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Thellungiella halophila ST103 enhances salt tolerance in *Gossypium hirsutum*

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Abstract

Background: Cotton (*Gossypium hirsutum*), the major textile fiber crop of the world, is negatively affected by salinity. It leads to the induction of adverse effects on growth and development of cotton. The overall yield of cotton faces major drawback once they are grown in saline soil. To improve cotton salt tolerance, transgenic approach offers a fast and effective way but it relies on the availability of salt tolerance genes.

Results: In this study, we have reported the evaluation of *ThST103*, a homologue of *Arabidopsis* ozone-induced protein (*AtOZI1*) in *Thellungiella halophila*, in enhancing salt tolerance in cotton. Overexpression of *ThST103* enabled cotton plants to germinate and grow better than the wild types under salt stress. The transgenic lines showed enhanced survival rate in the saline environment and experienced less oxidative damage compared with the wild types. In the field, the transgenic cotton lines produced higher yield than the wild type in saline soil. Transcriptomic comparison analyses of *ThST103* overexpression lines versus the wild type revealed upregulated genes enriched in salt stress tolerance and ion homeostasis.

Conclusions: Our results demonstrate that *ThST103* has the capability to improve salt tolerance in cotton. It can be used in cotton breeding for salt tolerance cultivars.

Keywords: Salinity, *Thellungiella halophila*, *ThST103*, Ozone-induced protein, Salt tolerance, Cotton (*Gossypium hirsutum*)

Background

Salt stress is one of the primary abiotic hazards that decreases the yield and production of crops (Wang and Li 2008; Hasan et al. 2020). In saline soil, high salt concentration around roots reduces the plants' ability to absorb water (Munns 2002), thus leading to dehydration (Mahajan and Tuteja 2005). Salinity stress induces ionic toxicity due to excessive uptake of Cl^- and Na^+ and deficiency of Ca^{2+} and K^+ (Waraich et al. 2011). In addition, salt stress accelerates the accumulation of reactive oxidative species (ROS) (Heath 1998; Shulaev and Oliver 2006), which disturbs cellular redox homeostasis and oxygen metabolism and damages macromolecules. Plants combat salt stress by active exportation of Na^+ out of the cell by plasma membrane bound Na^+/H^+ antiporter and active importation of Na^+ in the vacuoles of plant by vacuolar membrane-bound Na^+/H^+ antiporter (Munns and Tester 2008; Agarwal et al. 2013); increasing potassium (K^+) uptake to cope with the deficiency of K^+ (Ghars et al. 2008); synthesizing osmolytes or osmoprotectants to balance osmotic pressure and counteract the ionic toxicity of high NaCl (Zulfiqar et al. 2019); and

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increasing ROS scavenging ability (Hasanuzzaman et al. 2020; Nadarajah 2020).

The halophytes have developed mechanisms to avoid surplus Na^+ accumulation in cytoplasm that involves Na^+ extrusion or/and intracellular compartmentalization (Peng et al. 2016). *Thellungiella halophilla* (salt cress), a close relative of *Arabidopsis*, is an important and suitable halophytic model (Wang et al. 2004). It was demonstrated that the H^+ transport and hydrolytic activity of plasma membrane H^+-ATPases from leaves and roots is enhanced under saline conditions in *Thellungiella halophilla* (Vera-Estrella et al. 2005). The expression of *SOS1* (plasma membrane Na^+/H^+ exchanger), *NHX1* (tonoplast Na^+/H^+ exchanger), and vacuolar H^+-translocating ATPase subunit (*VHA-E*) is also increased in saline environment (Vera-Estrella et al. 2005). These studies suggest that *Thellungiella halophilla* has an enhanced ability to allocate and store Na^+ by a controlled movement of ions across plasma and tonoplast membrane (Vera-Estrella et al. 2005).

The gene *ThST103* was isolated by Du et al. as a cDNA of 630 bp in length that confers improved salt tolerance in *Arabidopsis* (Du et al. 2008). Its nucleotide sequence has 82.9% similarity with *Arabidopsis* homolog *At1G01170* and 97% resemblance at amino acid level. The gene *At1G1170* is annotated to be analogous to *AtOZI1*, encoding an ozone-induced protein that accumulates in response to ROS (Sharma and Davis 1995). The *AtOZI1* mRNA accumulation was higher in plants exposed to ozone stress than the untreated ones, suggesting its role as a stress related protein in response to ROS (Sharma and Davis 1995). *AtOZI1* is upregulated in response to oxidative stress (Li et al. 2009). Razzaque et al. (2019) revealed the upregulation of *AtOZI1* (*LOC_Os11g06240.1*) under salinity stress in rice (Razzaque et al. 2019). However, detailed information regarding *ThST103* gene and its role in the salinity tolerance is not available yet, neither it has been demonstrated to improve crop salt tolerance by heterologous expression.

Cotton holds a great significance in terms of its agricultural value; therefore, it is necessary to find ways to increase its tolerance against abiotic stresses for better yield. Transgenic technology is a powerful approach to enhance abiotic stress tolerance in plants (Abdelraheem et al. 2019). Progress to elevate the tolerance of cotton against salinity have been made in the past through transgenic technology. For example, it was demonstrated that the upregulation of *Arabidopsis* vacuolar H^+-pyrophosphatase (*AVP1*) gene resulted in enhanced proton electrochemical gradient, that aids in increase of sugars isolation and ions in vacuoles, thus reducing water potential and increasing salt and drought tolerance (Gaxiola et al. 2001). A study used the same approach and expressed *AVP1* in cotton, that resulted in dynamic growth of transgenic lines as opposed to wild types in saline environment (Pasapula et al. 2011). Another study showed that the overexpression of *AVP1* in cotton led to salinity tolerance, as *AVP1* stimulated auxin polar transport which resulted in development of root (Zhang et al. 2012). The augmented root system in transgenic lines enabled cotton plants to uptake more water and withstand salinity. Likewise, a study revealed that the overexpression of *Thellungiella halophilla,* H^+-PPase gene, *TsVP*, in cotton enhanced root, shoot and photosynthesis under salt conditions. Under salinity, the transgenic cotton accumulated more soluble sugars, Ca^{2+}, Cl^-, K^+, and Na^+ in leaves and roots. The toxic effect of salinity is minimized in transgenic cotton by reduced membrane ion leakage and MDA (malondi-aldehyde), which suggested that H^+-PPase caused vacuolar Na^+ and Cl^- accumulation rather than cytoplasmic (Lv et al. 2008). Thus enhanced water uptake by increase accumulation of sugars and ions resulted in reduction of cell water potential, which is vital for increasing tolerance to salt (Lv et al. 2008). Similarly, a study showed that expression of *Thellungiella halophilla*, H^+-PPase gene, *TsVP*, in cotton improved the emergence rate, survival rate and yield in transgenic plants as compared with the wild types (Zhang et al. 2016). Another study demonstrated that *Arabidopsis* gene *AtNHX1*, when expressed in cotton, resulted in more biomass and fibers under salinity as opposed to controls (He et al. 2005). It was demonstrated that the simultaneous overexpression of *Arabidopsis AtNHX1* and *AVP1* in cotton resulted in enhanced boll number, plant height and yield of fiber under salt conditions (Shen et al. 2015). In another study, the co-expression of *AtNHX1* and *TsVP* genes from *Arabidopsis* and *Thellungiella halophilla,* respectively, in cotton improved salt tolerance. The cotton co-expressing *AtNHX1* and *TsVP* genes demonstrated improved seed yield under saline environment (Cheng et al. 2018). A choline monooxygenase (CMO) gene, *AhCMO*, from *Atriplex hortensis* was transferred to cotton, the resultant transgenic cotton showed lower electrolyte leakage and accumulation of MDA in leaves, enhanced protection to cell membranes and higher seed yield under saline stress (Zhang et al. 2009). Overexpression of a rice NAC gene (*SNAC1*) increased proline content and reduced MDA level in the transgenic cotton plants under salt treatment. The *SNAC1* overexpressed cotton lines displayed increased root development and boll number, and reduced rate of transpiration under saline conditions (Liu et al. 2014).

In this study, we aimed to evaluate whether *ThST103* can improve salt tolerance in cotton. We generated transgenic cotton lines overexpressing the *ThST103* gene from *Thellungiella halophilla*. Our work showed that

the expression of *ThST103* gene in the transgenic cotton enhanced seed germination and survival under salt stress. The *ThST103* cotton produced higher yield under saline soil environment in the field trial. Transcriptome analyses revealed that the transgenic lines had the upregulated genes enriched in ion homeostasis and salt stress tolerance. Together, our results demonstrated that *ThST103* conferred enhanced salt tolerance in cotton and may serve as a candidate gene for cotton improvement.

Results

ThST103 enhances seed germination of cotton under salt stress

To investigate whether *ThST103* confers salt tolerance in cotton, we created transgenic lines overexpressing *ThST103* (OE) (Additional file 1: Fig. S1). The expression of *ThST103* in OE lines was confirmed via RT-PCR (Additional file 1: Fig. S1B). We checked the germination of transgenic (*ThST103*-OE) and the wild type (CK) seeds under 0 mmol·L^{-1} and 250 mmol·L^{-1} NaCl in soil. Figure 1A demonstrates that under 0 mmol·L^{-1} NaCl, there was no difference in germination between the wild type and transgenic lines (*ST103*-OE1 and *ST103*-OE2). In contrast under 250 mmol·L^{-1} NaCl, germination of transgenic lines initiated at the 3rd day of sowing, one day prior to CK. The germination of transgenic lines was ~ 30% to 40% higher than that of CK over the time period of 8 days.

Similarly, we examined germination in soil over the period of 2 weeks. CK and transgenic lines germinated under normal conditions displayed no difference in the survival rate, however with the treatment of 250 mmol·L^{-1} NaCl, the survival rate of transgenic lines was ~ 30% to 40% higher than that of the CK (Fig. 1B, C).

ThST103 enhances seedling growth under salt stress

To evaluate the growth and survival of transgenic cotton seedlings under saline environmnet, we hydroponically grew cotton seedlings. The seeds were grown in soil for 4 days and then transferred to hydroponic culture for 1 week. After that they were treated with 140 mmol·L^{-1} NaCl for 3 days. In the absence of NaCl in hydroponic medium, CK and transgenic lines (*ST103*-OE1 and *ST103*-OE2) showed no difference in the survival. However, under 140 mmol·L^{-1} NaCl, CK displayed ~ 20% survival rate while *ST103*-OE1 and *ST103*-OE2 displayed ~ 80% and ~ 79%, respectively (Fig. 2A, B). This showed that *ThST103* could enhance salt tolerance in cotton.

Similarly, OE and CK cotton seeds were grown in soil for 20-days and then treated with 0 mmol·L^{-1} and 300 mmol·L^{-1} NaCl in soil for another 10 days in order to evaluate its tolerance towards salt stress in soil. Under 0 mmol·L^{-1} NaCl conditions, no obvious difference was seen between OE and CK plants. However, under 300 mmol·L^{-1} NaCl conditions, OE plants (*ST103*-OE1) showed ~ 80% survival rate as compared with CK with ~ 40% (Fig. 2C, D).

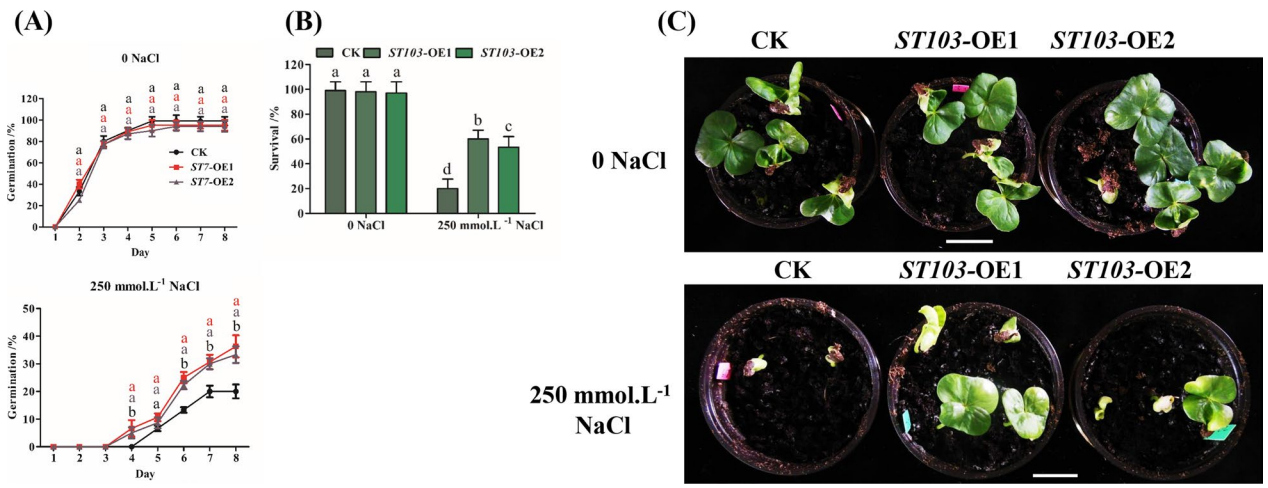

Fig. 1 *ThST103* confers salt tolerance phenotype in seed germination in cotton. **A** Curve of seed germination. The seeds overexpressing *ThST103* genes and CK were germinated for 8 days in the soil augmented with 0 and 250 mmol·L^{-1} NaCl and the germination rate was recorded daily. Values are mean ± SD (10 seeds per replicate, five replicates per treatment). Different letters denote significant differences ($P < 0.05$) from Duncan's multiple range tests. **B** Ratio of seedling survival. The germinated seedlings were grown for 2 weeks prior to the recording of seedling survival ratio. Values are mean ± SD (10 plants per replicate, five replicates per treatment). Different letters denote significant differences ($P < 0.05$) from Duncan's multiple range tests. **C** Growth of germinated seeds. Bar = 4 cm

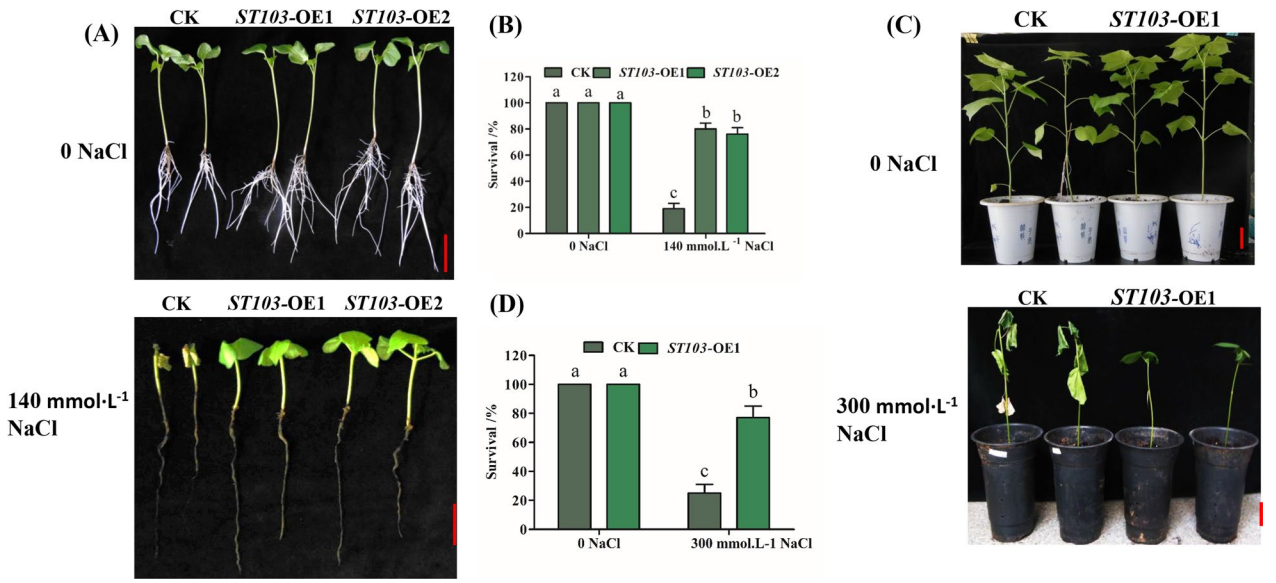

Fig. 2 The overexpression of *ThST103* enhances the salt tolerance of 2 weeks old cotton seedling grown in the hydroponic medium and in soil under saline environment. **A**, **B** The seeds germinated for 4 days in soil, relocated to hydroponic culture for a week and exposed to 140 mmol·L^{-1} NaCl for 3 further days. Bar = 4 cm. Values are mean ± SD (7 plants per replicate, four replicates per treatment). Different letters denote significant differences ($P < 0.05$) from Duncan's multiple range tests. **C** Plants grown in soil for 20 days and treated with 0 NaCl or 300 mmol·L^{-1} NaCl for another 10 days. Bar = 4 cm. **D** Survival rate of seeds germinated in the soil for 20 days and then treated with 0 and 300 mmol·L^{-1} NaCl for another 10 days. Values are mean ± SD (10 plants per replicate, five replicates per treatment). Different letters denote significant differences ($P < 0.05$) from Duncan's multiple range tests

ThST103 improves cotton yield in field

To investigate the role of *ThST103* against salt stress, we performed field trails in the field experimental station of Shanxi Agricultural Academy, Yuncheng, Shanxi Province, China. They were performed under normal and 0.4% NaCl conditions as described in Methods. Our results showed that under no salt treatment (control), the yield of CK and OE lines was similar. However, under 0.4% NaCl treatment transgenic lines (*ST103*-OE1 and *ST103*-OE2) produced higher yield than the CK (Fig. 3A). The boll weight of transgenic lines under similar saline conditions was significantly higher than that of CK, whereas under normal conditions there was no difference between OE lines and CK (Fig. 3B).

ThST103 augments the tolerance to oxidative stress

In order to analyze the role of *ThST103* in withstanding salinity induced oxiadtive stress, histochemical analysis was carried out. The results showed more DAB staining in CK leaves treated with 140 mmol·L^{-1} NaCl as

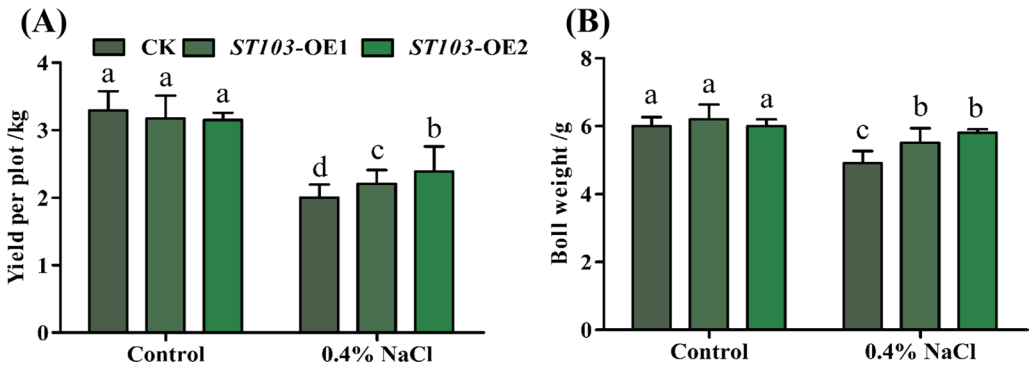

Fig. 3 The overexpression of *ThST103* increases the cotton yield under saline conditions in field. **A** Yield of CK and transgenic cotton lines (*ST103*-OE1 and *ST103*-OE2) under control and 0.4% NaCl conditions. **B** Average boll weight

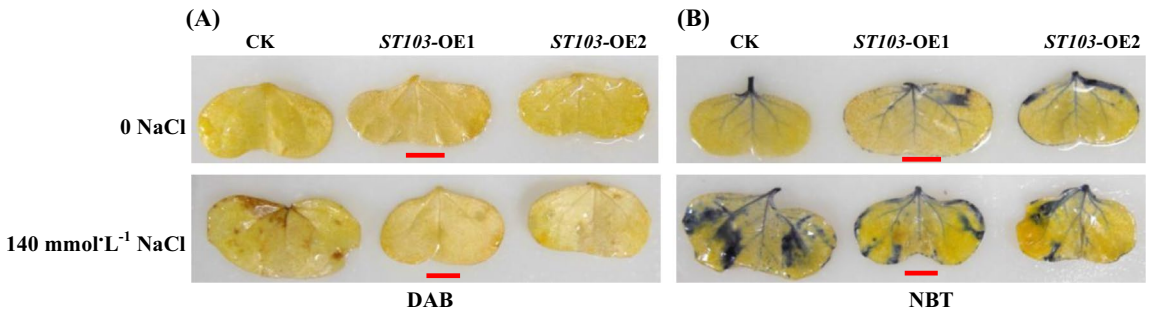

Fig. 4 The histochemical staining analysis with DAB (3,3′ diaminobenzidine) and NBT (nitroblue tetrazolium chloride); the leaves of CK and transgenic cotton lines (*ST103*-OE1 and *ST103*-OE2) were grown in the soil for a week, moved to the hydroponic culture for 4 days and treated with either 0 mmol·L^{-1} NaCl or 140 mmol·L^{-1} NaCl for a day prior staining **A** CK and transgenic cotton lines (*ST103*-OE1 *and ST103*-OE2) treated with DAB staining. Bar = 1 cm **B** CK and transgenic cotton lines (*ST103*-OE1 *and ST103*-OE2) treated with NBT staining. Bar = 1 cm

compared with OE leaves, indicating less H_2O_2 accumulation (Fig. 4A). Similarly, more NBT staining was seen in CK leaves treated with 140 mmol·L^{-1} NaCl compared with OE leaves, indicating a lower endogenous O_2^- level (Fig. 4B).

RNA-seq analyses reveals ion homeostasis and salt stress tolerance related genes are upregulated in the OE cotton

To explore molecular mechanisms underlying *ThST103*-mediated salinity tolerance in cotton, we investigated the genome-wide transcriptional landscape that is impacted by *ThST103*-OE in response to salinity stress. Under saline condition, the expression profiling after ontology assessment showed that major stress tolerance categories were differentially expressed. The total number of differentially expressed genes (DEGs) under normal and salt conditions were 2 114 and 7 272, respectively. Amongst them, the upregulated genes under normal and saline conditions were 1 057 and 4 229, respectively. On the other hand, the down-regulated genes under normal and salt environment were 1 057 and 3 043, respectively (Fig. 5A).

Pairwise comparisons (CK-N vs. *ThST103*-OE1-N, CK-N vs. CK-S, and CK-S vs. *ThST103*-OE1-S) were done for determination of transcriptomic differences between CK and *ThST103*-OE. Three groups were created on the basis of pairwise comparisons: the number of total genes found in CK-N versus *ThST103*-OE1-N were 285, amongst which 122 genes overlapped with CK-N versus CK-S. Similarly, total number of genes belonging to CK-N versus CK-S were 8 703, amongst which 1 499 overlapped with CK-S versus *ThST103*-OE1-S. Likewise, total number of genes belonging to CK-S versus *ThST103*-OE1-S were 628, amongst which 39 overlapped with CK-N versus

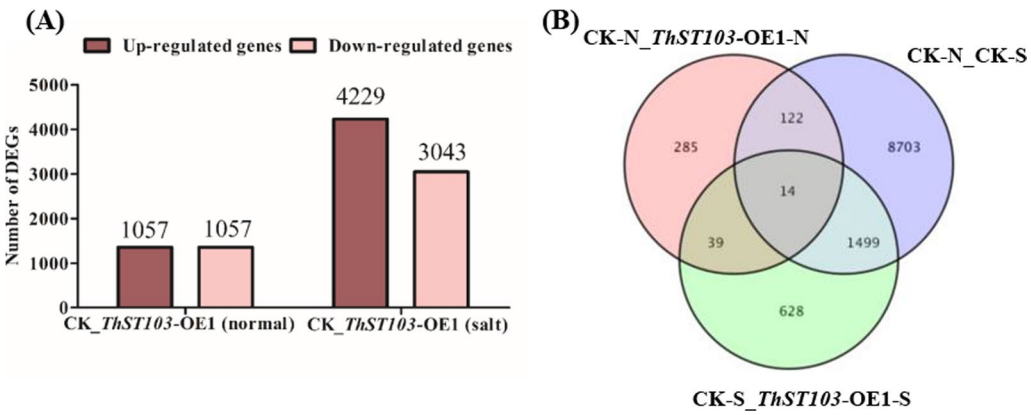

Fig. 5 Expression profiling of cotton seedlings under normal and saline stress. **A** Number of differentially expressed genes (DEGs) in CK and *ThST103*-OE under normal and salt stress. **B** Venn diagram analysis of the common and specific genes that are unique and shared between the different pairwise comparisons

ThST103-OE1-N. Only14 genes overlapped in all three groups (Fig. 5B).

The genes upregulated under normal conditions were identified in biological processes including transcription factor activity, signal transduction mechanisms, carbohydrate transport and metabolism, inorganic ion transport and metabolism, plant–pathogen interaction, regulation of sulfur utilization, translation, and lipid transport and metabolism (Fig. 6A). On the other hand, a large number of genes were upregulated under saline conditions, which were identified in biological processes including carbohydrate transport and metabolism, metabolite biosynthesis, photosynthesis, signal transduction mechanism, inorganic ion transport and mechanism, translation, regulation of sulfur utilization, defense mechanisms, lipid transport and metabolism, plant hormone signal transduction (Fig. 6B).

Additional file 1: Tables S1 and S2 represent the genes related to various abiotic stresses, which were upregulated under normal and saline environment, respectively. Interestingly, heat map of DEGs revealed that under saline conditions the transcript levels of genes related to salt tolerance like *CAX3 (GH_D01G2538)* (Cheng et al. 2005; Zhao et al. 2008), *PETH (GH_A12G0591)* (Song et al. 2014; Zhao et al. 2017; Chen et al. 2020), *APX3 (GH_D01G1910)* (Wang et al. 1999; Mittova et al. 2002), *CAT2 (GH_D07G1879)* (Kim et al. 2005; Bueso et al. 2007; Awaly et al. 2020) were upregulated (Fig. 7). These genes play a role in anti-oxidation, ion homeostasis and photosynthesis. Similarly, Additional file 1: Table S2

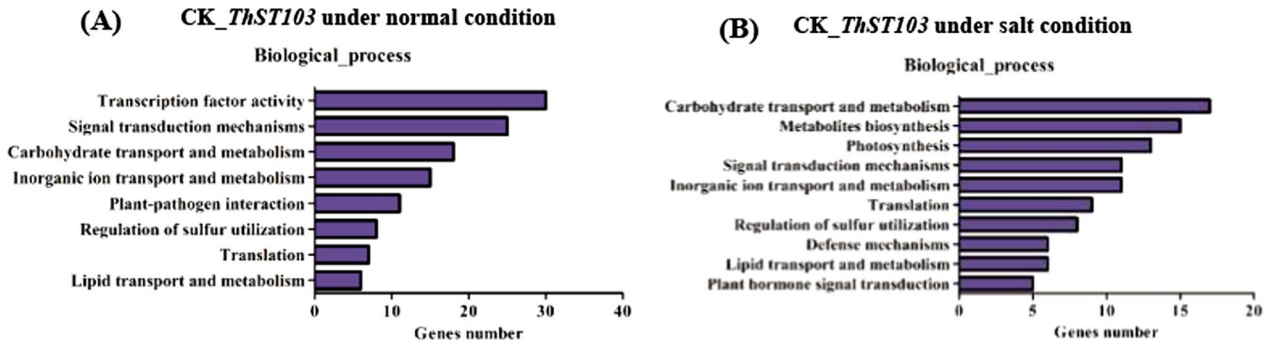

Fig. 6 The GO grouped up-regulated genes in one main category: biological processes. **A** The number of genes involved in biological processes under normal conditions. **B** The number of genes involved in biological processes under salt conditions

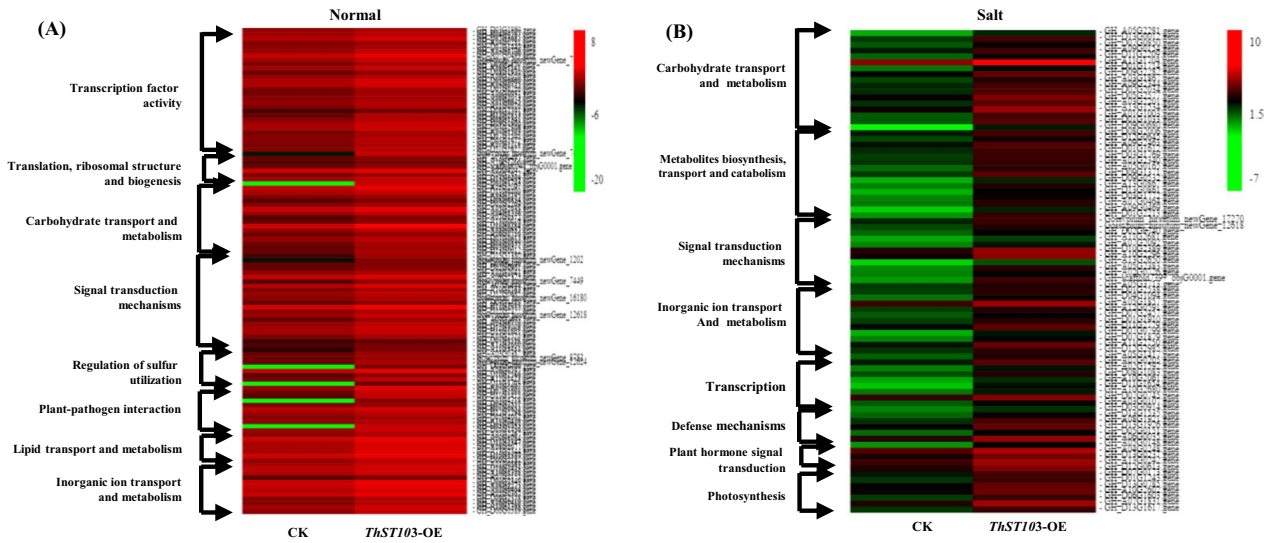

Fig. 7 Heat maps of DEGs in normal and saline conditions. **A** Heat map of common differentially expressed genes in CK and *ThST103*-OE under normal condition. **B** Heat map of common differentially expressed genes in CK and *ThST103*-OE under salt stress. Red and green colors indicate up- and down- regulated genes, respectively, from both control and salt treated plants. The results were presented as log2 ratios. Only results with a *P* value < 0.05 and that have been confirmed in three independent experiments were included

shows the upregulation of antioxidant enzymes genes that are vital for tolerance against salt stress like *2-CYS PEROXIREDOXIN BAS1-LIKE, CATALASE ISOZYME 2, GLUTATHIONE PEROXIDASE, L-ASCORBATE PEROXIDASE 3, PROTEIN DETOXIFICATION 16, PROTEIN DETOXIFICATION 29, REDOXIN; AHPC/TSA FAMILY, SUPEROXIDE DISMUTASE AND SULFIREDOXIN*. These results demonstrated that *ThST103* could impact transcription levels of genes involved in tolerance against salt stress.

In order to figure out homologues of *ThST103* in genome of cotton, we carried out a BLAST search using *ThST103* protein sequence against cotton genome. The outcome of BLAST displayed that there were 51 genes with homology to *ThST103* in *Gossypium hirsutum* L. genome. Out of those 51 genes, only 5 genes display high similarity index of 90% to *ThST103*, namely uncharacterized proteins LOC107889402, LOC107891306, LOC107891309, LOC107943169, and LOC107921082 (Additional file 1: Table S4).

Discussion

Cotton is a vital crop in terms of being a source of biofuel and fibers (Reddy and Yang 2009; Parveen et al. 2017). This crop faces various adverse environmental stresses, among which salinity is a major threat (Ahmad et al. 2002). It is urgent to breed crops that have tolerance against salinity stress. For crop improvement with salt tolerance and better yield, transgenic approach has been demonstrated to be a faster and effective way.

In this study we evaluated the role of *ThST103* in cotton against salt stress. The transgenic cotton plants overexpressing *ThST103* displayed improved salinity tolerance in cotton.

In the midst of all stages of cotton development, germination and seedling stage are the two most sensitive to salinity (Ahmad et al. 2002). When salt content of soil is higher than 0.3%, it can cause osmotic stress, ion toxicity and other injuries that delay the germination of seed, reduce the rate of germination and inhibit the seedling growth (Dong et al. 2008; Abbas et al. 2011). Another study showed that high concentration salt caused a complete arrest of germination in cotton (Sattar et al. 2010). Our study displayed that the *ThST103* overexpressing lines demonstrated early and enhanced germination compared with the wild type (Fig. 1). That depicted that *ThST103* could confer salt tolerance in cotton germination.

Seedling stage is very vulnerable to salt stress (Tiwari et al. 2013). In our study we observed that the overexpression of *ThST103* in transgenic lines enhanced the growth and survival of cotton grown in soil and hydroponic culture as compared with the wild type (Fig. 2), demonstrating that overexpression of *ThST103* could induce salt tolerance in seedlings.

Salinity reduces the weight, size, and the number of cotton bolls (Zhang et al. 2012). It is the ultimate objective of crop genetic engineering to improve overall yield without much drawback of growth (Cattivelli et al. 2008). It has been demonstrated that salt exposure imparts adverse effects on vegetative growth that may delay the process of flowering, which in turn leads to reduction of cotton yield. Our field trials showed that transgenic lines overexpressing *ThST103* produced higher yield and boll weight compared with the wild type under saline environment (Fig. 3), showing that *ThST103* is a good candidate to improve salt tolerance in cotton.

Production of ROS takes place as a result of salinity exposure and is extremely harmful to cell as they can disturb redox homeostasis of cell (Li et al. 2017). The accumulation of ROS can cause degradation of enzymes and proteins, lipid peroxidation, DNA mutation along with damage to PSII system and electron transport system (Pitzschke et al. 2006; Li et al. 2017). In our study the levels of H_2O_2 and O_2^- in transgenic lines overexpressing *ThST103* were decreased under saline environment as shown by DAB and NBT staining (Fig. 4). Increased level of H_2O_2 and O_2^- was observed in cotton with increased salinity (Zhang et al. 2013). In relation to this, *AtOZI1*, a homolog of *ThST103*, was seen to be activated in response to production of ROS upon ozone exposure, indicating that it may play a role in oxidative response (Sharma and Davis 1995). A rice homolog of *AtOZI1* (*LOC_Os11g06240.1*) was upregulated under salinity stress in salt tolerant lines of rice (Razzaque et al. 2019). Our results showed that transgenic lines overexpressing *ThST103* exhibited enhanced ROS scavenging capability.

The exact molecular mechanisms underlying the *ThST103*-confered salt tolerance in cotton are not clear at present. However, from our study the improved tolerance against oxidative stress of OE lines should contribute to enhanced salt tolerance (Fig. 4). It is consistent with our RNA-seq data that genes participating in oxidative response are upregulated (Additional file 1: Tables S1 and S2). Moreover, our RNA-seq results revealed that a significant number of DEGs were upregulated upon salt stress in *ThST103*-OE as compared with CK. Among those, the genes involved in ion homeostasis and salt tolerance were enriched (Additional file 1: Tables S1 and S2), which may collectively contribute to enhanced salt tolerant phenotype observed in *ThST103* OE lines.

Conclusions

Our results showed that *ThST103* was able to enhance salt tolerance in cotton, especially in seed germination and early seedling establishment. Furthermore,

ThST103-OE lines exhibited improved yield in saline soil environment as compared with the wild type. Thus, *ThST103* is a promising candidate for the improvement in cotton salt tolerance.

Methods

Construction of vectors and transformation in plants

The cDNA of *ThST103* was sub-cloned into binary expression vector pCB2004 under the control of *CaMV* (cauliflower mosaic virus) 35S promoter by GATEWAY cloning system. *Agrobacterium tumefaciens*-mediated cotton (*Gossypium hirsutum,* cultivar R15) transformation was done by using the overexpression vectors (Additional file 1: Fig. S1A) (Li et al. 2002). The obtained vector was electroporated in LBA4404 strain of *A. tumefaciens*. The cotton seeds were sterilized by using 70% ethanol and 10% H_2O_2 for 1 min each. Washing of seeds was done for 3∼4 times with sterile water. Seeds were germinated in culture room on half-strength MS medium under 8 h dark/16 h light photoperiod at 28 °C. The transformation of cotton was done by using hypocotyl sections of length 2∼3 cm of 7-day old seedlings as explants. The dip of explants was done for 15 min to *Agrobacterium* suspension containing pCB2004-*ThST5* vector with OD_{600} from 0.4 to 0.5. Culture of explants was performed in co-cultivation MS medium for 2 days in dark at 28 °C. Hypocotyls were transferred to selection medium for 4∼6 weeks. Antibiotic (hygromycin) resistant calli were relocated to MS medium to restore their growth for 2 weeks. Embryogenic calli were positioned on embryo maturation selection medium for 5 months. Culture of developed healthy embryogenic calli was done for 3 months on germination medium. After the germination of somatic embryos, the regenerated plantlets were transplanted in soil.

With the grafting of shoots of primarily transformed plants (T_0) into well-developed wild type plants, T_1 seeds were attained. For salinity assays, homozygous T_3 plants were utilized. We created 6 lines expressing *ThST103*, from which 2 lines were analyzed using RT-PCR for *ThST103* to ensure that they contain *ThST103* gene (Additional file 1: Fig. S1B).

Isolation of RNA and RT-PCR (qualitative real time PCR)

By using the Eastep Super Total RNA Extraction Kit (Promega Biotech Co. Ltd. Beijing), cellular RNA was extracted from plant materials (i.e., 10-day old plants grown in soil under long-day conditions with 16-h light/8-h dark cycle at 28 °C). For the reverse transcription reactions, 1 μg of the total RNA from each sample were utilized. A cDNA template of 0.5 μL was used for the RT-PCR with primers explicit for genes in Additional file 1: Table S3, for the determination of *ThST103* expression levels. Cotton *GhHis3* (AF024716) were amplified as internal controls.

Germination of cotton seeds in soil

The transgenic cotton seeds along with the controls were germinated in soil under long-day conditions with 16-h light/8-h dark cycle at 28 °C. For pots preparation, Pindstrup Substrate was used as soil medium. Totally 10 seeds per pot, five pots per treatment were used. The plants were irrigated with 0 mmol·L^{-1} NaCl and 250 mmol·L^{-1} NaCl, respectively, from the beginning for 2 weeks before the photos were taken and survival counted.

Survival of cotton plants in soil and hydroponic culture

The seeds were germinated in soil under conditions mentioned before and then the healthy controls and transgenic cotton seedlings were transferred to Hoagland solution (hydroponic culture) (Hoagland and Arnon 1950) for 1 week with aeration in greenhouse at 28 °C day/light with 70% relative humidity, photoperiod of 8 h dark and 16 h light, and 250 mmol·m^{-2}·s^{-1} light intensity. The wild type and transgenic cotton lines were exposed to salinity by adding 140 mmol·L^{-1} NaCl for 3 days in the nutrient solution (7 plants per replicate, four replicates per treatment).

Similarly, both the wild type and transgenic lines were grown in the soil for 20 days under established conditions. Then, irrigated with 0 mmol·L^{-1} and 300 mmol·L^{-1} NaCl, respectively, for further 10 days (10 plants per replicate, five replicates per treatment).

Histochemical staining analysis

The wild type and transgenic lines were grown in soil for a week and then transferred to the hydroponic culture for 4 days and treated with 140 mmol·L^{-1} for 1 day. The histochemical assay of H_2O_2 anion and O_2^- was done by the usage of DAB (diaminobenzidine) and NBT (nitroblue tetrazolium chloride) as chromogenic substrate, respectively. For the visualization of H_2O_2 accumulation in plants (Alvarez et al. 1998), leaves of transgenic cotton lines and the wild type under salinity treatments were covered with 0.1% (w/v) DAB for 18 h in the dark, then engrossed in 96% (v/v) ethanol until the chlorophyll is eradicated. The superoxide radicals content was identified by NBT staining (Wohlgemuth et al. 2002). The cotton leaves were covered with 10 mmol·L^{-1} phosphate buffer (pH 7.8) comprising 1 g·L^{-1} for 12 h and then dipped in 96% (v/v) ethanol to remove chlorophyll. The purple colored formazan deposition within the leaves shows superoxide production.

Field trials

For salinity tolerance analysis of transgenic cotton in field, transgenic cotton lines and the wild type were grown with 0.4% NaCl stress throughout the life from April to September 2020 in Shanxi Agricultural Academy, Yuncheng, Shanxi Province, China. The plants were grown in salt ponds with saline soil. The salt ponds had a cement pool bottom with the width of 2.5 m, depth of 0.5 m, and length of 20 m. Salt was sprinkled evenly on soil surface according to the calculated demand and later sprayed with water to aid the easy dissolving of salt on the surface.

The experiment was organized in 3 replicate plots per line per treatment in size of 2.5×6 m, with random arrangements. However, controls were grown in soil planted in a field with < 0.1% NaCl for whole life next to salt stress ponds.

RNA-sequencing analysis

The plants were grown hydroponically with conditions described above as control and salt treated lines (140 mmol·L^{-1} NaCl). 16 days old seedlings were sampled for RNA sequencing. Twenty seedlings of salt treated and controls were collected. RNA library construction and sequence analysis were conducted as already described (Qi et al. 2016).

Statistical analysis

Statistically significant differences were computed based on the one-way analysis of variance (ANOVA).

Accession numbers

The sequence data seen in this article can be found in The National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/) or *Arabidopsis* TAIR database (https://www.arabidopsis.org/), by the following accession numbers: *ThST103-2: EU714076, ST6-66: EU714068, ST225*: *EU714080, GhHIS3: LOC107951735, AT1G01170: NM_099999_4, AtOZI1:AT4G00860, GhNHX1: LOC107891968, GhAnn1: KM062523, HDG11: NM_105996.*

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42397-022-00120-z.

Additional file 1: Fig. S1. Overexpression vector and expression analysis of *ThST103* in transgenic cotton (*ST103*-OE1 and *ST103*-OE2). **Table S1**. Lists of genes significantly upregulated in the OE plants compared to control under normal growth conditions. **Table S2**. Lists of genes significantly upregulated in the OE plant compared to control under salt growth condition. **Table S3**. List of primers. **Table S4**. *ThST103* homologues in cotton genome.

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Authors' contributions

Xiang CB and Alfatih A designed the experiments. Javaid A, Nazish T, 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. Javaid A and Nazish T wrote the manuscript. Wu SJ, Xiang CB, and Alfatih A revised the manuscript. Wu S and Xiang C supervised the project. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its additional files].

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The manuscript has not been published, or submitted for publication elsewhere.

Competing interests

The authors declare no competing interests.

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