

Study on the Effect of *AtTTG1* on Salt Tolerance of *Arabidopsis Thaliana*

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Abstract: Soil salinization, caused by natural processes or human activities, is characterized by excessive soluble salts in the soil. It severely impairs normal plant growth and development. The TRANSPARENT TESTA GLABRA1 (*TTG1*) gene encodes a WD40 repeat protein that participates in multiple key biological processes, including trichome development, cuticular wax biosynthesis, photosynthetic efficiency regulation, and salt stress response. These functions enable *TTG1* to support the growth adaptation of *Arabidopsis thaliana* under different environmental conditions. In this study, CRISPR-Cas9-mediated *TTG1* knockout lines of *A. thaliana*, generated in a previous study, were used as experimental materials. In this study, CRISPR-Cas9-generated *TTG1* knockout lines (from a prior experiment) were used as experimental materials. Genomic DNA was extracted from candidate lines, followed by nested PCR validation and Sanger sequencing to eliminate false-positive individuals. Phenotypic characterization of confirmed positive mutants was conducted to dissect the role and regulatory mechanism of *TTG1* in plant salt stress tolerance.

Keywords: *TTG1*; CRISPR-Cas9; Mutant Screening; *Arabidopsis Thaliana*; Salt Stress.

1. Introduction

Saline soils contain high levels of soluble salts that induce osmotic stress and ionic toxicity in plants, leading to cell dehydration, plasma membrane damage, and even plant death[1]. Additionally, soil salts compete with plants for binding sites and transport channels of essential nutrients, disrupting nutrient uptake and utilization and causing nutritional imbalance. Thus, long-term exposure to saline-alkaline environments severely inhibits plant growth and development.

The *TTG1* gene of *A. thaliana* encodes a transcription factor belonging to the MYB transcription factor family. Previous studies have demonstrated that *TTG1* is involved in multiple cell differentiation processes, such as trichome, pollen, flower, and seed coat development, and plays a pivotal role in regulating epidermal morphogenesis[2]. Under high-salt conditions, *TTG1* expression is dynamically regulated by salt concentration and plant physiological status: enhanced expression strengthens cell wall stability and defense capacity, while reduced expression prioritizes plant resources for salt stress response. Mechanistically, *TTG1* protein interacts with other regulatory proteins to modulate the composition and structure of cell walls, enhancing their robustness against salt-induced osmotic shock[3].

Despite extensive research on *TTG1* in epidermal development, its role in salt stress tolerance remains incompletely understood. The association between *TTG1*-mediated trichome formation and plant salt adaptation, as well as the underlying molecular mechanisms, requires further investigation. In this study, CRISPR-Cas9-derived *TTG1* mutants were characterized to clarify the function of *TTG1* in salt stress response, providing a theoretical basis for improving plant salt tolerance through genetic engineering.

2. Materials and Methods

2.1. Experimental Materials

Plant materials included wild-type (WT) *A. thaliana* ecotype Columbia (Col-0) and homozygous *TTG1* knockout mutants constructed via CRISPR-Cas9. All materials were preserved by Qilu University of Technology (Shandong Academy of Sciences).

2.2. Experimental Methods

2.2.1. Genomic DNA Extraction

Genomic DNA was extracted from young leaves of candidate mutants using the CTAB method, with minor modifications. DNA quality and concentration were determined by 1% agarose gel electrophoresis and a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

2.2.2. PCR Validation

PCR was performed to verify the integration of the CRISPR vector pHEC401 into the *A. thaliana* genome. The reaction system (50 μ L total volume) included 4 μ L dNTP (2.5 mM each), 5 μ L 10 $\times$ EX Buffer, 2 μ L forward primer (TTG-F-OUT-7, 10 μ M), 2 μ L reverse primer (pHEC401-R, 10 μ M), 1 μ L EX Taq Enzyme (5 U/ μ L), 1 μ L template DNA (50 ng/ μ L), and 35 μ L ddH₂O. The PCR program was as follows: initial denaturation at 94 $^{\circ}$ C for 5 min; 34 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 55 $^{\circ}$ C for 30 s, and extension at 72 $^{\circ}$ C for 1 min; final extension at 72 $^{\circ}$ C for 10 min; storage at 4 $^{\circ}$ C.

2.2.3. Nested PCR and Sequencing

Positive lines identified by primary PCR were subjected to nested PCR for target region amplification. PCR products were purified using the AxyPrep DNA Gel Extraction Kit

(Axygen, USA) and sent to Tsingke Biotechnology Co., Ltd. (Qingdao, China) for Sanger sequencing. Sequences were aligned with the WT *TTG1* gene using DNAMAN 9.0 software to identify mutation types.

2.2.4. Phenotypic Observation

Seeds of validated *TTG1* mutants and WT plants were surface-sterilized with 75% ethanol for 30 s and 10% NaClO for 5 min, rinsed 5 times with sterile water, and sown on Murashige and Skoog (MS) medium (0 mM NaCl, salt-free) or MS medium supplemented with 100 mM NaCl (high-salt). Seedlings were cultured in a growth chamber at 22°C with a 16 h light/8 h dark photoperiod (light intensity: 120 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, relative humidity: 70%). Trichome morphology was observed at the 4-leaf stage using a stereomicroscope. Root hair length and density were measured at 7 days post-germination using ImageJ software ($n = 30$ per line, 3 biological replicates).

3. Results

3.1. Verification of Candidate Mutant Lines

Genomic DNA was extracted, genomic DNA was used as a template, guide RNA was designed as a primer to verify whether the CRISPR vector pHEC401 was successfully introduced into the genome, and six plants to be tested were obtained according to the results obtained from agarose gel electrophoresis. Agarose gel electrophoresis showed that 6 candidate lines (Line1–Line6) had a clear target band (Fig. 1). This indicated successful integration of the CRISPR vector pHEC401 into the genome of the 6 candidate lines, confirming they were preliminary positive lines.

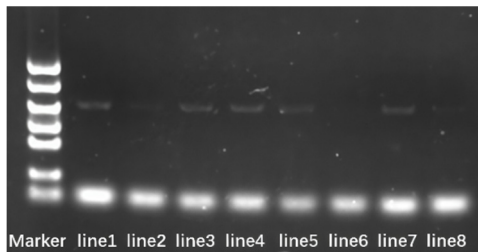


Fig 1. Agarose gel electrophoresis of verification PCR products. Marker: DNA Marker DL2000; 1–8: Candidate *TTG1* mutant lines

The PCR products of the 6 candidate lines were sequenced, and sequence alignment revealed different distinct mutation types. Line1 showed a single-base substitution (C→T) in the non-coding region, with no significant impact on *TTG1* expression. No sequence variations were found in Line2, Line3 and Line5 compared to WT, suggesting either incomplete knockout or off-target effects followed by DNA repair. In Line4, a 2-base deletion (position 345–346 of the *TTG1* coding sequence), resulting in a frameshift mutation. In Line 6, there was an 11-base deletion (position 289–299 of the *TTG1* coding sequence), leading to premature termination of translation. Line4 and Line6 were selected as functional *TTG1* knockout mutants for subsequent phenotypic analysis due to the severe disruption of *TTG1* gene structure.

3.2. Phenotypic Observation of *TTG1* Mutants

Stereomicroscopic observation showed that Line6 mutants lacked trichomes entirely on rosette leaves, with a smooth leaf surface (Fig. 2). In contrast, WT leaves had dense trichomes

(Fig. 3b). Line4 mutants exhibited sparse trichomes (average density: 2.3 ± 0.5 trichomes per leaf, $n = 30$) compared to WT (18.7 ± 1.2 trichomes per leaf, $P < 0.001$), confirming that *TTG1* is essential for trichome formation.

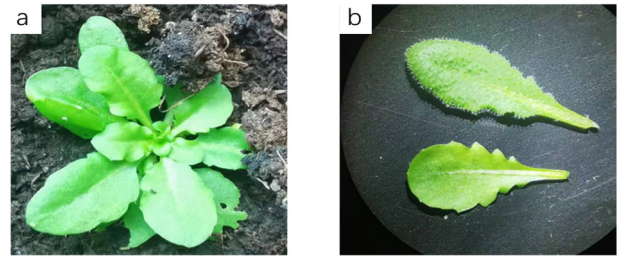


Fig 2. Trichome morphology of Arabidopsis leaves. (a) *TTG1* mutant Line 6 plant; (b) Line6 mutant (lower one) and wild type (upper one).

3.3. Identification of Salt Tolerance in *TTG1* Mutants

3.3.1. Phenotype Under Salt-Free and High-Salt Conditions

At the morphological level, the number of epidermal hairs of *ttg1* mutants was significantly less than that of wild type under both 0 mM and 100 mM NaCl treatment (Fig. 3), which was consistent with the results of previous studies on *ttg1* gene function[4]. As the main stomatal accessory structure of plants, the reduction of epidermal hairs directly affects the transpiration of plants, resulting in a decrease in the water retention ability of *ttg1* mutants in high-salt environments.

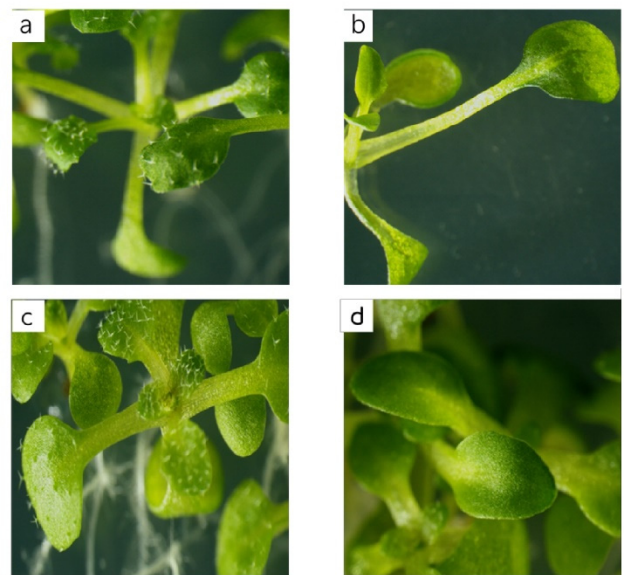


Fig 3. Growth status of epidermal hairs in salt-free condition of (a) Wild-type Arabidopsis thaliana (b) *ttg1* mutant and in salt-treated condition of (c) Wild-type Arabidopsis thaliana (d) *ttg1* mutant

The root hair growth of *ttg1* mutant plants was extremely vigorous in both salt-free and high-salt environments. In the salt-free environment, the root hair length and density of *ttg1* mutants were significantly higher than those of wild-type plants (Fig. 4). In a high-salt environment, this advantage is even more obvious, and the root hairs of the *ttg1* mutant not only grow longer, but also branch more profusely, greatly increasing the surface area of plant roots. We accurately

measured the root hairs of both types of *Arabidopsis thaliana* and found that the root hairs of the *ttg1* mutants significantly exceeded those of the wild type. Root hairs greatly increase the surface area of plant roots, allowing plants to absorb more water and nutrients from soil solutions, so we speculate that their vigorous growth is mainly used to compensate for excessive water evaporation caused by the loss of epidermal hairs in plants.

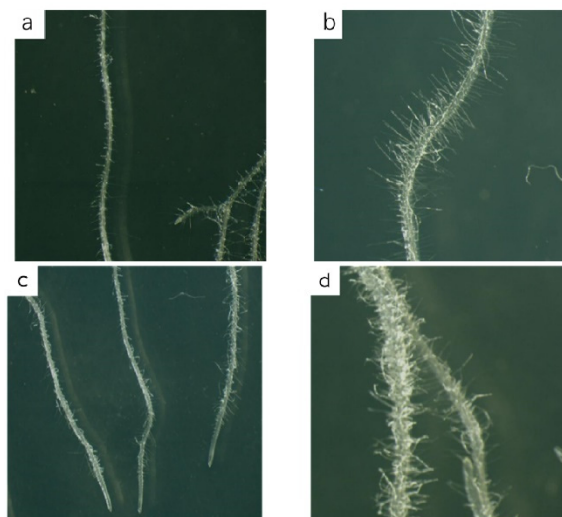


Fig 4. Growth status of root hairs in salt-free condition of (a) Wild-type *Arabidopsis thaliana* (b) *ttg1* mutant and in salt-treated condition of (c) Wild-type *Arabidopsis thaliana* (d) *ttg1* mutant

3.3.2. Differences in Root Hair Length Under Salt Stress

Root hair length exhibited significant differences between

WT and *ttg1* mutant plants under both salt-free and salt-treated conditions, with distinct responses to salt stress observed in the two genotypes (Figure 5A). Under salt-free conditions, the root hair length of *ttg1* mutant was significantly higher than that of WT plants, representing an 18.1% increase. After salt treatment, the root hair length of WT plants had a slightly 4.7% increase compared to salt-free treatment, showing no statistical significance. In contrast, the root hair length of *ttg1* mutants significantly increased by 8.6% and was significantly longer than that of salt-treated WT plants. These results indicate that the root hair growth of *ttg1* mutants has a stronger adaptive response to salt stress, while the root hairs of WT plants show a weak response to salt stress.

3.3.3. Differences in Fresh Weight Under Salt Stress

Plant fresh weight is a core indicator reflecting growth status under salt stress, and significant differences in fresh weight were observed between WT and *ttg1* mutants under different salt treatments (Fig. 5B). Under salt-free conditions, the fresh weight of *ttg1* mutants was 0.13 g/plant, which was significantly higher than that of WT plants (0.09 g/plant, $P < 0.05$). This suggests that *TTG1* gene knockout has already exerted a positive effect on plant biomass accumulation under normal growth conditions. After salt treatment, the fresh weight of WT plants significantly decreased to 0.06 g/plant (a 33.3% decrease compared to salt-free treatment, $P < 0.01$). In contrast, the fresh weight of *ttg1* mutants only slightly decreased to 0.1 g/plant (a 23.1% decrease compared to salt-free treatment, $P < 0.05$) and remained significantly higher than that of salt-treated WT plants ($P < 0.01$). These results demonstrate that the growth inhibition of *ttg1* mutants under salt stress is significantly lower than that of WT plants, indicating enhanced salt tolerance in *ttg1* mutants.

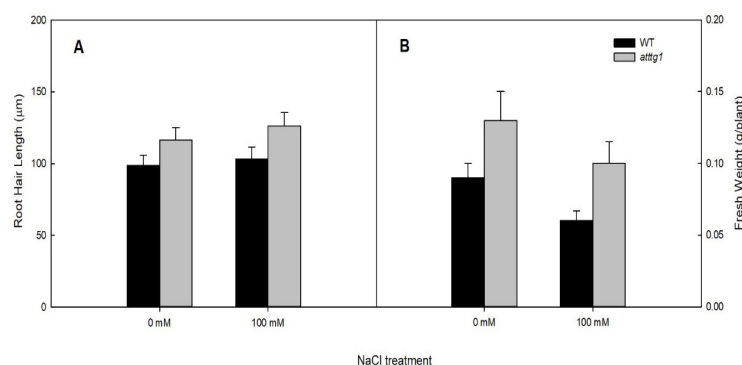


Fig 5. Effects of salt stress on root hair length (A) and fresh weight (B) in WT and *ttg1* mutants

4. Discussion

The data from this study showed that the root hair length of *ttg1* mutants is significantly higher than that of WT plants under both salt-free and salt stress conditions, and root hair length further increases after salt treatment, while the response of WT root hairs to salt stress is weak. This result is consistent with the previous study [4], confirming that the *TTG1* gene negatively regulates root hair development. *TTG1* knockout relieves its inhibition on root hair growth-related genes, thereby promoting root hair elongation and increasing density.

As the core structure for plants to absorb water and nutrients, increased root hair length can significantly expand the contact area between the root system and the soil [5].

Under salt stress, the longer root hairs of *ttg1* mutants can more efficiently absorb water from the saline environment, alleviating osmotic drought caused by salt stress. Meanwhile, the expanded root surface area helps enhance the absorption of essential ions such as K^+ , reduce the intracellular Na^+/K^+ ratio, and mitigate ionic toxicity [6]. This adaptive trait provides a structural basis for mutants to maintain growth under salt stress and explains why the decrease in fresh weight is significantly lower than that of WT plants.

Limitations of this study include the lack of physiological data on ion homeostasis and the limited number of mutant lines. Future work should expand the mutant library and measure ion accumulation in roots and shoots to clarify the role of *TTG1* in ionic stress tolerance.

Acknowledgments

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