyped by PCR analysis. Similarly, salt/osmotic stress-sensitive seedlings from the SALK_87417, SALK_63943, and SALK_57389 pools were genotyped. PCR-based genotypic analysis was performed as described (Koiwa et al., 2006) using primers TTL1F (5′-TGG ACT CAC CAC CAC CAC TA-3′) and TTL1R (5′-ACC GAG TCT GCG AAC AAG AT-3′) for *ttl1-1*, SALK_87417, SALK_63943, and SALK_57389; LB3 for *ttl1-1*; and LBa1 (5′-TGG TTC ACG TAG TGG GCC ATC G-3′) for SALK_87417, SALK_63943, and SALK_57389 (*ttl1-2*, *ttl1-3*, and *ttl1-4*, respectively).

RT-mediated PCR analysis for *TTL1* was performed as described (Koiwa et al., 2002) using primer pair QTTL1F (5′-CTC CGA TCA TCT CCG TGA TT-3′) and QTTL1R (5′-CTG TCG GTA CAA CCC TCA CA-3′).

Promoter *TTL1*::GUS-GFP Construct and Histochemical Analysis

For the *TTL1* promoter::GUS-GFP fusion, 2.0 kb of the genomic sequence upstream of the *TTL1* translation start site was amplified by PCR. The following primers were used: PROTTL1F, 5′-TGG TAC CTT GAG TGG AAG AAG GAA-3′; and PROTTL1R, 5′-ACC ATG GTG AGT GTT GTG GTG AGT GAA-3′. The genomic fragment amplified was cloned into the binary vector PCAMBIA1303. The recombinant plasmid was used to transform Agrobacterium strain GV3101. The resulting transformant was used to transform wild-type and *ttl1* mutant plants. Agrobacterium strain GV3101 transformed with pCAMBIA 1303 empty vector also was used to transform the wild-type and *ttl1* mutant plants as a control. GUS activity was detected in situ as described (Jefferson et al., 1987). Mannitol-treated wild-type and *ttl1*-transformed seedlings were grown as described above and harvested 3 d later. Care was taken to ensure that controls and transformed plants were mannitol treated, harvested, and stained for GUS activity simultaneously.

Semiquantitative RT-PCR

Two-week-old plants were transferred from MS agar plates to petri dishes and placed over a filter paper soaked with liquid MS media in control plates or MS supplemented with 300 mM NaCl in salt treatments. For ABA treatments, 2-week-old plants were directly sprayed in the MS-agar plate with a 0.1% Triton X-100 solution in water with or without 100 μM ABA. Plates were sealed and incubated at 23°C with light in a growth chamber. Whole plants were collected for RNA isolation 3 h later, immediately frozen in liquid nitrogen, and stored at −80°C. Each sample of plant material was weighed immediately prior to being homogenized in 1.5-mL Eppendorf tubes containing 500 μL of Trizol (GIBCO/BRL) and incubated for 5 min at room temperature. RNA was chloroform extracted, isopropyl alcohol precipitated, and resuspended in water. Genomic DNA was removed by adding 5 units of DNase I (GIBCO/BRL) and incubated for 30 min at 37°C and then for 10 min at 70°C to inactivate the enzyme. RNA was ethanol precipitated and resuspended in 50 μL of nuclease-free water. For the first-strand cDNA synthesis, total RNA (1 μg) was used as template and the retrotranscription was performed using the SuperScript III First Strand synthesis system (Invitrogen) following the protocol modifications performed in the Gen Expression Lab and published online at http://www.protocolsonline.org. Primer sets used in the semiquantitative RT-PCR and the number of cycles used in the amplification are indicated in Supplemental Table S1. Each of these oligonucleotide pairs was designed, if possible, to span at least one intron to distinguish between genomic DNA and cDNA amplification products. PCR amplifications were performed in 20-μL reaction mixes of 200 μM for each dNTP and 2 mM $MgCl_2$, containing 0.4 units of BioTaq enzyme (Bioline), 2 mL of 10× reaction buffer (Bioline), and 1 μL of the 25-μL cDNA solution obtained from each sample of plant material. The final concentration of each oligonucleotide in the reaction mixture was 0.5 μM, which was reached by taking 1 μL from a master mixture containing the oligonucleotides listed in the supplemental data, each at a concentration of 10 μM. The thermocycling program started with an initial 90-s denaturation step at 94°C, followed by 25, 30, or 35 cycles (30 s at 94°C, 15 s at

55°C, 90 s at 70°C) to elucidate the exponential amplification cycle for each primer combination, and final 7-min incubation at 70°C. As a control for mRNA quantity, the constitutive gene tubulin β-5 chain (*At1g20010*) was used.

Water Loss Assays in Detached Shoots

Detached shoots of wild-type and *ttl1* mutant plants at the rosette stage were placed on weighing trays and allowed to dry slowly at constant temperature (23°C) and humidity (approximately 50%). Weights of shoots were determined over a 330-min period. Plants were watered to saturation and shoots were sprayed with a 0.1% Triton X-100 solution in water with or without 100 μM ABA 2 h before the weighing measurements.

Supplemental Data

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

Supplemental Figure S1. *ttl1* and wild-type leaves display similar transpirational water loss.

Supplemental Figure S2. *ttl1-4* shows wild-type responses to osmotic stress and ABA.

Supplemental Table S1. Primers and conditions used for semiquantitative RT-PCR.

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