Thellungiella halophila ST5 improves salt tolerance in cotton

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Abstract

Background: Salinity is a major abiotic stress to global agriculture which hampers crop growth and development, and eventually reduces yield. Transgenic technology is an effective and efficient approach to improve crop salt tolerance but depending on the availability of effective genes. We previously isolated Salt Tolerance5 (ThST5) from the halophyte Thellungiella halophila, an ortholog of Arabidopsis SPT4-2 which encodes a transcription elongation factor. However, SPT4-2-conferred salt tolerance has not been evaluated in crops yet. Here we report the evaluation of ThST5-conferred salt tolerance in cotton (Gossypium hirsutum L.).

Results: The ThST5 overexpression transgenic cotton plants displayed enhanced tolerance to salt stress during seed germination and seedling stage compared with wild type. Particularly, the transgenic plants showed improved salinity tolerance as well as yield under saline field conditions. Comparative transcriptomic analysis showed that ThST5 improved salt tolerance of transgenic cotton mainly by maintaining ion homeostasis. In addition, ThST5 also orchestrated the expression of genes encoding antioxidants and salt-responsive transcription factors.

Conclusion: Our results demonstrate that ThST5 is a promising candidate to improve salt tolerance in cotton.

Keywords: Salinity, Thellungiella halophila Salt Tolerance5 (ThST5), Salt tolerance, Cotton (Gossypium hirsutum L.), Ion homeostasis

Background

Salinity is a critical abiotic stress which not only restricts the plant growth and its survival but also decreases worldwide distribution of plants (Liang et al. 2018). According to a recent estimation, around 1 billion hectares (20%) of arable land is affected by salt (Ivushkin et al. 2019). To minimize the loss from salinity, there is an utmost need to develop crop varieties that are salt tolerant with acceptable yield under stress conditions.

Cotton, a member of Malvaceae family, is the world most important source of fiber and supplies 35% of the world total textile fiber. It is grown in tropical, semi-tropical and other areas of more than 80 countries/regions of the globe (Rojo-Gutiérrez et al. 2020). The allotetraploid upland cotton (Gossypium hirsutum L.) is the most important cultivated species providing more than 90% of the global cotton production (Abdelraheem et al. 2019). Cotton is moderately tolerant of salt, however, excessive salinity decreases the seed germination and restricts the growth of seedlings, hence being a serious menace to cotton growth and productivity (Wang et al. 2019).

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The development of transgenic insect-resistant and herbicide-tolerant cotton was a revolution of genetically-modified crops in the world (Cheema et al. 2015; Raman 2017). Since the last decade, transgenic cotton has been applied to improve abiotic stress tolerance. In order to reduce the effects of salt generated toxicity, plants trigger the response mechanism to accumulate osmoprotectants (glycine betaine, amino acids, polyols, sugars, and polyamines, etc.) in cells, resulting in enhanced salt tolerance (Naidoo and Naidoo 2001; Rontein et al. 2002). In order to maintain the intracellular ion homeostasis, one important mechanism is to sequester the sodium (Na\(^+\)) ions to the vacuoles or extracellular space by various ion transporters. Studies have shown that overexpression of Arabidopsis tonoplast-located Na\(^+\)/H\(^+\) antiporter AtNHX1 (He et al. 2005), Arabidopsis vacuolar H\(^+\)-pyrophosphatase AVP1 gene (Pasapula et al. 2011; Zhang et al. 2011), Thellungiella halophila H\(^+\)-pyrophosphatase gene TsVP (Lv et al. 2008; Zhang et al. 2016a, b), plasma membrane-localized sodium/proton antiporter K2-NhaD (Guo et al. 2020), and co-expression of TsVP and AtNHX1 (Cheng et al. 2018) improved salt tolerance in transgenic cotton plants. Transcription factors are key regulators of multiple stress-responsive genes (Yang et al. 2010) and are considered potential candidates for improving plant stress tolerance by genetic engineering. For example, the overexpression of rice SNAC1 (Liu et al. 2014), Arabidopsis EDT1/HDG11 (Yu et al. 2016), and maize ABRE binding protein 9 (ABP9) (Wang et al. 2017) in cotton improved the salt tolerance in transgenic plants compared with WT.

Thellungiella halophila or Thellungiella salsuginea (salt cress), recently has been named as Eutrema salsugineum, is a close relative of Arabidopsis thaliana (Koch and German 2013). This halophyte is a native species growing at extremely saline coastal areas of Eastern China, and it exhibits an extraordinary characteristic of salt tolerance (Amtmann 2009; Wu et al. 2012; Yang et al. 2013). We have previously isolated a number of salt tolerance genes via the functional gene-mining approach from salt cress (Ni et al. 2007; Du et al. 2008). Thellungiella halophila Salt Tolerance5 (ThST5), one of the isolated salt tolerance genes, is highly homologous to SPT4-2 gene in Arabidopsis. SPT4 is encoded by SPT4-1 and SPT4-2 in Arabidopsis. This zinc-finger protein through its N-terminal NusG (NGN)-binding domain interacts with another transcription factor SPT5 to form SPT4/SPT5 complex which together acts as transcription elongation factor by binding to RNA polymerase II (Guo et al. 2008; Hartzog and Fu 2013; Dürr et al. 2014). SPT4 is proven to be involved in root growth and its RNA interference (RNAi) knockdown Arabidopsis mutants displayed retarded root growth and reduced the number of lateral roots compared with wild type (Dürr et al. 2014; Wang et al. 2019). However, the role of SPT4 has not yet been explored in abiotic stress tolerance.

In the present study, we aim to evaluate whether ThST5 improves salt tolerance in cotton by generating transgenic cotton overexpressing ThST5 in upland cotton (Gossypium hirsutum L.). Our results clearly depict that overexpression of ThST5 improved the salt tolerance of transgenic plants under both laboratory and field conditions. The transgenic plants exhibited enhanced germination rate and seedling emergence in laboratory, and have improved yield in the field. Transcriptomic analyses demonstrate that ThST5 positively regulates an array of salt tolerance-associated genes. Together, our results demonstrate that ThST5 confers salt tolerance in cotton and is a promising candidate for enhancing cotton salt tolerance. Our findings will help the future researchers to improve salt tolerance in crops using ThST5 without any compromise on yield and agronomic traits.

**Results**

**ThST5 enhances salt tolerance in germination of cotton**

ThST5 was isolated as a salt tolerance candidate gene from Thellungiella halophila (Du et al. 2008). Based on sequence similarity, ThST5 is a homologue of Arabidopsis SPT4 (Additional file 1: Fig. S1). To evaluate its role in salt tolerance in cotton, we generated transgenic cotton overexpressing ThST5 (Additional file 1: Fig. S2) and used one overexpression (OE) line in the subsequent experiments.

In order to test whether the overexpression of ThST5 improves the germination rate of transgenic cotton under salt stress, the seeds of transgenic ThST5 overexpressing line and transgenic explant R15 (WT) were sown in the soil and irrigated with water or 250 mmol·L\(^{-1}\) NaCl. After one week, under water treatment, the germination rate of ThST5-OE as well as control plants displayed no difference and reached 100% by day 5, while under salt stress, the average germination rate in transgenic plants was significantly higher than that of CK and plateaued at nearly 50% by day 7 compared with wild type plateaued at 20% (Fig. 1). This result demonstrated that ThST5 indeed improved salt tolerance in seed germination, and played a critical role for cotton seedling establishment in saline soil.

**ThST5 improves cotton seedling growth under salt stress**

In order to evaluate the effect of ThST5 on cotton growth under salt stress, we germinated the seeds of both CK and transgenic line in soil. And 4 days after germination plants were transferred to hydroponic culture. Seven days after shifting to hydroponic culture, both the control and transgenic plants were subjected to 0 or
140 mmol·L\(^{-1}\) NaCl treatment for 3 consecutive days. Under 0 mmol·L\(^{-1}\) NaCl control conditions, the seedlings of both genotypes grew well showing no apparent difference between two phenotypes (Fig. 2A). Whereas under salt stress, the transgenic cotton exhibited improved salt tolerance compared with CK which showed leaf-wilting, chlorosis and growth retardation (Fig. 2B). Importantly, the transgenic line displayed a significantly higher survival rate of 78% than the survival rate of only 23% in CK (Fig. 2C).

We also evaluated salt stress tolerance of soil-grown transgenic plants at vegetative stage. Twenty days old T\(_3\) homozygous transgenic and CK cotton plants grown in soil under greenhouse conditions were irrigated with 0 or 300 mmol·L\(^{-1}\) NaCl for 10 days. Under 0 mmol·L\(^{-1}\) NaCl conditions, the transgenic and CK plants displayed no significant difference regarding growth (Fig. 2D). However, under salt stress conditions, CK plants displayed withered and dead leaves due to acute salt toxicity and eventually most of them died. In contrast, the transgenic plants exhibited two top green leaves with reduced wilting phenotype and a doubled survival rate than that of CK plants (Fig. 2E, F). Taken together, our results clearly demonstrated that overexpression of ThST5 significantly improved the salt tolerance in transgenic plants during vegetative growth.

**ThST5 increases the lateral root number and root biomass**

Lateral root plays a pivotal role in the uptake of nutrients and water, and participates in keeping plants fitness in fluctuating environment (Hochholdinger et al. 2004; Coudert et al. 2010), which ultimately improves crop yield. The elevated expression of ThST5 increased the number of lateral root per plant by 33% relative to CK, when the CK and overexpression (OE) plants were grown in soil for 1 week, under normal conditions (Fig. 3A, B). Moreover, fresh root weight was significantly increased in the transgenic plants (Fig. 3C). Our results are consistent with those of Dürr et al. which showed that down-regulation of SPT4 in Arabidopsis decreased the number of lateral roots (Dürr et al. 2014).

**ThST5 overexpression cotton plants exhibit decreased ROS (reactive oxygen species) accumulation**

Hydrogen peroxide (H\(_2\)O\(_2\)) and superoxide (O\(_2^−\)) are the two most prominent reactive oxygen species accumulated
Fig. 2  ThST5 significantly enhanced salt tolerance in transgenic cotton at seedling stage. A, B Growth phenotype of CK and ST5-OE under saline hydroponic conditions. Four days old seedlings were shifted to hydroponic culture for 7 days and treated with 140 mmol·L⁻¹ NaCl for 3 days before photographs were captured. Bar = 4 cm. C Survival rate of transgenic plants under saline hydroponic culture. Values are mean ± SD of three biological replicates (16 seedlings of each genotype per replicate). Different letters represent significant difference (P < 0.05) as calculated by Duncan’s multiple range tests. D, E Growth phenotype of CK and ST5-OE in soil. Twenty days-old seedlings were irrigated with either water or 300 mmol·L⁻¹ NaCl for 10 days. Photographs were captured at the 10th day after the treatment. Bar = 4 cm. F Survival rate of transgenic seedlings in saline soil. Values are mean ± SD of three biological replicates (16 seedlings of each genotype per replicate). Different letters represent significant difference (P < 0.05) as calculated by Duncan’s multiple range tests.

Fig. 3  Overexpression of ThST5 significantly increased lateral root number and improved the root biomass in cotton. A Root phenotype of 7 days old CK and transgenic plants from soil under normal condition Bar = 4 cm. B The number of lateral roots and C Fresh root weight of plants of 7 days after sowing. Values are mean ± SD of three biological replicates (16 plants of each line/replicate). Different letters represent significant difference (P < 0.05) as calculated by Duncan’s multiple range tests.
in response to oxidative stress (Zhang et al. 2015). Both CK and ThST5 overexpressing plants were grown in soil for 7 days followed by growing under hydroponic conditions for 4 days. Leaves of similar stage from control and transgenic plants were treated with 0.6% (100 mmol·L⁻¹) NaCl for 24 h and incubated in DAB (3, 3’-diaminobenzidine) solution as well as NBT (nitro blue tetrazolium) solution for the detection of H₂O₂ and O₂⁻ radicals, respectively. Less accumulation of H₂O₂ (brown precipitates) and O₂⁻ (blue precipitates) was detected in transgenic leaves than CK under saline condition (Fig. 4). These results indicated that ThST5 enhanced ROS-scavenging capability in transgenic plants under salt stress.

**ThST5 improves cotton yield under saline field conditions**

Apart from salinity tolerance assays in laboratory, we assessed the salt tolerance of transgenic cotton in field. The field trials were carried out in Yuncheng, Shanxi Province, from April to September of 2020. We used one ThST5 overexpression line and CK in the field trial which contained 3 replica plots (22.5 m² each) for each line. Under non-saline condition, there was no significant difference in yield between CK and transgenic cotton. However, under 0.4% (68 mmol·L⁻¹) salinity, the transgenic plants exhibited 24% increased yield than CK (Fig. 5A). Moreover, boll weight was less affected in transgenic plants than CK in saline conditions. The weight per boll of the transgenic cotton was slightly lower than that of CK under non-saline conditions. In saline circumstances, the average boll weight of transgenic cotton was slightly higher than that of control (Fig. 5B). The number of boll setting branches per plant was almost the same between CK and ST5-OE line under regular growth condition. However, in saline soil, ST5-OE plants showed higher number of boll setting branches per plant than that of CK (Fig. 5C). The number of first boll setting branches per plant was slightly higher in CK under normal conditions, but this number was similar between ST5-OE and CK under 0.4% (68 mmol·L⁻¹) NaCl (Fig. 5D). Thus, our data indicated that overexpression of ThST5 improves cotton yield in saline field.

**Transcriptome analysis uncover genome-wide ThST5-regulated genes**

For the exploration of regulatory network of ThST5-mediated salinity tolerance, transcriptomic comparison between 14 days old transgenic and CK cotton, under control and saline conditions, was performed in order to evaluate the upregulated and downregulated differentially expressed genes (DEGs) (>1.5 fold change, \(P<0.05\)). The transcriptome data was analyzed in two distinct sets by using aligned reads: control and transgenic cotton under normal condition (CK-N/ThST5-OE-N), control and transgenic cotton under salt conditions (CK-S/ThST5-OE-S). DEGs identified between transgenic cotton and CK under salinity were different from normal growth condition, representing ThST5 has distinct impact on transcriptome in response of salinity (Fig. 6A, B).

In order to obtain the detailed information about regulatory pathways involved, we performed the Gene Ontology (GO) enrichment analysis. The GO analysis revealed a number of biological processes were significantly enriched in DEGs between CK and ST5-OE under normal condition, including the transcription, signal transduction, and inorganic ion transport (Fig. 6C). On the other hand, under salt stress, GO analysis identified the biological processes related to signal transduction, amino
acid transport and metabolism, cell wall/membrane/envelope biogenesis, inorganic ion transport, defense mechanism, lipid transport and metabolism, carbohydrate metabolism, replication, recombination and repair, and coenzyme transport and metabolism were significantly enriched in DEGs between CK and ST5-OE under saline condition (Fig. 6D).

Additionally, we performed hierarchical clustering for analyzing the expression pattern of DEGs under salt stress. The DEGs for heatmap were selected on the bases of ones involved in ion homeostasis, salt tolerance and root formation. It is obvious from heatmap (Fig. 6E, F) that ThST5 positively regulates the expression of ion transporters such as NHX2, AKT1, CHX25, P-ATPases, and KUP11 and genes involved in potassium channels such as KAT1 and KAT3. Apart from transporters and channels, the expression level of key genes encoding antioxidants (peroxidases and glutathione S-transferase) was significantly higher in OE than CK. Furthermore, various transcription factors regulating salt tolerance such as WRKY, bZIP, and ZFP were differentially expressed in ST5-OE and CK plants. Furthermore, the genes related to auxin-signaling pathways were differently expressed in ST5-OE and CK, which may contribute to the improved root system of OE plants. Taken together, our transcriptomic analyses depict that ThST5 is a positive regulator of salinity-responsive genes.

Discussion
Although cotton is regarded as a salt tolerant crop, soil salinity negatively impacts the germination, seedling growth as well as yield (Khorsandi and Anagholi 2009; Higbie et al. 2010; Dong 2012). Hence, there is an urgent need to breed the cotton cultivars with enhanced salinity tolerance without any compromise on yield. In this regard, transgenic technology provides a promising way for cotton improvement. In this study, we evaluated the role of ThST5, previously isolated from Thellungiella halophila (Du et al. 2008), in salt tolerance in cotton. We have demonstrated that overexpression of ThST5 enhance salt tolerance in cotton during germination and seedling stage, as well as improve yield in saline soil.
The ThST5 overexpression cotton display improved salt tolerance at morphological as well as physiological level. Firstly, the root in the ThST5 overexpression plants have a greater number of lateral root and increased fresh root biomass. As the distribution of nutrients and water within the soil is uneven, the spatial positioning of root is of uttermost importance for the utilization of these resources. The plant capability to change its root system architecture plays a vital role in its response to various abiotic stresses (Smith and De Smet 2012; Koevoets et al. 2016). Hence the increased number of lateral root and fresh root biomass (Fig. 3) may benefit the development of transgenic cotton under salt stress. The homologue of ThST5 in Arabidopsis SPT4 has been proven to be involved in lateral root development by regulating auxin-signaling genes (Dürr et al. 2014). Auxin is a main regulator of lateral root development in plants (Blakely et al. 1988). When we explored the transcriptomic data, a number of genes involved in auxin-signaling were up- or down-regulated in ThST5-OE compared with CK. For example, genes involved in auxin biosynthesis (YUC and TAR2) were upregulated whereas repressors of auxin synthesis (Aux/IAA) were down-regulated in OE, both under control and salt conditions (Fig. 6E, F, Additional file 1: Table S1-4). It is conceivable that ThST5 may modulate auxin signaling to regulate lateral root formation but the exact mechanism is not known at present. Furthermore, the positive transcriptional regulator of lateral organ boundaries-domain16(LBD16) (Goh et al. 2012) was among the significantly upregulated DEGs between CK and ST5-OE under control conditions (Fig. 6E), which may contribute to the enhanced number of lateral root in ThST5 transgenic cotton.

Plants have adapted plenty of approaches to deal with salt stress, such as growth modulation, detoxification of toxic substances, osmotic adjustment, and ion homeostasis (Liang et al. 2018). Among these strategies, maintaining ion homeostasis by decreasing Na\(^+\) concentration and increasing K\(^+\) concentration (reduced Na\(^+\)/K\(^+\) ratio) in cytosol plays a crucial role in enhancing salinity tolerance of plants (Cui et al. 2020). The sequestration of Na\(^+\) into vacuole by sodium/hydrogen exchangers (NHXs) helps to maintain cellular Na\(^+\) homeostasis (Zhang et al. 2016a, b). In our transcriptomic data, NHX2 was significantly upregulated in transgenic plants compared with CK, under salt stress. The role of NHX2 in vacuolar sequestration of Na\(^+\) and enhancing salt tolerance has already been discussed (Cao et al. 2011; Yarra et al. 2012; Bulle et al. 2016; Yarra and Kirti 2019). Moreover, P-type ATPases, which are responsible for
creating the H⁺ gradient on two sides of plasma membrane by the ATP hydrolysis, driving the transmembrane transport of numerous ions and nutrients (Falhof et al. 2016), were also upregulated in the transgenic plants (Fig. 6F, Additional file 1: Table S1). The genes associated with maintaining high intracellular K⁺ content, such as AKT1, KAT1, KAT3, and KUP11 were detected to be upregulated in ThST5-OE. Collectively, our results depict that ThST5 confers salt tolerance by maintaining lower intracellular Na⁺ and higher K⁺ concentration in transgenic plants.

Salinity gives rise to eruption of oxidative stress via increased generation of ROS (Choudhury et al. 2017). The elevated levels of ROS wreak havoc on cellular physiology by damaging DNA, lipids, proteins, ultimately leading to cell death (Zhu 2002; Baxter et al. 2014). In our experiments, the ROS mediated damage was exacerbated in CK under salinity in contrary to the transgenic plants which exhibited reduced levels of ROS as depicted by DAB and NBT staining (Fig. 4A, B). Plants have been equipped with antioxidant mechanisms to scavenge extra ROS produced in cells, in response to abiotic stress. Ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and glutathione S-transferase (GST) are chief enzymatic players of cell’s antioxidant mechanism (Mittler et al. 2004; Foyer and Noctor 2005). The RNA-seq data demonstrated that the expression of genes encoding APX, POD, and GST (Fig. 6F; Additional file 1: Table S1) was significantly enhanced in ST5-OE under saline conditions, implicating that ThST5 buffered the salinity-triggered ROS burden by triggering antioxidant mechanism. Hence, the increased scavenging of ROS in transgenic cotton (Fig. 4) is in agreement with our RNA-seq data. Cumulatively, ThST5 confers salt tolerance by scavenging cellular ROS.

Transcription factors control the expression of downstream genes by binding to their regulatory regions and mediate crosstalk between phytohormones and stress-responsive genes (Golldack et al. 2014; Todeschini et al. 2014; Fernando et al. 2018). A number of salt-responsive transcription factors were enriched in our transcriptome data (Fig. 6F, Additional file 1: Table S1, S3). For example, some positive regulators of salinity, such as WRKY57 (Jiang et al. 2016), bZIP53 (Hartmann et al. 2015), bZIP44 (Liao et al. 2008), ZFP1 (Guo et al. 2009) were upregulated in OE under salt stress. On the other hand, the expression of negative regulators of salinity such as WRKY6 (Ivushkin et al. 2019) and MPT1 (Zhu et al. 2012) was downregulated in OE.

Conclusions
In conclusion, our results indicate that ThST5 is a positive regulator of salinity tolerance mainly by improving root system, maintaining intracellular ion homeostasis, cellular ROS detoxification, and regulating salt-related transcription factors. Therefore, ThST5 is a promising candidate gene for future crop improvement.

Methods
Plant materials and growth conditions
Cotton (Gossypium hirsutum L.) variety R15 was used in the present study. R15 is an upland cotton variety and is highly responsive to genetic manipulations (Ji et al. 2021). ThST5 overexpressing cotton was obtained by transferring ThST5:pCB2004 construct into CK by Agrobacterium-mediated transformation method. The full-length coding region of ThST5 was subcloned into binary vector pCB2004 under the control of CaMV (cauliﬂower mosaic virus) 35S promoter by GATEWAY cloning system. The resulting vector (Additional file 1: Fig. S2) was then electroporated into LBA4404 strain of Agrobacterium tumefaciens via electroporation. The surface-sterilization of R15 seeds was performed using 70% ethanol for 1 min and followed by 10% H₂O₂ for 1 min. The seeds were then washed with sterile water for 3–4 times and germinated on half-strength MS medium in the culture room having 28 °C and 8 h dark/16 h light. The cotton was transformed by using the hypocotyl Sects (2–3 cm) of 7 days old seedlings as explants; the explants were added for 15 min to Agrobacterium suspension harboring pCB2004-ThST5 vector having OD₆₀₀ from 0.4 to 0.5. The explants were then cultured on co-cultivation MS medium (MS salts, 100 μg·L⁻¹ kinetin, 60 μg·L⁻¹ aceotylsyringone, 2.5 g·L⁻¹ phytalge, 30 g·L⁻¹ glucose, pH 5.7–5.8) in dark at 28 °C, for 2 days. Then the hypocotyls were shifted to selection medium (MS salts, 100 μg·L⁻¹ kinetin, 30 g·L⁻¹ glucose, 15 mg·L⁻¹ hygromycin, 500 mg·L⁻¹ cefotaxime, 2.5 g·L⁻¹ phytalge, pH 5.7–5.8) for a duration of 4–6 weeks. The hygromycin resistant calli were shifted to MS medium (MS salts, 30 g·L⁻¹ glucose, 250 mg·L⁻¹ cefotaxime, 2.5 g·L⁻¹ phytalge, pH 5.7–5.8) for growth restoration, for two weeks. Then the embryogenic calli were placed on embryo maturation selection medium (MS salts, 1.9 g·L⁻¹ KNO₃, 30 g·L⁻¹ glucose, 2.5 g·L⁻¹ phytalge, 15 mg·L⁻¹ hygromycin, pH 5.7–5.8) for a duration of 5 months to obtain embryogenic calli. The healthy embryogenic calli were cultured on germination medium (MS salts, 1.9 g·L⁻¹ KNO₃ but no NH₄NO₃, 0.5 g·L⁻¹ asparagus, 1 g·L⁻¹ glutamine, 2.5 g·L⁻¹ phytalge, pH 5.7–5.8) for 3 months. Ultimately, the germination of somatic embryos occurred and the regenerated plantlets were shifted to the soil pots for subsequent growth. The T₁ seeds were obtained by grafting of the shoots of primarily transformed plants (T₀) into fully-grown WT plants. All of these developmental stages were kept in a controlled condition (28 °C, 16 h light/8 h dark). The homozygous T₃ plants were used for salinity assays.
RNA extraction and real-time (RT) PCR analyses
The total RNA was extracted from cotton seedlings using Trizol reagent (Invitrogen, Carlsbad, CA, USA). From each sample, 1 μg of the total RNA was used to perform reverse transcription reactions. The expression level of ThST5 was analyzed by taking 1 μL of synthesized cDNA by RT-PCR using the gene-specific primers (Additional file 1: Table S5). In the RT-PCR analysis, the cotton GhHis3 (AF024716) was used as an internal control. The qualitative RT-PCR was performed according to the protocol reported previously (Yu et al. 2013).

Salt tolerance assays of transgenic plants in laboratory
For cotton seeds germination assay under salt stress, approximately 50 seeds of each line were sown in soil under greenhouse conditions with a temperature of 28 °C in 16 h light/8 h dark. The CK and transgenic lines were either irrigated with distilled water or 500 mL of 250 mmol·L⁻¹ salt solution for two weeks and germination rates (emergence of radicles) were calculated at indicated time points. The post-germination evaluation of salinity tolerance was performed in the hydroponic culture and soil. The seeds of each line were germinated in the soil for 4 days and shifted to Kimura B solution for hydroponic cultivation. The young seedlings were raised in the growth chamber having 28 °C and 8 h dark/16 h light with a photon density of 0.3 μmol·m⁻²·s⁻¹ and the medium was refreshed on alternate days. The 140 mmol·L⁻¹ NaCl solution was prepared in Hoagland solution and subjected to 11 days old seedlings for 3 days. For salinity-tolerance evaluation of cotton seedlings in soil, the seeds of each line were germinated in soil under normal growth conditions in the greenhouse. After germination, 500 mL of 300 mmol·L⁻¹ salt solution was subjected to 20-day old seedlings for 10 consecutive days. The plant phenotype was observed and captured with Nikon D700 digital camera.

Determination of root architecture in transgenic cotton
In order to compare the root architecture of transgenic cotton and CK, both genotypes were germinated in soil under greenhouse condition and irrigated as usual. After one week, 16 uniform seedlings of each line were dug out of soil, roots were washed with distilled water, and lateral roots were counted. The fresh weight of root was measured using digital weighing balance. The phenotype was captured with Nikon D700 digital camera.

Field trials of ThST5 overexpressing plants
In order to assess the function of ThST5 in salinity tolerance of cotton in field, field trials were performed in Shanxi province under water-proof sheds in 2020. Before the plantation of cotton, soil was extensively irrigated with water followed by plowing and harrowing after the surface water was completely infiltrated. According to the calculated salt demand, NaCl was evenly sprinkled over the soil surface and water was sprayed for even distribution of the salt. The average salinity content of field was 0.4% (68 mmol·L⁻¹) and the average pH was detected to be 8.5. The seeds of CK and transgenic plants were sown manually and the experiment was performed in completely randomized block design. Each plot was 2.5 X 6 m and 0.5 m deep; there was one row of ThST5 overexpressing plants and one row of CK in each plot. The control experiment used the plot of the same dimensions as described above and was situated next to the experimental plot. The salinity level in control field was 0.1% (17 mmol·L⁻¹). The distance between two rows was 30 cm and the distance between two plants was 12.5 cm, and each plot was occupied by 600 plants. There was normal germination of seeds followed by normal irrigation. After 5 months of sowing, the plants were selected randomly from each plot for the evaluation of important agronomic traits as well as yield analysis. Approximately 27 plants of each CK and transgenic cotton were selected for trait evaluation.

DAB and NBT assays
Three leaves per plant were taken from three random selected plants of CK and ThST5-OE line for each treatment (described below). The leaves were first treated with 0.6% (100 mmol·L⁻¹) NaCl for one day. For H₂O₂ staining, leaves were incubated with 0.1 mg·mL⁻¹ solution of DAB with pH 3.8 prepared in phosphate buffer (0.2 mmol·L⁻¹, pH 7). The O₂⁻ level was measured by incubating the salt-treated leaves in 0.1 mg·mL⁻¹ solution of NBT prepared in phosphate buffer (10 mmol·L⁻¹, pH 7.0). The incubation was conducted at 25 °C in dark for 24 h. The staining was followed by overnight soaking of stained leaves in bleaching solution (acetic acid:glycerol:ethanol = 1:1:3) to abolish the chlorophyll. Leaves were then placed in boiling water (95 °C) for 20 min. The decolorization procedure was repeated with fresh bleaching solution. The brown precipitates formed by reaction between H₂O₂ + DAB and blue precipitates formed by reaction between O₂⁻ + NBT were visible on the surface of leaves.

Statistical analyses
All of above-mentioned assays were performed thrice and data were presented as mean ± standard deviation (SD). The statistical significance of effect of treatments on genotypes was determined by Duncan’s multiple range tests. Different letters depict significance difference.
Transcriptome-sequencing analysis
For transcriptome-seq analysis, seedlings of transgenic and CK cotton were raised hydroponically in the growth chamber and treated with 140 mmol-L⁻¹ salt solution for three consecutive days. The 14 days old seedlings of each genotype (from salt treated and non-salt treated group) were collected for transcriptome sequencing at the same time. Each treatment had three independent replicates, and each replicate contained a sample of 20 seedlings. The extraction of RNA was conducted by using RNAprep Pure Kit (TIANGEN, Beijing, China) and the integrity of total RNA was analyzed using Bioanalyzer 2100 (Agilent, California, USA). The RNA sequencing was performed by Beijing Biomarker Technologies (Beijing, China). The construction of the RNA library as well as sequence analyses were performed according to previous protocol (Zhang et al. 2016a, b).

Supplementary Information
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Authors’ contributions
Xiang CB and Alfatih A designed the experiments. Nazish T, Javaid A, Ali M, Wu J, and Alfatih A performed most of the experiments and data analyses. Zhu YH, Li J, Zhang HY, and Wu SJ conducted field trials and data analyses. Nazish T and Alfatih A wrote the manuscript. Xiang CB, Wu SJ, and Alfatih A revised the manuscript and supervised the project. All authors have read and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests
The authors declare that they have no competing interests.

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