Overlapping and specific function of the *TTL* gene family

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Environmental Stress and Adaptation
The *Arabidopsis thaliana* TETRATRICOPEPTIDE THIOREDOXIN-LIKE gene family is required for osmotic stress tolerance and male sporogenesis

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Abstract

Tetratricopeptide thioredoxin-Like (TTL) proteins are characterized by the presence of 6 tetratricopeptide repeats (TPR) in conserved positions and a C-terminal region known as thioredoxin-like (TRXL) domain with homology to thioredoxins. In *Arabidopsis thaliana*, the TTL gene family is composed by four members, and the founder member, *TTL1*, is required for osmotic stress tolerance. Analysis of sequenced genomes indicates that *TTL* genes are specific to land plants. In this study, we report the expression profiles of *Arabidopsis TTL* genes using data mining and promoter-reporter GUS fusions. Our results show that *TTL1*, *TTL3*, and *TTL4* display ubiquitous expression in normal growing conditions but differential expression patterns in response to osmotic and NaCl stresses. *TTL2* shows a very different expression pattern, being specific to pollen grains. Consistent with the expression data, *ttl1*, *ttl3*, and *ttl4* mutants show reduced root growth under osmotic stress and the analysis of double and triple mutants indicate that *TTL1*, *TTL3* and *TTL4* have partially overlapping yet specific functions in abiotic stress tolerance while *TTL2* is involved in male gametophytic transmission.
Introduction

Root growth is dependent on proper meristem activity and cell cycle regulation. This is particularly important under drought conditions in which plant roots need to get deep into soils to access water supply. Extensive efforts have been devoted towards the elucidation of sensory and signal transduction mechanisms that perceive osmotic stress and control cellular homeostasis in plants (Xiong and Zhu, 2001; Borsani et al., 2002; Borsani et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2006; Munns and Tester, 2008). However, little is known about the mechanism(s) coordinating osmosensing and plant cell cycle regulation, as well as the adaptive processes required to maintain the meristem function under osmotic stress conditions.

In Arabidopsis, several genes required for cell cycle progression under osmotic and drought stresses have been identified using genetic approaches. For instance, the salt overly-sensitive5 (sos5) characterized by root tip swelling and root growth arrest under salt stress was isolated in a screen for Arabidopsis salt-hypersensitive mutants (Shi et al., 2003). The predicted SOS5 protein contains an N-terminal signal sequence for plasma membrane localization, two arabinogalactan protein–like domains, two fasciclin-like domains and a C-terminal glycosyolphosphatidylinositol lipid anchor signal sequence. The presence of fasciclin-like domains, which typically are found in animal cell adhesion proteins, suggests a role for SOS5 in cell-to-cell adhesion in plants (Shi et al., 2003). The Arabidopsis STT3 encodes an essential subunit of the oligosaccharyltransferase complex that is involved in protein N-glycosylation. Osmotic stress disturbs the cell cycle progression in stt3a root meristematic cells, indicating the need of proper protein glycosylation and folding for osmotic stress adaptation (Koiwa et al., 2003). Mutations in the Arabidopsis TETRATRICOPEPTIDE-REPEAT THIOREDOXIN-LIKE 1 gene (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 (Rosado et al., 2006b).

TTL1 is the first characterized member of a novel protein family defined by the presence of six tetratricopeptide (TPR) motifs and a region in the C terminus with homology to class h thioredoxins, but lacking essential cysteine residues required for thioredoxin activity (Rosado et al., 2006b; Ceserani et al., 2009). The 34-amino acid
TPR motif is conserved in all organisms studied and is present in proteins involved in numerous cellular processes (D'Andrea and Regan, 2003; Cliff et al., 2005; Schapire et al., 2006; Schreiber et al., 2011). TPR domains have been described as protein-protein interaction modules defined by a pattern of small and large amino acids forming an all-helical secondary structure (Blatch and Lässle, 1999; D'Andrea and Regan, 2003; Yang et al., 2005).

In Arabidopsis, more than 235 proteins containing TPR motifs, usually arranged in tandem repeats of three to sixteen, have been identified (D'Andrea and Regan, 2003; Prasad et al., 2010). Many of these proteins show a modular structure containing additional domains that mediate interactions with downstream proteins acting as co-chaperones or have enzymatic activity that amplify and transduce signals (D'Andrea and Regan, 2003; Davies and Sánchez, 2005). Proteins containing TPR domains are involved in protein folding and mRNA stability, processing, and translation, and have been also found to be essential for responses to hormones such as ethylene, cytokinin, gibberellins, auxin, and abscisic acid (Prodromou et al., 1999; Gounalaki et al., 2000; Fedoroff, 2002; Wang et al., 2004; Greenboim-Wainberg et al., 2005; Yang et al., 2005; Silverstone et al., 2007). Thus, TPR proteins are emerging as essential components of signal transduction pathways mediated by most plant hormones.

Of all proteins containing TPR domains, Hsp90 is arguably the best studied (Sangster and Queitsch, 2005; Whitesell and Lindquist, 2005; Pratt et al., 2008; Taipale et al., 2010). The highly conserved and abundant molecular chaperone Hsp90 is distinct from other chaperones in that it plays a key role in signal transduction networks, cell cycle control, protein degradation, and genomic silencing (Taipale et al., 2010). Hsp90 lies at the center of a multiprotein chaperone complex that facilitates the folding of client proteins into their stable or active conformations (Krishna and Gloor, 2001). In Arabidopsis, TTL proteins have been identified as putative chaperone receptors based on the identification of conserved amino acids in their TPR motifs known as carboxylate clamps, opening the possibility that TTL proteins act in concert with Hsp90 chaperone complexes (Prasad et al., 2010). In addition TTL3 was identified as an interacting partner of the activated (phosphorylated) cytoplasmic domain of VH1/BRL2, a receptor-like kinase of the BRI1 family with a role in vascular development (Ceserani et al., 2009).
In this study we have performed a functional analysis of the four Arabidopsis *TTL* genes. Genetic analysis indicates that TTLs are essential for plant viability. *TTL* expression patterns and physiological characterization of *ttl* mutants indicate that TTLs have overlapping and yet distinct functions in the maintenance of the root meristem integrity under osmotic stress, as well as in male gametophyte and vasculature development.

**Results**

**TTL Proteins are Characteristic of Land Plants**

The tetratricopeptide thioredoxin like (TTL) comprise a family of plant-specific proteins with a common modular architecture containing six Tetratricopeptide Repeat (TPR) domains distributed in specific positions throughout the sequence, and a C terminal sequence displaying homology to thioredoxins termed the TRXL motif (Rosado et al., 2006b). A distinctive feature of the TRXL motif is the lack of essential cysteine residues conserved in all thioredoxins and required for reducing activity (Rosado et al., 2006b; Cesarani et al., 2009).

Extensive searches in a variety of sequenced genomes using the typical distribution of TPRs and TRXL domains of the Arabidopsis TTLs failed to identify any members of this protein family in microorganisms and animals but showed that they are present in all plant genomes analyzed. Next, we investigated the phylogeny of TTL proteins using the genomic data available from the phytozome database v7.0 (Goodstein et al., 2011), and genomic data from barley (*Hordeum vulgare*) and tomato (*Solanum lycopersicum*) (Supplemental Table S1). Using the strict criteria detailed in Material and Methods, three and one genes encoding TTLs were identified in the primitive land plant species *Physcomitrella patens* and *Selaginella moellendorffii* respectively. The absence of *TTL* genes in algae indicated that this gene class is restricted to land plants. We also identified TTL-like proteins containing the TRXL motif and 7-8 TPR motifs that could possibly function as TTL proteins but were not considered in our analysis.

Figure 1 shows the phylogenetic analysis of the 4 Arabidopsis TTLs, the 4 TTLs from rice (both model species for dicots and monocots respectively) and TTL1 homologs from selected species included in Supplemental Table 1. The analysis
grouped separately monocot and dicot TTLs and, as expected, the ancestral TTLs from *P. patens* and *S. moellendorffii* were outgrouped.

In Arabidopsis, at least four different large-scale duplication events occurred 100 to 200 million years ago favoring the diversification of this species (Vision et al., 2000). To test the contribution of the Arabidopsis segmental duplications in the number of TTL genes, we searched the Arabidopsis genome for duplicated chromosomal segments containing TTLs using the Plant Genome Duplication Database (http://chibba.agtec.uga.edu/duplication/). As shown in Supplemental Figure S1, two independent duplicate blocks containing the genomic pairs TTL1/TTL2 and TTL3/TTL4 were identified. Considering the function of the Arabidopsis TTL1, a protein required for osmotic stress tolerance, the large scale duplication and retention of TTL genes in plants suggests that TTL genes may be a source of diversity important for proper responses to stressful environments.

We next investigated whether TTL genes were originated from an ancestral gene in land plants and subsequent duplications or resulted from convergent evolution in species with several TTL genes. Because TPRs are located in similar positions in all TTLs proteins identified, we aligned the 6 TPRs motifs of TTLs from phylogenetically distant species and obtained the corresponding cladogram. As shown in Figure 2, TPRs in equivalent position are more homologous among them than TPRs within a TTL protein. Thus, TPR1 from Arabidopsis TTL1 is more closely related to TPR1 from *P. patens* than to any other TPR within TTL1. This indicates that an original TTL protein expanded among different species by duplication and TPRs have been relatively well conserved throughout evolution, probably due to functional constrains. A phylogenetic analysis using the TRXL sequences provides similar results to those using the complete TTL sequence (Figure 3A). We did not find any TTL protein containing the consensus sequence required for thioredoxin activity (Figure 3B).

**Arabidopsis TTL genes display overlapping and divergent functions in abiotic stress tolerance**

Plant genes that respond to environmental stimuli tend to have a higher rate of retention after duplication than non-responsive genes, however it is not clear which mechanisms contribute to their retention (Hanada et al., 2008). After duplication, the
predominant fate of duplicated genes is pseudogenization (Taylor and Raes, 2004), still, a significant fraction of gene duplicates in plants are preserved and follow different evolutionary scenarios including retention, loss, gain, and switch of functions. To investigate the specific evolutionary path(s) followed by the different members of the TTL family in Arabidopsis, we studied T-DNA mutants for the four Arabidopsis TTL genes, and evaluated their specific role in abiotic stress responses. The previously characterized ttl1-2 mutant allele (SALK_063943, hereafter referred as ttl1) in the Col-0 background was used in this study (Rosado et al., 2006b). In addition, homozygous mutant lines for TTL2 (SALK_106516), TTL3 (SALK_193805), and TTL4 (SALK_026396) in the Col-0 background were isolated (Figure 4A). In every case, transcripts for the TTL gene in its corresponding mutant background were not detected by RT-PCR analysis indicating that none of the ttl mutant alleles were able to produce intact full-length mRNAs (Figure 4B).

Mutations in TTL1 caused reduced tolerance to NaCl and osmotic stress characterized by altered seedling development, reduced root elongation, disorganization of the root meristem, and impaired responses during germination. However, no differences between ttl1 and WT plants were observed under non-stressing conditions (Rosado et al., 2006b). Similarly, we did not detect abnormalities in any of the ttl single mutants based on detailed analyses of root elongation under non-stressing conditions (Supplemental Figure S2). Next, we investigated seedling development of the ttl mutant genotypes at increasing concentrations of mannitol. As shown in Figure 5, while roots of ttl1, ttl3 and ttl4 single mutants showed hypersensitivity to mannitol at high concentrations (300 and 400 mM), ttl2 did not show any difference compared to WT. These results suggest that the ancestral TTL had a role in osmotic stress tolerance and after the TTL1 segmental duplication TTL2 changed its original function.

In addition, ttl1, ttl3 and ttl4 mutants also exhibited hypersensitivity to NaCl at concentrations above 150 mM, known to also generate osmotic stresses (Borsani et al., 2002). Surprisingly, at moderate NaCl concentrations (120 mM), ttl4 seedlings displayed improved root growth and increased cotyledon fresh weight compared to WT while no differences were observed non-stress conditions (Figure 6A, 6B). Next, we investigated whether the higher cotyledon fresh weight in the ttl4 mutant correlated with differences in Na\(^+\) accumulation. For that purpose, Na\(^+\) content was analyzed in WT and ttl4 seedlings grown for 1 week in MS medium with or without 120 mM NaCl. As
shown in Figure 6C, no differences in Na\(^+\) content were detected in WT and \(ttl4\) seedlings in control medium, while \(ttl4\) accumulated significantly more Na\(^+\) than WT in medium supplemented with NaCl. This result suggests that \(ttl4\) improve seedling tolerance to NaCl by either improving long distant Na\(^+\) transport or increasing the seedling capacity to compartmentalize Na\(^+\). Finally, an extended analysis of the \(ttl\) mutants and WT responses against oxidative, sugar, pH and nutrient deprivation stresses using root elongation bioassays showed no significant differences (Supplemental Figure S3). These results indicate that TTL genes are specifically involved in the response to osmotic and NaCl stresses.

**TTL promoters differentially respond to abiotic stress treatments**

Based on the phenotypic analysis, TTL genes have different roles in NaCl and osmotic stress tolerance. While \(ttl1\), \(ttl3\), and \(ttl4\) display hypersensitivity to osmotic stress in roots, \(ttl4\) shows increased tolerance to NaCl and \(ttl2\) do not show altered responses to abiotic stresses. Mutations in cis-regulatory elements in the promoter regions is a common mechanism for gene subfunctionalization (Van de Peer et al., 2001; Haberer et al., 2004), and differences in gene expression are mainly the result of divergence in cis-regulatory motifs (Evangelisti and Wagner, 2004). Therefore we determined whether the different roles of the TTL genes in stress responses correlate with differential expression patterns. We first compiled expression data for the four TTL Arabidopsis genes using the Genevestigator database (Hruz et al., 2008). As shown in Supplemental Figure S4, the analysis revealed two distinct gene expression patterns for TTL genes. Whereas \(TTL1\), \(TTL3\), and \(TTL4\) were ubiquitously expressed throughout most tissues, \(TTL2\) expression was confined to pollen/stamen. \(\beta\)-glucuronidase reporter gene fusions (\(pTTL::GUS\)) were constructed using ~2kb of the upstream regulatory region for each TTL gene. Seedlings of at least 10 independent transgenic lines per construct were stained for GUS activity and lines with single insertions harboring homozygous \(pTTL::GUS\) were used to analyze expression patterns.

Figure 7 shows representative expression profiles for all \(pTTL::GUS\) transgenic lines in 2-days-old seedlings, 10-days-old seedlings and flowers. The first phylogenetic pair \(TTL1/TTL2\) shows very different expression patterns with \(pTTL1\) showing ubiquitous expression in seedlings while \(pTTL2\) expression was restricted to pollen. The
second phylogenetic pair *TTL3/TTL4* displayed similar expression patterns with both genes ubiquitously expressed in vegetative tissues and strong signal in the vasculature. Thus, the expression patterns obtained from the promoter-GUS constructs corroborate the available microarray data (Supplemental Figure S5).

Next, we analyzed the *TTL* expression patterns in response to abiotic stresses (Figure 8). For the first phylogenetic pair *TTL1/TTL2*, NaCl treatments induced *pTTL1::GUS* expression in roots and cotyledons, while mannitol stress induced *pTTL1::GUS* expression in roots but suppressed it in cotyledons. *TTL2* expression was never detected in vegetative tissues. The second phylogenetic pair *TTL3/TTL4*, showed an expression pattern very similar to *TTL1*, with strong induction by NaCl and drastic repression by mannitol in cotyledons.

These results indicate that individual members of the *TTL* family have differential abiotic stresses responses and that changes in cis-regulatory elements might have been instrumental in the subfunctionalization within the *TTL* genes.

**TTL2 functions in male gametophyte development**

We next examined whether *TTL* genes act redundantly or sinergically, and for that purpose we aimed to produce all possible mutant combinations of the *TTL* genes (Figure 4A). In total 6 double mutants (*ttl1/2, ttl1/3, ttl1/4, ttl2/3, ttl2/4, ttl3/4*) and 4 triple mutants (*ttl1/2/3, ttl1/2/4, ttl1/3/4, ttl2/3/4*) were obtained. We were unable to obtain plants containing homozygous mutations in all *TTL* genes (quadruple *ttl1/2/3/4* mutant) despite numerous attempts, suggesting that *TTL* function is essential for plant viability.

During the generation of the different mutant combinations, we noticed that *ttl2* homozygous mutants in F2 segregant populations appeared at lower frequencies than the theoretically expected for a Mendelian recessive trait. Considering that *TTL2::GUS* was strongly and specifically expressed in pollen grains, we hypothesized that *TTL2* might be required for male gametophytic transmission. Reciprocal crosses between WT and *ttl2* were performed and our results indicated that no significant differences in the number of seeds per silique exist when *ttl2* was used as a female parent, but the number of seeds per silique was significantly reduced when *ttl2* was used as the male parent.
This result indicates that *ttl2* has defects in the male gametophyte and confirms that *ttl2* mutant pollen is responsible for the reduced fertility.

We next analyzed the progenies derived from WT plants, *ttl2* plants and reciprocal crosses between WT and heterozygous (*TTL2/*ttl2*) plants. According to Mendelian inheritance for recessive traits the result of the cross between a WT and a heterozygous *TTL2/*ttl2* plant should render 50% of the population with WT (*TTL2*) genotype, and 50% with heterozygous *TTL2/*ttl2* genotype. Since *TTL2/*ttl2* plants are resistant to kanamycin due to the presence of this selectable marker in the T-DNA of the *ttl2* allele, we carried out the genetic analysis of F2 seeds in MS plates supplemented with kanamycin. As shown in Table 2, ~50% of the seeds were resistant to kanamycin when WT was used as the pollen donor, suggesting that there are no defects in the transmission of the *ttl2* female gametophyte. However, when pollen from heterozygous (*TTL2/*ttl2*) plants was used, only a ~10% of the progeny was resistant to kanamycin, further confirming that mutations in *TTL2* decrease the efficiency of the male gametophyte transmission.

As previously indicated, we were unable to obtain plants that contained homozygous mutations in all four *TTL* genes; however we were able to obtain viable plants with a *ttl1/ttl2/ttl3/TTL4/ttl4* genotype albeit at very low rate. To further investigate the role of *TTL* genes in embryogenesis, we analyzed the progeny of *ttl1/ttl2/ttl3/TTL4/ttl4* plants in more detail. Based on Mendelian inheritance, a frequency of 25% quadruple homozygous mutants was expected, however a large proportion of the pollen obtained from *ttl1/ttl2/ttl3/TTL4/ttl4* plants showed striking morphological distortions (Figure 9A), and the number of aborted seeds on the *ttl1/ttl2/ttl3/TTL4/ttl4* progenies was approximately 42%, which is significantly higher than the expected 25% (Figure 9B). This result indicates that a single copy of *TTL4* can, in some cases, provide enough TTL4 protein for proper seed development. The requirement of *TTL4* for seed development is consistent with the large progeny required to obtain an individual *ttl1/ttl2/ttl3/TTL4/ttl4* plant from a *ttl1/2/3* x *ttl1/2/4* cross. Interestingly, once the *ttl1/ttl2/ttl3/TTL4/ttl4* plant was obtained, it did not show obvious differences in their vegetative growth, indicating that *TTL* genes are particularly important for gametophytic transmission.
The function of *TTL1, TTL3* and *TTL4* in abiotic stress responses is not fully redundant

To determine whether the contribution of *TTL* genes to abiotic stress responses is fully redundant, partially redundant, or additive, we examined the responses of the different *ttl* mutant combinations against mannitol and NaCl stresses. We did not find differences in germination or seedling development between WT and any of the mutant combinations under control conditions (Supplemental Figure S2). As indicated previously, *ttl1, ttl3* and *ttl4* but not *ttl2* single mutants were hypersensitive to high mannitol concentrations (Figure 5). The double mutant combinations *ttl1/ttl3, ttl1/ttl4* and *ttl3/ttl4* displayed higher sensitivity to 200 mM mannitol than their respective single mutant counterparts, suggesting that those genes have an additive effect in their tolerance to osmotic stress. This was confirmed by the triple *ttl1/ttl3/ttl4* mutant being the most sensitive genotype analyzed, showing reduced root growth at mannitol concentration as low as 100 mM. The enhanced sensitivity observed in the triple mutant relative to the double mutants, and double mutants relative to single mutants indicate that TTLs have similar roles in osmotic stress tolerance but they are not fully redundant.

We further investigated the redundancy and/or additive effects of *TTL* genes by generating triple-heterozygous mutants for the *TTL1, TTL3* and *TTL4* genes. We reasoned that if the responses of the different *TTLs* to osmotic stress were dependent of a dosage effect, we should observe certain degree of hypersensitivity in triple heterozygous plants. As shown in Supplemental Figure S6, the response of the triple heterozygous plants to mannitol was identical to WT (Supplemental Figure S6), suggesting that the osmotic stress response is not controlled by total *TTL* gene dosage but by the individual contributions of the different *TTL* genes.

Finally, *ttl4* but not *ttl1, ttl2*, and *ttl3* seedlings displayed improved cotyledons development and higher fresh weight than WT at low and moderate NaCl concentrations. To determine whether the mutation in *TTL4* was the only contributor to this increased NaCl tolerance, we evaluated the different *ttl* mutant combinations in the presence of 120 mM NaCl. As shown in Figure 6, only those genotypes carrying the *ttl4* mutation showed improved growth in NaCl comparable to the improvement observed in the *ttl4* single mutant. This result confirms that *TTL4* is the only one affecting NaCl
tolerance and therefore we can assign a non-redundant function to TTL4 in responses to high NaCl.

**Root meristem and vascular defects are enhanced in plants with multiple mutations in TTL genes**

The main feature caused by osmotic stress in *ttl1* mutants was swollen root tips and meristem disorganization (Rosado et al., 2006b) therefore we analyzed in detail the root developmental defects in plants harboring different *ttl* mutant combinations. Thus, under mannitol stress, all mutants (with the expected exception of *ttl2*) showed root meristem disorganization similar to that previously reported for *ttl1* (Figure 10A). Correlating with the osmotic sensitivity previously reported, the degree of root meristem disorganization increased with the number of *ttl* mutations, being maximal in the triple *ttl1/ttl3/ttl4* mutant (Figure 10B).

To gain insight into the changes that caused the root tip swelling at cellular level, we analyzed transverse-sections of the primary roots in WT and triple *ttl1/ttl3/ttl4* mutant grown on control media or media supplemented with 400 mM mannitol. No differences in radial organization between WT and *ttl1/ttl3/ttl4* were observed in control conditions, however a reduced differentiation of vascular tissues was observed in *ttl1/ttl3/ttl4* mutants suggesting a role for TTL genes in vasculature development (Figure 10C, 10D). After mannitol treatment, WT roots decreased in diameter, and the typical shrink effect induced by low water potential was observed (Figure 10E). In contrast roots of *ttl1/ttl3/ttl4* showed a significant increase in size of all cell layers, indicating that all root cell types were affected by the *ttl* mutations (Figure 10F). We further analyzed the radial organization of roots grown in control media or media supplemented with 400 mM mannitol using scanning electron microscopy (Figure 10G to 10J). The analysis confirmed the disorganization in vascular bundles and aberrant thickness and diameter of the meristematic cells in the *ttl1/ttl3/ttl4* background compared with WT. Thus, cell diameters for cortex, endodermis, pericycle, xylem and phloem in *ttl1/ttl3/ttl4* under osmotic stress conditions were ~twice bigger than WT cells after mannitol treatment (Supplemental Figure S7). These results indicate a role for TTLs in the control of meristematic cell size during osmotic stress.
DISCUSSION

The role of TTL duplication in Arabidopsis

In Arabidopsis, the TTL family is composed by 4 members derived from two syntenous genomic regions with collinear features suggesting the existence of a common ancestor. This aspect is further supported by the evolutionary conservation of the internal modular domains among Arabidopsis TTLs and phylogenetically separated TTL proteins, and by the identification of a single TTL gene in Selaginella moellendorffii (SmTTL1), an important model organism in comparative genomics representing the oldest extant division of the vascular plants. The presence of a single TTL gene in S. moellendorffii strongly suggests that an ancestral TTL protein can perform all TTL functions required for plant viability. We therefore propose that successive duplications of an ancestral TTL gene led towards the neofunctionalization and subfunctionalization of the TTL gene family in Arabidopsis contributing to the fine-tuning of the Arabidopsis responses against abiotic stresses. Thus, the combination of gene duplication and selection pressure imposed by stress conditions likely led to substantial changes in TTL gene expression and functional changes of the TTL gene duplicates over time that likely contributed to the physiological complexity of the Arabidopsis TTL response against abiotic stress. Our results are a clear example supporting the hypothesis that gene duplication is an important evolutionary mechanism in the generation of novel functions and phenotypes, contributing to the adaptation of land plants to stressful environments (Hanada et al., 2008; Dassanayake et al, 2011).

Neofunctionalization and subfunctionalization in the Arabidopsis TTL family

Phylogenetic studies within the Arabidopsis TTL family indicate that TTL1 and TTL2 proteins cluster together and form a separated cluster than TTL3 and TTL4 (Figure 1). Although closely related to TTL1 based on amino acid sequence, the observed differences in TTL2 expression patterns compared to the other TTL genes suggests that TTL2 subfunctionalization is driven by mutations in its regulatory sequence. Moreover, TTL2 expression is confined to pollen grains and our genetic studies
confirmed that TTL2 performs a distinct function among the TTLs in male gametophyte development.

TTL1 expression pattern closely resembles that of TTL3 and TTL4, based not only on microarray analysis but also in promoter-GUS studies. Accordingly, the phenotypic consequences of mutations in TTL1, TTL3, and TTL4 affect similar Arabidopsis responses to abiotic stress. A plausible explanation for the TTL1, TTL3, and TTL4 gene duplications is that TTL duplications are required to increase the bulk of TTL proteins amounts without changing protein function. This seems to be the case for mannitol tolerance, in which an increment in the number of TTL mutations results in additive hypersensitivity mannitol. In this scenario, the increased hypersensitivity to mannitol could be explained as a result of a decrease in gene dosage, independently of the TTL genes mutated. However, the triple heterozygous mutant for TTL1, TTL3, and TTL4 is not hypersensitive to mannitol in conditions where the roots of single mutants are clearly affected. Thus, our results suggest that although some redundancy within the TTL family may occur, the roles of TTL genes cannot be simply explained as TTL gene dosage defects and suggest specific functions. Furthermore, the fact that TTL4 increased the shoot growth at moderate NaCl indicates that TTL4 followed a process of neofunctionalization that differentiates it from the rest of the TTLs.

In this study, we have shown that independent TTL genes are essential for root growth and integrity under osmotic stress, having a role on root meristem organization and vascular genesis, and that TTL4 controls the osmotic/ionic stress responses by regulating Na⁺ homeostasis. Since colonization of different habitats by plants is determined by morphological and physiological adaptation designed to deal with multiple abiotic stresses such as drought and salinity, it is very attractive to hypothesize that specialization of TTLs function contributed to Arabidopsis adaptation to very different environmental conditions. Still, an important question that needs to be addressed is how TTL proteins are mechanistically involved in these adaptive processes.

**Protein-protein interactions mediating TTL function**

TTL proteins play important roles in different aspects of plant development, being essential for both gametophytic viability and root growth and integrity under osmotic stress. The TPR units are common modules in molecular chaperones and are
required for the establishment of protein–protein interaction during the formation of multiprotein complexes (Blatch and Lässle, 1999).

Recently, TTL proteins have been proposed as potential interactors of the Hsp90 and Hsp70 chaperone complexes (Prasad et al., 2010). This is based on the finding that TTLs, like confirmed Hsp90 co-chaperones, such as Hsp70-Hsp90 organizing protein (Hop), high molecular weight immunophilins [cyclophilin 40 (Cyp40) and FK506-binding proteins 51 and 52 (FKBP51 and FKBP52)], protein phosphatase 5 (PP5) and the carboxyl terminus of Hsc70 interacting protein (CHIP), contain a subset of basic residues in the TPR binding pocket, known as the carboxylate clamp (CC). The CC domain interacts with the acidic side chains of the highly conserved EEVD motif at the C-terminal ends of Hsp90 and Hsp70 (Carrigan et al., 2006; Prasad et al., 2010).

Hsp90 and Hsp70 complexes perform key roles in signal transduction by regulating maturation, localization, stability and protein interactions of a large number of signaling proteins in eukaryotes (Pearl et al., 2008). Alterations in these complexes lead to morphological abnormalities, such as altered leaf shape, organ number and pigment accumulation, indicating an important role for these chaperone complexes in plant development (Sangster et al., 2007). Since the overexpression of the cytosolic Hsp90 complex alters the sensitivity to drought and NaCl in Arabidopsis (Song et al., 2009), and the HSP70/HSP90 machinery is important in plants to integrate signals from their biotic and abiotic environments through stomatal regulation (Clément et al., 2011), we hypothesize that TTL proteins could regulate abiotic stress responses through their specific expression under stress conditions and their interaction with the Hsp90 or Hsp70 complexes.

**Chaperone function of TTL proteins**

In Arabidopsis, the proteins with highest homology to TTL are AtTPR12, AtTPR13 and AtTPR14. These proteins show a similar TPR domain localization as TTL proteins but lack the C-terminal region with homology to thioredoxin. Another protein with high homology is AtTDX (Lee et al, 2009; Vignols et al, 2003), a protein that contains 3 TPR domains and a C terminal *bona fide* thioredoxin domain. AtTDX exhibits multiple functions, acting as a disulfide reductase, foldase chaperone, and holdase chaperone (Lee et al, 2009). The disulfide reductase and foldase chaperone...
functions predominate when AtTDX occurs in the low molecular weight (LMW) form, whereas the holdase chaperone function predominates in the high molecular weight (HMW) complexes. Chaperones are largely classified into 2 groups: foldase chaperones, which support the folding of denatured proteins to their native state, and holdase chaperones, which bind to folding intermediates, thereby preventing their nonspecific aggregation (Beissinger and Buchner, 1998).

This study reveals that TTL genes are specific for land plants and play a regulatory role in different aspects of plant development and stress responses. Although the molecular mechanisms regulated by TTLs need to be elucidated, this study provide important insights in the understanding of a novel and essential protein family with very little available functional information.

MATERIAL AND METHODS

Sequence Analysis

Genes encoding TTL proteins in different plant species were identified using the phytozome web page (www.phytozome.net) with the exception of tomato and barley where their genome database were employed. We used the BLASTP algorithm against the putative proteome derived from the predicted genes. Later the TPR motif (Pfam:00505) and TRLX motif (Pfam:00085) were analyzed individually in each sequence using both, the tool available in phytozome and the Conserved Domain Database at National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/structure/ccd/ccd.shtml) and discarded those sequencing without 6 TPR motifs in conserved positions or lacking the TRLX domain. Protein data matrices were aligned using ClustalX multiple sequence alignment program with default gap penalties.

Phylogenetic Analysis and Genomic Structure

Multiple sequence alignments were performed with the ClustalW program. The unrooted phylogenetic tree was generated with the ClustalX2 program and visualized using Treeview. Alignment analysis of Arabidopsis, rice, moss and lycophyte TTLs was
obtained using MegAlign 4 software. TTL in silico analysis were performed using DNASTAR Lasergene 8 software. All parameter values correspond to default definitions.

**Plant Material**

The plants used were Arabidopsis thaliana L. ecotype Col-0 wild type (WT) and the T-DNA insertion lines Salk_063943 (for TTL1), Salk_106516 (for TTL2), Sail_193_B05 (for TTL3) and Salk_026396 (for TTL4). The Col-0 T-DNA insertion mutants were identified using the SIGnal World Web site at http://signal.salk.edu. Seeds of the T-DNA insertion lines were obtained from the ABRC.

**Analysis of the T-DNA Insertion lines and Generation of Mutant Combinations**

PCR-based genotypic analysis was performed as described (Koiwa et al., 2006) using the following primers: LB SAIL (5’-TAG CAT CTG AAT TTC ATA ACC AAT CTC GAT ACA C-3’); LBSALK (5’-TGG TTC ACG TAG TGG GCC ATC G-3’); TTL1DPCRF (5’-TGG ACT CAC CAC CAC CAC TA-3’); TTL1DPCRR (5’-ACC GAG TCT GCG AAC AAG AT-3’); TTL2DPCRF (5’-GAG CTC TCC CAC ATT CCA AC-3’); TTL2DPCRR (5’-CCG TTT AGC CTC ATT GCT TC-3’); TTL3DPCRF (5’-AGA GAG CTG CGA TGC TTG AT-3’); TTL3DPCRR (5’-ATG CTC TCC TCC ACA TCC AC-3’); TTL4DPCRF (5’-AGA TCG GTG ATT GGA GAA CG-3’); and TTL4DPCRR (5’-TCG ATA GAG CGT TCC TTG CT-3’). Mutant combinations generated in this work were obtained by classical genetic crosses and further PCR genotypic analysis.

**Arabidopsis Seed Germination and Root Elongation Measurements**

Arabidopsis seeds were surface sterilized with 70% (v/v) ethanol for 5 min, followed with 20% sodium hypochlorite for 15 min and then washed three times with sterile water. For in vitro germination, seeds were stratified for two days at 4ºC in the dark, and plated onto petri dishes containing basal MS medium (Murashige and Skoog, 1962). Seedlings were grown four days in MS medium and then transferred to petri dishes containing the different concentration of the stress agent or hormone concentrations (Rosado et al., 2006a). Plates were located under long-day regime (16 h light/8 h dark.), 25 µE at 23ºC (day/night). For root elongation measurements, eight
seeds were used per replicate, and three replicates were made for each treatment. Seedlings with 1- to 1.5-cm-long roots were transferred from vertical agar plates containing MS medium onto a second agar medium supplemented with different concentrations of salts and osmotic stress agents as described above. Root length and plants weight were scored 7 days after transferred to plate with the different treatments.

**Ion Content Determination**

Seedlings were grown as indicated previously. For ion content determination, 15-day-old *ttl4* and WT seedlings grown in 120 mM NaCl after the transfer from MS medium were used. The shoot was removed and washed three times with bi-distilled H₂O. To determine tissue Na⁺ concentrations, the dried plant material was boiled for 20 min in 1 mL of 100 mM nitric acid and analyzed using a flame photometer at the Laboratorio de Ionómica (CEBAS-CSIC, Murcia, Spain).

**Promoter TTL::GUS Analysis**

Approximately 2.0 kb of the genomic sequence upstream of the TTLs translation start site was amplified by PCR. The following primers were used: PRO TTL1F, 5’-TGG TAC CTT GAG TGG AAG AAG GAA-3’; and PRO TTL1R, 5’-ACC ATG GTG AGT GTT GTG GTG AGT GAA-3’ for TTL1::GUS; PRO TTL2F, 5’-AGG TAC CTT GAA TAA ATC CGA-3’; and PRO TTL2R, 5’-ACC ATG GTT AGC AAG ATT ACA AAA AAG-3’ for TTL2::GUS; PRO TTL3F, 5’-AGG TAC CGC AAC ACC CTT CTA TTT-3’; and PRO TTL3R, 5’-ACC ATG GTG GTC GTC ACT TCC TCG TGA GCT-3’ for TTL3::GUS; PRO TTL4F, 5’-TGG TAC CCA AAT CTT GTT ATT TTG-3’; and PRO TTL4R, 5’-AGG TAC CGC AAC ACC CTT CTA TTT-3’ for TTL4::GUS. The amplified fragments were cloned into the binary vector pCAMBIA1303. The recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 and used to transform WT plants. Control plants consisted in WT transformed with the pCAMBIA 1303 empty vector. GUS activity was detected *in situ* as previously described (Jefferson et al., 1987). Histochemical analysis for TTLs promoter-GUS constructs under stress treatments was performed using 4–day-old seedlings treated with 120 mM NaCl and 300 mM Mannitol for 24 hours. Special care was taken to ensure that controls and transformed plants were mannitol and NaCl treated and stained for GUS activity simultaneously.
**TTLs Expression Analysis using RT-PCR**

Two-week old plants were transferred from MS agar plates to soil, grown at 23°C under long-day regime in a growth chamber during one month until flowering. Whole flowers were collected from each mutant for RNA isolation, immediately frozen in liquid nitrogen, and stored at -80°C until use. RNA extraction and RT-PCR was performed as previously described (Rosado et al., 2006b).

**Microscopy Analysis**

*Arabidopsis* roots were fixed overnight at 4°C in 4% (v/v) paraformaldehyde in 0.1 M phosphate buffer saline buffer, pH 7.4. Samples were dehydrated gradually in a series of ethanol, and subsequently followed of dry acetone, 1:1 Araldite 502/dry acetone during 1 h, and Araldite 502 overnight (EMS, Pais). The Araldite 502 resin is a mixture of 9.2 g Araldite 502, 10.8 g DDSA, and 0.4 g DMP-30. After embedding in Araldite, blocks were left for polymerization at 60°C during 72 h. Transverse sections of the roots (1 μm thick) were obtained using an ultramicrotome (Reichert Jung-Ultracut E), stained with 0.1% (w/v) Toluidine blue and visualized with a Leika DM 1000 microscope. For each line, an average of 10 plants was used for analysis.

Vascular bundles were visualized using a Ranvier type microtome. Sections were dehydrated in absolute ethanol (99.9% v/v), which was removed in a critical point dryer system (BAL-TEC, CPD030). Then, samples were mounted on specimen holders and covered with a fine gold coat in a metallization unit (Jeol JCC 1100). A scanning electron microscopy (SEM) equipped with a digital acquisition system (Jeol JSM-840) was used for visualization of the samples.

Quantification of the diameter of vascular cells, root apex cells, and number of cells was performed using ImageJ software (http://rsb.info.nih.gov/ij/). Measurements were performed on eight different cells for each tissue (cortex, endocycle, pericycle, xylem and phloem) from three independent plants.

Silques examination was performed by clearing them in 95% ethanol for 1 hr, hydrated in 50% glycerol and examined under a magnifying glass.
Bioinformatics Analysis

Bioinformatic analyses was performed using the following databases: GENEVESTIGATOR (www.genevestigator.com); The Bio-Array Ressource for Arabidopsis Functional Genomics (www.bar.utoronto.ca); TAIR (www.arabidopsis.org); NCBI (www.ncbi.nlm.nih.gov); and EBI (www.ebi.ac.uk/Tools/clustalw2/index.html).

Tetratricopeptide-Repeat Thioredoxin-Like (TTLs) Analysis

The mining of TTLs genes was performed by searching sequences homologous to the TTL1 protein using the phytozome Database (www.phytozome.net). Redundant sequences, as well as those sequences that did not present six TPRs and the TRLX domain were discarded. Sequences were also analyzed using NCBI Conserved Domain Database (CDD, http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Generation of the unrooted phylogenetic tree was performed by alignment of full-length amino acid sequences using ClustalW and the BIOEDIT software, followed by manual adjustments.

ACKNOWLEDGMENTS

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LITERATURE CITED


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FIGURE LEGENDS

Figure 1. Phylogenetic tree of 29 TTL protein from 21 plant species. TTL proteins from moss, lycophyte (Lyc), monocots, and dicots were used in the analysis. All TTL proteins identified in P. patens, S. moellendorffii, O. sativa, and A. thaliana were included in the analysis. From the rest of the species only the TTL protein most homologous to A. thaliana TTL1 was included. The phylogenetic tree was generated using ClustalW software and bootstrap values are shown.

Figure 2. Phylogenetic relationship among individual TPR motifs. An unrooted tree was built using the 6 TPR sequences of the four Arabidopsis thaliana proteins, and TPR sequences from TTL orthologs from phylogenetically distant species such moss, lycophytes, and monocots.

Figure 3. Phylogenetic and sequence analysis of TRXL domains. (A) Phylogenetic analysis of the TRXL domain of the TTLs from A thaliana, O sativa, P. patens and S. moellendorffii is shown. In the analysis the protein sequence of At3g17880, a genuine thioredoxin, was included. (B) Detail of the alignment of the TRXL domains including the two cysteines motif (WCGPC) required for thioreductase activity. As shown, homologous regions from the TRXL domains of TTLs lack at least one of the two cysteines that are essential for reductase activity.

Figure 4. Molecular characterization of ttl mutants (A) (a) ttl1, ttl2, ttl3 and ttl4 mutant alleles. The corresponding T-DNA line from SALK is indicated on top. Solid boxes represent exons and lines represent introns. ATG and stop codons are indicated. Open boxes indicate the 5’ and 3’ untranslated regions. T-DNA insertion sites (not drawn to scale) are represented by triangles. The primers used for PCR genotyping are indicated by arrows (see Material and Methods for primer sequences). (B) Expression analysis of the four TTL genes in wild-type (WT) and ttl mutants by RT-PCR. RNA was extracted from flowers, which is the only tissue where TTL2 expression was detected. The gene-specific primers designed to amplify cDNA fragments are detailed in Material and Methods. Tubulin gene was used as a positive control for the RT-PCR.

Figure 5. Root growth responses of ttl mutants to mannitol stress. (A). Root elongation of single, double, and triple ttl mutants was measured and root growth was
expressed as the percentage relative to WT seedlings grown on the same conditions. Results are the means of three independent experiments (±SD). Asterisks indicate a significant difference between samples, as determined by t-tests: *, P < 0.1; **, P < 0.05.

**Figure 6. **TTL4 loss-of-function increases tolerance to mild NaCl stress. (A) Relative fresh weight of single *ttl* mutants, and selected double and triple *ttl* mutants in MS medium or MS medium supplemented with 120 mM NaCl. All genotypes containing the *ttl4* mutation displayed improved growth in medium supplemented with NaCl. Asterisks indicate a significant difference between samples, as determined by t-tests: *, P < 0.05. (B) Pictures of seedlings grown on MS agar medium for 1 week and transferred to MS agar medium for 7 additional days without (left) or with (right) 120 mM NaCl. (C) Sodium content in the shoot of seedlings depicted in (B). Asterisks indicate a significant difference between samples, as determined by t-tests: *, P < 0.05.

**Figure 7. **Tissue-Specific expression pattern of TTL genes by promoter-reporter (GUS) fusions. GUS histological staining in transgenic Arabidopsis lines containing *pTTL::GUS* constructs. The samples depicted are 2-day-old seedlings (A-D), 10-day-old seedlings (E-H) and flowers (I-M).

**Figure 8. **TTL genes show differential expression responses to NaCl and mannitol stresses. Transgenic lines harboring TTL promoter::GUS fusions were grown for 4 days and then transferred to control medium or medium supplemented with 120 mM NaCl or 300 mM mannitol for 24 hours before staining for GUS activity. The pictures show whole seedlings, roots and cotyledons.

**Figure 9. **TTL genes are required for male gametophytic development. Pollen and siliques from WT and (*ttl1/ttl2/ttl3/TTL4/ttl4*) plants were analyzed by light microscopy. *ttl1/ttl2/ttl3/TTL4/ttl4* plants showed abnormal, shrunken pollen grains (indicated by arrowheads). *ttl1/ttl2/ttl3/TTL4/ttl4* plants do not support normal seed development showing approximately a 50% of aborted seeds.

**Figure 10. **Osmotic stress effects on root morphology of the triple mutant *ttl1/ttl3/ttl4*. (A) *ttl* mutants root swelling induced by 7 days treatments in growth media supplemented with 400 mM mannitol (B) Root growth hypersensitivity of the triple *ttl1/ttl3/ttl4* mutant after 400 mM mannitol treatments. (C-F) Transverse sections of the
roots of WT and triple *ttl1ttl3ttl4* mutant in control conditions (C and D) or after treatment with mannitol (E and F). Magnification 40X. (G-J) Scanning electron microscopy of WT (G and I) and *ttl1ttl3ttl4* mutant (H and J) root meristematic tissue after osmotic stress treatments. Mutant shows vascular bundle disorganization, large endodermis and cortex cells, altered pericycle and damage in vascular tissue with highly lignified xylem. (G) and (H) 700X magnification. (I) and (J) 3000X magnification.

**Supplemental Figure S1. Schematic representation of TTL containing duplicated segments identified in the Arabidopsis Genome.** Arabidopsis *TTLs* syntenic relationships were identified using the Plant Genome Duplication Database. (A) *TTL1* (Chromosome 1) and *TTL2* (Chromosome 3) (red arrow) belong to a large inverted duplicated segment containing 94 additional duplicated genes or anchors (blue arrows). (B) *TTL3* (Chromosome 2) and *TTL4* (Chromosome 3) (red arrow) belong to a very large duplicated segment containing 290 additional duplicated genes or anchors (blue arrows). Graphs represent ±100 kb duplicated regions centered in the *TTL* genes.

**Supplemental Figure S2. ttl mutants show identical root growth control medium.** (A) Root growth was expressed as the percentage relative to WT seedlings grown on control medium. Results are the means of three independent experiments (±SD). No significant difference between samples, as determined by t-tests. (B) Ten-day-old seedlings of WT and all *ttl* mutants grown on MS medium.

**Supplemental Figure S3. Root growth of triple *ttl* mutants are not affected by pH, low nutrients or reactive oxygen species generators.** Root growth was expressed as the percentage relative to WT seedlings grown on the same conditions. Results are the means of three independent experiments (±SD). No significant difference between samples, as determined by t-tests

**Supplemental Figure S4. Expression pattern of A. thaliana TTL genes based on data available from Genevestigator database (http://www.genevestigator.ethz.ch).** The data is expressed as the total number of pixels.

**Supplemental Figure S5. Expression pattern of A. thaliana TTL genes in response to NaCl stress based on data available from Genevestigator database (http://www.genevestigator.ethz.ch).** The expression levels of *TTL1* (A), *TTL2* (B),...
TTL3 (C), and TTL4 (D) are shown. A gene responsive to NaCl (RD29A) (E) is shown as a control.

Supplemental Figure S6. Root growth inhibition is not caused by reduce gene dosage of TTL genes. Root elongation of seedlings of the triple ttl1/ttl3/ttl4 compared with the triple F1 heterozigous TTL1, TTL3, and TTL4 plants. Root growth was expressed as the percentage relative to WT seedlings grown on the same conditions. Results are means of three independent experiments (±SD). Asterisks indicate a significant difference between samples, as determined by t-tests: *, P < 0.1.

Supplemental Figure S7. Root cell diameters of WT and ttl1/ttl3/ttl4 triple mutant after 400 mM mannitol stress. (A) pericycle cells, (B) cortex, (C) endodermis, (D) xylem, (E) phloem. The analysis was performed using transversal root meristem sections. Values are means of 8 cells from 3 independent roots. Error bars represent standard deviation. All cell types displayed significant differences P-values<0,01.
Table I. Effect of mutation in *TTL2* on the number of seeds per silique.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Obtained</th>
<th>T-test</th>
<th>Nº of siliques</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>TTL2/ttl2</em></td>
<td>13.3 ± 6.34</td>
<td>P &lt; 0.001</td>
<td>10</td>
</tr>
<tr>
<td><em>ttl2/TTL2</em></td>
<td>22.8 ± 9.41</td>
<td>P &gt; 0.001</td>
<td>10</td>
</tr>
<tr>
<td><em>TTL2/TLL2</em></td>
<td>29.5 ± 10.61</td>
<td>-</td>
<td>10</td>
</tr>
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</table>
Table II. Genotypic analysis using kanamycin resistance. Expected and experimental percentage of germination in kanamycin selection medium of the indicated genotypes.

<table>
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<tr>
<th>Genotypes</th>
<th>Expected</th>
<th>Obtained</th>
<th>Nº of seeds</th>
</tr>
</thead>
<tbody>
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<td>100 % ± 0</td>
<td>116</td>
</tr>
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<tr>
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<td>50%</td>
<td>9.7 % ± 8.4</td>
<td>110</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3
Figure 4

(A) Diagrams of the T-DNA insertion sites in the TTL1, TTL2, TTL3, and TTL4 genes. Each diagram shows the ATG start codon, the T-DNA insertion site labeled with the corresponding SALK or SAIL accession number, and the TAA stop codon. The Fw and Rv arrows indicate the forward and reverse primers used for PCR analysis.

(B) Gel electrophoresis results showing the absence of TTL1, TTL2, TTL3, and TTL4 expression in the mutant lines (ttl1, ttl2, ttl3, and ttl4) compared to the wild type (WT). The Tubulin (Tub) gene is used as a control for equal loading of the samples.
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10